Chemical-induced allergy and autoimmunity

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Chemisch geïnduceerde allergie en auto-immuniteit

(met een samenvatting in het Nederlands en in het Duits)

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Contents

Abbreviations

ANOVA	analysis of variance
APC	antigen presenting cell
BB	Bandrowski's base
BQ	benzoquinone
CYP	cytochrome P450
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
HAPA	*N*-hydroxylamino-procainamide
ICAM	intracellular adhesion molecule
IFA	incomplete Freund's adjuvant
IFN	interferon
IL	interleukin
mAb	monoclonal antibody
MACS	magnetic activated cell sorter
MAO	monoamine oxidase
MHC	major histocompatibility complex
MPO	myeloperoxidase
NAT	*N*-acetyltransferase
NK	natural killer (cell)
NKT	natural klller T (cell)
^{inv}NKT	natural killer T cell using the invariant Vα14-Jα281 TCR
PA	procainamide
PAP	3-(*N*-phenylamino)-1,2-propanodiol
pAP	para-aminophenol
PBS	phosphate buffered saline
PE	phycoerythrine
PGE_2	prostaglandine E_2
PGHS	prostaglandine H synthase
PLN	popliteal lymphnode
PMA	phorbol myristate acid
PMN	polymorphonuclear leukocyte
PMφ	macrophage
pPD	para-phenylenediamine
RT-PCR	reverse transcriptase-polymerase chain reaction
SD	standard deviation
SI	stimulation index
SLE	systemic lupus erythematosus
STZ	streptozotocin
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCR	T cell receptor
Thd	thymidine
TMB	3,3'-5,5'-tetramethyl benzidine
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNCB	2,4,6-trinitrochlorobenzene
TNF	tumor necrosis factor
TNP	2,4,6-trinitrophenyl
TOS	toxic oil syndrome
WBMC	white bone marrow cells

CHAPTER 1

General introduction

Principles of innate and adaptive immunity

The immune system is the body's defense against invading pathogens and microorganisms. The immune response in mammals is classically divided into *innate* and *adaptive* immunity. The *phagocytes* of the *innate* immune system provide a fast, unspecific defense mechanism, but cannot always eliminate infectious organisms. The *lymphocytes* of the *adoptive* immune system provide a more specific defense and, in addition, memorize their 'defense strategy', so that subsequent infections with the same organism can be handled more efficiently. The two key features of the adaptive immune response are thus *specificity* and *memory* (1).

Antigen recognition by cells of the innate and adaptive immune system

The innate immune system can only combat bacteria carrying highly conserved surface molecules, that can be recognized by receptors on the surface of phagocytes (2). Unsurprisingly, many bacteria have evolved ways to disguise these molecules, so that they no longer are recognized by phagocytic cells. The recognition mechanism used by the lymphocytes of the adaptive immune system has overcome these problems. Lymphocytes do not recognize conserved microbial surface molecules, but instead, each lymphocyte entering the blood

stream bears unique receptors with a certain *specificity*. The specificity of a lymphocyte is randomly generated by a unique genetic mechanism called *gene rearrangement*. As there are more than a thousand million lymphocytes in a human body, the adaptive immune system is capable of recognizing a huge diversity of antigens (1-3).

Clonal expansion and lymphocyte memory

As each single lymphocyte carries a unique specific receptor, the number of lymphocytes that recognize a given antigen is very small. To enable the adaptive immune system to effectively combat invading organisms, there must be an amplification mechanism; this mechanism is called *clonal expansion*. Upon encounter with their specific antigen lymphocytes proliferate and produce around a thousand daughter cells of identical specificity which then differentiate into effector cells. Lymphocytes can be divided in two major categories: *T lymphocytes*, or *T cells,* and *B lymphocytes*, or *B cells*, which have a different role in the immune response to microorganisms. T cells can, after activation and expansion, differentiate into cytotoxic effector cells or into T helper cells which provide help to B cells to enable them to proliferate and produce *antibodies*. The antibodies produced by B cells bind to the microorganism and thereby facilitate recognition and uptake of these organisms by the phagocytes of the innate immune system. After eliminating the organism from the body most of the involved lymphocytes die. However, some persist and form the basis of *immunological memory*, which ensures a more rapid and effective response upon a second encounter with the same pathogen (1).

Lymphocyte activation

Recognition of antigen alone is not sufficient to activate lymphocytes. To fully arm a lymphocyte it takes two signals, called *signal 1* and *signal 2* (4,5). The first signal is the specific binding of the receptor to the antigen, the second signal an unspecific costimulatory signal. T cells receive signal 2 from professional antigen presenting cells (APC), i.e., dendritic cells, macrophages, and B cells (6,7). After activation, T cells can provide signal 2 to B cells, thereby helping them to proliferate and produce antibodies (8,9). The signals that lead to "activation", i.e. upregulation of costimulatory molecules of APC

and subsequent signal 2 delivery to T cells are not completely elucidated; they may involve recognition of conserved microbial structures by the APC, or induction of stress, e.g., through free radicals formed by chemical transformation (6,7,10). Lymphocytes that receive signal 1 without co-stimulation are deleted or anergized, a term used for the non-responsive state of lymphocytes (11). The requirement for signal 2 is one of the mechanisms protecting man against lymphocytes that have an autoimmune potential (12).

Self tolerance and autoimmunity

Thus, activation and subsequent clonal expansion of lymphocytes expressing randomly generated receptors is the main principle of adaptive immunity. However, this principle in its most basic form bears a significant danger: recognition of self-antigens on the tissues of the body and subsequent reaction towards them (4). This is partially prevented by clonal deletion, a mechanism by which maturing lymphocytes in the thymus are tested for potential auto-reactive behavior and consequently deleted (13-15). The adaptive immune system, therefore, consists of lymphocytes that recognize a wide variety of different foreign-antigens without reacting to self-antigens. However, some auto-reactive lymphocytes escape clonal deletion and become activated during their lifespan, thereby initiating autoimmune diseases like diabetes (16), systemic lupus erythematosus (SLE) (17), or autoimmune arthritis (18). In the periphery, autoreactive lymphocytes normally encounter their self-antigens without costimulation, leaving them unresponsive. The mechanism by which these auto-reactive lymphocytes do become activated after years of slumbering in the body is not always known, but in some cases viruses, chemicals or trauma are thought to be involved (18-21).

NKT cells, the bridge between the innate and adaptive immune system?

Recently a specialized population of T cells was discovered, that coexpress receptors of the natural killer (NK) cell lineage (22-24). These NKT cells have unique potential to very rapidly secrete large amounts of cytokines (25), providing early help for effector cells and regulating the adaptive immune response. Murine NKT cells have a biased TCR repertoire; 85% of all murine NKT cells are Vα14-Jα281^{+} (26). NK T cells do not recognize peptides on

MHC molecules, like classical T cells do, but instead recognize hydrophobic antigens on transmembrane molecules distantly related to MHC-encoded antigen-presenting molecules. These molecules, called CD1, can present lipid antigens, e.g., glycolipids from mycobacteria, to NKT cells (27). As NKT cells can rapidly secrete cytokines upon recognition of bacterial glycolipids, they seem to straddle the adaptive and innate immune system (28). It was shown recently that NKT cells do not only play a role in induction of immunity but also in tolerance (29,30). Since NKT cells can recognize non-classical antigens and play an ambiguous role in the induction of immune responses, they may be involved in the pathogenesis of drug-induced autoimmunity.

Induction of allergy and autoimmunity by chemicals

Chemical induced allergy or autoimmunity is often observed after administration of certain drugs, e.g., procainamide (20), sulfonamides (31), and diphenylhydantoin (32), or after contact with industrial or environmental chemicals like $HgCl_2$ (21) or azo-dyes (33). T cells play a central role in the development of drug-induced adverse immune reactions (34-37). If the TCR of a certain T cell recognizes its cognate antigen on the surface of an APC, the T cell will respond with clonal expansion, cytokine production, and / or cytotoxicity (Fig. 1). A major difficulty in studying T-cell reactions to sensitizing chemicals is the fact that in most cases the ultimate neoantigen recognized by "drug-specific" T cells is unknown.

Neoantigen formation

As most T cells can only recognize peptides on MHC molecules, chemicals have to bind to a protein carrier in order to be recognized by T cells (38,39). The neoantigen thus formed is called the *hapten*-carrier complex which can be degraded by APCs. Parts of the neoantigen are presented on MHC molecules on the surface of the APC and can be engaged by T cells (40-42). Two different possibilities arise during processing and presentation of hapten-carrier complexes: (i) the part which is processed and presented on the surface of APCs is the part which has bound the hapten (43), or (ii) binding of the hapten to the

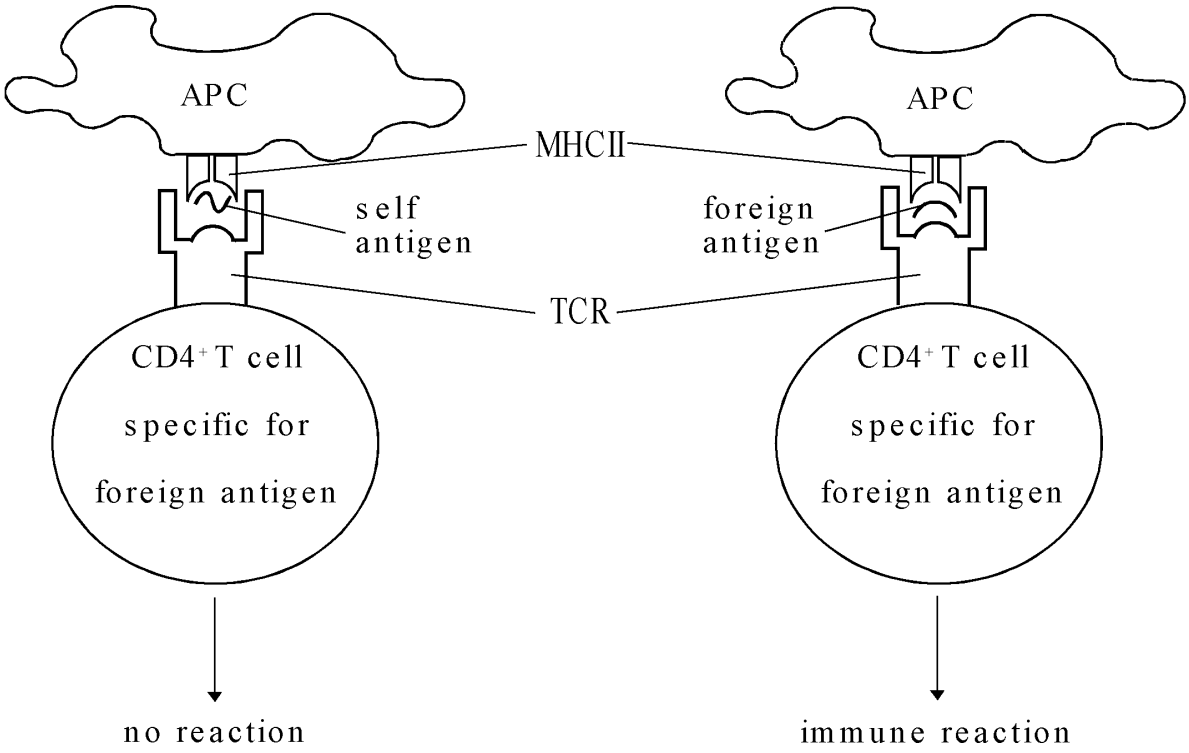

Figure 1. $CD4^+$ T cells can only be activated by peptide-MHC-complexes presented to them on APCs. Proteins, including self-proteins, are processed, cleaved and the resulting peptides presented on MHCII molecules on the surface of APC. $CD4^+$ T cells that engage self-peptides do not react upon contact with APC (left part), whereas peptides from pathogens, i.e. foreign peptides, elicit a T cell dependent immune reaction (right part).

self-protein influences the processing of this carrier (44,45). In the latter case, self-peptides which are normally not presented can be presented on the APC's surface. Because these so called *cryptic self-peptides* (46) are normally not presented, T cells are not tolerant against them and would react upon encounter with these "foreign" peptides. The two above mentioned possibilities, presentation of *hapten-peptide adduct* or *cryptic peptide*, respectively, are depicted in Fig. 2.

Metabolism of prohaptens

Chemicals that need to be activated in order to bind to proteins are called prohaptens (47). In their prohaptenic form they enter the human body where they are either metabolized in the liver, or in other cells that contain a panel of drug-metabolizing enzymes. The liver is believed to be an immune-privileged site concerning drug-induced adverse effects. Two mechanisms may account for

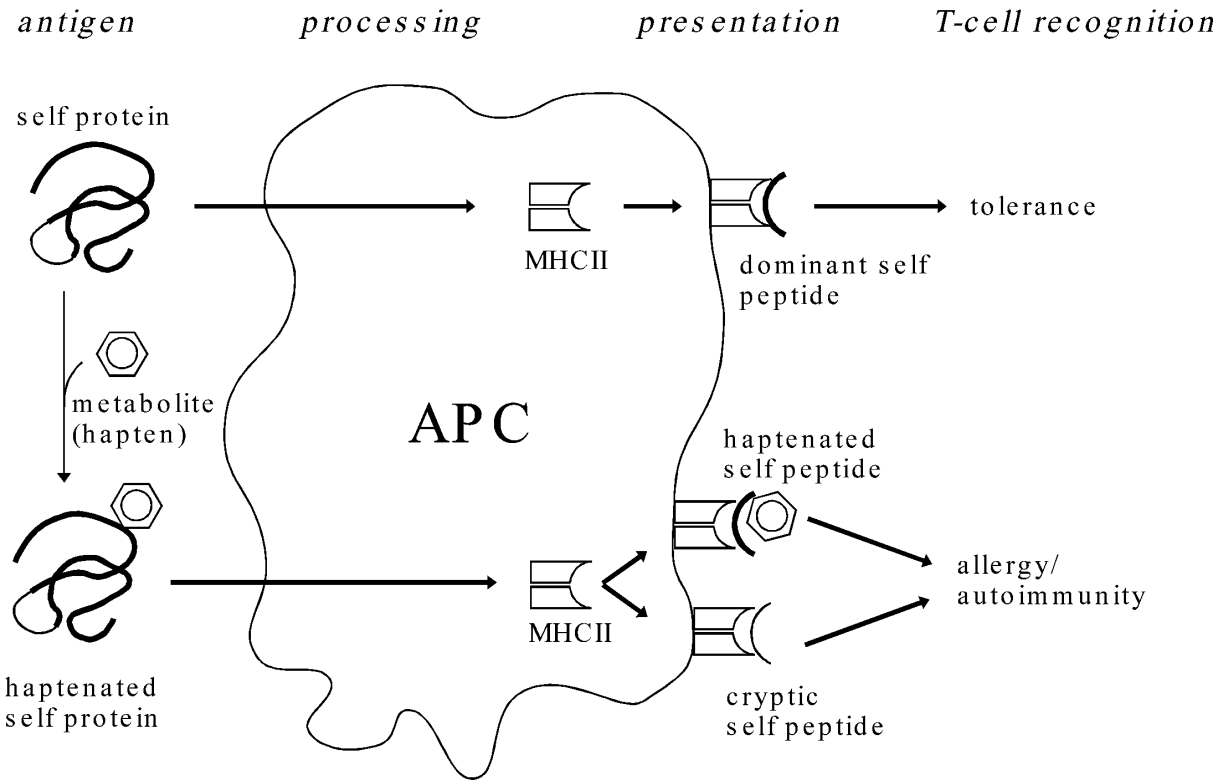

Figure 2. Processing and presentation of self-proteins and altered self-proteins, respectively. Self-proteins that are taken up by APC are processed and one ore more dominant peptides are presented on MHC molecules on the surface (upper part). Self-proteins that are altered by, e.g., haptens, are processed differently. This may lead to either presentation of an haptenated self-peptide, or presentation of a self-peptide, that normally is not presented after processing of the self-protein. As the T cells did not come in contact with this cryptic peptide before, tolerance does not exist and T cells are activated upon contact with it.

this: (i) the specialized function of the liver in detoxifying possibly harmful compounds; protein-reactive metabolites formed in the liver are quickly bound to special molecules like glutathion and acetyl, abundantly present in the liver (48); (ii) the environment in the liver is thought to be tolerogenic rather than immunogenic (49,50); T cells that come into contact with their antigen in this environment, would not be activated but rather deleted or anergized. Although the liver is specialized in drug-metabolism (48), it therefore does not seem to play an important role in drug-induced allergy and autoimmunity. In contrast, cells from the immune system itself can do both, oxidize the prohapten into the hapten, *and* present the so formed neoantigen together with the proper costimulation to T cells (37). Several studies have shown that neutrophils and monocytes are involved in drug-metabolism prior to drug-induced adverse immune effects (51-53).

Specificity versus cross-reactivity of single T cell clones

As mentioned before, specificity is one of the key-features of adaptive immunity. A few years ago it was generally accepted that a single T cell can only recognize one single peptide. Such specificity can be compared with a key fitting only a single key-hole. Recent publications, however, undermined this so called "one clonotype, one specificity" dogma. Theoretical considerations led to the conclusion that one single T cell clone must be able to react with a few thousand different peptides in order to efficiently react to the multitude of invading pathogens (54). Experimental evidence for this theory was provided by several other groups (55-57). Although T cells seem to recognize several hundreds or thousands of different peptides, this has not been described with haptens. On the contrary, T cell clones seem to specifically distinguish between changes in hapten side-chains (43,58) or even between stereoisomers (59).

Scope of this thesis

In this study we investigated the mechanisms involved in chemical-induced allergy and autoimmunity. Although from a first point of view there is a difference between allergy and autoimmunity, there is no clear-cut border between these two, especially when chemicals are the causative agent. Chemical-induced allergy can develop into autoimmunity by mechanism like molecular mimicry or presentation of cryptic peptides. On the other hand, symptoms of chemical-induced autoimmune diseases like procainamide-induced lupus disappear after discontinuation of drug-therapy, implying allergy instead of autoimmunity. A difficulty in studying the mechanism of drug-induced adverse immune effects is the fact that the ultimate neoantigens are unknown. Most chemicals have to be metabolized before they are capable of eliciting immune reactions. Another phenomenon that is not completely understood is cross-sensitization, which means that patients allergic to a given compound react positive in patch tests to compounds that are similar, but with which they had not been in contact with before.

In this thesis we have tried to elucidate some of the mechanisms involved in chemical induced adverse immune effects. **Chapter 2** reviews several aspects of neoantigen formation by xenobiotics. It deals with metabolism of chemicals, the polymorphism of metabolizing enzymes involved, induction of

costimulatory signals, and sensitization of T cells. In **Chapter 3** we show, that specific T cell reactions in the popliteal lymph node (PLN) assay could be obtained by injection of the protein-reactive metabolites hydroquinone and benzoquinone, but not by the parent compound benzene. The missing link in this chapter, metabolism, is shown in **Chapter 4**, were we used the lupus-causing drug procainamide (PA) to show that phagocytes metabolize PA to its protein-reactive metabolite *N*-hydroxyl-amino-procainamid (HAPA), which consecutively forms adducts with self-proteins. Furthermore, T cells from long-term PA-treated mice reacted to both, the metabolite HAPA, as well as the neoantigen formed in PA pulsed peritoneal macrophages. **Chapter 5** also deals with metabolism and with non-classical haptens: fatty acid anilides and phenylaminopropanodiol (PAP)-esters of fatty acids. They are suspected to be the cause of the toxic oil syndrome (TOS), an epidemic-like disease in Spain in 1981. This disease induced a graft-vs-host-like disease in several thousand people after ingestion of rape seed oil contaminated with aniline. In **Chapter 6**, our hypothesis of NKT cell involvement in the pathogenesis of TOS was tested using mice deficient in NKT cells. In **Chapter 7** we investigated the principles of cross-sensitivity to chemicals by studying single T cell clones specific for a given hapten coupled to a model self-protein. Three different mechanisms that can account for cross-sensitization and their possible consequences for autoimmunity are discussed. In **Chapter 8**, a new faster method is described to screen T cell hybridomas for specificity, the CellELISA. Finally, **Chapter 9** summarizes and discusses the contents of this thesis.

References

1. **Janeway, C. A., P. Travers, M. Walport, and J. D. Capra**. 1999. *Immunobiology*. Current Biology Publications, New York.

2. **Roitt, I. M.** 1988. *Essential Immunology*. Blackwell Scientific Publishers, London.

3. **Roitt, I., J. Brostoff, and D. Male**. 1989. *Immunology*. Gower Medical Publishing, New York.

4. **Matzinger, P.** 1994. Tolerance, danger, and the extended family. *Annu.Rev.Immunol. 12*: 991-1045.

5. **Bretscher, P.** 1992. The two-signal model of lymphocyte activation twenty-one years later. *Immunol.Today 13*: 74-76.

6. **Ibrahim, M. A. A., B. M. Chain, and D. R. Katz**. 1995. The injured cell: the role of the dendritic cell system as a sentinel receptor pathway. *Immunol.Today 16*: 181-186.

7. **Weaver, C. and E. Unanue**. 1990. The costimulatory function of antigen-presenting cells. *Immunol.Today 11*: 49-55.

8. **Lederman, S., M. J. Yellin, A. Krichevsky, J. Belko, J. J. Lee, and L. Chess**. 1992. Identification of a novel surface protein on activated $CD4^+$ T cells that induces contact-dependent B cell differentiation (help). *J.Exp.Med. 175*: 1091-1101.

9. **Noelle, R. J., E. C. Snow, J. W. Uhr, and E. S. Vitetta**. 1983. Activation of antigen-specific B cells: role of T cells, cytokines and antigen in induction of growth and differentiation. *Proc.Natl.Acad.Sci.USA 80*: 6631.

10. **Janeway, C. A.** 1992. The immune system evolved to disciriminate infectious nonself from non-infectious self. *Immunol.Today 13*: 11-16.

11. **Jenkins, M. K., C. A. Chen, G. Jung, D. L. Mueller, and R. H. Schwartz**. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J.Immunol. 144*: 16-22.

12. **Gallucci, S. and P. Matzinger**. 2001. Danger signals: SOS to the immune system. *Curr.Opin.Immunol. 13*: 114-119.

13. **Scott, D. W.** 1984. Mechanisms in immune tolerance. *Crit.Rev.Immunol. 5*: 1-25.

14. **Abe, R., C. A. Kozak, M. Foo-Philips, J. Roberts, and M. A. Principator**. 1993. Involvement of multiple factors in the clonal deletion of self-reactive T cells. *Cell.Immunol. 151*: 425-436.

15. **Heath, V. L., A. Saoudi, B. P. Seddon, N. C. Moore, D. J. Fowell, and D. W. Mason**. 1996. The role of the thymus in the control of autoimmunity. *J.Autoimmun. 9*: 241-246.

16. **Yoshida, K. and H. Kikutani**. 2000. Genetic and immunological basis of autoimmune diabetes in the NOD mouse. *Rev.Immunogenet. 2*: 140-146.

17. **Kammer, G. M. and N. Mishra**. 2000. Systemic lupus erythematosus in the elderly. *Rheum.Dis.Clin.North.Am. 26*: 475-492.

18. **Albani, S., D. A. Carson, and J. Roudier**. 1992. Genetic and environmental factors in the immune pathogenesis of rheumatoid arthritis. *Rheum.Dis.Clin.North.Am. 18*: 729-740.

19. **Kissler, S., S. M. Anderton, and D. C. Wraith**. 2001. Antigen-presenting cell activation: a link between infection and autoimunity. *J.Autoimmun. 16*: 303-308.

20. **Adams, L. E., C. E. Sanders, R. A. Budinsky, R. J. Donovan-Brand, S. M. Roberts, and E. V. Hess**. 1989. Immunomodulatory effects of procainamide metabolites: Their implications in drug related lupus. *J.Lab.Clin.Med. 113* : 482-492.

21. **Powell, J. J., J. van de Water, and M. E. Gershwin**. 1999. Evidence for the role of environmental agents in the initiation or progression of autoimmune conditions. *Environ.Health Perpect. 107 Suppl 5*: 667-672.

22. **Bendelac, A.** 1995. Mouse $NK1^+$ T cells. *Curr.Opin.Immunol. 7*: 367-374.

23. **MacDonald, H. R.** 1995. NK1.1+ T cell receptor a/b cell: new clues to their origin, specificity, and function. *J.Exp.Med. 1892*: 633-638.

24. **Bix, M. and R. M. Locksley**. 1995. Natural T cells. Cells that co-express NKRP-1 and TCR. *J.Immunol. 155*: 1020-1022.

25. **Nanda, N. K., E. E. Sercarz, D. H. Hsu, and M. Kronenberg**. 1994. A unique pattern of lymphokine synthesis is a characteristic of certain antigen-specific suppressor T cell clones. *Int.Immunol. 6*: 731-737.

26. **Lantz, O. and A. Bendelac**. 1994. An invariant T cell receptor α chain is used by a unique subset of MHC class I specific $CD4^+$ and $CD4^-8^-$ T cells in mice and humans. *J.Exp.Med. 180*: 1097-1106.

27. **Prigozy, T. I. and M. Kronenberg**. 1998. Presentation of bacterial lipid antigens by CD1 molecules. *Trends.Microbiol. 6*: 454-458.

28. **Bendelac, A., M. N. Rivera, S.-H. Park, and J. H. Roark**. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu.Rev.Immunol. 15*: 535-562.

29. **Sonoda, K.-H., M. Exley, S. Snapper, S. P. Balk, and J. Stein-Streilein**. 1999. CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune priviliged site. *J.Exp.Med. 190*: 1215-1225.

30. **Hong, S. and L. van Kaer**. 1999. Immune privilege: keeping an eye on natural killer T cells. *J.Exp.Med. 190*: 1197-1200.

31. **Martin-Munoz, F., A. Moreno-Ancillo, C. Dominguez-Noche, J. M. Diaz-Pena, C. Carcia-Ara, T. Bovano, and J. A. Oieda**. 1999. Evaluation of drug-related hypersensitivity reactions in children. *J.Investig.Allergol.Clin.Immunol. 9*: 172-177.

32. **Haltiner, A. M., D. W. Newell, N. R. Temkin, S. S. Dikmen, and H. R. Winn**. 1999. Side effects and mortality associated with use of phenytoin for early posttraumatic seizure prophylaxis. *J.Neurosurg. 91*: 588-592.

33. **Seidenari, S., L. Mantovani, B. M. Manzini, and M. Pignatti**. 1997. Cross-sensitizations between azo dyes and para-amino compound. A study of 236 azo-dye-sensitive subjects. *Contact Dermatitis 36*: 91-96.

34. **Padovan, E., D. Mauri-Hellweg, W. J. Pichler, and H. U. Weltzien**. 1996. T cell recognition of penicillin G: structural features determining antigenic specificity. *Eur.J.Immunol. 26*: 42-48.

35. **Mauri-Hellweg, D., F. Bettens, D. Mauri, C. Brander, T. Hunziker, and W. J. Pichler**. 1995. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazipine. *J.Immunol. 155*: 462-472.

36. **Kubicka-Muranyi, M., R. Goebels, C. Goebel, J. Uetrecht, and E. Gleichmann**. 1993. T lymphocytes ignore procainamide, but respond to its reactive metabolites in peritoneal cells: demonstration by the adoptive transfer popliteal lymph node assay. *Toxicol.Appl.Pharmacol. 122*: 88-94.

37. **Griem, P., M. Wulferink, B. Sachs, J. B. Gonzalez, and E. Gleichmann**. 1998. Allergic and autoimmune reactions to xenobiotics: how do they arise? *Immunol.Today 19*: 133-141.

38. **Kohler, J., S. Martin, U. Pflugfelder, H. Ruh, J. Vollmer, and H. U. Weltzien**. 1995. Cross-reactive trinitrophenylated peptides as antigens for class II major histocompatibility complex-restricted T cells and inducers of contact sensitivity in mice. Limited T cell receptor repertoire. *Eur.J.Immunol. 25*: 92-101.

39. **Weltzien, H. U., C. Moulon, S. Martin, E. Pardovan, U. Hartmann, and J. Kohler**. 1996. T cell immune responses to haptens. Structural models for allergic and autoimmune reactions. *Toxicology 107*: 141-151.

40. **Allen, P. M. and E. R. Unanue**. 1984. Antigen processing and presentation by macrophages. *Am.J.Anat. 170*: 483-490.

41. **Rammensee, H.-G.** 1996. Antigen presentation - recent developments. *Int.Arch.Allergy Appl.Immunol. 110*: 299-307.

42. **Martin, S. and H. U. Weltzien**. 1994. T cell recognition of haptens, a molecular view. *Int.Arch.Allergy Immunol. 104*: 10-16.

43. **Kohler, J., U. Hartmann, R. Grimm, U. Pflugfelder, and H. U. Weltzien**. 1997. Carrier-independent hapten recognition and promiscuous MHC restriction by CD4 T cells induced by trinitrophenylated peptides. *J.Immunol. 158*: 591-597.

44. **Jang, Y. S., K. H. Lim, and B. Kim**. 1991. Analysis of T cell reactivities to phosphorylcholine-conjugated hen egg lysozyme in C57Bl/6 mice: hapten-conjugated specificity reflects ann altered expression of a major carrier epitope. *Eur.J.Immunol. 21*: 1303-1310.

45. **Griem, P., K. Panthel, H. Kalbacher, and E. Gleichmann**. 1996. Alteration of a model antigen by Au(III) leads to T cell sensitization to cryptic peptides. *Eur.J.Immunol. 26*: 279-287.

46. **Sercarz, E. E., P. V. Lehman, A. Ametani, G. Benichou, A. Miller, and K. Moudgil**. 1993. Dominance and crypticity of T cell antigenic determinants. *Annu.Rev.Immunol. 11*: 729-766.

47. **Landsteiner, K. and J. Jacobs**. 1936. Studies on the sensitization of animals with simple chemical compounds. *J.Exp.Med. 64*: 625-639.

48. **Parkinson, A.** 1996. Biotransformation of xenobiotics. In *Casarett & Doull's toxicology*. 5th ed. C.D. Klaassen, ed. McGraw-Hill, New York, p. 113-186.

49. **Trop, S., D. Samsonov, I. Gotsman, R. Alper, J. Diment, and Y. Ilan**. 1999. Liver-associated lymphocytes expressing NK 1.1 are essential for oral immune tolerance induction in a murine model. *Hepatology 29*: 746-755.

50. **Meyer, D., C. Otto, C. Rummel, H. J. Gassel, W. Timmermann, K. Ulrichs, and A. Thiede**. 2000. "Tolerogenic effect" of the liver for a small bowel allograft. *Transplant.Int. 13 Suppl 1*: S123-S126.

51. **Uetrecht, J. P.** 1992. The role of leukocyte-generated reactive metabolites in the pathogenesis of idiosyncratic drug reactions. *Drug Metab.Rev. 24*: 299-366.

52. **Jiang, X., G. Khursigara, and R. L. Rubin**. 1994. Transformation of lupus-inducing drugs to cytotoxic products by activated neutrophils. *Science 266*: 810-813.

53. **Goebel, C., M. Kubicka-Muranyi, T. Tonn, J. Gonzalez, and E. Gleichmann**. 1995. Phagocytes render chemicals immunogenic: oxidation of gold(I) to the T cell-sensitizing gold(III) metabolite generated by mononuclear phagocytes. *Arch.Toxicol. 69*: 450-459.

54. **Mason, D.** 1998. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol.Today 19*: 395-404.

55. **Ausubel, L. J., K. D. Bieganowska, and D. A. Hafler**. 1999. Cross-reactivity of T-cell clones specific for altered peptide ligands of myelin basic protein. *Cell.Immunol. 193*: 99-107.

56. **Joshi, S. K., P. R. Suresh, and V. S. Chauhan**. 2001. Flexibility in MHC and TCR recognition: degenerate specificity at the T cell level in the recognition of promiscuous Th epitopes exhibiting no primary sequence homology. *J.Immunol. 166*: 6693-6703.

57. **Grogan, J. L., A. Kramer, A. Nogai, L. Dong, M. Ohde, J. Schneider-Mergener, and T. Kamradt**. 1999. Cross-reactivity of myelin basic protein-specific T cells with multiple microbial peptides: experimental autoimmune encephalomyelitis induction in TCR transgenic mice. *J.Immunol. 163*: 3764-3770.

58. **Schnyder, B., C. Burkhart, K. Schnyder-Frutig, S. von Greyerz, D. J. Naisbitt, M. Pirmohamed, B. K. Park, and W. J. Pichler**. 2000. Recognition of sulfamethoxazole and its reactive metabolites by drug specific $CD4^+$ T cells from allergic individuals. *J.Immunol. 164*: 6647-6654.

59. **Nagata, N., U. Hurtenbach, and E. Gleichmann**. 1986. Specific sensitization of LYT-1^+2^- T cells to spleen cells modified by the drug D-penicillamine or a stereoisomer. *J.Immunol. 136*: 136-142.

CHAPTER 2

Allergic and autoimmune reactions to xenobiotics: how do they evolve?

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Induction of allergic and autoimmune reactions by drugs and other chemicals constitutes a major public health problem. Elucidation of the mechanisms might help improve diagnostic tools and therapeutic approaches. Here, Peter Griem and colleagues focus on several aspects of neoantigen formation by xenobiotics: metabolism of xenobiotics into reactive, haptenic metabolites; polymorphisms of metabolizing enzymes; induction of costimulatory signals; and sensitization of T cells.

Introduction

Immune reactions to xenobiotics (i.e. drugs, metals, industrial and naturally occurring chemicals) can give rise to allergy and autoimmunity. These reactions are frequent and encompass a broad spectrum of different diseases and organs. In order to decrease the health risks associated with exposure to xenobiotics, it is important to understand the pathogenic mechanisms involved and to identify human populations at risk. In view of the striking clinico-pathological similarity between adverse immune reactions to xenobiotics and graft-versus-host reactions, there is little doubt that adverse reactions to xenobiotics are initiated and maintained by T cells (1). For immunologists, a major difficulty in trying to study T-cell reactions to sensitizing xenobiotics is the fact that the ultimate neoantigens formed by xenobiotics are not known, even though considerable progress has recently been made in this respect using the classical hapten trinitrophenyl (TNP) (Refs. 2,3) and 3-pentadecyl-catechol, a representative catechol derivative in urushiol, the sensitizing component of poison ivy (4). In extension of these findings, it is assumed that adverse immune reactions to other xenobiotics also involve formation of protein adducts (in the toxicological terminology) or hapten-carrier conjugates (in the immunological terminology).

Reactive organic compounds most often bind covalently; that is, their electrophilic properties enable them to react with protein nucleophilic groups such as thiol, amino and hydroxyl groups (reviewed in Ref. 5). Examples of such reactive, haptenic compounds that frequently lead to sensitization after dermal contact or inhalation are toluene diisocyanate, trimellitic anhydride, phthalic anhydride, benzoquinone, formaldehyde, hexyl cinnamic aldehyde, ethylene oxide, dinitrochlorobenzene, picryl chloride, penicillins, and *D*-penicillamine. Sensitizing metal ions react somewhat differently in that they oxidize proteins or form stable protein-metal chelate complexes by undergoing multipoint binding with several amino acid side-chains (Fig. 1). Since all of these compounds have long been known as sensitizers, protection measures are being taken in order to decrease the risk of sensitization (e.g. at workplaces).

In contrast to haptenic compounds, most xenobiotics eliciting adverse immune reactions are unable to bind to proteins when entering the body. However, they can do so after conversion to reactive metabolites (Table 1).

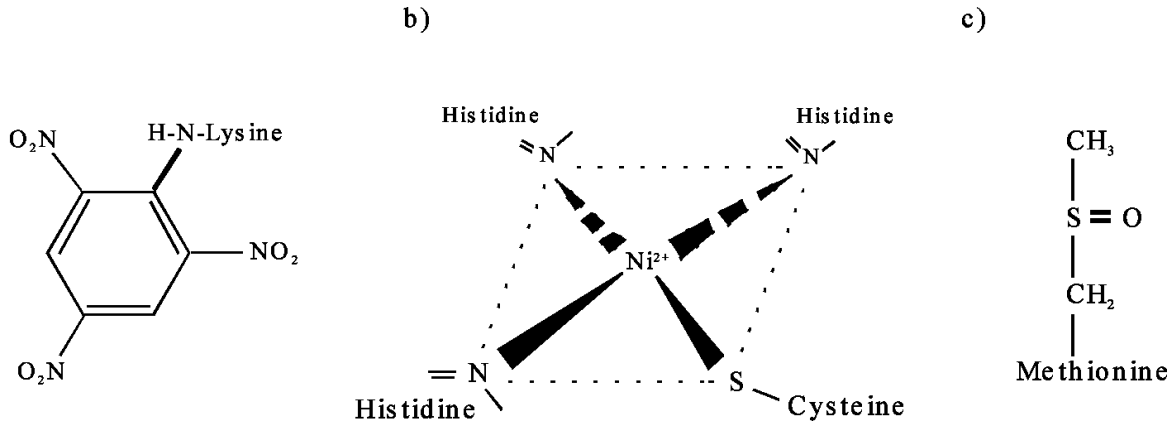

Figure 1. Haptens comprise organic compounds as well as metal ions and bind to proteins forming either covalent bonds (a) or coordination complexes (b). These two types of chemical bonds differ in the amount of energy required to break the bond (bond strength). (a) Organic haptens forming covalent bonds bind to a single amino acid side-chain. Depicted is the covalent binding of trinitrophenyl (TNP) to lysine. (b) Metal complexes consist of a center placed metal ion and a set of atoms, ions or small molecules, regarded as ligands. These ligands are aligned in a characteristic geometric form, e.g., a plane square or a octahedral. The interactions between a metal ion and ligands allow the electron-rich ligands to transfer part of their electron densitiy to the positively charged metal ion (coordination bond) in order to increase complex stability. Depicted is a square planar complex of nickel with three histidines and one cysteine. (c) Alternatively, reactive chemicals can irreversibly oxidize protein side-chains, such as those of cysteine and methionine. Shown is a methionine monosulfone.

These xenobiotics can be considered as prohaptens. This article takes into account that neoantigen formation by prohaptens involves an initial pharmaco-toxicological phase that is determined by metabolic conversion of xenobiotics. This phase precedes the T-cell-sensitization phase, and this, in turn, is followed by an immune-effector phase that leads to the various clinico-pathological manifestations of adverse immune reactions to xenobiotics. There are several model xenobiotics for each phase in this pathogenic cascade. The reader should be aware, however, that no single xenobiotic has yet been analyzed so extensively that it could serve as a universal example for the entire cascade. Therefore, different xenobiotics will have to illustrate the individual phases described.

Preimmunological phase

Hepatic metabolism of xenobiotics

As the main organ for metabolism of xenobiotics, the liver is well-equipped

Table 1. Examples of adverse immune reactions to xenobiotics that involve reactive metabolites [a]

Parent compound	Adverse immune reaction	Candidate metabolite involved	Ref.
Procainamide	Drug-induced lupus	*N*-Hydroxyprocainamide (M,H)	(6,7)
Propylthiouracil	Vasculitis, drug-induced lupus	Propyluracilsulfonic acid (M,H)	(8,9)
Halothane	Autoimmune hepatitis	Trifluoroacetylchloride (R,H)	(10,11)
Tienilic acid	Autoimmune hepatitis	Thiophene sulphoxide (H)	(12)
Dihydralazine	Drug-induced lupus, autoimmune hepatitis	Hydralazine radical (R,H)	(13)
Gold(I) antirheumatics	Dermatitis, glomerulonephritis	Gold(III) (M,H)	(14,15)
Practolol	Oculomucocutaneous syndrome	Practolol epoxide (H)	(16)
Urushiol	Contact dermatitis	3-pentadecyl-*o*-quinone (M)	(4)
p-Phenylenediamine	Contact dermatitis	Bandrowski's base (H)	(17)

[a] The adverse immune reactions listed in the table were observed in humans, while identification of candidate metabolites was achieved in: M, mice; R, rats; H, humans

with xenobiotic-metabolizing enzymes and is prepared for detoxifying reactive metabolites. Compared with its high metabolic activity, adverse immune reactions in the liver are relatively rare. Nevertheless, such reactions do occur, one example being the autoimmune hepatitis caused by long-term treatment with the diuretic drug tienilic acid (a prohapten). This side-effect is associated with the production of autoantibodies directed against the cytochrome P450 (CYP) isoenzyme 2C9 (CYP2C9), and interestingly this is the very enzyme that catalyzes hepatic metabolism of the prohapten to its reactive metabolite (12,18). This short-lived, haptenic metabolite was found to bond covalently to CYP2C9. A similar mechanism is assumed for other cases of drug-induced autoimmune hepatitis, such as those caused by halothane (10) or dihydralazine (13), in which autoantibodies are directed against the enzymes converting these prohaptens to the respective haptens (CYP2E1 and CYP1A2, respectively). T-cell recognition of the haptenated enzymes in drug-induced hepatitis is likely, but has not been formally demonstrated.

Extrahepatic metabolism of xenobiotics

In quantitative terms, extrahepatic metabolism of xenobiotics is less important than hepatic metabolism. However, as far as adverse immune reactions to prohaptens are concerned, extrahepatic metabolism appears to play a crucial role. Rather than being metabolized to reactive, haptenic metabolites in the

liver, and subsequently traveling to distant extrahepatic sites, such as the skin, lung, or bone marrow, it is likely that reactive metabolites are formed at the very sites where adverse immune reactions to xenobiotics manifest themselves. Hence, the xenobiotic-metabolizing capacity of extrahepatic tissues merits special attention in the present context. One example here is the skin, a barrier organ which has a considerable metabolic capacity in conjunction with immunological competence, and is often involved in adverse immune reactions to xenobiotics, be it after dermal or systemic application (19). Interestingly, dermal Langerhans cells contain CYP1A isoenzymes and are able to metabolize prohaptenic xenobiotics, such as the polyaromatic hydrocarbon dimethylbenz-[a]-anthracene, to haptens. They can activate specific T cells that mediate contact hypersensitivity, presumably by presentation of haptenated peptides (20). Similarly, urushiol, a mixture of allergenic 3-alkyl and 3-alkenyl catechols from the plants poison ivy and poison oak, can be oxidized in the skin to reactive *o*-quinones that can elicit specific T-cell responses after adduct formation with protein (4,21,22). Another chemical frequently involved in allergic contact dermatitis is *p*-phenylenediamine, which is oxidized to a reactive metabolite termed Bandrowski's base. Specific T-cell reactions to this hapten have been demonstrated *in vitro:* peripheral mononuclear cells of sensitized patients responded to Bandrowski's base, but not to the prohapten *p*-phenylenediamine (17).

Xenobiotic metabolism in phagocytes

Phagocytes include polymorphonuclear leukocytes (PMN), monocytes, macrophages, and resident Langerhans cells. While the latter three can themselves act as antigen-presenting cells (APC), PMN die after they have been activated in inflammatory sites; the dead cells and debris are phagocytosed and processed by APC. Hence, the capacity of phagocytes to metabolize xenobiotics is particularly relevant in the present context. For instance, there is indirect evidence that metabolism in phagocytic cells may be involved in systemic adverse immune reactions caused by procainamide (PA) (6), propylthiouracil (8) and disodium gold(I) thiomalate (14). Whereas the respective parent compounds, or prohaptens, themselves proved unable to elicit T-cell reactions in mice, their reactive metabolites generated in macrophages were able to do so.

Generation of reactive metabolites in neutrophils and monocytes has been attributed to metabolizing enzymes with a broad substrate specificity, such

as myeloperoxidase (MPO), prostaglandin H synthase, and various CYP isoenzymes (19,23-25). For PA, it has been shown in mice that T cells sensitized to the reactive metabolite *N*-hydroxy-PA, which can easily be further oxidized to nitroso-PA (another reactive, unstable metabolite), recognized macrophages incubated with the nonreactive parent compound (i.e. the prohapten), indicating generation of the hapten and hapten-protein adducts in these cells (6). Additionally, *in vivo* bioactivation of PA in macrophages to *N*-hydroxy-PA and nitroso-PA was indirectly demonstrated by successful restimulation of *N*-hydroxy-PA-primed T cells with peritoneal cells of long-term PA-treated mice (6) (Fig. 2). Similar findings were obtained with a chemically different compound, the antirheumatic drug gold(I) thiomalate. Using gold(III)-specific T cells as detection probes in *in vivo* and *in vitro* assays, indirect evidence was provided for the generation of the short-lived, reactive metabolite gold(III) in macrophages (14,26). Hence, in view of the multiple functions they can fulfill, macrophages, and presumably other types of APC, appear to serve as a connecting link between the preimmunological phase, which includes regional xenobiotic bioactivation and neoantigen formation, and the phase of T-cell sensitization to these neoantigens.

Genetic polymorphisms of xenobiotic-metabolizing enzymes

Metabolism of xenobiotics can be divided into two phases. Phase I reactions, such as those carried out by CYP isoenzymes, usually lead to insertion of functional groups into xenobiotics, or lead to demasking of such groups, and thus can result in formation of reactive metabolites (part of which can act as haptens). In phase II reactions, metabolites are conjugated with small endogenous molecules, such as glucuronic acid, glutathione, acetate, or sulfate in order to increase water solubility and facilitate elimination from the body. Unlike larger haptenated peptides, these conjugates are too small to make stable contact with the MHC binding groove and thus are unable to cause sensitization. Several genetic polymorphisms of xenobiotic-metabolizing enzymes have been identified, some of which cause expression of defective enzymes, or enzymes with a reduced (or increased) metabolic activity (30). These inter-individual differences in the generation of reactive metabolites among humans may influence formation of protein adducts and, hence, may result in a different susceptibility to chemically induced allergy and autoimmunity.

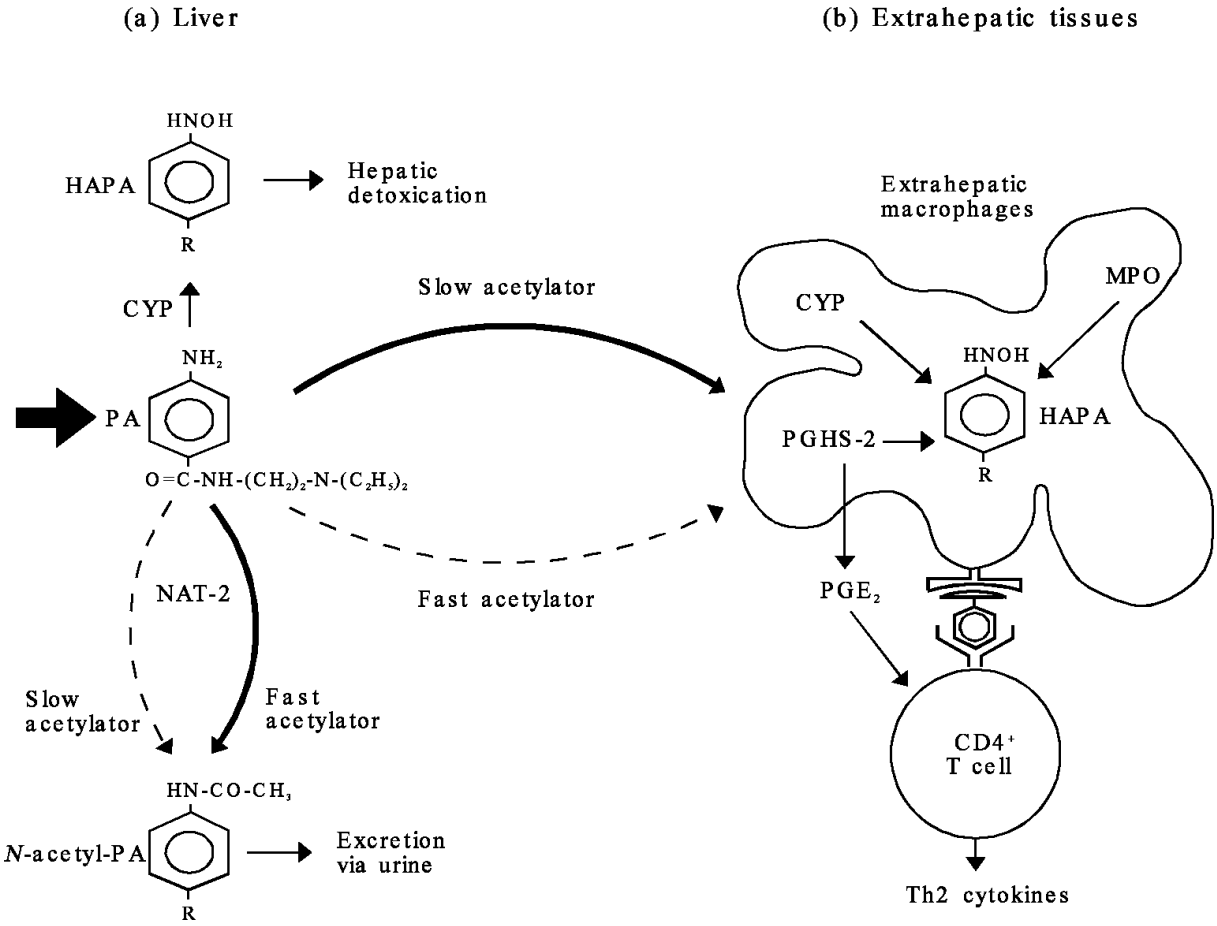

Figure 2. Hypothetical scheme of the initial immunotoxic steps underlying PA-induced SLE, based on experimental results (Ref. 6, C. Goebel, C. Vogel, B. Sachs, S. et al., unpublished). (a) Hepatic metabolism of the arylamine PA consists of two competing pathways (27,28). N-acetylation of the amino group, catalyzed by NAT-2, leads to formation of N-acetyl PA, a stable metabolite that can be eliminated. By contrast, oxidation of the amino group by CYP isoenzymes yields HAPA and, through further oxidation, yields nitroso-PA (not shown); the latter can haptenate proteins and thus can induce the adverse immune reactions seen in PA-induced SLE. However, in the liver, probably due to its high detoxicating capacity, PA-induced adverse immune reactions fail to develop. Because of genetic polymorphism, individuals differ in their NAT-2 activity, resulting in the slow- and fast-acetylator phenotype. In slow-acetylator individuals, hepatic acetylation of PA is reduced, thereby increasing the amount of substrate available for extrahepatic PA metabolism. (b) Extrahepatic metabolism of PA can occur in phagocytic cells containing enzymes with a broad substrate specificity, such as PGHS-1, PGHS-2, MPO and CYP isoenzymes (19,23-25). Importantly, phagocytic cells, like monocytes and macrophages, which are capable of oxidizing PA to HAPA and further to nitroso-PA, can process proteins and present hapten-conjugated peptides to T cells. Interestingly, PA was shown to induce expression of PGHS-2 in mouse macrophages and thus can probably enhance its own oxidation to HAPA and nitroso-PA. Moreover, the PA-induced enhancement of the generation of PGE_2 by PGHS-2 might skew the immune reaction toward a Th2-type response (29), thereby favoring formation of (auto)antibodies.

Abbreviations: CYP, cytochrome P450 isoenzyme; HAPA, N-hydroxylamine PA; MPO, myeloperoxidase; NAT-2, N-acetyl transferase 2; PA, procainamide; PGE_2, prostaglandin E_2; PGHS, prostaglandin H synthase; SLE, systemic lupus erythematosus; Th2, T helper 2.

Whether a genetic polymorphism of a xenobiotic-metabolizing enzyme has clinical relevance depends on its functional role in the metabolism of a given compound (i.e. its bioactivation or detoxication), and on whether other enzymes can compensate for the defect. Individuals carrying certain genetic polymorphisms, especially combined phase I and phase II defects, might be at higher risk for allergic and autoimmune disorders induced by xenobiotics (31-33). However, it should be noted that, besides genetic determination, the individual activity of xenobiotic-metabolizing enzymes can also be influenced by nongenetic factors such as drugs, diet, alcohol, smoking and cytokines.

Table 2 presents selected examples of polymorphic enzymes that metabolize drugs associated with adverse immune reactions in humans. Thus far, the clearest association between a genetic polymorphism and adverse immune reactions to certain drugs has been found for *N*-acetyltransferase-2 (Fig. 2). Approximately half of the Caucasian population is homozygous for the mutant alleles and exhibits the slow-acetylator phenotype. In individuals exhibiting the slow-acetylator phenotype, the incidence of dihydralazine- or PA-induced systemic lupus erythematosus (SLE) is higher than in those exhibiting the fast-acetylator phenotype (27,28). Furthermore, of patients developing severe erythema multiforme variants (Stevens-Johnson syndrome and toxic epidermal necrolysis) following sulphonamide treatment, 90% compared with 45% in controls exhibited the slow-acetylator phenotype (34). It remains to be studied whether or not adverse immune effects caused by the other xenobiotics listed in Table 2 are also associated with certain polymorphisms of the metabolizing enzymes listed. As far as idiopathic autoimmune diseases are concerned, associations with genetic polymorphisms of xenobiotic-metabolizing enzymes would indirectly point to xenobiotics as etiological agents of such diseases and provide information as to the type of chemical compound to be searched for.

Sensitization phase

Only few of the different hapten-protein conjugates formed in the body will induce a clinically manifest allergy or autoimmunity. Whether an immune response is initiated depends on several factors such as dose, metabolism, protein binding, type and activation state of APC, antigen processing and

Table 2. Polymorphisms of xenobiotic-metabolizing enzymes[a]

Enzyme	Substrates associated with adverse (immune) reactions
Phase-I enzymes	
CYP1A2[h]	Aromatic amines[b,e]
CYP2A6[g]	Coumarin[b]
CYP2C9[g]	Nonsteroidal anti-inflammatory drugs (diclofenac[b], tienilic acid[d] , piroxicam[b,c], tenoxicam[b,c], ibuprofen[b], naproxen[b]), phenytoin[b,b,e], tolbutamide[b,c]
CYP2C19[g]	Omeprazol[b], proguanil[b,c], propranolol[b,c], imipramine[b,c], citalopram[b], moclobemide[b], diazepam[b], hexobarbital[b]
CYP2D6[g]	Antiarrhythmics[f], beta-blockers[f], antihypertensives[f], neuroleptics[f], tricyclic antidepressants[f], MAO inhibitors[f], analgetics[f], miscellaneous agents[f]
CYP2E1[g]	Dapsone[b,c,e], carbamazepine[b,e], quinidine[e], acetaminophene[b], halothane[d]
Phase-II enzymes	
N-Acetyltransferase-2[g] dapsone[b,c,e],	Isoniazid[b,e], dihydralazine[e], procainamide[e], sulfasalazine[c,e]
Glutathion-S-transferases M1 and T1[g]	Halothane[d]
NAD(P)H-quinone reductase[g]	Azo dyes[b], nitroaromates[b], quinones[b]
Phenolsulfphotransferase (P-PST)[h]	Aromatic hydroxylamines[b]

[a] Data are from Refs 30,35, and 36; selected adverse (immune) reactions to single drugs or certain, not necessarily all, members of classes of compounds are as follows: [b] skin reactions (e.g. exanthema, urticaria, dermatitis); [c] hematological adverse effects (aplastic anemia, leukopenia, agranulocytosis); [d] autoimmune hepatitis; [e] drug-induced lupus; [f] chemically heterogeneous drugs with versatile adverse effects; polymorphisms influencing enzyme activity as follows: [g] characterized at molecular level; [h] mutation not discovered yet, described by distinct phenotypes.

Abbreviations: CYP, cytochrome P450 isoenzyme; MAO, monoamine oxidase; NAD(P)H, reduced form of nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate.

peptide density on APC, some of which are presented in this article in more detail.

T cells are activated when they receive both signal 1 and signal 2 (37,38). Signal 1 is triggered by T-cell recognition of peptides embedded in major histocompatibility complex (MHC) molecules on the surface of APC and involves signal transduction via the T-cell receptor (TCR)-associated CD3 complex and coreceptors such as CD4. Signal 2 is an abstract, generic term for a variety of different accessory or costimulatory signals transmitted during T cell-APC interaction. During this crosstalk, exchange of signal 1 and signaling via the CD40-CD40L interaction, upregulates membrane molecules on the APC, such as intercellular adhesion molecule 1 (ICAM-1), CD80 and CD86, that contribute to signal 2 for T-cell activation.

Dendritic cells residing in tissues have to get activated in order to migrate to lymph nodes and prime T cells. While residing in nonlymphoid organs, dendritic cells such as skin Langerhans cells efficiently take up and process material, including haptenated protein, from their vicinity, but their T-cell-stimulating capacity during this developmental stage is poor. Specific signals, such as tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1), which may originate, for example, from activated or damaged keratinocytes, can switch the functional state of dendritic cells. Dendritic cells then migrate to the draining lymph nodes, lose their capacity to take up and process antigen, upregulate MHC class I and class II molecules as well as accessory molecules on the cell surface, and thereby differentiate into immunostimulatory dendritic cells that can efficiently trigger naive T cells (38,39).

Activation of dendritic cells following exposure to xenobiotics

The two-signal requirement for T-cell activation poses the interesting question of how sensitizing xenobiotics can induce activation of dendritic cells. Dendritic-cell maturation might be triggered by a casual infection at the site of exposure, implicating activation of APCs by, for instance, lipopolysaccharide, glycans, double-stranded RNA and *N*-formylmethionyl peptides (39). Presumably, however, xenobiotics themselves can act in a similar way by inducing keratinocytes to produce TNF-α, IL-1α, IL-6 and other cytokines. Keratinocyte activation could be achieved by cytotoxicity of the sensitizing chemical itself or its reactive metabolite (24), or by concomitant exposure to chemical or physical noxae, such as sodium dodecyl sulfate, dimethylsulfoxide, phorbol myristate acetate, and ultraviolet light (39-41). In addition to exerting these unspecific toxic effects, sensitizing xenobiotics may lead to activation of

dendritic cells and more efficient costimulation of T cells by specific mechanisms (Table 3). Having outlined how xenobiotics can activate dendritic

Table 3. Selected examples of how xenobiotics can contribute to costimulation of T cells

Chemical	Effects observed	Ref.
TNCB, DNCB	Induction of IL-1β mRNA in Langerhans cells, induction of IL-1α and TNF-α mRNA in keratinocytes	(40)
DNFB, nickel sulfate	Induction of IL-1β in Langerhans cells	(42)
Nickel sulfate	Stabilizing TNF-α mRNA in keratinocytes	(41)
Nickel sulfate, cobalt sulfate	Expression of adhesion molecules on endothelial cells	(43)
Mercuric chloride	Secretion of IL-1 from macrophages	(44)

Abbreviations: DNCB, dinitrochlorobenzene; DNFB, dinitrofluorobenzene; IL-1, interleukin-1; TNCB, trinitrochlorobenzene; TNF-α, tumor necrosis factor α

cells (i.e. elicit signal 2 for T-cell activation) this article will now discuss signal 1 - the mode of how T cells 'see' xenobiotics or, more exactly, their footprints on self-proteins.

T-cell reactions to haptenated peptides

Activation of αβ T cells that recognize peptides in the context of MHC class I or class II molecules involves signaling through the TCR. This requires the 3-dimensional structure of its antigen-binding site to be complementary to that of the peptide-MHC complex and, thus, allows ionic, dipole, aromatic and hydrophobic interactions. Bonding of a xenobiotic to a peptide-MHC complex alters its structure and the number, type and distribution of possible interactions with the TCR. A neoantigen is thus created and can be specifically recognized by T cells.

TNP derivatives were the first haptens for which T-cell reactions against haptenated peptides presented by MHC class I or class II molecules were demonstrated (2,3,45-47). These studies clearly demonstrated that both MHC class I- and class II-restricted, hapten-specific T cells recognize TNP-conjugated peptides irrespective of the exact amino acid sequence of the peptides. The only requirements were 1) that the haptenated peptides carried appropriate side-chains for anchoring in the MHC groove and 2) that the TNP-coupled lysine

side-chain of the peptide was correctly positioned relative to these anchors so that TNP could make contact with the TCR. The first requirement by definition is fulfilled by all self-peptides presented and the second requirement is apparently met by a variety of different self-peptides. Thus, after exposure to trinitrobenzene sulfonic acid (TNBS) or trinitrochlorobenzene (TNCB) both of which form TNP-protein conjugates, a large pool of MHC-bound, haptenated self-peptides becomes available for T-cell recognition (2,47). A recent publication suggests that some TNP-specific human T cells can even recognize TNP in context of different MHC class II molecules (47). Taken together, these findings may explain the extraordinary strength of immune reactions against TNP derivatives, which are comparable with those seen in alloreactions (2). T-cell reactions to the 3-alkyl and 3-alkenyl catechols contained in urushiol follow the same rules laid down for TNP, namely recognition of the correctly positioned hapten irrespective of the amino acid sequence of the peptide (4).

Specific reactions of human T cells from patients with drug allergies have been shown for a large number of drugs, such as β-lactam antibiotics (penicillin) (48-51), sulphonamides, nonsteroidal anti-inflammatory drugs, and aromatic anticonvulsants such as phenytoin and carbamazepine (52). There is evidence that the TCR of penicillin-specific T cells can interact with both the thiazolidin ring, which is common to all β-lactam antibiotics, and the penicilloyl side-chain, which is specific for a particular antibiotic (49-51). T-cell reactions to structurally-defined haptenated peptides have also been shown for diazotized *p*-aminobenzene arsonic acid (53) and photoreactive azido compounds (54). Interestingly, in some autoimmune disorders T-cell responses against endogenously haptenated peptides, i.e., physiologic protein modifications, were found (55,56). MHC-restricted recognition of noncovalently bound organic xenobiotics is rare and has so far only been proposed for sulfamethoxazole (57). Despite the fact that various metal salts can induce hypersensitivity and/or autoimmune reactions (58), knowledge of how metal ions elicit the specific T-cell reactions underlying these conditions is very limited. Although there is experimental evidence that nickel(II) (59,60), beryllium(II) (61), gold(I) (62) and some other metals might act as haptens in that they are recognized as metal-peptide complexes, demonstration of T-cell recognition of a structurally defined metal-peptide-MHC complex is still lacking. Theoretically, reactive chemicals

and metal ions could also elicit specific T-cell responses by binding to the MHC molecule itself rather than to an embedded peptide. Beryllium ions are good candidates for this alloreactive-like T-cell reactions: it has been proposed that beryllium ions might bind directly to the critical glutamate residue in position 69 of the HLA-DPB1*0201 molecule, which shows a strong positive correlation with berylliosis (61); but again conclusive experimental proof is lacking.

T-cell reactions to cryptic peptides uncovered by xenobiotics

Chemical modification of self-proteins can change their processing in APCs and lead to presentation of cryptic peptides that may elicit autoimmune T-cell reactions. While T-cell reactions to cryptic peptides have been shown in humans, the induction of T-cell responses to cryptic peptides by xenobiotics as so far only been demonstrated in mouse models. Analysis of T-cell hybridomas prepared after immunization of mice with phosphorylcholine-conjugated hen egg lysozyme revealed that some clones reacted against a cryptic lysozyme peptide such that modification of the protein led to presentation of a novel peptide which itself was not haptenated (63). The same peptide was presented when lysozyme was pretreated with other diazotized aromatic amines, all of which bind to tyrosine side-chains, but not when lysine-reactive fluorescein isothiocyanate was used.

Similar results were obtained following investigation of the murine T-cell response to bovine ribonuclease A that had been pretreated with gold(III) this being the reactive metabolite of gold(I)-containing antirheumatic drugs (26). T-cell hybridomas reacting specifically against gold(III)-pretreated ribonuclease recognized one of two cryptic peptides of this protein. When these clones were tested with ribonuclease pretreated with other metals, they only showed crossreactivity with palladium(II), palladium(IV), nickel(IV), and platinum(IV) salts indicating these metals, but not others, induced presentation of the same cryptic peptides to T cells. A conformational change of ribonuclease A treated with the crossreacting metals was detectable by circular dichroism spectrospopy, suggesting that these changes are the molecular basis for the observed alteration of antigen processing (P. Griem, K. Panthel, S.L. Best, P.J. Sadler, and C.F. Shaw III, unpublished).

Experimental evidence suggests that *in vivo* treatment with mercury(II) can lead to the presentation of cryptic peptides of fibrillarin (64), a nucleolar protein recognized by autoantibodies of mice treated with mercury(II), gold(I),

or silver(I) and also by autoantibodies of scleroderma patients (58). The observation that mercury(II) can alter the protein structure of fibrillarin (65), could explain the presentation of cryptic fibrillarin peptides.

By definition, T cells recognizing cryptic self-peptides are autoreactive and, moreover, some hapten-specific T cells also recognize the nonhaptenated peptide after priming (46). Hence, the possibility arises that an immune response may be extended and lead to overt autoimmunity even if the offending xenobiotic has been cleared from the body. On the other hand, some xenobiotics, especially metals, can persist for years in the body and might continuously activate T cells.

$CD4^+$ versus $CD8^+$ T-cell responses to xenobiotics

Defining rules as to whether a given xenobiotic will predominantly activate $CD4^+$ or $CD8^+$ T cells is a difficult task. From current knowledge about hapten recognition by T cells, we can conclude that reactivity and lipophilicity of xenobiotics will determine in which extra- or intracellular compartment haptenated proteins will be formed and which presentation pathway these will enter. Reactive xenobiotics that can directly bind to proteins and modify peptide-MHC complexes, seem to induce both $CD4^+$ and $CD8^+$ T-cell responses, as has been observed for TNP derivatives (2,66), penicillins (48) and nickel (59,60). Likewise, nonreactive xenobiotics, such as urushiol, that can be converted into reactive metabolites nonenzymatically or extracellularly were found to activate both $CD4^+$ and $CD8^+$ T cells (4,22).

Xenobiotics such as PA and propylthiouracil, that are metabolized inside APCs by enzymes localized along the exogenous processing pathway might be preferentially presented in the context of MHC class II molecules. The same is true for xenobiotics that can be metabolized extracellularly, such as during the oxidative burst of phagocytes (23), and then bind to extracellular proteins or membrane proteins. This explanation might account for $CD4^+$ T-cell help to B cells and thus for the production of autoantibodies. However, other drugs such as sulphonamides, carbamazepine and phenytoin, which can also be metabolized via myeloperoxidase-dependent oxidation in phagocytes, have been shown to induce specific activation of both $CD4^+$ and $CD8^+$ T cells (52).

Xenobiotics that are lipophilic enough to cross the cell membrane and are metabolized inside the cell (e.g. by CYP isoenzymes at the endoplasmic reticulum) tend to modify proteins inside the cytoplasm that preferentially enter the class I-processing pathway. Examples of this type of xenobiotic are

polyaromatic hydrocarbons (20). Similarly, tienilic acid is metabolized by CYP2C9 into reactive metabolites that haptenate this CYP isoenzyme.

Considering the intracellular localization of the CYP2C9 isoenzyme at the endoplasmic reticulum, one would expect involvement of $CD8^+$ T cells in the immune reactions against tienilic acid. However, in tienilic acid-induced autoimmune hepatitis, anti-CYP2C9 IgG autoantibodies were found, implying participation of $CD4^+$ T helper cells and the class II-processing pathway (12,18). The proposed involvement of $CD4^+$ T cells in this situation could result from hepatocytes that were killed by highly reactive, toxic metabolites of tienilic acid or by hapten-specific $CD8^+$ cells, and that were subsequently taken up by APC, thus entering the class II pathway.

Direct recognition of xenobiotics by γδ *and* αβ *T cells ?*

Recent investigation of the antigen recognition of T cells expressing γδ TCRs revealed that these cells, unlike most αβ T cells, recognize antigens in an immunoglobulin-like fashion. Interestingly, human γδ T cells can react to nonproteinaceous microbial components, such as isopentenyl pyrophosphate and γ-substituted 5′-triphosphorylated thymidine (67). Moreover, it has been established that human $CD4^-$ $CD8^-$ (double-negative) αβ T cells can also react to hydrophobic nonpeptide antigens, such as lipoarabinomannan and mycolic acids bound to MHC-related CD1 molecules on APC (67). These findings open up the possibility that T cells might also recognize 'free' xenobiotics, which are not reactive enough to bind covalently to proteins. This hypothesis is supported by recent publications describing human γδ T cells specific for lidocain (68) and human $CD8^+$ αβ T cells recognizing a pollen antigen-derived carbohydrate on CD1 molecules (69).

Effector phase in adverse immune reactions to xenobiotics

Specific T-cell reactions to xenobiotic-induced neoantigens comprise both T helper 1 (Th1) and Th2 responses and can trigger an array of effector mechanisms that are not different from those of immune reactions to conventional protein antigens. As with protein antigens, factors such as the route of administration, dose and genetic background of individuals play a role in determining the type of effector mechanisms triggered by xenobiotics. Some examples underlining the importance of these factors will be mentioned below.

Administration route

It has long been known that cutaneous sensitization of mice to the classical hapten dinitrofluorobenzene (DNFB) does not occur if the animals are orally pretreated with DNFB (70), and the same is true for Ni(II) (71). In contrast to feeding DNFB to normal littermates, feeding of either MHC class II-deficient or CD4-depleted mice with DNFB did not tolerize but primed to the hapten (72). This indicates that oral application of DNFB, and possibly of other haptens as well, generates both hapten-specific $CD8^+$ T cells, which act as effector cells in contact hypersensitivity, and hapten-specific $CD4^+$ T cells, which suppress activity of the $CD8^+$ T cells in normal mice so that tolerance results. This is in line with other studies on contact hypersensitivity to DNFB and oxazolone that have shown that cutaneous application of sensitizing doses of these haptens induces two opposing T-cell populations: interferon γ (IFN-γ)-producing Th1-like $CD8^+$ T cells as effector cells; and IL-4- and IL-10-producing $CD4^+$ Th2 cells as downregulatory cells (73).

Dose

The type of effector mechanism induced by xenobiotics is also dose-dependent: while cutaneous application of sensitizing doses of oxazolone induces effector mechanisms leading to contact hypersensitivity in mice, cutaneous application of low, subsensitizing doses of oxazolone induces tolerance that is solely mediated by specific $CD8^+$ cells expressing a Th2-like cytokine pattern (74). It is proposed that this mechanism may be valid for other xenobiotics as well, and that this might explain why most individuals fail to show signs of sensitization after continuous exposure to low concentrations of xenobiotics on the skin although an immune response is induced. However, exposure to relatively high concentrations of xenobiotics would break tolerance and lead to sensitization.

Genetic background

The genetic background also influences the probability of immune reactions and the kind of immunopathological lesions. The importance of polymorphisms of xenobiotic-metabolizing enzymes has long been known in chemical carcinogenesis and their relevance in immune reactions to xenobiotics has already been mentioned above. Another illustrative example is the striking MHC dependence of susceptibility to the systemic autoimmune syndrome induced by mercury and gold salts in mice and rats (1,58). Treatment with mercuric chloride or the antirheumatic drug gold(I) thiomalate induces a Th2-like effector

response in susceptible H-2^s mice, causing production of autoantibodies and increased levels of serum IgG1, IgG2A and IgE, whereas resistant H-2^d mice showed a Th1-like response (1,75). The susceptibility of

H-2^s mice is dependent on the presence of the MHC class II A^s molecule (76). In the rat, mercury induces activation of autoreactive T-cells in the Brown Norway strain and the Lewis strain. However, the latter does not develop the autoimmune syndrome because the activated T cells produce tumor necrosis factor α (77). As mentioned above, the beryllium-induced lung disease in humans is strongly associated with the HLA-DPB1*0201 molecule (61).

Outlook

In chemically induced carcinogenesis, the role of reactive metabolites acting as ultimate carcinogens has been firmly established decades ago. Accordingly, metabolite-generating systems are used in mutagenicity screening tests. By analogy, reactive metabolite-generating systems that can render prohaptens into haptens, should be used in tests designed to detect the sensitizing potential of xenobiotics. Thus, liver microsomes have been successfully used for bioactivation of nonreactive xenobiotics to haptens in the lymphocyte transformation test in humans (19) and in the popliteal lymph node assay (78). Conceivably, the blood monocytes present in the routine lymphocyte transformation test fulfill a similar function within the limits of their xenobiotic-metabolizing capacity. The development of prognostic tools based on genetic polymorphisms of xenobiotic-metabolizing enzymes could help protect people with an increased risk for adverse immune reactions to certain classes of xenobiotics that are substrates of those enzymes.

Finally, immune responses to xenobiotics, just like those to conventional antigens, can be subject to tolerance induction. In mouse models, tolerance was induced by oral administration of DNCB (70) and nickel (71), parenteral treatment with a peptide haptenated with 3-pentadecyl catechol from urushiol (4) and topical application of low doses of oxazolone (74). Furthermore, TNP-specific T cells could be inhibited by altered peptide ligands carrying alterations either in the peptide sequence or the hapten (79). In view of these findings, new therapeutic approaches such as tolerance induction and modulation of immune responses by altered peptide ligands might also be feasible with xenobiotics.

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References

1. **Goldman, M., P. Druet, and E. Gleichmann**. 1991. Th2 cells in systemic autoimmunity: insights from allogeneic diseases and chemically-induced autoimmunity. *Immunol.Today 12*: 223-227.

2. **Kohler, J., S. Martin, U. Pflugfelder, H. Ruh, J. Vollmer, and H. U. Weltzien**. 1995. Cross-reactive trinitrophenylated peptides as antigens for class II major histocompatibility complex-restricted T cells and inducers of contact sensitivity in mice. Limited T cell receptor repertoire. *Eur.J.Immunol. 25*: 92-101.

3. **Cavani, A., C. J. Hackett, K. J. Wilson, J. B. Rothbard, and S. I. Katz**. 1995. Characterization of epitopes recognized by hapten-specific CD4(+) T cells. *J.Immunol. 154*: 1232-1238.

4. **Gelber, C., L. Gemmell, D. McAteer, M. Homola, P. Swain, A. Liu, K. J. Wilson, and M. Gefter**. 1997. Down-regulation of poison ivy/oak-induced contact sensitivity by treatment with a class II MHC binding peptide:hapten conjugate. *J.Immunol. 158*: 2425-2434.

5. **Roberts, D. W. and J.-P. Lepoittevin**. 1998. Hapten-Protein Interactions. In *Allergic Contact Dermatitis*. J.-P. Lepoittevin, D.A. Basketter, A. Goosens and A.-T. Karlberg, eds. Springer-Verlag, Berlin, Heidelberg, p. 81-111.

6. **Kubicka-Muranyi, M., R. Goebels, C. Goebel, J. P. Uetrecht, and E. Gleichmann**. 1993. T lymphocytes ignore procainamide, but respond to its reactive metabolites in peritoneal cells: demonstration by the adoptive transfer popliteal lymph node assay. *Toxicol.Appl.Pharmacol. 122*: 88-94.

7. **Uetrecht, J. P. and B. Sokoluk**. 1992. Comparative metabolism and covalent binding of procainamide by human leukocytes. *Drug Metab.Dispos. 20*: 120-123.

8. **Schmiedeberg, S. v., U. Hanten, C. Goebel, H. C. Schuppe, J. Uetrecht, and E. Gleichmann**. 1996. T cells ignore the parent drug propylthiouracil but are sensitized to a reactive metabolite generated in vivo. *Clin.Immunol.Immunopathol. 80*: 162-170.

9. **Waldhauser, L. and J. P. Uetrecht**. 1991. Oxidation of propylthiouracil to reactive metabolites by activated neutrophils. Implications for agranulocytosis. *Drug Metab.Dispos. 19*: 354-359.

10. **Eliasson, E. and J. G. Kenna**. 1996. Cytochrome P450 2E1 is a cell surface autoantigen in halothane hepatitis. *Mol.Pharmacol. 50*: 573-582.

11. **Kharasch, E. D., D. Hankins, D. Mautz, and K. Thummel**. 1996. Identification of the enzyme responsible for oxidative halothane metabolism: implications for prevention of halthane hepatitis. *Lancet 347*: 1367-1371.

12. **Lecoeur, S., C. Andre, and P. H. Beaune**. 1996. Tienilic acid-induced autoimmune hepatitis: anti-liver and -kidney microsomal type 2 autoantibodies recognize a three-site conformational epitope on cytochrome P4502C9. *Mol.Pharmacol. 50*: 326-333.

13. **Bourdi, M., M. Tinel, P. H. Beaune, and D. Pessayre**. 1994. Interactions of dihydralazine with cytochromes P4501A: a possible explanation for the appearance of anti-cytochrome P4501A2 autoantibodies. *Mol.Pharmac. 45*: 1287-1295.

14. **Goebel, C., M. Kubicka-Muranyi, T. Tonn, J. Gonzalez, and E. Gleichmann**. 1995. Phagocytes render chemicals immunogenic: oxidation of gold(I) to the T cell sensitizing gold(III) metabolite generated by mononuclear phagocytes. *Arch.Toxicol. 69*: 450-460.

15. **Verwilghen, J., G. H. Kingsley, L. Gambling, and G. S. Panayi**. 1992. Activation of gold-reactive T lymphocytes in rheumatoid arthritis patients treated with gold. *Arthritis Rheum. 35*: 1413-1418.

16. **Davies, G. E.** 1979. Adverse reactions to practolol. In *Drugs and Immune Responsiveness*. J.L. Turk and D. Parker, eds. Macmillan, London, p. 199-211.

17. **Krasteva, M., J. F. Nicolas, G. Chabeau, J. L. Garrigue, H. Bour, J. Thivolet, and D. Schmitt**. 1993. Dissociation of allergenic and immunogenic functions in contact sensitivity to para-phenylenediamine. *Int.Arch.Allergy Immunol. 102*: 200-204.

18. **Lecoeur, S., E. Bonierbale, D. Challine, J. C. Gautier, P. Valadon, P. M. Dansette, R. Catinot, F. Ballet, D. Mansuy, and P. H. Beaune**. 1994. Specificity of in vitro covalent binding of tienilic acid metabolites to human liver microsomes in relationship to the type of hepatotoxicity: Comparison with two directly hepatotoxic drugs. *Chem.Res.Toxicol. 7*: 434-442.

19. **Merk, H. F.** 1998. Skin Metabolism. In *Allergic Contact Dermatitis*. J.-P. Lepoittevin, D.A. Basketter, A. Goosens and A.-T. Karlberg, eds. Springer Verlag, Berlin, Heidelberg, p. 68-80.

20. **Anderson, C., A. Hehr, R. Robbins, R. Hasan, M. Athar, H. Mukhtar, and C. A. Elmets**. 1995. Metabolic requirements for induction of contact hypersensitivity to immunotoxic polyaromatic hydrocarbons. Metabolic requirements for induction of contact hypersensitivity to immunotoxic polyaromatic hydrocarbons. *J.Immunol. 155*: 3530-3537.

21. **Liberato, D. J., V. S. Byers, R. G. D. Ennick, and N. Castagnoli**. 1981. Regiospecific attack of nitrogen and sulfur nucleophiles on quinones derived from poison oak/ivy catechols (urushiols) and analogues as models for urushiol-protein conjugate formation. *J.Med.Chem. 24*: 28-33.

22. **Kalish, R. S., J. A. Wood, and A. LaPorte**. 1994. Processing of urushiol (poison ivy) hapten by both endogenous and exogenous pathways for presentation to T cells in vitro. *J.Clin.Invest. 93*: 2039-2047.

23. **Uetrecht, J. P.** 1992. The role of leukocyte-generated reactive metabolites in the pathogenesis of idiosyncratic drug reactions. *Drug Metab.Rev. 24*: 299-366.

24. **Jiang, X., G. Khursigara, and R. L. Rubin**. 1994. Transformation of lupus-inducing drugs to cytotoxic products by activated neutrophils. *Science 266*: 810-813.

25. **Eling, T. E., D. C. Thompson, G. L. Foureman, J. F. Curtis, and M. F. Hughes**. 1990. Prostaglandin H synthase and xenobiotic oxidation. *Annu.Rev.Pharmacol.Toxicol. 30*: 1-45.

26. **Griem, P., K. Panthel, H. Kalbacher, and E. Gleichmann**. 1996. Alteration of a model antigen by Au(III) leads to T cell sensitization to cryptic peptides. *Eur.J.Immunol. 26*: 279-287.

27. **Weber, W. W.** 1987. *The Acetylator Genes and Drug Response*. Oxford University Press, New York.

28. **Woosley, R. L., D. E. Drayer, M. M. Reidenberg, A. S. Nies, K. Carr, and J. A. Oates**. 1978. Effect of acetylator phenotype on the rate at which procainamide induces antinuclear antibodies and the lupus syndrome. *New Engl.J.Med. 298*: 1157-1159.

29. **Hilkens, C. M. U., A. Snijders, H. Vermeulen, P. H. Vandermeide, E. A. Wierenga, and M. L. Kapsenberg**. 1996. Accessory cell-derived IL-12 and

prostaglandin E_2 determine the IFN-(gamma) level of activated human $CD4^+$ T cells. *J.Immunol. 156*: 1722-1727.

30. **Daly, A. K.** 1995. Molecular basis of polymorphic drug metabolism. *J.Mol.Med. 73*: 539-553.

31. **Schmiedeberg, S. v., E. Fritsche, A. C. Rönnau, K. Golka, C. Specker, H. C. Schuppe, O. Döhr, B. Sachs, H. M. Bolt, P. Lehmann, T. Ruzicka, C. Esser, J. Abel, and E. Gleichmann**. 1996. Polymorphisms of drug-metabolizing enzymes in patients with idiopathic systemic autoimmune diseases. *Exp.Toxic.Pathol. (Suppl.)*: 349-353.

32. **Flockhart, D. A., D. J. Clauw, E. Buchert Sale, J. Hewett, and R. L. Woosley**. 1994. Pharmacogenetic characteristics of the eosinophilia-myalgia syndrome. *Clin.Pharmacol.Ther. 56*: 398-405.

33. **Ollier, W., E. Davies, J. Alldersea, A. Fryer, P. Jones, and R. Strange**. 1996. Association of homozygosity for glutathione-S-transferase GSTM1 null alleles with the Ro+/La- autoantibody profile in patients with sytemic lupus erythematosus. *Arthritis Rheum. 39*: 1763-1764.

34. **Wolkenstein, P., V. Carriere, D. Charue, S. Bastuji-Garin, J. Revuz, J. C. Roujeau, P. H. Beaune, and M. Bagot** . 1995. A slow acetylator genotype is a risk factor for sulphonamide-induced toxic epidermal necrolysis and Stevens-Johnson syndrome. *Pharmacogenetics 5*: 255-258.

35. **Yung, R. L., K. J. Johnson, and B. C. Richardson**. 1995. Biology of disease: New concepts in the pathogenesis of drug-induced lupus. *Lab.Invest. 73*: 746-759.

36. **Gleichmann, E., I. Kimber, and I. F. H. Purchase**. 1989. Immunotoxicology: suppressive and stimulatory effects to drugs and environmental chemicals on the immune system. *Arch.Toxicol. 63*: 257-273.

37. **Matzinger, P.** 1994. Tolerance, danger, and the extended family. *Annu.Rev.Immunol. 12*: 991-1045.

38. **Cella, M., F. Sallusto, and A. Lanzavecchia**. 1997. Origin, maturation and antigen presenting function of dendritic cells. *Curr.Opin.Immunol. 9*: 10-16.

39. **Ibrahim, M. A. A., B. M. Chain, and D. R. Katz**. 1995. The injured cell: the role of the dendritic cell system as a sentinel receptor pathway. *Immunol.Today 16*: 181-186.

40. **Enk, A. H. and S. I. Katz**. 1992. Early molecular events in the induction phase of contact sensitivity. *Proc.Natl.Acad.Sci.U.S.A. 89*: 1398-1402.

41. **Lisby, S., K. M. Muller, C. V. Jongeneel, J. H. Saurat, and C. Hauser**. 1995. Nickel and skin irritants up-regulate tumor necrosis factor- alpha mRNA in keratinocytes by different but potentially synergistic mechanisms. *Int.Immunol. 7*: 343-352.

42. **Rambukkana, A., F. H. Pistoor, J. D. Bos, M. L. Kapsenberg, and P. K. Das**. 1996. Effects of contact allergens on human Langerhans cells in skin organ culture: migration, modulation of cell surface molecules, and early expression of interleukin-1β protein. *Lab.Invest. 74*: 422-436.

43. **Goebeler, M., G. Meinardus-Hager, J. Roth, S. Goerdt, and C. Sorg**. 1993. Nickel chloride and cobalt chloride, two common contact sensitizers, directly induce expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule (ELAM-1) by endothelial cells. *J.Invest.Dermatol. 100*: 759-765.

44. **Zdolsek, J. M., O. Soder, and P. Hultman**. 1994. Mercury induces in vivo and in vitro secretion of interleukin-1 in mice. *Immunopharmacol. 28*: 201-208.

45. **Ortmann, B., S. Martin, A. von Bonin, E. Schiltz, H. Hoschutzky, and H. U. Weltzien**. 1992. Synthetic peptides anchor T cell-specific TNP epitopes to MHC antigens. *J.Immunol. 148*: 1445-1450.

46. **Martin, S., A. von Bonin, C. Fessler, U. Pflugfelder, and H. U. Weltzien**. 1993. Structural complexity of antigenic determinants for class- I MHC-restricted, hapten-specific T-Cells - 2 qualitatively differing types of H-2K(B)-restricted TNP epitopes. *J.Immunol. 151*: 678-687.

47. **Kohler, J., U. Hartmann, R. Grimm, U. Pflugfelder, and H. U. Weltzien**. 1997. Carrier-independent hapten recognition and promiscuous MHC restriction by CD4 T cells induced by trinitrophenylated peptides. *J.Immunol. 158*: 591-597.

48. **Brander, C., D. Mauri-Hellweg, F. Bettens, H. Rolli, M. Goldman, and W. J. Pichler**. 1995. Heterogeneous T cell responses to beta-lactam-modified self- structures are observed in penicillin-allergic individuals. *J.Immunol. 155*: 2670-2678.

49. **Mauri-Hellweg, D., M. Zanni, E. Frei, F. Bettens, C. Brander, D. Mauri, E. Padovan, H. U. Weltzien, and W. J. Pichler**. 1996. Cross-reactivity of T cell lines and clones to beta-lactam antibiotics. *J.Immunol. 157*: 1071-1079.

50. **Padovan, E., D. Mauri-Hellweg, W. J. Pichler, and H. U. Weltzien**. 1996. T cell recognition of penicillin G: structural features determining antigenic specificity. *Eur.J.Immunol. 26*: 42-48.

51. **Padovan, E., T. Bauer, M. M. Tongio, H. Kalbacher, and H. U. Weltzien**. 1997. Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy. *Eur.J.Immunol. 27*: 1303-1307.

52. **Mauri-Hellweg, D., F. Bettens, D. Mauri, C. Brander, T. Hunziker, and W. J. Pichler**. 1995. Activation of drug-specific CD4(+) and CD8(+) T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J.Immunol. 155*: 462-472.

53. **Nalefski, E. A. and A. Rao**. 1993. Nature of the ligand recognized by a hapten- and carrier- specific, MHC-restricted T cell receptor. *J.Immunol. 150*: 3806-3816.

54. **Romero, P., J. L. Casanova, J. C. Cerottini, J. L. Maryanski, and I. F. Luescher**. 1993. Differential T cell receptor photoaffinity labeling among H-2K^d restricted cytotoxic T lymphocyte clones specific for a photoreactive peptide derivative. Labeling of the alpha-chain correlates with J alpha segment usage. *J.Exp.Med. 177*: 1247-1256.

55. **Champion, B. R., K. R. Page, N. Parish, D. C. Rayner, K. Dawe, G. Biswas-Hughes, A. Cooke, M. Geysen, and I. M. Roitt**. 1991. Identification of a thyroxine-containing self-epitope of thyroglobulin which triggers thyroid autoreactive T cells. *J.Exp.Med. 174*: 363-370.

56. **Michaelsson, E., J. Broddefalk, A. Engstrom, J. Kihlberg, and R. Holmdahl**. 1996. Antigen processing and presentation of a naturally glycosylated protein elicits major histocompatibility complex class II-restricted, carbohydrate-specific T cells. *Eur.J.Immunol. 26*: 1906-1910.

57. **Schnyder, B., D. Mauri-Hellweg, M. Zanni, F. Bettens, and W. J. Pichler**. 1997. Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alpha-beta T cell clones. *J.Clin.Invest. 100*: 136-141.

58. **Griem, P. and E. Gleichmann**. 1995. Metal ion induced autoimmunity. *Curr.Opin.Immunol. 7*: 831-838.

59. **Sinigaglia, F., D. Scheidegger, G. Garotta, R. Scheper, M. Pletscher, and A. Lanzavecchia**. 1985. Isolation and characterization of Ni-specific T cell clones from patients with Ni-contact dermatitis. *J.Immunol. 135*: 3929-3932.

60. **Moulon, C., J. Vollmer, and H. U. Weltzien**. 1995. Characterization of processing requirements and metal cross- reactivities in T cell clones from patients with allergic contact dermatitis to nickel. *Eur.J.Immunol. 25*: 3308-3315.

61. **Richeldi, L., R. Sorrentino, and C. Saltini**. 1993. HLA-DPB1 glutamate 69: a genetic marker of beryllium disease. *Science 262*: 242-244.

62. **Romagnoli, P., G. A. Spinas, and F. Sinigaglia**. 1992. Gold-specific T cells in rheumatoid arthritis patients treated with gold. *J.Clin.Invest. 89*: 254-258.

63. **Jang, Y. S., K. H. Lim, and B. S. Kim**. 1991. Analysis of T cell reactivities to phosphorycholin-conjugated hen egg lysozyme in C57BL/6 mice: hapten-conjugate specificity reflects an altered expression of a major carrier epitope. *Eur.J.Immunol. 21*: 1303-1310.

64. **Kubicka-Muranyi, M., P. Griem, B. Lübben, N. Rottman, R. Lührmann, K. Beyer, and E. Gleichmann**. 1995. Mercuric chloride-induced autoimmunity in mice involves an upregulated presentation of altered and unaltered nucleolar self antigen. *Int.Arch.Allergy Immunol. 108*: 1-10.

65. **Pollard, K. M., D. K. Lee, C. A. Casiano, M. Bluthner, M. M. Johnston, and E. M. Tan**. 1997. The autoimmunity-inducing xenobiotic mercury interacts with the autoantigen fibrillarin and modifies its molecular and antigenic properties. *J.Immunol. 158*: 3521-3528.

66. **Kempkes, B., E. Palmer, S. Martin, A. von Bonin, K. Eichmann, B. Ortmann, and H. U. Weltzien**. 1991. Predominant T cell receptor gene elements in TNP-specific cytotoxic T cells. *J.Immunol. 147*: 2467-2473.

67. **Porcelli, S. A., C. T. Morita, and R. L. Modlin**. 1996. T-cell recognition of non-peptide antigens. *Curr.Opin.Immunol. 8*: 510-516.

68. **Zanni, M. P., D. Mauri-Hellweg, C. Brander, T. Wendland, B. Schnyder, E. Frei, S. von Greyerz, A. Bircher, and W. J. Pichler**. 1997. Characterization of lidocaine-specific T cells. *J.Immunol. 158*: 1139-1148.

69. **Corinti, S., R. De Palma, A. Fontana, C. Gagliardi, C. Pini, and F. Sallusto**. 1997. Major histocompatibility complex-independent recognition of a distinctive pollen antigen, most likely a carbohydate by human $CD8^+$ α/β T cells. *J.Exp.Med. 186*: 899-908.

70. **Bour, H., E. Peyron, M. Gaucherand, J. L. Garrigue, C. Desvignes, D. Kaiserlian, J. P. Revillard, and J. F. Nicolas**. 1995. Major histocompatibility complex class I-restricted CD8(+) T cells and class II-restricted CD4(+) T cells, respectively, mediate and regulate contact sensitivity to dinitrofluorobenzene. *Eur.J.Immunol. 25*: 3006-3010.

71. **Van Hoogstraten, I. M., C. Boos, D. Boden, M. E. von Blomberg, R. J. Scheper, and G. Kraal**. 1993. Oral induction of tolerance to nickel sensitization in mice. *J.Invest.Dermatol. 101*: 26-31.

72. **Desvignes, C., H. Bour, J. F. Nicolas, and D. Kaiserlian**. 1996. Lack of oral tolerance but oral priming for contact sensitivity to dinitrofluorobenzene in major histocompatibility complex class II-deficient mice and in CD4(+) T cell-depleted mice. *Eur.J.Immunol. 26*: 1756-1761.

73. **Xu, H., N. A. Diiulio, and R. L. Fairchild**. 1996. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: Interferon gamma- producing (Tc1) effector CD8(+) T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulatory CD4(+) T cells. *J.Exp.Med. 183*: 1001-1012.

74. **Steinbrink, K., C. Sorg, and E. Macher**. 1996. Low zone tolerance to contact allergens in mice: A functional role for CD8(+) T helper type 2 cells. *J.Exp.Med. 183*: 759-768.

75. **Doth, M., M. Fricke, F. Nicoletti, G. Garotta, M.-L. van Velthysen, J. A. Bruijn, and E. Gleichmann**. 1997. Genetic differences in immune reactivity to mercuric chloride ($HgCl_2$): immunosuppression of H-2^d mice is mediated by interferon-gamma (IFN-g). *Clin.Exp.Immunol. 109*: 149-156.

76. **Hanley, G. A., J. Schieffenbauer, and E. Soebel**. 1997. Class II haplotype differentially regulates immune response in $HgCl_2$-treated mice. *Clin.Immunol.Immunopathol. 84*: 328-337.

77. **Bridoux, F., A. Badou, A. Saoudi, I. Bernard, E. Druet, R. Pasquier, P. Druet, and L. Pelletier**. 1997. Transforming growth factor beta (TGF-beta)-dependent inhibition of T helper cell 2 (Th2)-induced autoimmunity by self-major histocompatibility complex (MHC) class II- specific, regulatory CD4(+) T cell lines. *J.Exp.Med. 185*: 1769-1775.

78. **Katsutani, N. and H. Shionoya**. 1992. Popliteal lymph node enlargement induced by procainamide. *J.Immunopharmacol. 14*: 681-686.

79. **Preckel, T., R. Grimm, S. Martin, and H. U. Weltzien**. 1997. Altered hapten ligands antagonize trinitrophenylspecific cytotoxic T cells and block internalization of hapten-specific receptors. *J.Exp.Med. 185*: 1803-1813.

CHAPTER 3

T Cell-Dependent Immune Reactions to Reactive Benzene Metabolites in Mice

Susanne Ewens, Marty Wulferink, Carsten Goebel, and Ernst Gleichmann

Using the popliteal lymph node (PLN) assay in mice, we studied the sensitizing potential of benzene and its metabolites. Whereas benzene and phenol failed to induce a PLN reaction, catechol and hydroquinone induced a moderate, and p-benzoquinone a vigorous response. Following a single injection of the reactive metabolite p-benzoquinone (100 nmol/mouse), cellularity in the draining PLN was increased more than 15-fold, and it took about 100 days until it reverted back to normal. Although the PLN response was T cell-dependent, flow cytometric analysis revealed that the increased cellularity in the PLN after a single injection of p-benzoquinone was mainly due to an increase in B cells. Mice primed to p-benzoquinone and challenged with a small dose of p-benzoquinone (0.1 nmol/mouse) mounted a secondary PLN reaction, indicating hapten specificity of the reaction; this was confirmed by results obtained in the adoptive transfer PLN assay. An unexpected finding was the secondary PLN response to benzene (1 nmol/mouse) observed in mice primed to p-benzoquinone. This finding suggests that some of the benzene (at least 10%) was locally converted into p-benzoquinone which then elicited the secondary response observed. In conclusion, the reactive intermediate metabolites hydroquinone and p-benzoquinone can act as haptens and sensitize, their precursors, benzene and phenol, may be considered as prohaptens.

Introduction

Due to the presence of benzene in petrol and its use as industrial solvent there is significant benzene emission into the environment, resulting in continuous, relevant uptake by humans living in industrialized areas. In the body, benzene is metabolized in several steps, starting in the liver by epoxidation through cytochrome P450 2E1 (1-3) and subsequent conversion into phenol. Phenol can be further oxidized by cytochrome P450 into catechol and hydroquinone. Via the bloodstream these intermediates reach other organs, such as the bone marrow, where they are further oxidized by the myeloperoxidase of phagocytes, and perhaps other peroxidases, into the highly reactive benzoquinones, *p*- and *o*-benzoquinone (4) (Fig. 1). Benzene metabolites have been shown to bind covalently to proteins in blood, liver, spleen, and bone marrow (5-7), and they are likely to exert much of the toxicity of benzene (8,9).

Little is known about the effects of benzene and its metabolites on the immune system. Nonspecific immunotoxic effects were reported by MacEachern and Laskin (10) who noticed modulation of cytokine production in bone marrow leukocytes of benzene-exposed mice. A different question is whether benzene metabolites are contact sensitizers. This was first investigated by Benezra *et al.* (11) in a systematic search for structure-activity relationships of skin contact sensitizers. Using a database, they were unable to find evidence for the assumption that benzoquinones are sensitizers. This evidence was provided by Basketter and Goodwin (12) who studied dermal sensitization to 1,4-substituted benzene derivatives in guinea pigs. With three different, adjuvant-based test methods they showed that hydroquinone and *p*-benzoquinone possess sensitizing potential. Subsequently, Basketter and Liden (13) investigated the sensitizing potential of benzene derivatives in humans using the patch test. Surprisingly, reactions to hydroquinone were negative, but *p*-benzoquinone yielded a number of positive test results. However, in the latter reactions it proved difficult to distinguish between the toxic and the sensitizing potential of *p*-benzoquinone. From their results one cannot deduce that the positive patch test reactions were specific recall responses, because it was unknown whether or not prior sensitization to *p*-benzoquinone had taken place in these indivuals, prior to challenge.

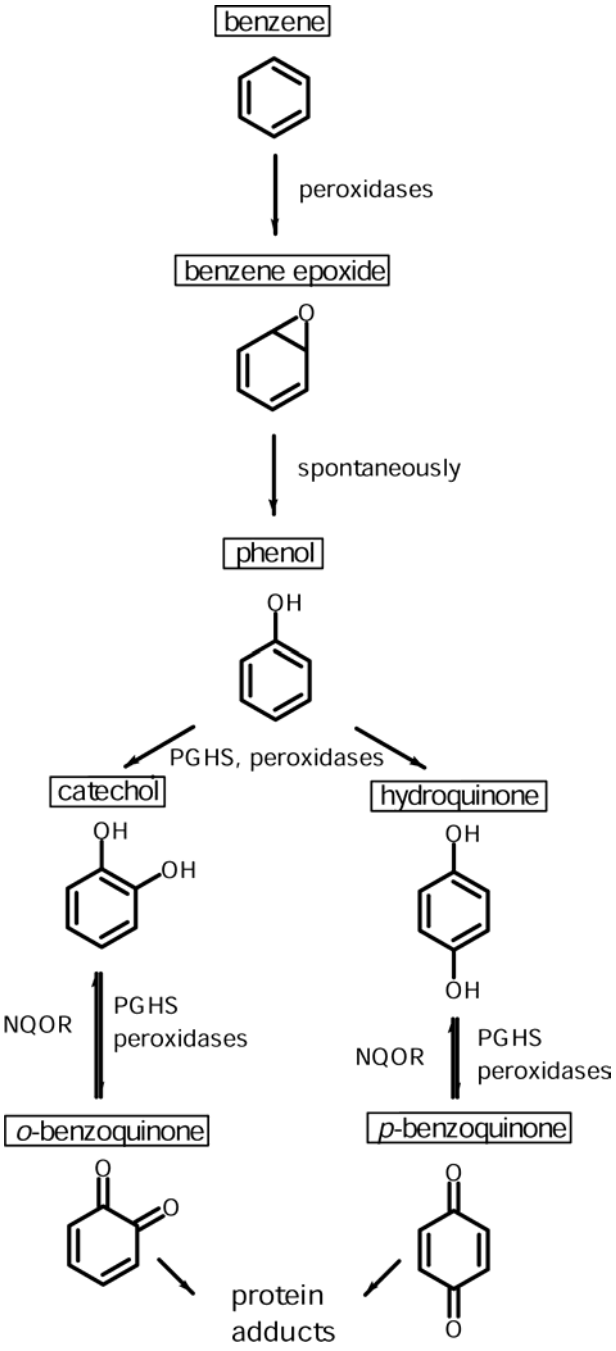

Figure 1. Pathway of bioactivation of benzene. In the liver, benzene epoxide, formed by cytochrome *P*-450 monooxigenases, spontaneously converts into phenol which is further oxidized to catechol and hydroquinone, respectively. Extrahepatically, catechol and hydroquinone are converted by the myeloperoxidase of phagocytes into the highly reactive benzoquinones, *o*- and *p*-benzoquinone. NQOR: NADPH-quinone-oxidoreductase; PGHS: prostaglandin H synthase

In view of this somewhat scanty knowledge of the sensitizing potential of the widespread pollutant benzene and its metabolites, we studied their sensitizing potential, using the PLN assay in mice. In contrast to sensitization tests in guinea pigs, the PLN assay allows to quantify immune responses to sensitizing chemicals, does not require the use of adjuvant, and can measure both primary and secondary T cell-dependent immune responses to such compounds (14).

Materials and Methods

Chemicals Benzene, *p*-benzoquinone, hydroquinone, catechol, phenol, and streptozotocin were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); ethanol was purchased from E. Merck (Darmstadt, Germany); sterile, pyrogen-free phosphate-buffered saline (PBS) was obtained from Gibco GmbH (Karlsruhe, Germany); and sterile, pyrogen-free 0.9% NaCl was purchased from Fresenius AG (Bad Homburg, Germany).

Mice Specific pathogen-free female C57BL/6J mice, obtained from Harlan Winkelmann GmbH (Borchen, Germany), were used throughout, unless mentioned otherwise. In one experiment female BALB/c mice (wildtype), BALB/c *nu/nu* mice and their BALB/c *nu/+* littermates, obtained from Harlan CPB (Austerlitz, Netherlands), were used. Animals were kept under specific pathogen-free conditions and had free access to standard diet (Ssniff Spezialdiäten GmbH, Soest, Germany) and tap water; they were 6 to 8 weeks old at the onset of experiments.

Antibodies Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Thy1.2 (clone 53-2.1), FITC-conjugated rat anti-mouse CD4 (clone RM4-5), phycoerythrin (PE)-conjugated rat anti-mouse CD8a (clone 53-6.7), PE-conjugated rat anti-mouse B220 (clone RA 3-6B2), and PE-conjugated anti-mouse NK cells (clone 2B4) monoclonal antibodies for FACScan analyses were purchased from Pharmingen (Hamburg, Germany). Anti-mouse-B220 monoclonal antibodies with magnetic microbeads were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

Primary response PLN assay This assay was performed as described (14-16). Briefly, test compounds were dissolved in ethanol and diluted in PBS to a concentration of 0.1 % ethanol; this solvent is referred to as ethanol/PBS. On day 0, animals (5 to 6 mice per group) received a single sc injection (50 µl) of the compound indicated into the left hindfoot pad, control animals received ethanol/PBS only. On day 6, PLNs of both treated and untreated sides were removed and cell numbers of individual PLNs were determined using a Casy 1 automatic cell counter (Schärfe Systems GmbH, Reutlingen, Germany). The PLN cell count index from each mouse was calculated by dividing the cell count of the treated side by that of the untreated side.

Secondary response PLN assay In order to determine secondary PLN responses of mice, groups of animals were primed with the test compounds on day 0, as described above. After complete regression of the primary PLN reaction, mice were challenged by a second sc injection into the same hindfoot pad. The dose of test compounds used for recall was suboptimal, that means it was just too small to induce a primary PLN response. Four days after the second sc injection, PLN cell count indices were determined. For control of

specificity, streptozotocin was used for priming (0.5 mg/mouse) and challenge (0.05 mg/mouse), as described before (17).

Treatment of prospective T cell donors On days -14 and -7 before the adoptive cell transfer, C57BL/6J donor mice received an iv injection of *p*-benzoquinone (50 nmol/mouse) into the tail vein, and on day -6 they received an sc injection of *p*-benzoquinone (100 nmol/mouse) at the base of tail. Control groups of prospective T cell donors were treated with ethanol/PBS. On day 0, spleens were removed, and splenic T cells enriched as described below.

Enrichment of splenic T cells For enrichment of T cells, B cells were depleted from spleen cell suspensions using a magnetic cell separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), as described (18). In short, 1 x 10^8 spleen cells were incubated (15 min at 4 °C) in 1 ml PBS containing 50 µl anti-mouse-B220 antibody coupled with magnetic microbeads (Miltenyi Biotec GmbH). Stained cells were withdrawn from the cell suspension in a high gradient magnetic field. After separation, the cell fraction was tested for T cell purity with FITC-labeled monoclonal anti-Thy1.2 antibody (Pharmingen) using a FACScan flow cytometer (Beckton Dickinson, San Jose, California, USA). Cells in the unstained fraction after separation contained 85 to 95% Thy1.2-positive cells and are referred to as enriched T cells. These enriched T cells were used in the adoptive transfer PLN assay.

Adoptive transfer PLN assay This test system allows to detect anamnestic T cell responses of donor animals to chemicals of low molecular weight (14,15,17,19). Enriched T cells of donor animals were irradiated (20 Gy) using a ^{137}Cs source (Gammacell 2000, Molsgaard, Copenhagen, Denmark); this step was introduced in order to decrease the nonspecific PLN reaction occasionally seen after transfer of unirradiated T cells from recently immunized donors (20). On day 0, 50 µl PBS containing 1 x 10^7 irradiated enriched T cells was injected sc into the left hindfoot pad of syngeneic recipients. On day 1, these animals received an additional sc injection (50 µl) into the same foot pad: these injections contained a suboptimal dose of *p*-benzoquinone or streptozotocin, or the control compounds indicated. On day 6, PLN cell count indices were determined.

Flow cytometric analysis PLN cells (2 x 10^5 cells/well) were transferred into 96-well plates and marked with the FITC- and PE-labeled antibodies indicated. Antibody-marked probes were incubated for ten minutes at 4 °C, washed twice with PBS, and analysed in a FACScan flow cytometer (Becton Dickinson).

Statistical analysis All experiments were performed twice to assure reproducibility of the data. Results of individual experiments are shown as arithmetic means + standard deviation

(SD) of 5 to 6 animals. One-way analysis of variance (ANOVA) with Newman-Keuls comparison was used to calculate statistically significant differences.

Results

Differential capacity of benzene and Its metabolites to elicit primary PLN responses

Primary PLN responses to benzene (100 nmol/mouse) and equimolar doses of the benzene metabolites indicated were determined in C57BL/6J mice (Fig. 2). Benzene, phenol, and catechol failed to induce a PLN reaction, whereas hydroquinone elicited a weak and *p*-benzoquinone a vigorous PLN response. When compared with hydroquinone, *p*-benzoquinone was found to induce a five times higher PLN response at the same dose of 100 nmol. Both metabolites showed a dose-response relationship in the primary PLN assay.

Kinetics of primary PLN responses to benzene and *p*-benzoquinone

As shown in Fig. 3, benzene failed to elicit increased PLN cell count indices at any of the ten time points tested. In marked contrast, *p*-benzoquinone induced a PLN response that was 15-fold above normal on day 6 and still 6-fold on day 14; from then on it gradually decreased reaching normal values at day 105 after injection.

T-Cell dependence of the PLN response to benzene metabolites

To answer the question if the observed PLN reaction to hydroquinone and *p*-benzoquinone is T cell-dependent, the PLN assay was performed in BALB/c *nu/nu* mice and BALB/c *nu/+* littermates. Hydroquinone and *p*-benzoquinone failed to induce a PLN response in *nu/nu* mice, whereas they elicited significant PLN reactions in *nu/+* littermates (Fig. 4). Hence, the PLN enlargement observed after injection of hydroquinone and *p*-benzoquinone, respectively, is a T cell-dependent reaction.

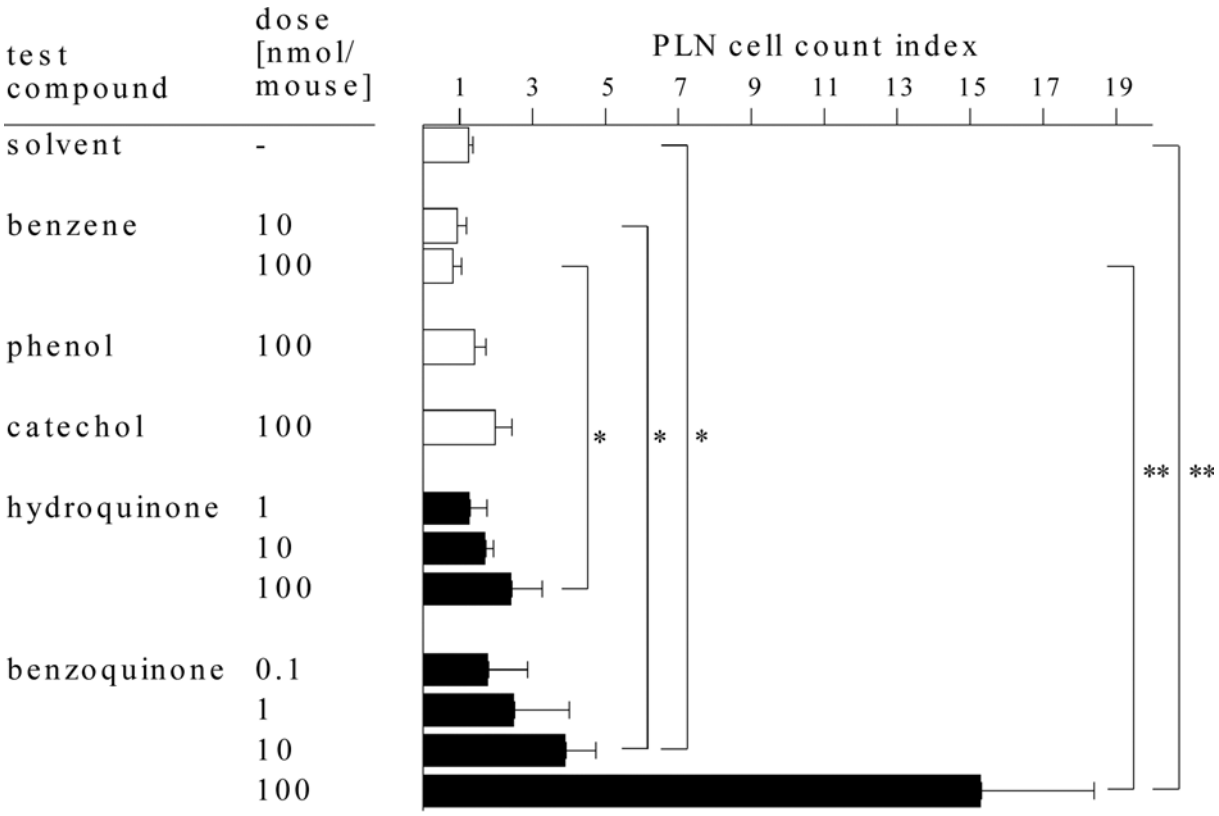

Figure 2. Primary PLN response to reactive benzene metabolites. On day 0, groups of mice received a single sc injection of the indicated test compound at the dose specified into one hindfoot pad; control animals received a single sc injection of solvent (ethanol/PBS) only. On day 6, PLN cell count indices were determined. Asterisks denote significant difference between groups marked with brackets (*p<0.05, **p<0.01).

Flow cytometric analysis of primary responses to *p*-benzoquinone

Cell populations and T-cell subpopulations involved in the primary PLN response to *p*-benzoquinone were analysed by immune-flow cytometry. Table 1 shows that up to a dose of 100 nmol *p*-benzoquinone/mouse the absolute numbers of B and T cells in the draining PLN were increased and that the increase in B cells was higher than that in T cells. Absolute numbers of $CD4^+$ and $CD8^+$ cells also increased, but the ratio $CD4^+/CD8^+$ did not change.

Secondary PLN responses to *p*-benzoquinone and benzene

The results described above established that reactive benzene metabolites, especially *p*-benzoquinone, are able to elicit significant PLN reactions that

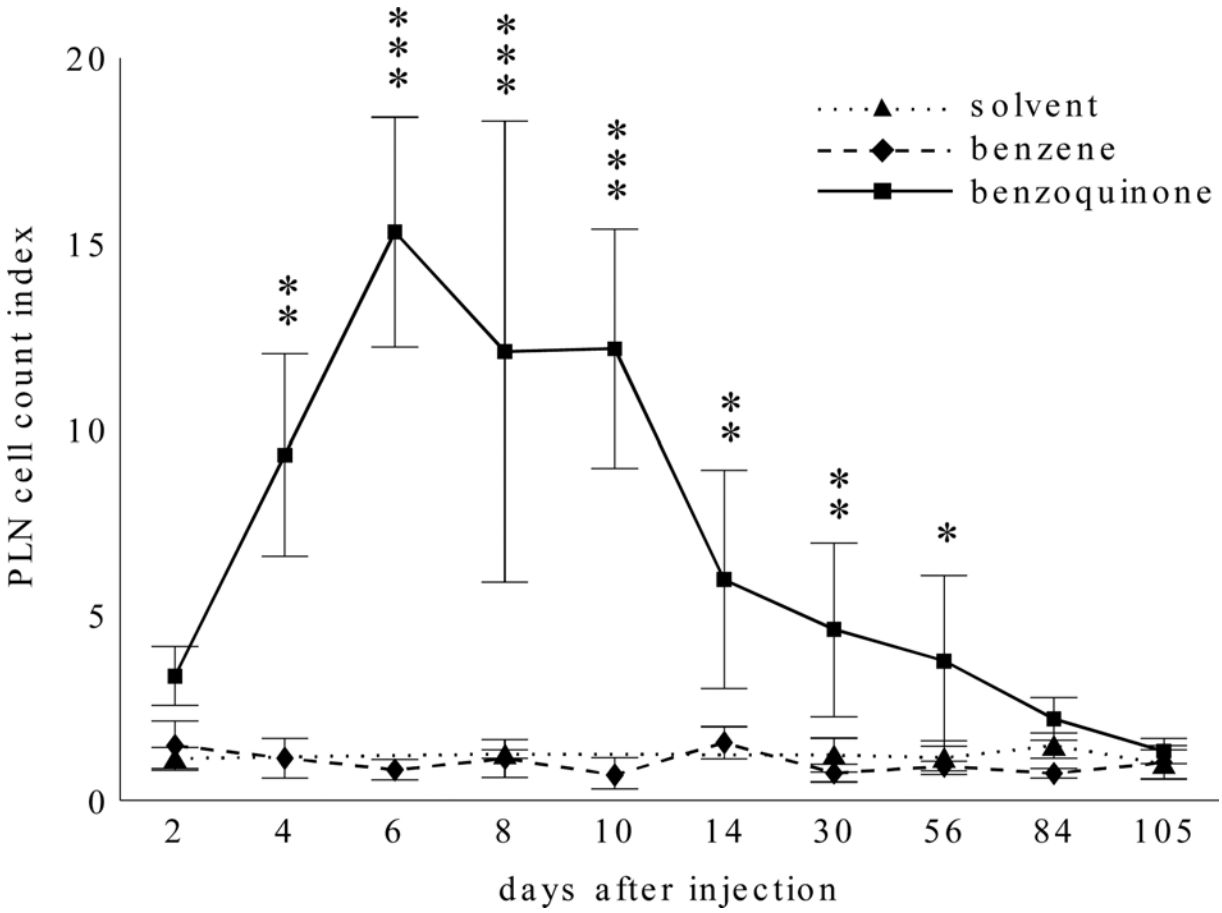

Figure 3. Kinetics of the primary PLN response to benzene and *p*-benzoquinone. On day 0, C57BL/6J mice received a single sc injection of 100 nmol benzene or *p*-benzoquinone into the left hindfoot pad; control animals received solvent only. At the time points indicated on the abcissae, PLN cell count indices were determined. Asterisks denote significant difference between the group injected with *p*-benzoquinone and both control groups (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

require participation of T cells. Next, we studied whether or not the observed PLN reactions to *p*-benzoquinone were due to specific T cell reactions to this metabolite. This was done by two different experimental approaches.

In the first approach, we asked whether mice primed to *p*-benzoquinone were able to mount a secondary response upon challenge with a suboptimal dose to induce specific secondary PLN reactions (17). Groups of C57BL/6 mice were primed with either *p*-benzoquinone (100 nmol/mouse) or, for control, benzene (100 nmol/mouse), streptozotocin (0.5 mg/mouse), or ethanol/PBS. After 13 weeks, when the primary PLN response seen in the *p*-benzoquinone-primed group had disappeared (cf. Fig. 3), groups of mice were challenged by

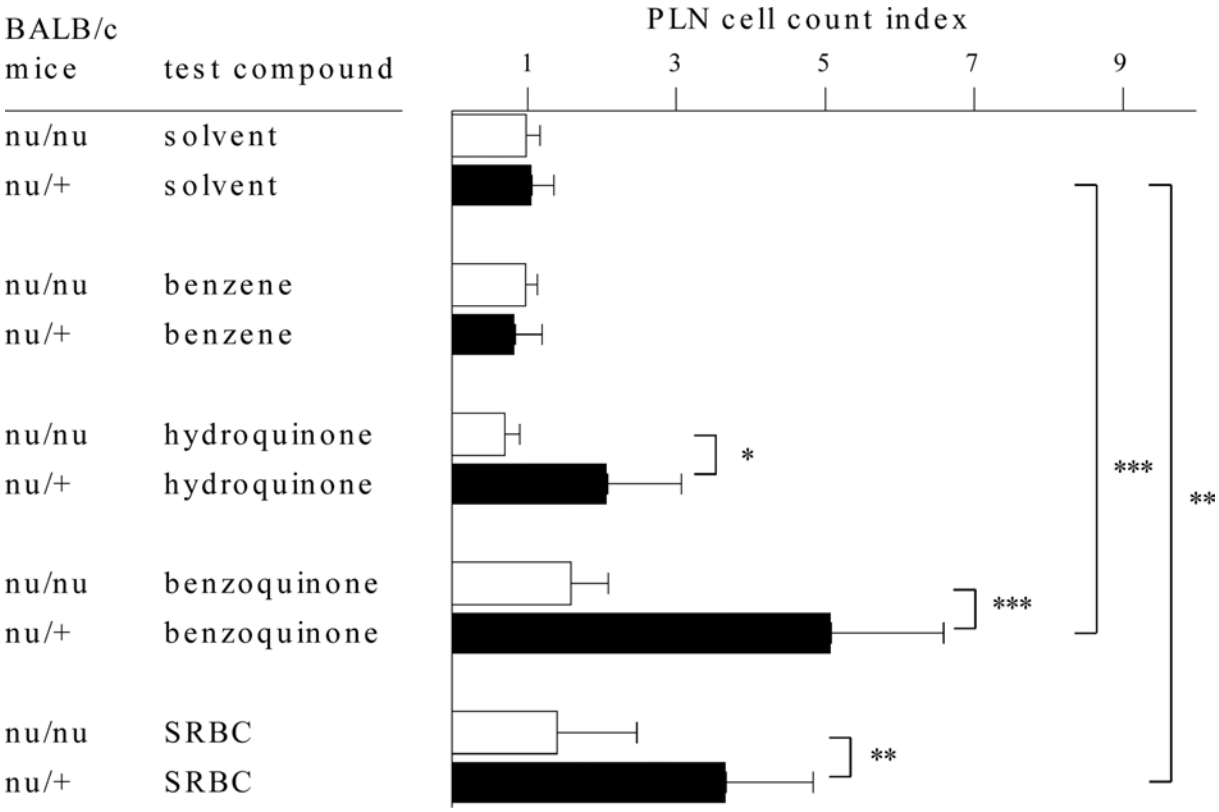

Figure 4. T cell dependence of the primary PLN response to hydroquinone and *p*-benzoquinone of benzene. On day 0, athymic (*nu/nu*; open bars) and euthymic (*nu/+*; closed bars) BALB/c received a single sc injection containing 100 nmol of either benzene, hydroquinone or *p*-benzoquinone, or 1 x 10^7 SRBC into the left hindfoot pad; control animals received solvent (ethanol/PBS) only. On day 6, the PLN cell count indices were determined. Asterisks denote a significant difference (**p*<0.05, ***p*<0.01, ****p*<0.001) between the groups marked with brackets.

Table 1. Flow cytometric analysis of PLN cells in the draining lymph nodes after injection of benzquinone or solvent[a]

Dose injected (nmol *p*-benzo-quinone/mouse)	PLN cell count (x10^5)	% B cells[b]	% T cells[c]	% $CD4^+$ of T cells[d]	% $CD8^+$ of T cells[e]
0 (solvent)	5.3 ± 2.2	19 ± 0.6	78.7 ± 0.8	52.6 ± 0.6	45.0 ± 0.7
0.1	6.4 ± 4.6	19.6 ± 3.6	78.0 ± 4.2	49.3 ± 5.4	45,8 ± 5.3
10	22.4 ± 9.9	36.9 ± 3.3	59.6 ± 3.2***[f]	47.6 ± 1.2	48,8 ± 0.3
100	41.8 ± 12.1*	46.9 ± 16.3***	48.6 ± 15.7***	52.2 ± 7.5*	47.2 ± 4.2

[a] Arithmatic means ± standard deviations of triplicate determinations. [b] Marked with PE-conjugated rat anti-mouse B220, [c] FITC-conjugated rat anti-mouse Thy1.2, [d] PE-conjugated rat anti-mouse CD4, [e] FITC-conjugated rat anti-mouse CD8. [f] Significantly different from solvent group: **p*<0.05, ****p*<0.001.

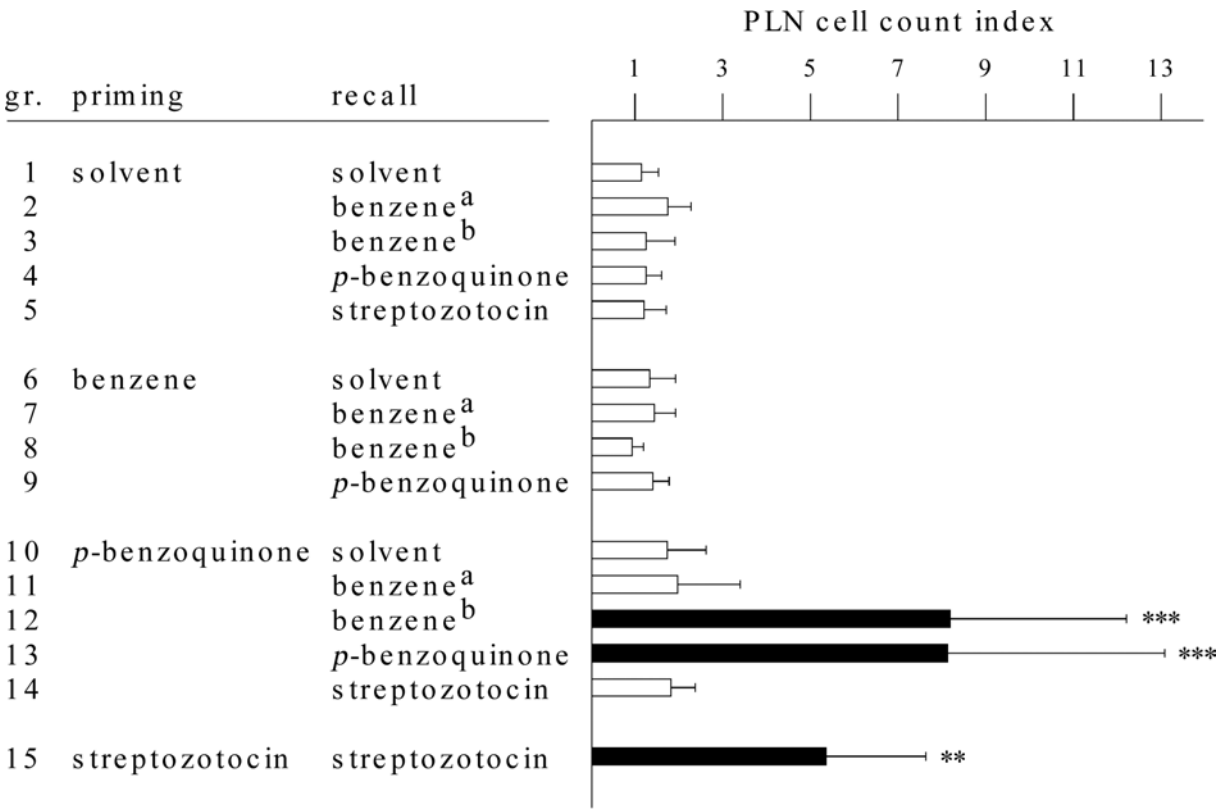

Figure 5. Secondary PLN response against *p*-benzoquinone. On day 0, groups of mice were primed with a single sc injection (100 nmol/mouse) of benzene or *p*-benzoquinone into the left hindfoot pad; control animals were primed with solvent or with streptozotocin (0.5 mg/mouse). After thirteen weeks, groups of mice received a second sc injection containing 0.1 or 1 nmol benzene (benzene[a] and benzene[b], respectively), 0.1 nmol *p*-benzoquinone, solvent, or 0.05 mg/mouse streptozotocin into the same hindfoot pad. Four days later the PLN cell count indices were determined.. Asterisks denote significant difference (**$p<0.01$, ***$p<0.001$) between the black bar indicated and any of the open bars.

injection of either *p*-benzoquinone (0.1 nmol/mouse), benzene (0.1 or 1 nmol/mouse), solvent, or streptozotocin (0.05 mg/mouse). Four days after recall, PLN cell count indices were determined. As can be seen in Fig. 5, animals primed with solvent or benzene failed to mount a significant PLN response to the compounds used for recall (groups 1 to 9). By contrast, mice which were primed with *p*-benzoquinone exhibited a secondary PLN response upon recall with the suboptimal dose of *p*-benzoquinone, but not of streptozotocin (cf. groups 13 and 14). An unexpected finding was the significant response to 1 nmol benzene/mouse detected in *p*-benzoquinone-primed animals (group 12). No response was elicited when the benzene dose used for recall was equimolar to that of *p*-benzoquinone, that is, 0.1 nmol/mouse (group 11). Whereas the dose of 1 nmol/mouse elicited a

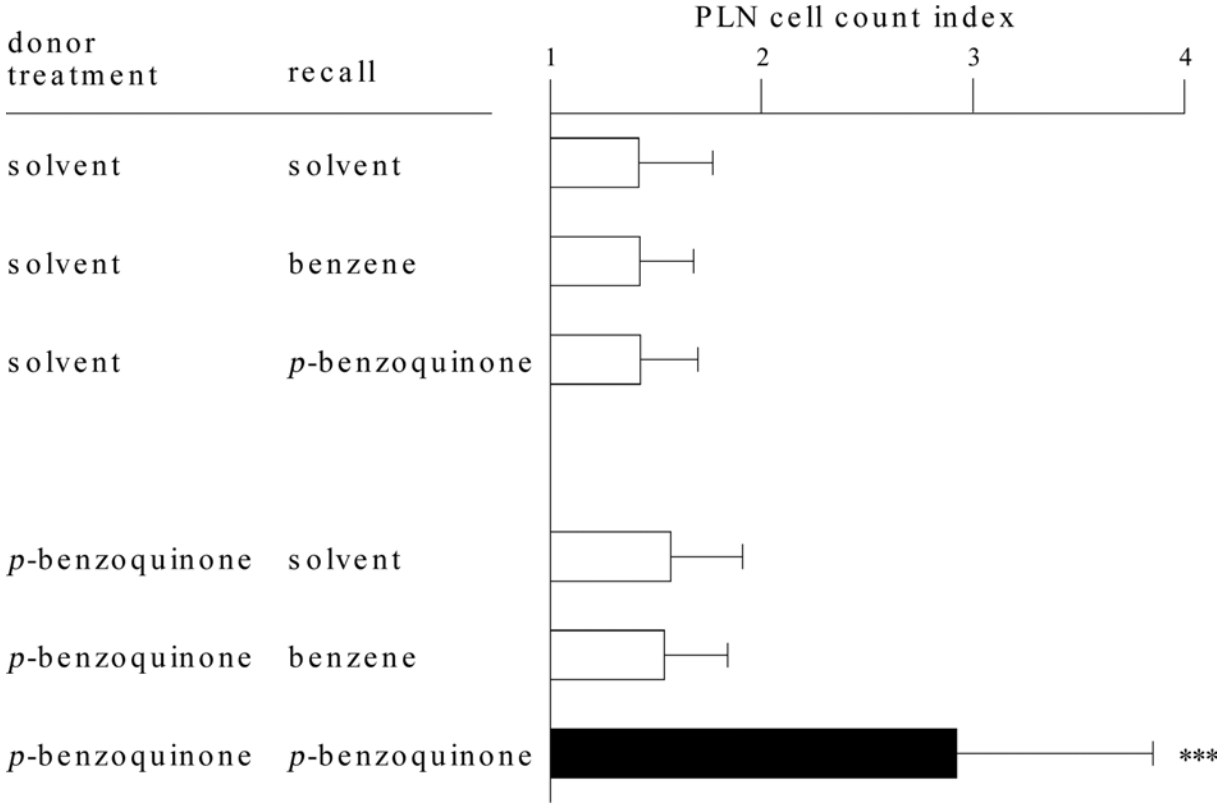

Figure 6. Adoptive transfer PLN assay demonstrating the transferability of T cell memory for *p*-benzoquinone. On day -14 and -7, the prospective T-cell donors received an injection of *p*-benzoquinone (50 nmol/mouse) into the tail vein. In addition, they were injected with 100 nmol *p*-benzoquinone/mouse sc at the root of the tail on day -6. On day 0, spleens were removed for T cell enrichment, and 1 x 10^7 T cells per mouse were injected sc in the left hindfoot pad of syngeneic recipient mice; control mice received T cells of untreated donor mice. On day 1, recipients received an sc injection into the same foot pad containing either benzene(0.1 nmol/mouse), a suboptimal dose of *p*-benzoquinone (0.1 nmol/mouse), or solvent. On day 6, PLN cell count indices were determined. Asterisks denote significant difference (***$p<0.001$) between the closed bar and any of the open bars.

significant secondary response in mice primed with *p*-benzoquinone, it failed to do so in animals primed with benzene or PBS (cf. groups 3, 8, and 12).

In the second experimental approach, the adoptive transfer PLN assay was used in order to test if memory T lymphocytes specific for *p*-benzoquinone can be transferred from primed donors to naive recipients. Prospective T-cell donors were primed with two iv injections of 50 nmol *p*-benzoquinone/mouse and, in addition, an sc injection of 100 nmol *p*-benzoquinone. Six days after the last injection (day 0), spleens of donor mice were obtained and T cells enriched by magnetic cell separation. Immediately after cell separation, enriched T cells (1 x 10^7/mouse) were transferred to syngeneic recipients by sc injection into one hindfoot pad. Control recipients received an sc injection containing enriched

splenic T cells (1 x 10^7/mouse) obtained from untreated donor mice. On day 1, groups of mice received an sc injection containing a suboptimal dose of either *p*-benzoquinone (0.1 nmol), benzene (0.1 nmol), or solvent into the same foot pad. On day 6, PLN cell count indices were determined. Fig. 6 shows that a secondary PLN response was only detectable in those mice which had received both *p*-benzoquinone-primed T cells and a suboptimal dose of *p*-benzoquinone for challenge.

Discussion

Benzene is a toxicologically relevant compound whose uptake, toxicokinetics, and metabolism have been well characterized in rodents as well as men. The results presented here confirm and extend the findings of Basketter and Goodwin (12), who established the sensitizing potential of hydroquinone and *p*-benzoquinone in guinea pigs. We found that the parent compound benzene and its early intermediate, phenol failed to induce primary immune reactions in the PLN assay, whereas the reactive metabolites hydroquinone and *p*-benzoquinone were able to do so. No adjuvants were used in this test system. Moreover, *p*-benzoquinone was found to elicit vigorous secondary reactions in animals sensitized to *p*-benzoquinone. These structure-activity relationships noted in the PLN assay conform with the protein reactivity of the test compounds: while protein reactivity of benzene and phenol is known to be low, that of catechol and hydroquinone and, in particular, *p*-benzoquinone is high (5-7,21). Hence, benzene and phenol may be considered as prohaptens and hydroquinone and *p*-benzoquinone as haptens in the terminology introduced by Landsteiner and Jacobs (22) and also used by others (23,24). Thus, benzene is comparable with other prohaptens tested in the PLN assay, such as procainamide (19,25), propylthiouracil (15), and gold(I) thiomalate (16,20). In all cases, the parent compounds failed to elicit a PLN reaction, whereas their reactive metabolites were able to do so. Hydroquinone and *p*-benzoquinone were found to sensitize in a dose-dependent and T cell-dependent fashion. Albeit T cell-dependent, the majority of cells in the enlarged PLN analyzed after injection of *p*-benzoquinone were found to consist of B cells. In this respect, too, the PLN reaction to *p*-benzoquinone resembles that to previously studied compounds, such as diphenylhydantoin (26) and D-penicillamine (27).

The PLN reaction to *p*-benzoquinone was unusually long-lasting and strong. Whereas the PLN enlargement seen after injection of other sensitizing chemicals mostly reverted to normal after 3-4 weeks (14), that seen after injection of *p*-benzoquinone persisted for more than 14 weeks. Injection of 100 nmol *p*-benzoquinone per mouse induced a 15-fold increase in cell number in the draining PLN. Such strong PLN reactions were rarely seen with other test compounds and certainly not at such low doses of test compound (14,28). In general, there is good correlation between the capacity of test compounds to evoke a response in the PLN assay and their known ability to induce allergy or autoimmunity in men (14). It is likely, therefore, that *p*-benzoquinone is a potent sensitizer not only in mice and guinea pigs, but in humans as well. This conclusion conforms with reports on contact allergy in photographic technicians exposed to developing agents containing hydroquinone (29).

Benzene and its metabolites are notorious for inducing aplastic anemia. However, the doses of benzene and *p*-benzoquinone used in the present study failed to induce anemia in C57BL/6 mice thus treated (data not shown), and this conforms with published data on the dose requirements for myelotoxicity of these compounds. In the present paper, the maximal doses of benzene, hydroquinone, and *p*-benzoquinone used for PLN assay were 100 nmol per mouse, corresponding to approximately 0.5 mg benzene, hydroquinone, or benzoquinone per kg b.wt. As far as benzene is concerned, the doses required for induction of aplastic anemia in mice (9,30) were orders of magnitude higher than those used in the present investigation. As far as *p*-benzoquinone is concerned, Rao *et al.* (31) were able to induce aplastic anemia in mice by ip injection of 2 mg *p*-benzoquinone/kg b.wt. once a day for six weeks, six days per week. Again, a single dose of 2 mg *p*-benzoquinone/kg b.wt. is already four times higher than the maximal dose used in the PLN assay. A different question in this context is, however, whether or not T cells sensitized to benzene metabolites might contribute to the myelotoxicity of benzene through production of myelosuppressive cytokines, such as interferon-γ, or through the activity of hapten-specific cytotoxic T cells. This question is not unreasonable in view of the myelosuppressive potential of activated T cells (32-34), but it has not been explored up to now.

Interestingly, mice which were primed to *p*-benzoquinone and 13 weeks later received an injection of 1 nmol benzene into the same hindfoot pad mounted a secondary response. This was unexpected, as benzene itself failed to

induce a primary response in the PLN assay. A possible explanation for this finding is that a certain amount of the benzene used for challenge was metabolically converted into *p*-benzoquinone in the anatomical region ranging from the injection site to the draining PLN. As a dose of 0.1 nmol *p*-benzoquinone per mouse was able to elicit specific secondary PLN responses in *p*-benzoquinone-primed mice, metabolic conversion of 10% of the benzene dose (1 nmol/mouse) used for challenge would be sufficient for elicitation of the anamnestic response observed. This hypothesis raises the question which extrahepatic enzymes are able to convert benzene into hydroquinone and further hydroquinone into *p*-benzoquinone. Benzene can be oxidized to hydroquinone by cytochrome P450 isoenzyme 2E1 which is constitutively expressed in murine skin (35). The conversion of hydroquinone to *p*-benzoquinone can be catalyzed by prostaglandin H synthase (PGHS) which is found in almost every mammalian tissue including lymph nodes and skin (36,37). Involvement of PGHS in benzene metabolism has been reported by Gaido and Wierda (30) and Pirozzi *et al.* (38), who noted that the myelotoxicity exerted by benzene and hydroquinone was ameliorated by PGHS inhibitors. Direct PGHS-driven metabolic conversion of hydroquinone into reactive metabolites was described by Schlosser and colleagues (39,40). Another issue relevant in this context is that activation of antigen-specific T cells is known to require a minimal number of identical epitopes on a given antigen-presenting cell (41,42). Apparently, local injection of *p*-benzoquinone is able to generate this threshold number of identical neoantigens. However, it is unknown whether or not this threshold number can also be reached after systemic exposure to benzene and local metabolism to *p*-benzoquinone.

Our results do not provide information as to the nature of neoantigens that are recognized by T cells specific for *p*-benzoquinone. In view of its low molecular weight and strong protein reactivity (5,21), *p*-benzoquinone is supposed to act as a hapten and as such follow the rules laid down for T cell recognition of the classical haptens trinitrochlorobenzene and trinitrobenzene sulfonic acid (43,44). These electrophiles can covalently bind to the nucleophilic amino acid lysine in protein, thereby forming trinitrophenyl (TNP)-adducts (45). It has been established that both $CD4^+$ and $CD8^+$ TNP-specific T cells recognize TNP when coupled to lysine in MHC-embedded self-peptides. The latter were found to carry lysine in a position such that the TNP coupled to it could make direct contact with the T cell receptor for antigen recognition (45). T cell

recognition of the hapten 3-pentadecyl-catechol, which is a major allergen in poison ivy, follows the rules established for TNP. The catechol component of this hapten can spontaneously be oxidized to the electrophilic quinone which, like *p*-benzoquinone, shows great affinity for covalent bonding to cysteine, another nucleophilic amino acid (46). For *p*-benzoquinone, adducts to rat liver protein and mouse hemoglobin sulfhydryl groups have been detected using specific antibodies and hapten-specific T cell clones, respectively (47,48). Characterization of the neoantigens generated by *p*-benzoquinone cannot be achieved by experiments *in vivo*, as performed in the PLN assay, but requires hapten-specific T cell clones for analysis *in vitro*. Such experiments are now in progress in our laboratory (48).

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References

1. **Chepiga, T. A., C. S. Yang, and R. Snyder**. 1991. Benzene metabolism in two purified, reconstituted rat hepatic mixed function oxidase systems. In *Biological reactive intermediates IV. Molecular and cellular effects and their impact on human health*. C.M. Wittmer, R. Snyder, D.J. Jollow, G.F. Kalf, J.J. Kocsis and I.G. Sipes, eds. Plenum Press, New York, London, p. 261-265.

2. **Nakajima, T., E. Elovaara, S. S. Park, H. V. Gelbain, E. Hietanen, and H. Vainio**. 1990. Monoclonal antibody-directed characterization of benzene, ethoxy-resorufin and pentoxy-resorufin in metabolism in rat liver microsomes. *Biochem.Pharmacol. 40*: 1255-1261.

3. **Johansson, I. and M. Ingelman-Sundberg**. 1988. Benzene metabolism by ethanol-, acetone-, and benzene-inducible cytochrome P-450 IIEI in rat and liver microsomes. *Cancer Res. 48*: 5387-5390.

4. **Kalf, G. F.** 1987. Recent advances in the metabolism and toxicity of benzene. *Crit.Rev.Toxicol. 18*: 141-159.

5. **Sun, J. D., M. A. Medinsky, and L. S. Birnbaum**. 1990. Benzene hemoglobin adducts in mice and rats: characterization of formation and physiological modeling. *Fund.Appl.Toxicol. 15*: 468-475.

6. **Longacre, S. L., J. J. Kocsis, and R. Snyder**. 1981. Influence of strain differences in mice on the metabolism and toxicity of benzene. *Toxicol.Appl.Pharmacol. 60*: 398-409.

7. **Irons, R. D., J. G. Dent, T. S. Baker, and D. E. Rickert**. 1980. Benzene is metabolized and covalently bound in bone marrow in situ. *Chem.Biol.Interact. 30*: 241-245.

8. **Ganousis, L. G., D. Goon, T. Zyglewska, K. K. Wu, and D. Ross**. 1992. Cell-specific metabolism in mouse bone marrow stroma: studies of activation and detoxification of benzene metabolites. *Mol.Pharmacol. 42*: 1118-1125.

9. **Tunek, A., B. Högstedt, and T. Olofsson**. 1982. Mechanism of benzene toxicity. Effects of benzene and benzene metabolites on bone marrow cellularity, number of granulopoietic stem cells and frequency of micronuclei in mice. *Chem.Biol.Interact. 39*: 129-138.

10. **MacEachern, L. and D. L. Laskin**. 1992. Increased production of tumor necrosis factor-alpha by bone marrow leukocytes following benzene treatment of mice. *Toxicol.Appl.Pharmacol. 113*: 260-266.

11. **Benezra, C., C. C. Sigman, L. R. Perry, C. T. Helmes, and H. I. Maibach**. 1985. A systematic search for structure-activity relationships of skin contact sensitizers: methodology. *J.Invest.Dermatol. 85*: 351-356.

12. **Basketter, D. A. and B. F. J. Goodwin**. 1988. Investigation of the prohapten concept: Cross reactions between 1,4-substituted benzene derivatives in the guinea pig. *Contact Dermatitis 19*: 248-253.

13. **Basketter, D. A. and C. Lidén**. 1992. Further investigation of the prohapten concept: reactions to benzene derivates in man. *Contact Dermatitis 27*: 90-97.

14. **Bloksma, N., M. Kubicka-Muranyi, H.-C. Schuppe, E. Gleichmann, and H. Gleichmann**. 1995. Predictive immunotoxicological test systems: Suitability of the popliteal lymph node assay in mice and rats. *Crit.Rev.Toxicol. 25*: 369-396.

15. **von Schmiedeberg, S., U. Hanten, C. Goebel, H.-C. Schuppe, J. Uetrecht, and E. Gleichmann**. 1996. T cells ignore the parent drug propylthiouracil but are sensitized to a reactive metabolite generated in vivo. *Clin.Immunol.Immunopathol. 80*: 162-170.

16. **Goebel, C., M. Kubicka-Muranyi, T. Tonn, J. Gonzalez, and E. Gleichmann**. 1995. Phagocytes render chemicals immunogenic: oxidation of gold(I) to the T cell-sensitizing gold(III) metabolite generated by mononuclear phagocytes. *Arch.Toxicol. 69*: 450-459.

17. **Klinkhammer, C., P. Popowa, and H. Gleichmann**. 1988. Specific immunity to the diabetogen streptozotocin: cellular requirements for induction of lymphoproliferation. *Diabetes 37*: 74-80.

18. **Miltenyi, S., W. Müller, W. Weichel, and A. Radbruch**. 1990. High gradient magnetic cell separation with MACS. *Cytometry 11*: 231-238.

19. **Kubicka-Muranyi, M., R. Goebels, C. Goebel, J. Uetrecht, and E. Gleichmann**. 1993. T lymphocytes ignore procainamide, but respond to its reactive metabolites in peritoneal cells: demonstration by the adoptive transfer popliteal lymph node assay. *Toxicol.Appl.Pharmacol. 122*: 88-94.

20. **Schuhmann, D., M. Kubicka-Muranyi, J. Mirtschewa, J. Günther, P. Kind, and E. Gleichmann**. 1990. Adverse immune reactions to gold: I. Chronic treatment with an Au(I) drug sensitizes mouse T cells not to Au(I), but to Au(III) and induces autoantibody formation. *J.Immunol. 145*: 2132-2139.

21. **Snyder, R., E. W. Lee, and J. J. Kocsis**. 1978. Binding of labeled metabolites to mouse liver and bone marrow. *Res.Comm.Chem.Pathol.Pharmacol. 20*: 191-194.

22. **Landsteiner, K. and J. Jacobs**. 1936. Studies on the sensitization of animals with simple chemical compounds. *J.Exp.Med. 64*: 625-639.

23. **Schmidt, R. J., L. Y. Chung, and J. Hadgraft**. 1993. New perspectives on prohapten activation in the preimmunologic phase of allergic contact dermatitis. In *Prediction of percutaneous penetration*. K.R. Brain, V.J. James and K.A. Walters, eds. STS Publishing, Cardiff, p. 670-678.

24. **Dupuis, G. and C. Benezra**. 1982. Allergic contact dermatitis to simple chemicals. In *Dermatology*. 1 ed. C.D. Calnan and H.I. Maibach, eds. Dekker,M., New York/Basel, p. 66-69.

25. **Goebel, C., C. Vogel, B. Sachs, J. Abel, and E. Gleichmann**. 1997. Procainamide treatment induces inflammatory prostaglandine H synthase in and formation of immunogenic metabolites by macrophages. *Naunyn-Schmiedebergs Arch.Pharmacol. 355(suppl)*: R136.(Abstract)

26. **Gleichmann, H. I., S. T. Pals, and T. Radaszkiewicz**. 1983. T cell-dependent B cell proliferation and activation induced by administration of the drug diphenylhydantoin to mice. *Hematol.Oncol. 1*: 165-176.

27. **Hurtenbach, U., H. Gleichmann, N. Nagata, and E. Gleichmann**. 1987. Immunity to D-penicillamin: genetic, cellular, and chemical requirements for induction of popliteal lymph node enlargement in the mouse. *J.Immunol. 139* : 411-416.

28. **Kammüller, M. E., C. Thomas, J. M. Bakker de, N. Bloksma, and W. Seinen**. 1989. The popliteal lymph node assay in mice to screen for the immune disregulating potential of chemicals - a preliminary study. *Int.J.Immunopharmacol. 11*: 293-300.

29. **Lidén, C.** 1984. Occupational dermatoses at a film laboratory. *Contact Dermatitis 10*: 77-87.

30. **Gaido, K. and D. Wierda**. 1987. Suppression of bone marrow stromal cell function by benzene and hydroquinone is ameliorated by indomethacin. *Toxicol.Appl.Pharmacol. 89*: 378-390.

31. **Rao, G. S., S. M. Siddiqui, K. P. Pandya, and R. Shanker**. 1988. Relative toxicity of metabolites of benzene in mice. *Vet.Hum.Toxicol. 30*: 517-520.

32. **Tong, J., A. Bacigalupo, G. Piaggio, O. Figari, G. Sogno, and A. Marmont**. 1991. In vitro response of T cells from aplastic anemia patients to antilymphocyte globulin and phytohemagglutinin: colony-stimulating activity and lymphokine production. *Exp.Hematol. 19*: 312-316.

33. **Zoumbos, N. C., P. Gascon, J. Y. Djeu, and N. S. Young**. 1985. Interferon is a mediator of hemopoietic suppression in aplastic anemia in vitro an possibly in vivo. *Proc.Natl.Acad.Sci.USA 82*: 188-192.

34. **Car, B. D., V. M. Eng, B. Schnyder, M. LeHir, A. N. Shakhov, G. Woerly, S. Huang, M. Aguet, T. D. Anderson, and B. Ryffel**. 1995. Role of interferon-gamma in interleukin 12-induced pathology in mice. *Am.J.Pathol. 147*: 1693-1707.

35. **Jugert, F. K., R. Agarwal, A. Kuhn, D. R. Bickers, H. F. Merk, and H. Mukhtar**. 1994. Multiple cytochrome P450 isoenzymes in murine skin: induction of P450 1A, 2B, 2E and 3A by dexamethasone. *J.Invest.Dermatol. 102*: 970-975.

36. **Degen, G. H.** 1993. Prostaglandin-H synthase containing cell lines as tools for studying metabolism and toxicity of xenobiotics. *Toxicology 82*: 243-256.

37. **Eling, T. E., D. C. Thompson, G. L. Foureman, J. F. Curtis, and M. F. Hughes**. 1990. Prostaglandin H synthase and xenobiotic oxidation. *Annu.Rev.Pharmacol.Toxicol. 30*: 1-45.

38. **Pirozzi, S. J., M. J. Schlosser, and G. F. Kalf**. 1989. Prevention of benzene-induced myelotoxicity and prostaglandin synthesis in bone marrow of mice by inhibitors of prostaglandin H synthase. *Immunopharmacology 18*: 39-55.

39. **Schlosser, M. J., R. D. Shurina, and G. F. Kalf**. 1990. Prostaglandin H synthase catalyzed oxidation of hydroquinone to a sulfhydril-binding and DNA-damaging metabolite. *Chem.Res.Toxicol. 3*: 333-339.

40. **Schlosser, M. J. and G. F. Kalf**. 1989. Metabolic activation of hydroquinone by macrophage peroxidase. *Chem.Biol.Interact. 72*: 191-207.

41. **Harding, C. V. and E. R. Unanue**. 1990. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. *Nature 346*: 574-576.

42. **Demotz, S., H. M. Grey, and A. Sette**. 1990. The minimal number of class II MHC-antigen complexes needed for T cell activation. *Science 249*: 1028-1030.

43. **Cavani, A., C. J. Hackett, K. J. Wilson, J. B. Rothbard, and S. I. Katz**. 1995. Characterization of epitopes recognized by hapten-specific CD4+ T cells. *J.Immunol. 154*: 1232-1238.

44. **Kohler, J., U. Hartmann, R. Grimm, U. Pflugfelder, and H. U. Weltzien**. 1997. Carrier-independent hapten recognition and promiscuous MHC restriction by CD4 T cells induced by trinitrophenylated peptides. *J.Immunol. 158*: 591-597.

45. **Weltzien, H. U., C. Moulon, S. Martin, E. Pardovan, U. Hartmann, and J. Kohler**. 1996. T cell immune responses to haptens. Structural models for allergic and autoimmune reactions. *Toxicology 107*: 141-151.

46. **Liberato, D. J., V. S. Byers, R. G. Dennick, and N. Castagnoli**. 1985. Regiospecific attack of nitrogen and sulfur nucleophiles on quinones derived from poison ivy/oak catechols (urushiols) and analogues as models. *J.Immunol. 139*: 411-416.

47. **Rombach, E. M. and R. P. Hanzlik**. 1997. Detection of benzoquinone adducts to rat liver protein sulfhydryl groups using specific antibodies. *Chem.Res.Toxicol. 10*: 1407-1411.

48. **Pape, T., P. Griem, and E. Gleichmann**. 1998. The hapten 1,4-benzoquinone, a reactive benzene metabolite, renders self-hemoglobin antigenic for T cells. *Arch.Dermatol.Res. 290*: 93.(Abstract)

CHAPTER 4

Procainamide, a Drug Causing Lupus, Induces Prostaglandin H Synthase-2 and Formation of T Cell-Sensitizing Drug Metabolites in Mouse Macrophages

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Procainamide (PA) may cause drug-induced lupus, and its reactive metabolites, hydroxylamine-PA (HAPA) and nitroso-PA are held responsible for this. Here, we show that N-oxidation of PA to these metabolites could take place in macrophages and lead to formation of neoantigens that sensitize T cells. Murine peritoneal macrophages (PMφ) exposed to PA in vitro generated neoantigens related to HAPA, as indicated by 1) their capacity to elicit a specific recall response of HAPA-primed T cells in the adoptive transfer popliteal lymph node (PLN) assay and 2) the appearance of metabolite-bound protein in PA-pulsed PMφ, as determined by Western blot. Analysis of five phase-I enzymes that might be responsible for HAPA formation by PMφ pointed to prostaglandin H synthase-2 (PGHS-2) as a likely candidate. Experimental evidence that PA can be oxidized to HAPA by PGHS was obtained by exposing PA to PGHS in vitro. The resulting metabolites were determined by mass spectral analysis and covalent protein binding in ELISA, respectively. In vitro, PA exposure of PMφ of slow acetylator A/J and fast acetylator C57BL/6 mice failed to show significant strain differences in enzyme mRNA expression, enzyme activities, or formation of HAPA-related neoantigens. By contrast, after long-term PA treatment in vivo only in slow acetylators the PMφ harbored HAPA-related neoantigens and T cells were sensitized

to them. PMφ of fast acetylator C57BL/6 mice only contained HAPA-related neoantigens and their T cells were only sensitized to them if, in addition to long-term PA treatment, their donors had received injections of phorbol myristate acetate (PMA), a known enhancer of oxidative enzymes in phagocytes. In conclusion, PA treatment leads to N-oxidation of PA by enzymes, in particular PGHS-2, present in antigen-presenting cells (APC) and, hence, to generation of neoantigens which sensitize T cells. The enhanced neoantigen formation and T cell sensitization seen in slow acetylators might be explained by their higher concentration of PA substrate that is available for extrahepatic N-oxidation in APC.

Introduction

The antiarrhythmic drug procainamide (PA) is frequently associated with drug-induced lupus, the pathogenesis of which is not understood (1-5). PA is an arylamine, a class of compound widely used in industry and medicine and notorious for its potential to cause allergy and autoimmunity (6) Arylamines can be considered as prohaptens, i.e., before they can act as haptens that form neoantigens and trigger adverse immune reactions, they first have to be *N*-oxidized to chemically reactive hydroxylamines and nitroso derivatives (7-9). The nitroso derivative of PA, nitroso-PA, is capable of covalent bonding to (self-)proteins, whereas PA is not (10). The aqueous solution of the hydoxylamine derivative of PA used in the present investigation actually consists of hydroxylamino-PA (HAPA) and nitroso-PA, because a portion of the former is readily oxidized to the highly reactive nitroso-PA, a compound that is difficult to isolate because of its instability (5,7). For the sake of brevity, this mixture is referred to as $HAPA_{aq}$. In a previous paper, we showed that a single injection of $HAPA_{aq}$ induced a specific T cell response in mice, whereas a single injection of the prohapten PA failed to do so (11). Moreover, T cells from mice sensitized against $HAPA_{aq}$ specifically reacted against homogenized peritoneal cells of syngeneic animals that had received long-term PA treatment, indicating that their peritoneal cells contained HAPA-related neoantigens formed after metabolic conversion of PA to HAPA and nitroso-PA, respectively. We will use the term HAPA-related neoantigens to describe hitherto unknown neoantigens that must have been generated in the presence of HAPA and/or nitroso-PA.

Several groups of phase-I enzymes have been implicated in *N*-oxidation of arylamines, in particular the cytochrome P4501A subfamily and cytochrome P4502D6 (12,13), as well as peroxidases, such as myeloperoxidase (MPO) and prostaglandin H synthase (PGHS), also termed cyclooxygenase (3). Cytochrome P4501A2 and cytochrome P4502D6 are constitutively expressed in the liver. In extrahepatic tissues, including macrophages and other immune cells, mainly cytochrome P4501A1 is expressed (14-16). In neutrophils and monocytes, MPO was shown to *N*-oxidize arylamine drugs, such as PA, to the reactive hydroxylamine and nitroso derivatives (9,17,18). PGHS-1 is constitutively expressed and present in nearly all cell types and tissues. In contrast, PGHS-2, also termed inflammatory PGHS, is expressed only after induction, for instance by LPS, and is confined to certain cell types, especially macrophages (19). Whereas *N*-oxidation by PGHS of arylamines to reactive intermediates such as nitroso derivatives has been described (20), it is unknown whether arylamines can induce PGHS-2, thereby increasing their extrahepatic bioactivation.

While *N*-oxidation converts arylamine prohaptens to haptens and thus toxifies them, the competing *N*-acetylation pathway usually prevents, or retards, formation of reactive metabolites (8). Due to genetic polymorphism of *N*-acetyltransferase-2 (NAT-2) in both humans and mice, carriers of certain alleles show reduced *N*-acetylation capacity for several arylamine substrates, including PA (14,21). In humans, the slow acetylator phenotype is associated with a higher incidence of extrahepatic adverse immune reactions to arylamine drugs (22) and a more rapid development of PA-induced lupus (23) than the fast acetylator phenotype. Consistent with this, in the previous paper (11) we reported that PA treatment of mice for a period of 16 weeks led to the appearance of HAPA-related neoantigens in peritoneal cells of the slow acetylator, but not of the fast acetylator mouse strain studied.

However, it was not determined in the previous paper whether macrophages were among the peritoneal cells carrying the HAPA-related neoantigens and, if so, whether peritoneal macrophages (PM_{φ}) themselves had generated them or whether these came from phagocytosis of proteins that were modified by HAPA- or nitroso-PA generated by other cells. Moreover, it was not investigated which enzymes might be involved in the *N*-oxidation of PA to HAPA in macrophages and whether the HAPA-related neoantigens detectable after chronic PA treatment of slow acetylators would, indeed, sensitize their T cells. Here, using RT-PCR and tests of enzymatic activity for analysis of drug-

metabolizing enzymes, and the murine popliteal lymph node (PLN) assay for assessment of T cell sensitization (11,24), we tried to answer these questions. We provide novel experimental evidence for *1)* induction of PGHS-2 mRNA expression and PGE_2 synthesis in PA-treated PMφ, *2) N*-oxidation by PGHS of PA to HAPA, *3)* generation of HAPA-related neoantigens in PA-treated PMφ, *4)* spontaneous T-cell sensitization to the HAPA-related neoantigens formed during chronic PA treatment in vivo, and *5)* the slow *N*-acetylator genotype as a predisposing factor for in vivo generation of HAPA-related neoantigens and T cell sensitization to them.

Materials and Methods

Mice Specific pathogen-free female C57BL/6J mice and female A/J mice were purchased from Harlan Olac Ltd. (Bicester, Oxon, UK). Animals were kept under specific pathogen-free conditions until the onset of the experiments. They had free access to a standard diet (no. 1324, Altromin GmbH, Lage, FRG) and tap water and were 6 to 8 weeks-old at the onset of the experiments. C57BL/6 mice are fast acetylators, whereas A/J mice are slow acetylators (25).

Chemicals and media PA, PMA, *N*-acetyl-PA, and casein were purchased from Sigma Chemicals (Taufkirchen, Germany); PA was obtained as PA/HCl. HAPA was prepared as previously described (7). In aqueous solution, HAPA undergoes spontaneous oxidation by molecular oxygen with approximately 50% conversion to nitroso-PA in 30 min at room temperature (7). We refer to the preparation of HAPA in pyrogen-free saline (0.9 %; Fresenius AG; Bad Homburg, Germany) as $HAPA_{aq}$ here. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained at 99.9 % purity from Oekometrie (Bayreuth, Germany) and kept as a stock solution in DMSO. Sterile, pyrogen-free phosphate-buffered saline (pH 7.2), RPMI 1640 medium, FCS, penicillin, streptomycin, pyruvate, and non-essential amino acids were purchased from Gibco (Eggenstein, Germany).

Preparation and cultivation of PMφ Prospective donors of PMφ were injected ip (500 µL) with sterile 6 % casein solution in distilled water (pH 7.4). Four days after the injection, mice were killed by CO_2 anesthesia and their cells collected by peritoneal lavage with 5 ml of ice-cold phosphate-buffered saline (pH 7.2). Cells were washed and suspended in RPMI 1640 medium (supplemented with 10 % FCS, 200 IU penicillin/streptomycin, 2 mmol/500 mL of L-glutamine, pyruvate, and nonessential amino acids) and cultured at a density of 1×10^6

cells/mL in plastic tissue culture flasks (500 mL) (Greiner, Frickenhausen, Germany). After 20 h, nonadherent cells were removed by vigorous washing; about 90 % of adherent cells were macrophages as determined by Pappenheim's stain.

PA treatment of PMφ in vitro After removal of nonadherent cells, PMφ were incubated in culture medium (see above) in the absence or presence of either 1 mM PA, 1 mM *N*-acetyl-PA, 1 μg/mL LPS plus 25 U/mL IFN-γ, or 10 nM TCDD. After 48 h, PMφ were washed extensively and used either for injection as antigenic material in the PLN assay, total RNA extraction and RT-PCR analysis, or Western blot analysis.

Preparation of homogenates of PA-treated PMφ for use in the PLN assay Once incubation of PMφ with PA was completed, cells were washed in phosphate-buffered saline (pH 7.2) and harvested using a rubber policeman (Greiner). Cells (5×10^6 in 1 mL of phosphate-buffered saline, pH 7.2) were stored in liquid nitrogen. Prior to injection, cells were kept on ice and homogenized by ultrasonication (10 x 10 sec at 50 kHz) using a Labsonic V 2000 (B. Braun Melsungen AG, Melsungen, Germany). Homogenized PMφ (50 μL) were used, because live cells, when transferred, might produce immunomodulators, such as PGE_2 (19) , that could affect the transferred T cells or the recipient tissue in an antigen-independent fashion and thus increase nonspecific background values in the PLN assay.

Direct PLN assay The assay was performed as described (11,24). Test compounds were dissolved in saline; homogenates of PMφ (see above) were prepared in phosphate-buffered saline (pH 7.2). On day 0, animals received a single sc injection (50 μL) of the test substance into the left hindfoot pad. On day 6, mice were sacrificed, and the PLN from both the treated and the untreated side were removed. Cell numbers in the individual PLN were counted using a CASY 1 TT automatic counter (Schaerfe System, Reutlingen, Germany). The PLN cell count index of each mouse was calculated by dividing the cell number obtained from the treated side by that obtained from the control side of the same animal.

Treatment of T cell donors Prior to the adoptive cell transfer, prospective T cell donors were treated with either $HAPA_{aq}$ or PA according to one of the following protocols, as reported previously (11). **$HAPA_{aq}$**: At intervals of 2 days, mice received three sc injections (100 μL) of either saline or 8 μmol $HAPA_{aq}$ at the base of tail. One day after the third injection, their spleens were removed for preparation of T cells. **PA**: For a period of 16 weeks, one group of fast acetylator C57BL/6 mice received three sc injections (100 μL) a week, each consisting of 16 μmol PA/ mouse. For the same period of time, another group of

C57BL/6 mice received an i. p. injection of 100 µL PMA/mouse/week in addition to the PA treatment. PMA was kept in a stock solution of 50 % ethanol/saline (1 mg/mL), and prior to use it was diluted with saline to the final of 600 ng/100 µL. Age-matched controls received the same number of injections consisting of saline (given sc) and PMA (given ip). Slow acetylator A/J mice, which fail to tolerate repeated doses of 16 µmol PA (11), were treated with three sc injections (100 µL) of 8 µmol PA/mouse/week, or of saline alone, over the same period of time. Mice were killed and their spleens removed for T cell separation and adoptive transfer one week after the last injection.

Enrichment of donor T cells to be used for adoptive transfer For preparation of T cells, splenic B cells were removed from spleen cell suspensions by using a magnetic cell sorter (MACS) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), as described (11). Minimal purity of the resulting T cell population was 90 %, as determined by staining with FITC-labeled anti-Thy1.2 mAb (Becton Dickinson, San Jose, CA, USA) and analysis in a FACScan flow cytometer (Becton Dickinson). Staining of T cells with PE-labeled anti-L3T4 and anti-Lyt-2 mAb (Becton Dickinson) yielded 50 ± 5 % $CD4^+$ cells and 35 ± 4 % $CD8^+$ cells.

Preparation of homogenates of peritoneal cells from PA-treated donor mice for use in the adoptive transfer PLN assay As described above for treatment of T cell donors (PA protocol), mice were treated by three sc injections of PA or saline a week, and certain groups of C57BL/6 mice received an additional, ip injection of PMA once a week. Two days after the last injection, mice were killed by CO_2 anesthesia and their peritoneal cells prepared by peritoneal lavage as described for PMφ. Collected peritoneal cells were counted and stored in liquid nitrogen until use. Prior to injection the peritoneal cells were sonicated (5×10^7 cell in 1 mL phosphate-buffered saline, pH 7.2), as described for PMφ.

Adoptive transfer PLN assay This test system is suitable for detection of secondary T cell responses to chemicals of low molecular weight (reviewed in (24)). It is based on the principle that specific T cells exposed to a sensitizing compound in the donor animal will, upon adoptive transfer to a syngeneic recipient, respond to small amounts of the sensitizer by a secondary response. When both the transferred T cells and the immunogen to be tested are injected locally, this response manifests itself in the draining PLN. The doses of sensitizing agent used for elicitation of the secondary response do not suffice to elicit a primary PLN response by the recipient mice.

Splenocytes of T cell donor animals were prepared in phosphate-buffered saline (pH 7.2) and T cells enriched, as described above. Prior to transfer to syngeneic mice, donor

T cells were irradiated (2000 rad) *in vitro* using a ^{137}Cs source (Gammacell 2000, Molsgaard, Denmark); this step served to decrease the nonspecific PLN reaction occasionally seen after transfer to syngeneic recipients of unirradiated T cells from recently immunized donors. On day 0, 1 x 10^7 irradiated splenic T cells in 50 µL phosphate-buffered saline (pH 7.2) were injected sc into the left hindfoot pad of syngeneic recipients. One day after the T cell transfer, the mice received a sc injection (50 µL) of the compound to be tested into the same hindfoot pad; these injections contained either 0.15 µmol of PA or HAPA dissolved in saline ($HAPA_{aq}$). Alternatively, the recipients were injected with 2.5 x 10^6 homogenized peritoneal cells, or 2.5 x 10^5 homogenized PMφ, prepared in phosphate-buffered saline (pH 7.2). On day 6, recipient mice were sacrificed, and the PLN cell count index of each mouse was calculated as described for the direct PLN assay.

Reverse transcriptase-polymerase chain reaction (RT-PCR) For each experiment, peritoneal cells from 2 mice were pooled and PMφ isolated by adherence as described above. Total RNA was extracted from PMφ and from liver tissue by modification of the single-step method using TRIzol^{TM} total RNA isolation reagent (Gibco BRL, Eggenstein, Germany) according to manufacturer's instruction, followed by digestion with Rnase-free Dnase I. For cDNA synthesis, 1 µg total RNA was heated in a final volume of 10 µL with 2 µg oligo$(dT)_{15}$ primer for 5 min at 60 °C, chilled on ice, and reversely transcribed in a final volume of 40 µL containing dNTP (1 mM of each), 8 µL 5x M-MLV buffer, 60 units RNase inhibitor (Rnasin, Gibco BRL), 10 mM DTT, and 400 units M-MLV reverse transcriptase. Samples were incubated for 1 h at 37 °C and subsequently denatured for 10 min at 70 °C.

PCR primers were synthesized with a 391 DNA synthesizer (Applied Biosystems, Weiterstadt, Germany) and purified over NAP-5 columns (Pharmacia, Freiburg, Germany). Primer sequences were from published sources or chosen using a primer selection program (Oligo, National Biosciences, Plymouth, MN, USA), and are given in Table 1. PCR was performed in a final volume of 50 µL as follows: 2.5 µL of RT reaction product was added to a PCR mix comprised of 5 µL of 10 x PCR buffer, 200 µM of dNTP (Pharmacia, Freiburg, Germany), 0.2 µM of each primer (sense and antisense), 2.5 units Taq DNA polymerase (Boehringer), and 1 µCi [α-^{32}P] dCTP (Amersham Buchler, Braunschweig, Germany). Amplification was induced using a DNA thermal cycler (Hybaid-Omnigene, MWG-Biotech, Ebersberg, Germany) with the following temperature profile: denaturation: 1 min at 94 °C (first cycle: 4 min); annealing: 1 min (the temperatures are given in Table 1); extension: 1 min at 72 °C (last cycle: 7 min). Amplification of cDNA in the linear range of the PCR reaction was controlled by three different cycle numbers for one cDNA concentration. PCR products were analyzed on 10 % polyacrylamide gels and visualized by autoradiography. For analysis

the respective bands from the autoradiograms were scanned with an OmniMedia scanner (Millipore, Ueberlingen, Germany). Values of enzyme transcription levels were correlated to the expression of β-actin and are given as relative induction. To test the isolated RNA for DNA contamination, 1 μg total RNA instead of synthesized cDNA was tested in the PCR (negative control).

Table 1. Primer Sequences for cDNA Amplification

Gene	Primer sequences	Annealing temp. (°C)	Fragment size (bp)	Cycle no.	Reference
β-*actin*	FP: CTACAATGAGCTGCGTGTGG RP: TAGCTCTTCTCCAGGGAGGA	60	450	20	(*51*)
CYP1A1	FP: CCCACAGCACCACAAGAGATA RP: AAGTAGGAGGCAGGCACAATGTC	62	499	29	(*51*)
CYP1A2	FP: CAAAGACAATGGCGGTCTCA RP: TCCCACTTGGCCGGGATCTC	58	515	21	(*52*)
NAT-2	FP: GGATTGTTTTTCTTGCCTTAG RP: CATACTGCTCTCTTCTGATTT	52	535	30	(*29*)
PGHS-1	FP: ACCACTCGCCTCATCCTTAT RP: GCACACGGAAGGAAACATAG	56	757	26	(*53*)
PGHS-2	FP: ATCCACAGCCTACCAAAACAG RP: AACCTCACAGCAAAAACCTAC	52	1101	30	(*54*)

Determination of PGE_2 The concentration of PGE_2 in supernatants of cultured PMφ (10^6 cells/mL) was assayed by a competitive enzyme immunoassay using monoclonal antibody to PGE_2 (Cayman Chemical, Ann Arbor, MI, USA). The lower detection limit of this assay is at 30 pg/mL.

MPO measurements MPO activity in supernatants of cultured PMφ (1×10^6 cells/mL) was measured by an improved 3,3',5,5'-tetramethylbenzidine (TMB) method using the liquid substrate system (Sigma, Taufkrichen, Germany). As a positive control for MPO activity supernatants of cultured bone marrow cells (1×10^6 cells/mL) were used. Briefly, 10^6 cells/mL were exposed (15 min at 37°C) to cytochalasin B (5 μg/mL) and fMet-Leu-Phe (10^{-6} M) in RPMI 1640 without phenol red (Gibco). Resulting supernatants were assayed for MPO activity as described (26).

HPLC determinations PA (200 μM) was incubated with PGHS (113 units/mL; ovine COX-1, specific activity 49005 units/mg; Cayman Chemical, Ann Arbor, Michigan) in the presence of hematin (1 μM), phenol (2 μM) and either arachidonic acid (100 μM, Cayman

Chemical) or hydrogen peroxide (0.2 mM) in 1 mL phosphate buffer (pH 7.7) for 30 min at 37°C. The mixture from 4 separate incubations was then applied to a Sep-Pak (Chromatographic Specialties; Brockville, Ontario), washed with water, and then eluted with methanol (10 mL). The methanol solution was evaporated with a stream of nitrogen and then reconstituted by adding 0.3 mL of methanol. The 20 µL aliquots were analyzed by LC/MS. The HPLC column was a Prodigy 5µ ODS(3) with dimensions of 2 X 100 mm (Phenomenex, Torrance, CA), and the mobile phase consisted of water, acetonitrile, acetic acid (89:10:1, v/v) containing 2 mM ammonium acetate with a flow rate of 0.2 mL/min and a splitter to decrease the flow rate into the mass spectrometer of ~20 µL/min. The mass spectrometer was a Sciex API III mass spectrometer (Perkin-Elmer, Sciex; Thornhill, Ontario) operated in the Ion Spray mode and selected ion monitoring.

Production of anti-PA serum PA (4.7 mg) was made reactive by dissolving it in hydrochloric acid (1 N, 6 mL) and after cooling the solution in an ice bath, sodium nitrite (1.5 mg in 1 mL of water) was added dropwise with stirring over a period of 10 min. This solution was then added dropwise to keyhole limpet hemocyanin (10 mg in 10 mL of 5 M phosphate buffer, pH 9) with stirring and cooled in an ice bath. The pH of the solution was monitored and sodium bicarbonate (20 %) was added as needed to keep the pH between 8.5 and 9. After 1 h the pH was adjusted to 7 with hydrochloric acid and after extensive dialysis it was lyophilized. Polyclonal anti-PA- keyhole limpet hemocyanin antibodies were raised in a 2 kg male, pathogen free New Zealand White Rabbit (Charles River Ltd., Quebec, Canada) housed in the animal care facility at The Hospital for Sick Children, Toronto. After pre-immune serum was obtained, each animal was immunized with the PA-keyhole limpet hemocyanin conjugate (1 mg in 0.5 mL PBS emulsified with an equal volume of Freund's complete adjuvant) sc at multiple sites. Injections with 500 µg PA-keyhole limpet hemocyanin in Freund's incomplete adjuvant, divided into 6-8 sc sites, were repeated 4, 6, 8 and 12 weeks after the initial immunization. Exsanguination under pentobarbital anesthesia was conducted 10 days after the final immunization. Blood was allowed to clot overnight at 4° C and then centrifuged at 400 g. The serum was recovered and heat inactivated at 56° C for 30 min before being aliquoted and stored at -20° C.

Covalent Binding of PA to PGHS PA (100 µM) was incubated with PGHS (160 units) or hematin (1 µM) in 0.4 mL of phosphate buffer pH 7.7, and H_2O_2 was added to make a final concentration of 1 mM at 37° C. After 60 min 100 µL aliquots were plated into ELISA plates (Costar, Cambridge, MA) and left at 4°C overnight. The plates were then emptied and washed with ELISA wash buffer (10 mM Tris-HCl, pH 7.5, 154 mM NaCl, 0.5 % (w/v) casein and

0.02 % (w/v) thimerosal). This wash was repeated 3 additional times. The plates were then tapped dry and PA- keyhole limpet hemocyanin antiserum (100 µL diluted 1:1000) was added to the plates which were then incubated for 3 h. The plates were washed 4 times with ELISA wash buffer and tapped dry and then alkaline phosphatase-conjugated goat anti-rabbit IgG (100 µL diluted 1:1000; Jackson Immunoresearch Laboratories, West Grove, PA) was added and the plates were incubated for 2 h at room temperature. The plates were again washed 4 times with ELISA wash buffer and 2 times with PBS. A stock solution of methyl umbelliferyl phosphate (10 mg/ml in DMSO) was diluted 1:100 in PBS, and 100 µL of this solution was added to the wells and incubated for 10 min at room temperature. Fluorescence was then measured with a Fluorescence Concentration Analyzer (Pandex, Mundelein, IL) set at 365/450 (excitation/emission).

Preparation of PMφ for Western blot analysis After incubation of C57BL/6 PMφ with PA, *N*-acetyl-PA, or saline (see above), cells were resuspended in lysis buffer (PBS, 0.1% Triton X-100) for 3 min on ice, vortexed for 1 sec and centrifuged at 10,000x g for 3 min to separate cytoplasmic proteins and nuclei. Nuclei were then lysed in SDS-PAGE sample buffer. 1 mM PA was added to one sample of saline-preincubated PMφ lysate. Equal amounts of cytoplasmic and nucleic cell equivalents were separated on SDS-polyacrylamid gels.

Western blot analysis Cytoplasmic proteins or nuclear proteins of 1 x 10^6 cell equivalent were separated on 8% SDS-PAGE (27). Electrophoretic transfer of proteins to nitrocellulose filters (Hybond-C Super, Amersham), which were preincubated with 20% ethanol, 25 mM TrisHCl, 192 mM glycine for 30 min, was performed with a semi-dry transfer apparatus (Biorad). Nitrocellulose filters were blocked with 4% dried non-fat milk powder and 0.5% Tween 20 in PBS (PBS-Tween), pH 8, for 1 h at room temperature. Rabbit anti-PA serum or control serum was diluted 1:3,000 in PBS-Tween containing 4 % dried milk powder and incubated for 1 h at room temperature. After 3 washes with PBS-Tween, filters were incubated with goat anti-rabbit IgG (Dianova, Hamburg, Germany), diluted in PBS-Tween containing 4% dried milk powder for 1 h at room temperature. Immunoreactions were visualized on X-ray films by chemoluminiscence using the enhanced chemoluminescence detection system supplied by Amersham.

Statistical analysis Statistical analyses were performed by analysis of variance (ANOVA). Assays were performed at least twice to ensure reproducibility.

Results

Incubating PMφ with PA in vitro rendered them immunogenic

Previous results have established that a single injection of PA is unable to induce a primary PLN response in either fast acetylator C57BL/6 (11,28) or slow acetylator A/J mice (11). To examine whether macrophages can convert the non-sensitizing PA into a sensitizing metabolite, PMφ were exposed to PA for 48 h in vitro. At a number of 5 x 10^6 cell equivalents, homogenates of PMφ thus treated induced a significant primary PLN response when compared to the homogenates of untreated PMφ (Fig. 1), suggesting a primary immune response toward reactive PA metabolites generated in vitro by PMφ. No difference between slow and fast acetylator strains in the generation of the metabolite-induced neoantigens was observed.

Using the adoptive transfer PLN assay, in the preceding study (11) we found that synthetic, cell-free $HAPA_{aq}$ elicited a specific secondary response by T cells from $HAPA_{aq}$-primed donor mice. Here, we used the specificity of this reaction as a probe to test if the T cell response to PA-treated PMφ was due to HAPA-related neoantigens. As shown in Fig. 2A, challenge of $HAPA_{aq}$-primed donor T cells with either $HAPA_{aq}$ or homogenized, PA-treated PMφ elicited a secondary response. A comparison of fast acetylator C57BL/6 mice and slow acetylator A/J mice did not reveal a strain difference, suggesting that the PA-oxidizing capacity of PMφ from the two strains is similar, at least at the saturating PA concentrations used in vitro. Challenge of T cells from saline-treated donors with either $HAPA_{aq}$ or PA-treated PMφ failed to induce a

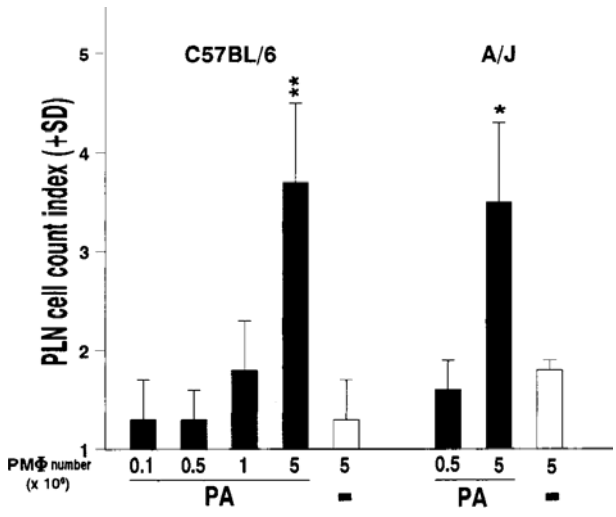


Figure 1. In vitro exposure of PMφ to the non-reactive PA generated neoantigens capable of inducing a primary PLN reaction. PMφ were incubated for 48 h in the presence of 1 mM PA (solid bars) or in culture medium without PA (open bars). Subsequently, cells were frozen and homogenized, and on day 0 syngeneic recipients were injected with the indicated cell number equivalents of PMφ into a hindfoot pad. On day 6, mice were sacrificed and the direct PLN assay was performed. Data represent arithmetic means + SD of 5 mice per group. (*p< 0.05; **p<

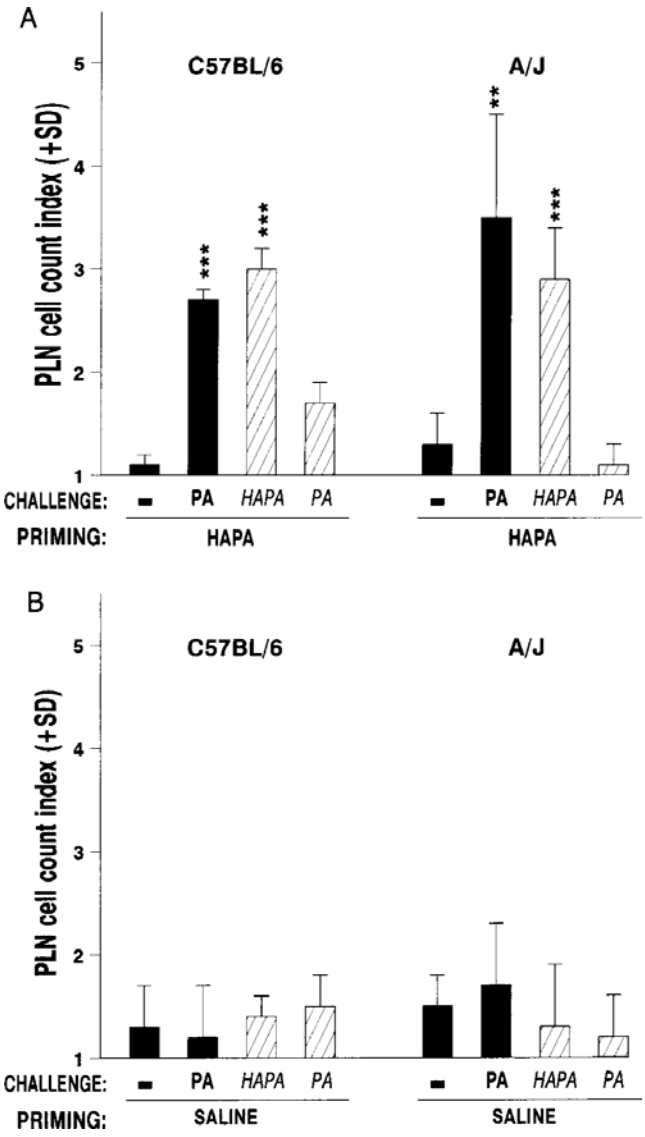
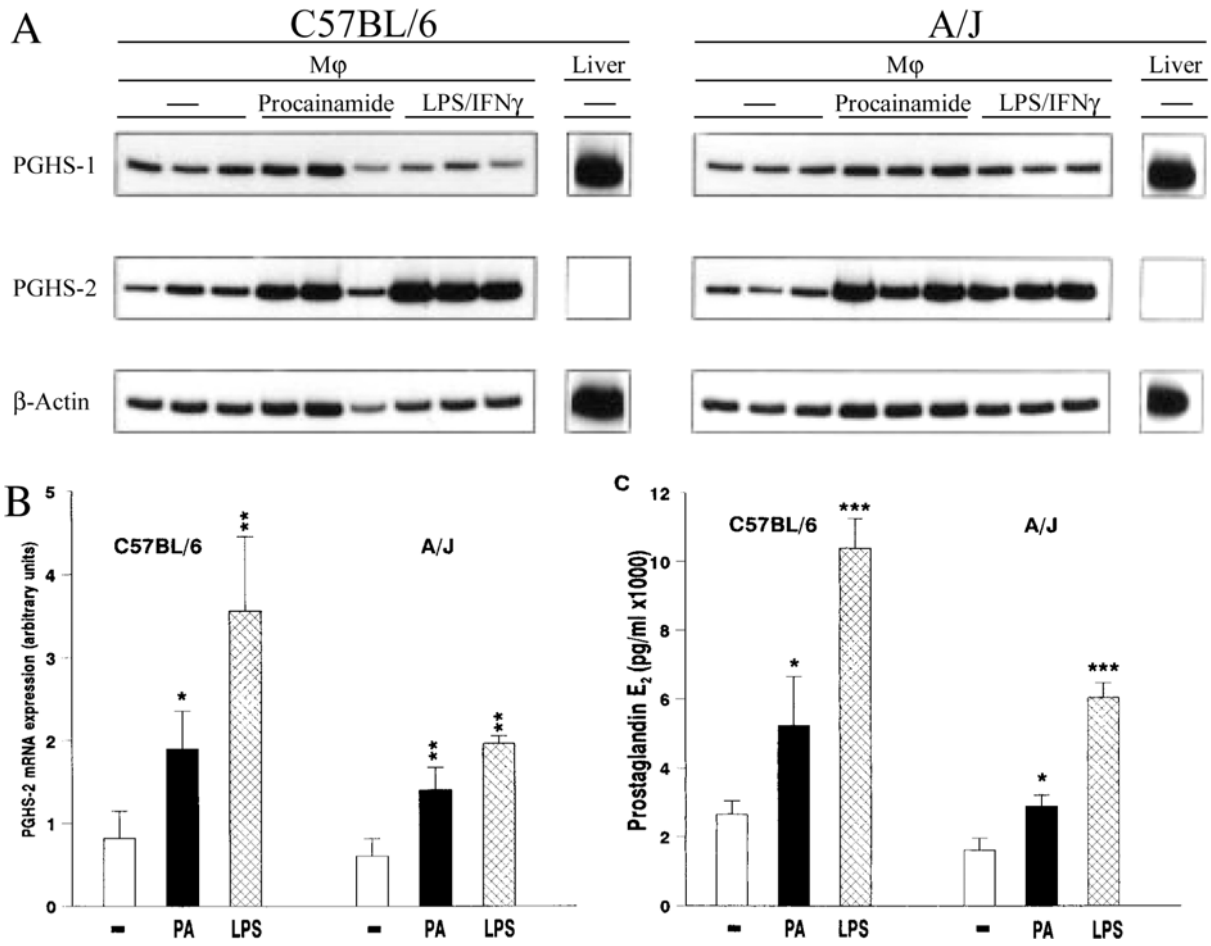


Figure 2. Capacity of in vitro PA-exposed PMφ to evoke a recall response by $HAPA_{aq}$-primed T cells. The adoptive transfer PLN assay was used. Donor T cells (1 x 10^7 cells/recipient) were adoptively transferred to syngeneic recipients by sc injection into one hindfoot pad. One day later, the transferred T cells were challenged by injecting into the same hindfoot pad either 2.5 x 10^5 homogenized PMφ (solid bars), or 0.15 µmol of either PA or $HAPA_{aq}$ indicated in italics (hatched bars) into the same hindfoot pad of recipients. Prior to injection, PMφ of either strain were cultured for 48 h in the absence (-) or presence of 1 mM PA. Data represent arithmetic means + SD of 5 to 7 mice per group; asterisks indicate significant differences between each of the the groups indicated by solid bars and hatched bars, respectively (**p< 0.01; ***p< 0.001). (A) Prospective T cell donor mice of C57BL/6 and A/J strain were primed by s. c. injections of $HAPA_{aq}$ (three dorsal injections of 8 µmol HAPA in saline). (B) T cell donor mice were treated with saline alone.

statistically significant increase of the PLN cell count index (Fig. 2B). This finding shows that PMφ of both strains had generated HAPA-related neoantigens in vitro and, hence, provides indirect evidence for HAPA formation by these cells.

Effect of PA exposure on inducibility of arylamine-metabolizing enzymes in PMφ

The experiments described above provided evidence that PMφ of slow and fast acetylator mice themselves can generate the reactive PA metabolite. In the next series of experiments, we tested whether enzymes capable of *N*-oxidizing or *N*-acetylating arylamines are differentially induced during exposure of PMφ of

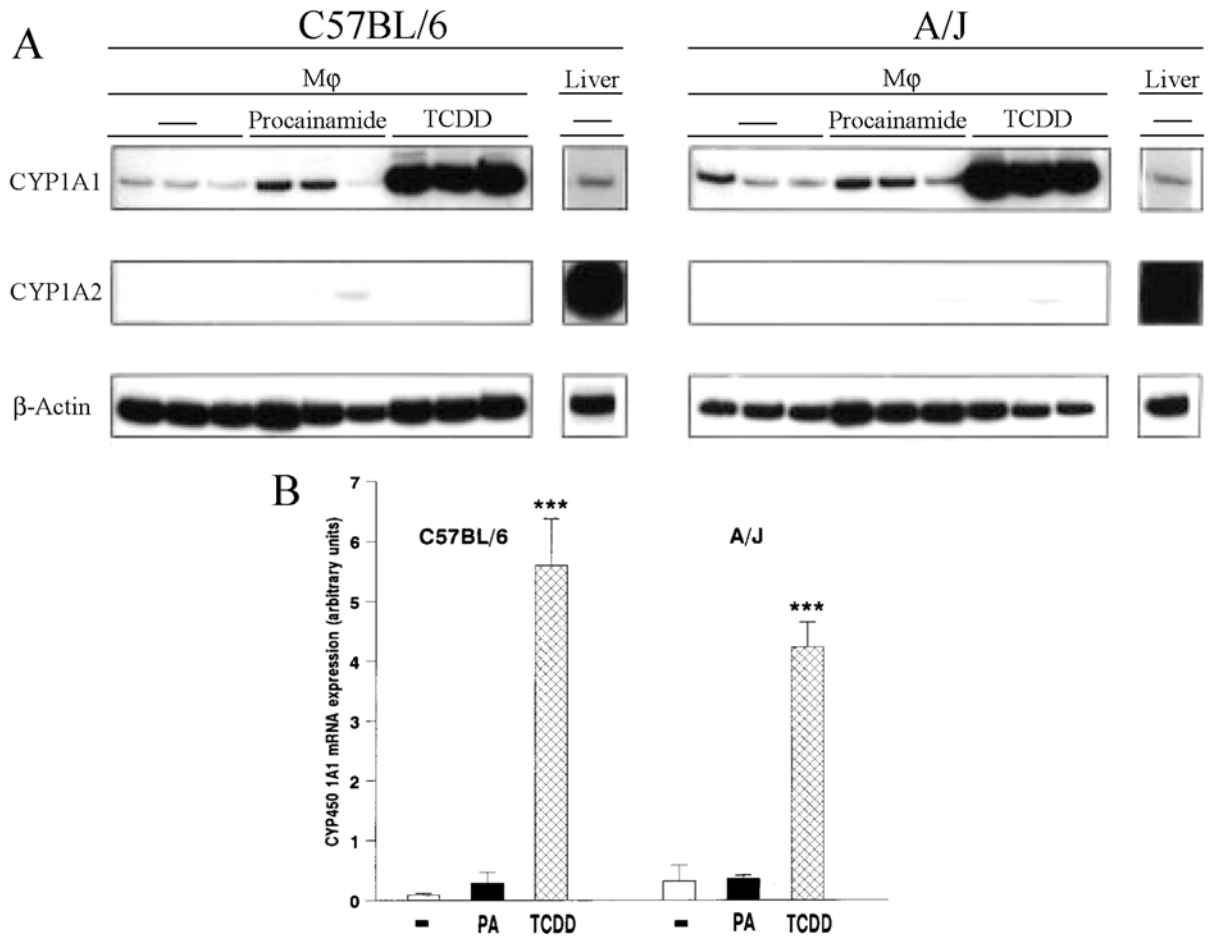


slow and fast acetylator mice to PA. For this purpose, mRNA expression levels

Figure 3. Enhancing effect of PA on PGHS-2 mRNA level in and PGE_2 secretion by PMφ exposed in vitro. (A) Detection by RT-PCR of mRNA expression of PGHS-1 and PGHS-2. PMφ (1 x 10^6 cells/mL) remained untreated or were exposed for 48 h to 1 mM PA or 1μg/mL LPS plus 25 U/mL IFN-γ, as indicated. β-actin mRNA expression by PMφ was studied for control. Radioactive PCR products were seperated on 10% polyacrylamide gels and visualized by autoradiography. (B) Relative changes in mRNA levels of PGHS-2, as quantified by densitometry. Results are given as band intensity ratios of PCR products of PGHS-2 divided by β-actin for untreated PMφ (open bars), PMφ exposed to 1 mM PA (solid bars), or 1μg/mL LPS plus 25 U/mL IFN-γ (cross-hatched bars). (C) Secretion of PGE_2 by PMφ in vitro. After 48h of treatment, supernatants were removed and assayed for PGE_2 levels. Results shown in (B) and (C) represent arithmetic means + SD obtained from three independent cultures of PMφ. Asterisks indicate significant differences compared to the control culture (open bars) of the respective mouse strain (*$p< 0.05$; **$p< 0.01$; ***$p< 0.01$).

of PGHS isoforms, cytochrome P4501A isoforms, and NAT-2 were determined by RT-PCR. In addition, enzymatic activity of MPO and PGHS were monitored, PGHS activity being measured by determination of PGE_2.

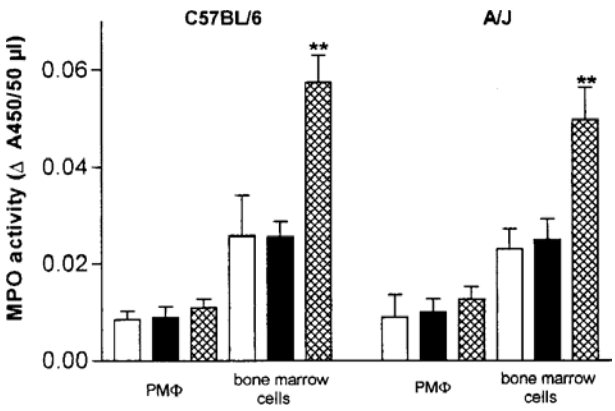
Exposure of PMφ to 1 mM PA for 48 h led to a 2-fold increase in PGHS-2 mRNA levels (Figs. 3A and 3B) and a 1.5-fold increase in PGE_2 secretion (Fig.



3C) when compared with untreated PMφ. In order to assess

Figure 4. Effect of PA on mRNA levels of cytochrome P4501A1 and cytochrome P4501A2 of PMφ exposed in vitro. (A) Detection by RT-PCR of mRNA expression. PMφ remained untreated or were exposed for 48h to 1 mM PA, or to 10 nM TCDD. β-actin mRNA expression was studied for control. mRNA detection was performed as described in the legend to Fig. 3. (B) Relative changes in mRNA levels of cytochrome P4501A1 as quantified by densitometry. Results are given as band intensity ratios of PCR products of cytochrome P4501A1 divided by β-actin for untreated PMφ (open bars), PMφ exposed to 1 mM PA (solid bars), or 10 nM TCDD (cross-hatched bars).

maximal inducibility of the enzyme during the 48 h culture period, PMφ were exposed to 1 μg LPS plus 25 U IFN-γ. mRNA levels of PGHS-2 in PMφ thus treated and the concentration of PGE_2 in their supernatants were increased 1.5 to 2-fold when compared with PA-exposed PMφ and 3 to 4-fold compared with untreated cells. In PMφ of C57BL/6 mice basal and inducible expression of PGHS-2 mRNA and PGE_2 were elevated compared to A/J mice. Exposure of PMφ to PA had no detectable effect on the mRNA level of the constitutively expressed isoenzyme PGHS-1 (Fig. 3A). In control experiments with liver tissue

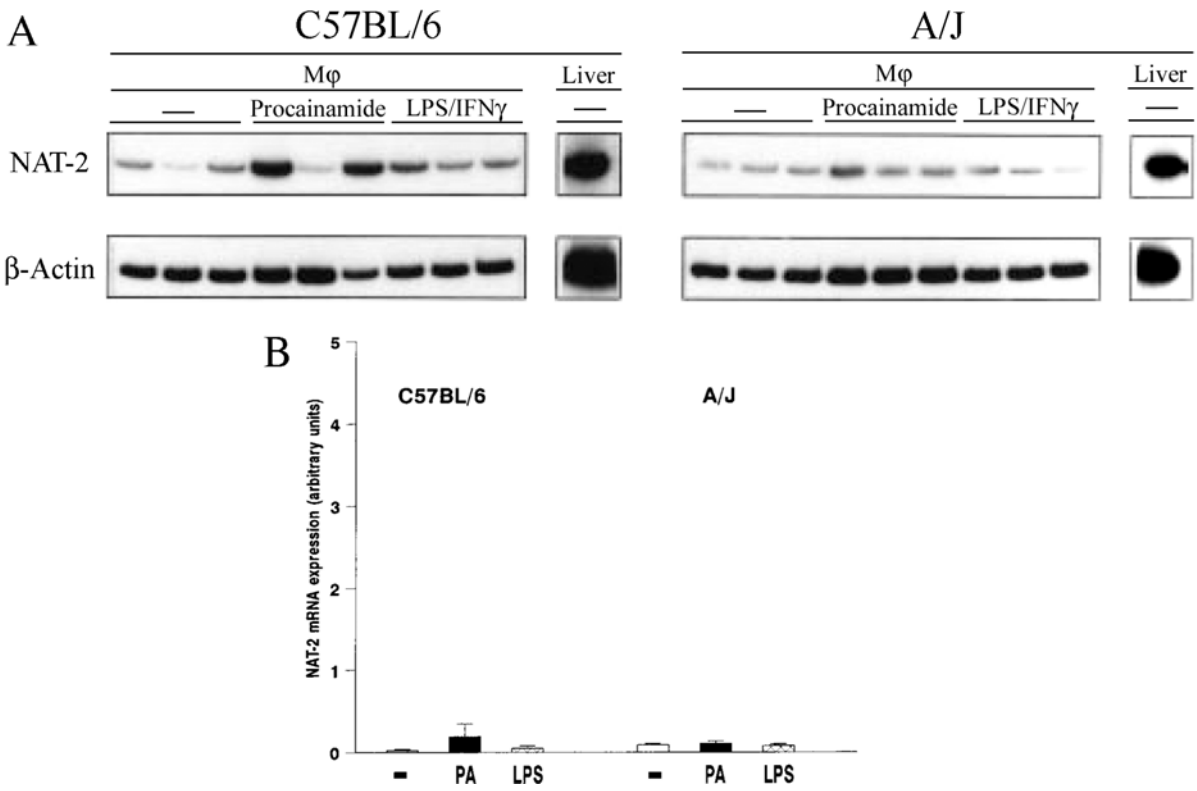


of untreated mice of both strains constitutive expression of PGHS-1 mRNA was detectable, whereas PGHS-2 mRNA was not expressed (Fig. 3A).

Figure 5. Comparing the induction of MPO activity in bone marrow cells and PMφ. Cells (1 x 10^6 cells/mL) were either cultured in medium only (open bars), in the presence of 1 mM PA (solid bars), or in the presence of 1 µg/mL LPS plus 25 U/mL IFN-γ (cross-hatched bars). After 48 h, supernatants were removed and assayed for MPO activity. Results represent arithmetic means + SD of three independent experiments. Asterisks indicate significant difference compared with the control culture (open bar) of the respective cell type and mouse strain (**p< 0.01).

Next, we tested the effect of in vitro PA exposure of PMφ on mRNA expression of cytochrome P4501A1 and cytochrome P4501A2, two candidates for bioactivation of arylamines to reactive species, showing overlapping substrate specificity. Relatively low expression of cytochrome P4501A1 mRNA was detected in untreated PMφ and PA-exposed PMφ, as opposed to the vigorous induction seen after exposure to 10 nM TCDD (Fig. 4). No strain differences were observed between C57BL/6 and A/J mice with regard to inducibility of cytochrome P4501A1 mRNA by PA and TCDD, respectively. As expected, cytochrome P4501A2 mRNA was clearly expressed in liver tissue of untreated mice. In PMφ, exposure to 1 mM PA or 10 nM TCDD failed to induce detectable cytochrome P4501A2 mRNA expression (Fig. 4).

We extended the analysis of enzymes that might be responsible for PA oxidation in PMφ by measuring MPO activity in supernatants of cultured PMφ (Fig. 5). Bone marrow cells were chosen to positively control the assay system, because they are rich in MPO-positive cells, such as granulocytes and monocytes. We found that basal MPO activity in supernatants of bone marrow cells was 2- to 3-fold higher than that of PMφ and that no significant strain difference was detectable between A/J and C57BL/6 mice. Whereas exposure to LPS plus IFN-γ failed to affect the MPO activity of PMφ, in bone marrow cells this in vitro treatment induced a 2.5-fold increase in MPO activity. PA, however, failed to induce MPO in either cell type. The enzyme NAT catalyzes *N*-acetylation of arylamines, such as PA (21,29). In vitro exposure of PMφ to PA



had no significant effect on the expression of NAT-2 mRNA in either strain tested (Fig. 6).

Figure 6. Effect of PA on mRNA levels of NAT-2 in PMφ in vitro. (A) Detection by RT-PCR of mRNA expression. PMφ were treated as described in the legend to Fig. 3. (B) Relative changes in mRNA levels of cytochrome P4501A1 as quantified by densitometry. Results are given as band intensity ratios of PCR products of NAT-2 divided by β-actin for untreated PMφ (open bars), PMφ exposed to 1 mM PA (solid bars), or 10 nM TCDD (cross-hatched bars). Asterisks indicate significant differences compared to the control culture (open bars) of the respective mouse strain (***$p < 0.01$).

Table 2. *N*-oxidation of PA and covalent binding to PGHS

	Constituents of the incubation mixture prior to assay[a]				
	PA PGHS Arachidonic acid / H_2O_2	PA PGHS - -	PA Albumin Hematin H_2O_2	PA Albumin - H_2O_2	- Albumin Hematin H_2O_2
Formation of HAPA[b] (μM)	0.0183 ± 0.003	< 0.001	n.d.	n.d.	n.d
Covalent binding[c] (fluorescence$_{365/450}$)	37,108 ± 1,572	2,749 ± 160	41,794 ± 1,369	5,991 ± 1,015	2,064 ± 30

[a] Values represent mean ± SD, [b] Determined by mass spectroscopy, [c] Determined by ELISA

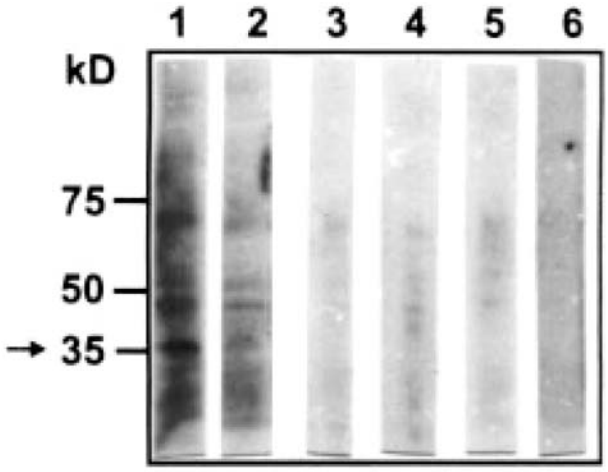
Taken together, analysis of arylamine-metabolizing enzymes in PMφ in vitro revealed that under the experimental conditions used *1)* no significant differences between strains C57BL/6 and A/J were detectable and *2)* out of the five *N*-oxidizing enzymes tested only PGHS-2 was inducible by PA.

PGHS is able to *N*-oxidize PA

The combination of PGHS and either arachidonic acid or hydrogen peroxide oxidized PA to HAPA and nitro derivatives. Under the HPLC conditions described in the Methods section, the retention time of HAPA was 1.5 min and that of the nitro derivative was 6.7 min. There was another peak in the ion current at m/z 252 with a retention time of 2.5 minutes; we suspect that this is the *N*-oxide of the tertiary amine, but this was not confirmed. As shown in Table 2, the concentration of HAPA in the incubations (n = 4) was 0.0183 ± 0.003 μM, while the level in the controls in which arachidonic acid was omitted from the incubation was less than 0.001 μM. The formation of HAPA was dependent upon both PGHS and either arachidonic acid or hydrogen peroxide. The identity of HAPA was confirmed by MS/MS and comparison with the MS/MS of synthetic hydroxylamine: the major fragments were at m/z 179, 162, and, 136 with smaller peaks at m/z 120 and 100. The peak due to HAPA also disappeared on addition of NaOH (to raise the pH above 10) which is characteristic of the hydroxylamine (30).

Covalent binding of metabolized PA

As shown in Table 2, PA was found to covalently bind to PGHS. The ELISA fluorescence from 3 experiments was 37,108 ± 1,572 while that of the control in which H_2O_2 was omitted from the incubation was found to be 2,749 ± 160. In order to make sure that the oxidation was not due simply to H_2O_2, another set of incubations was performed in which PGHS was replaced by bovine serum albumin (20 mg); in this case no significant binding ensued (fluorescence = 5,991 ± 1,015). However, the combination of hematin and H_2O_2 did lead to covalent binding of PA to the albumin (fluorescence = 41,794 ± 1,369). We



conclude that incubation of PA with PGHS or hematin results in the cooxidative activation of PA to a metabolite capable of covalent binding to protein.

Figure 7. Neoantigen formation in PA-treated PMφ. The cytoplasmic fraction of PMφ preincubated with either 1 mM PA (lane 1, 2 and 6), saline (lane 3 and 4), or 1 mM *N*-acetyl-PA (lane 5) were analyzed for neoantigen formation. After homogenization, one of the saline control fractions was incubated with PA (lane 4). The PA preincubated cell-lysates were stained with anti-PA serum (lane 1), anti-PA serum blocked with 4 mM PA (lane 2) or control serum (lane 6). Lanes 3, 4 and 5 were stained with anti-PA serum.

Neoantigen formation in PA-treated PMφ

As shown in Fig. 7, the cytoplasmic fraction of PA-preincubated PMφ (lane 1) gave a distinct band at 35 kD when stained with antiserum against PA, but no band when stained with control antiserum (lane 6). In the nuclear fraction, no band was seen (data not shown). Blocking of the anti-PA serum with 4 mM PA prior to staining in Western blot inhibited detection of the band at 35 kD (lane 2). Neither incubation of the PMφ with saline (lane 3) or *N*-acetyl-PA (lane 5), nor incubation of homogenated PMφ with PA (lane 4) showed a band at 35 kD. These results indicate that a putative neoantigen was formed after in vitro incubation of live PMφ with PA.

Chronic PA treatment sensitized T cells to HAPA-related neoantigens in slow, but not in fast acetylator mice

In a previous investigation, we found that PA treatment of slow acetylator A/J mice over a period of 4 months led to the formation of HAPA-related neoantigens detectable in peritoneal cells, whereas peritoneal cells of chronically PA-treated fast acetylator C57BL/6 mice failed to contain these antigens (11). Here, we asked whether the HAPA-related neoantigens induced by chronic PA treatment had sensitized T cells in vivo. Therefore, T cell recall responses were analyzed in the adoptive transfer PLN assay. Two types of antigen were used for

T cell challenge: *1)* cell-free, synthetic $HAPA_{aq}$ for control, and *2)* peritoneal cells from syngeneic mice chronically treated with

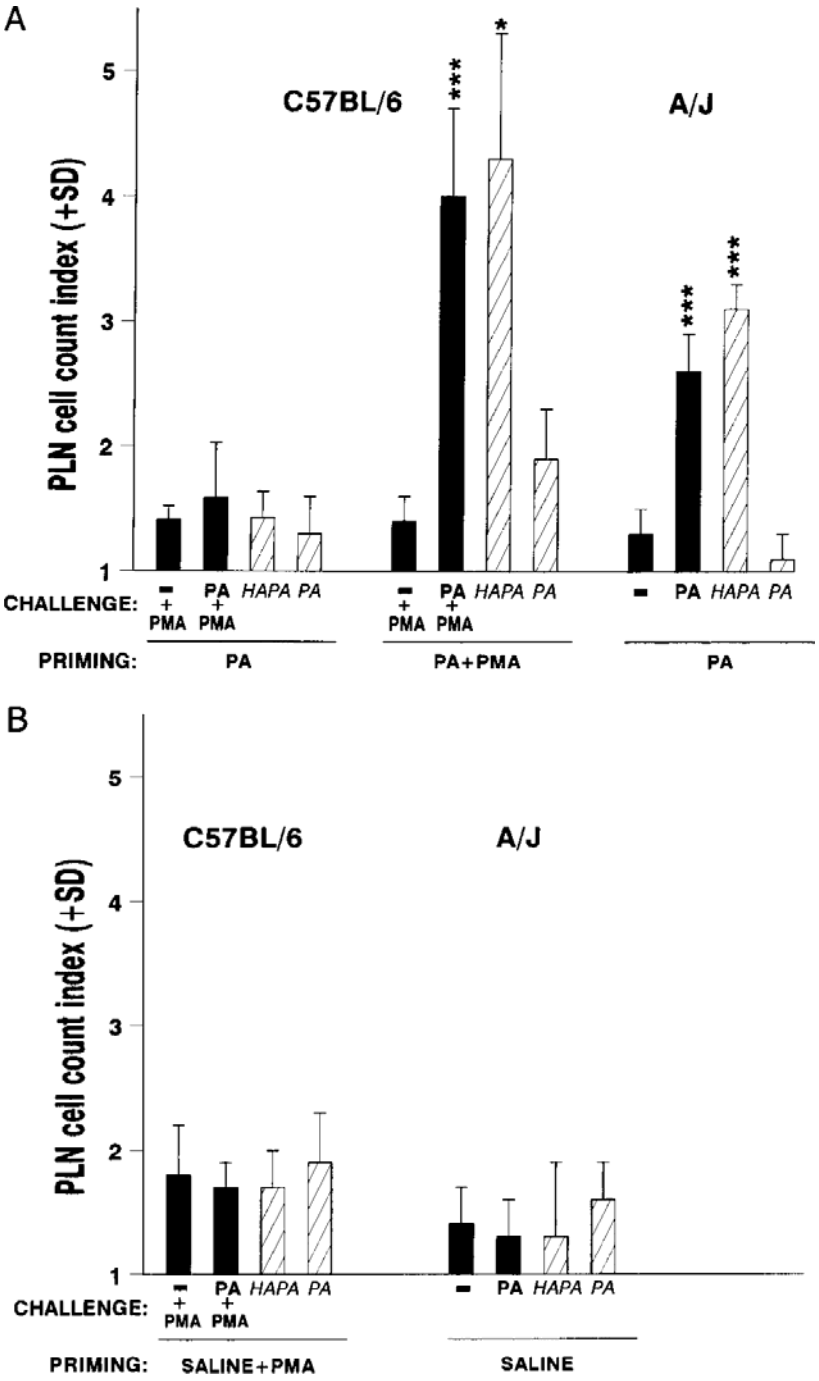


Figure 8. Evidence for generation of and T cell priming to HAPA-related neoantigens during long-term PA treatment of slow acetylator A/J mice; in fast acetylator C57BL/6 mice additional treatment with PMA was needed for these effects. T cell sensitization to HAPA-related neo-antigens, as detectable in peritoneal cells of PA-treated animals, was demonstrated by HAPA-specific recall responses in the adoptive transfer PLNA. Data represent arithmetic means + SD obtained from 5 to 7 mice per group; asterisks indicate significant differences between each of the two groups indicated by solid and hatched bars, respectively (*$p < 0.05$; ***$p < 0.01$). (A) <u>*Priming*</u>: T cell donor mice received three weekly sc injections of PA (8 µmol for A/J mice, and 16 µmol for C57BL/6 mice) over a period of 16 weeks. One group of C57BL/6 donors received an additional weekly ip injection of 600 ng PMA (PA + PMA). On day 0, 10^7 donor T cells were transferred to each syngeneic recipient. <u>*Challenge:*</u> One day after the T cell transfer, each recipient mouse received 5 x 10^5 homogenized peritoneal cells (solid bars) or 0.15 µmol of either PA or $HAPA_{aq}$ indicated in italics (hatched bars). Donors of peritoneal cells received either saline (-), saline (-) plus PMA, PA plus PMA, or PA only over a period of 16 weeks, as described above and indicated under the solid bars. (B) <u>*Priming*</u>: T cell donor mice of the A/J strain received three weekly sc injections of saline over a period of 16 weeks. For C57BL/6 T cell donors, this schedule of saline injections was supplemented by an additional weekly ip injection of 600 ng PMA (saline + PMA). On day 0, 10^7 donor T cells were transferred to each syngeneic recipient. <u>*Challenge:*</u> This was performed as decribed under (A).

PA. As shown in Fig. 7A, T cells from chronically PA-treated A/J donors reacted against both types of antigen, indicating T cells were primed to HAPA-related neoantigens formed in the slow acetylator strain during chronic PA treatment. By contrast, in fast acetylator C57BL/6 mice chronic PA treatment failed to prime T cells to HAPA-related neoantigens, even though double the amount of PA was administered compared to slow acetylator A/J mice.

Lack of T cell sensitization to HAPA-related neoantigens in fast acetylator mice was abrogated by additional PMA treatment

The lack of T cell sensitization in chronically PA-treated fast acetylator C57BL/6 mice was abrogated by additional weekly injection of 600 ng PMA/mouse (Fig. 8A); the latter is known to stimulate oxidizing enzymes, such as PGHS and myeloperoxidase, in vivo (31). For successful T cell recall, bothcell-free $HAPA_{aq}$ and peritoneal cells from C57BL/6 mice chronically treated with PA plus PMA could be used. Such peritoneal cells contain HAPA-related neoantigens, as previously described (11). In contrast, T cells from saline plus PMA-treated donors failed to give a statistically significant response to any of the materials indicated (Fig. 8B).

Discussion

Most investigators (1-3,11) agree that in the complex pathogenesis of PA-induced lupus a first, pre-immunologic step apparently consists of *N*-oxidation of PA to the reactive intermediates HAPA and nitroso-PA, respectively. As to the nature of the second step, our group proposed the hypothesis (11) that this may consist of T cell sensitization to neoantigens which are formed by these metabolites and presented by APC, such as macrophages. The structure of these metabolite-induced neoantigens has not yet been identified. As far as the cell type that generates reactive PA metabolites is concerned, polymorphonuclear phagocytes and monocytes were found capable of doing so (11,17), and in view of the results of the present investigation the same can be said about macrophages. Therefore, monocytes and macrophages could serve as a connecting link between the first, drug-metabolizing and the second, T cell-sensitizing step. Consistent with the hypothesis (11), our results indicate that T cells of slow acetylator A/J mice undergoing chronic treatment with the parent

compound PA were sensitized to HAPA-related neoantigens, as detectable in peritoneal cells of these animals. At the present time, we do not have data to show that these T cells reacting to HAPA-related neoantigens are, indeed, able to produce autoimmune disease.

With respect to the pre-immunologic step, we analyzed five different phase-I enzymes in mouse PMφ that could be implicated in *N*-oxidation of arylamines, such as PA. Apart from PGHS-2, PA exposure of PMφ did not alter mRNA expression or activity of the enzymes tested. In the case of PGHS-2, PA exposure led to enhanced mRNA expression and an increase in PGHS-dependent formation of PGE_2. It is known that during prostaglandin biosynthesis the peroxidase activity of PGHS reduces the endoperoxide-hydroperoxide substrate PGG_2 with electrons derived from oxidation of cosubstrates. The latter may be exogenous ones, such as arylamines (20,32,33). That PGHS, indeed, can *N*-oxidize PA was demonstrated by our finding that incubation of PA with ovine PGHS yielded HAPA and led to covalent binding.

In mice treated with TCDD an increase in mRNA levels of PGHS-2, but not of PGHS-1, was detected in lung and spleen (34). These observations are in line with our findings: exposure of PMφ to PA was found to induce PGHS-2 mRNA and a corresponding increase in PGE_2 levels, but did not affect PGHS-1. Both, TCDD and PA increase PGHS activity by induction of PGHS-2, but unlike PA, TCDD does not serve as a substrate for metabolic conversion by PGHS isoenzymes. Our results indicate that the arylamine PA can induce PGHS-2 in macrophages and, moreover, that the enhanced PGHS activity in these cells may account for, or contribute to, *N*-oxidation of PA and, hence, generation of HAPA-related neoantigens. Western blot analysis of PA-treated macrophages revealed formation of putative neoantigens. Since cell-lysates incubated with PA did not lead to covalent binding, we conclude that metabolism of PA is required for the formation of these neoantigens.

Generally, *N*-oxidation of arylamines can also be carried out by cytochrome P450 enzymes, including those of the cytochrome P4501A family (35). A recent investigation with human liver microsomes showed though that *N*-oxidation of PA is mainly due to the non-inducible cytochrome P4502D6 (12); in mice, however, the metabolizing activity of this enzyme was found to be low compared to rats and humans (36). Consistent with findings in humans (14), we were unable to detect expression of cytochrome P4501A2 mRNA in mouse PMφ. Cytochrome P4501A1, however, is expressed in extrahepatic tissues,

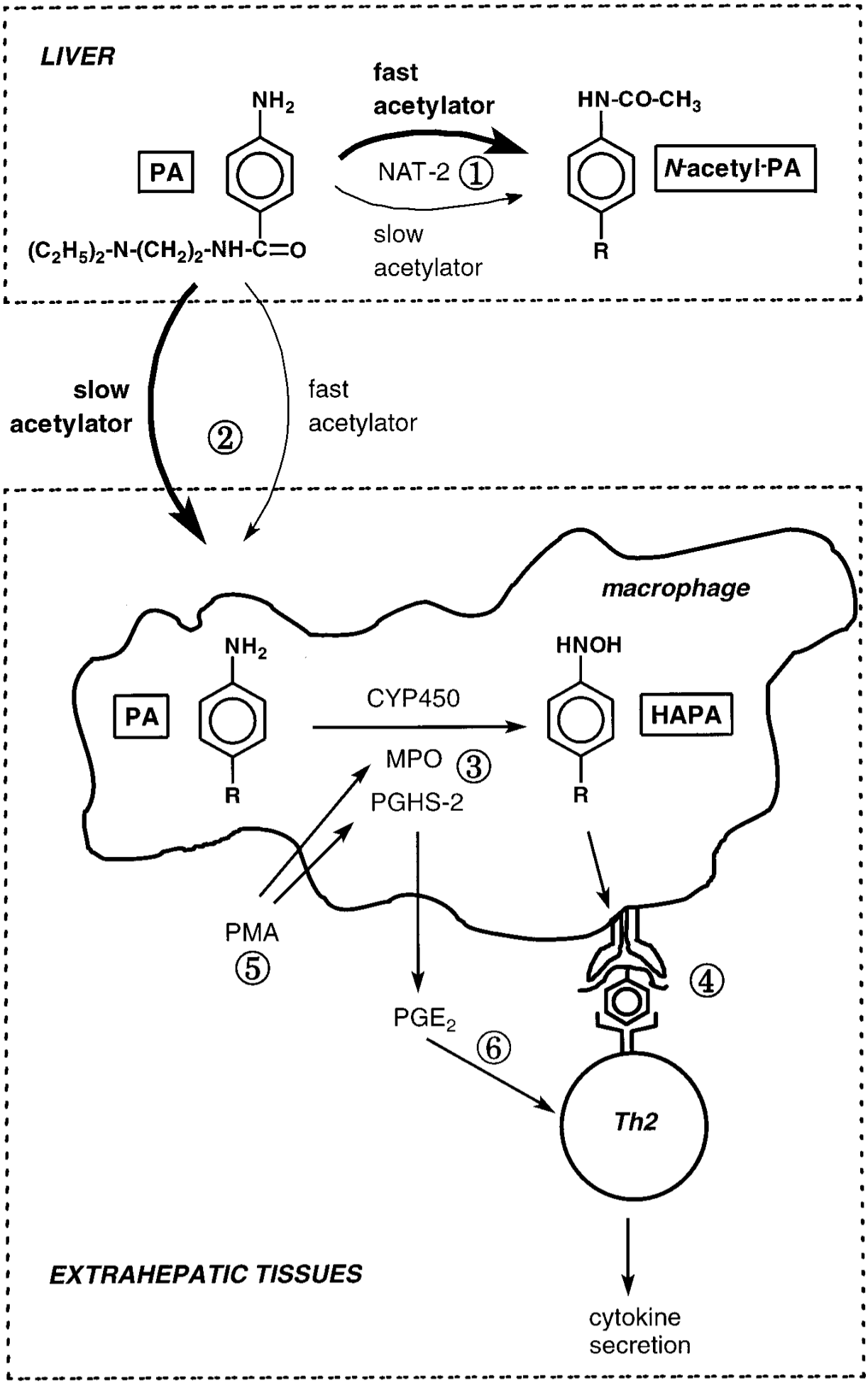
including macrophages (14-16). Unlike TCDD, a known inducer of cytochrome P4501A1, PA failed to enhance cytochrome P4501A1 (37) expression in PMφ cultured in vitro. Another enzyme known to be capable of *N*-oxidizing PA is the MPO of neutrophils and monocytes (9,17). However, confirming a previous report in the literature (38) we found that the basal activity of MPO in PMφ is relatively low and thus differs from that seen in mouse bone marrow cells. Moreover, incubation of PMφ with either PA or LPS plus IFN-γ failed to augment MPO activity, but did augment PGHS-2 activity in these cells. In conclusion, while PA exposure did not enhance enzyme activity or expression of either MPO or a member of the cytochrome P4501A family, we cannot rule out involvement of these enzymes in the *N*-oxidation of PA either.

Our results show that in vitro PA exposure of PMφ from both slow and fast acetylator mice did not alter their NAT-2 mRNA expression, and they strongly suggest that *N*-oxidation of PA can take place in PMφ of both strains (Fig. 2). Likewise, no differences in *N*-oxidation were detected between slow acetylator A/J and fast acetylator C57BL/6 mice, when murine mononuclear leukocytes were exposed to the arylamine 2-aminofluorene in vitro and DNA adduct formation was used as indicator of arylamine *N*-oxidation, even though C57BL/6 monocytes were 10-fold more active in *N*-acetylation (39). Admittedly, our data obtained by the adoptive transfer PLN assay do not allow a quantitative comparison of HAPA formation by PMφ of the fast and slow acetylator mouse strains used. Considering these limitations of detection method, the available results suggest that with the saturating substrate conditions used in vitro, macrophage-based *N*-oxidation of PA to HAPA or nitroso-PA was not affected by genetic differences in *N*-acetylation.

Since the capacity of fast *N*-acetylation does not prevent *N*-oxidation of PA by macrophages, what then can explain the lower risk for development of adverse immune reaction to PA and other arylamines observed in fast acetylator men and mice (2,11,21,22,40)? We propose that their lower risk is due to a lower concentration of PA available as substrate for extrahepatic *N*-oxidation by cells such as macrophages. Unlike slow acetylators, fast acetylators do not readily build up a serum concentration of PA that provides sufficient substrate for extrahepatic HAPA formation and, hence, for reaching the minimal number of identical neoantigens on APC required for T cell activation. This hypothesis is illustrated in Scheme 1, and it is based on the following three lines of evidence. First, in chronically PA-treated fast acetylator C57BL/6 mice

peritoneal cells failed to contain HAPA-related neoantigens (11) and their T cells failed to be sensitized to these antigens (Fig. 8A). In chronically PA-treated slow acetylator A/J mice, peritoneal cells did contain HAPA-related neoantigens (11) and T cells were sensitized to them (Fig. 8A), even though they had received only half the PA dose of that given to fast acetylator C57BL/6 mice. Second, in human fast acetylators the incidence of PA-induced lupus was equal to that seen in slow acetylators, when identical serum levels of PA were maintained in both groups (41). Third, in fast acetylator C57BL/6 mice, enhancing the rate of extrahepatic *N*-oxidation of PA could compensate for their high rate of *N*-acetylation. This statement is based on our observations that PMA stimulation of chronically PA-treated fast acetylator C57BL/6 mice sufficed *1)* to induce in vivo formation of HAPA-related neoantigens (11) and *2)* to spontaneously sensitize HAPA-specific T cells in vivo (Fig. 8A). Since PMA activates many oxidizing enzymes, such as PGHS and MPO, via the protein kinase C pathway (42), cells located at multiple sites of the body might be involved in enhanced HAPA formation and presentation of HAPA-related neoantigens. Besides the proposed enhancing effect on PA oxidation PMA was shown to downregulate induction of cytochrome P4501A1 in the liver of C57BL/6 mice (43,44). Downregulation of a potential PA metabolizing enzyme in the liver may contribute to higher extrahepatic PA levels and thus enhance extrahepatic HAPA formation. Interestingly, PMA has been reported to stimulate *de novo* synthesis of PGHS-2 in cultured murine fibroblasts, human monocytes, and rat and human epithelial cells (19,45,46). This suggests that the enhancing effects of adjunct PMA treatment on neoantigen formation and T cell sensitization, which were observed in the previous (11) and the present investigation performed in vivo, also were due to upregulation of PGHS-2.

In addition to the pathogenic concept of PA-induced lupus developed in the present paper (see Scheme 1), two other concepts are currently discussed for the second, immunologic step of the disease. Richardson and coworkers (4) assume a direct, autoimmunizing effect of PA on preactivated $CD4^+$ T cells, irrespective of the specificity of their T cell receptor for antigen. PA treatment would enable the T cells to proliferate in response to normal APC, not treated with the drug or pulsed with antigen. In contrast, the concept of Rubin and coworkers (5), like our concept, is based on metabolic conversion of PA to HAPA. They found that injection of HAPA into the thymus of normal mice


LIVER
fast
acetylator
NH2
HN-CO-CH3
PA
NAT-2 ①
N-acetyl-PA
slow
acetylator
(C2H5)2-N-(CH2)2-NH-C=O
R
slow
acetylator
②
fast
acetylator
macrophage
NH2
HNOH
PA
CYP450
HAPA
MPO ③
PGHS-2
R
R
PMA
⑤
④
PGE2
⑥
Th2
EXTRAHEPATIC TISSUES
cytokine
secretion

Scheme 1. *Scheme depicting the initial metabolic and immunogenic steps thought to be involved in the pathogenesis of PA-induced lupus*. Two compartments of the body are shown, the liver and extrahepatic tissues. In the latter, two types of cell are presented, a macrophage and a T helper-2 (Th2) cell. In essence, the hypothesis postulates that due to conversion into the reactive metabolites HAPA and nitroso-PA (not shown) neoantigens are generated and presented by APC, such as macrophages, which are recognized by specific T cells and activate them. The T cells then would secrete cytokines and thus activate other cells, including autoreactive B cells (not shown), in a fashion analogous to chronic graft-versus-host reaction in mice (47,51).

In the liver, PA can be either converted into *N*-acetyl-PA or HAPA (not shown). Hepatic HAPA probably is not a sensitizing agent as the liver is rich in detoxifying mechanisms and, moreover, relatively poor in APC. Consistent with this, hepatitis is not part of PA-induced lupus. Due to expression of a defective NAT-2 in slow acetylators, their hepatic elimination of PA via the nonreactive metabolite *N*-acetyl-PA is reduced (1); this defect increases the amount of substrate available for extrahepatic PA metabolism (2).

Extrahepatic *N*-oxidation of PA can be mediated by a variety of different enzymes (3). One of them is PGHS-2, which is present in monocytes and macrophages and, perhaps, other types of APC and wihich can be induced by PA. In monocytes and macrophages, *N*-oxidation of PA to the protein-reactive, haptenic species HAPA and nitroso-PA (not shown) can lead to presentation of HAPA-related neoantigens and subsequent T cell sensitization (4). In slow acetylators, the concentration of extrahepatic PA (2) is high enough to readily evoke sufficient generation of HAPA-related neoantigens by APC and, hence, sensitization of T cells. In fast acetylators, effective *N*-acetylation of PA in the liver (1) decreases the amount of PA available for extrahepatic *N*-oxidation (2). As a consequence, extrahepatic formation and presentation of HAPA-related neoantigens is suboptimal or remains even below the threshold required for activation of neoantigen-reactive T cells. Activation of oxidative enzymes in phagocytes, for instance by PMA (5), enhances extrahepatic *N*-oxidation of PA and, hence, T cell sensitization to HAPA-related neoantigens (4). PA-induced expression of PGHS-2 and the subsequent increase in PGE_2 release might shift the T cell response to HAPA-related neoantigens towards a Th2-like cytokine pattern (6).

induced formation of autoantibodies to histones (H2A-H2B) similar to those seen in PA-induced lupus in humans. They suggest that a loss of central T cell tolerance to chromatin underlies autoimmunity in PA-induced lupus, since chromatin-reactive T cell responses were detected in the spleen and in thymus organ culture after exposure to HAPA ex vivo. While all three models conceive a central role of T cells in PA-induced lupus, ours is the only one that considers drug-induced neoantigens as the trigger for T cell activation, analogous to the situation seen in SLE-like disease induced by chronic graft-versus-host reaction in mice (47).

Finally, it is noteworthy that the observed induction of PGHS-2 by PA in macrophages can have two different effects, each of which could contribute to

the induction of PA-induced lupus (11,48). First, PGHS-2 may account for, or contribute to, the observed generation of HAPA-related neoantigens in macrophages, as discussed. Second, the PA-induced stimulation of PGHS-2 in macrophages enhances the production of PGE_2, as shown in the present investigation. PGE_2 released from macrophages, in turn, could skew the T cell response to HAPA-related neoantigens towards a Th2-like cytokine secretion profile (49,49). In this way, PGE_2 itself might be involved in the pathogenesis of PA-induced lupus, because human SLE (50) as well as the SLE-like disease induced by chronic graft-versus-host reaction in mice (47,51) are dominated by Th2 cytokines.

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References

1. **Adams, L. E., C. E. JR. Sanders, R. A. Budinsky, R. J. Donovan Brand, S. M. Roberts, and E. V. Hess**. 1989. Immunomodulatory effects of procainamide metabolites: Their implications in drug-related lupus. *J.Lab.Clin.Med. 113*: 482-492.

2. **Rubin, R. L.** 1989. Autoimmune reactions induced by procainamide and hydralazine. In *Autoimmunity and Toxicology. Immune disregulation induced by drugs and chemicals.* M.E. Kammüller, N. Bloksma and W. Seinen, eds. Elsevier Science Publishers B.V., Amsterdam, New York, Oxford, p. 119-150.

3. **Uetrecht, J. P.** 1992. The role of leukocyte-generated reactive metabolites in the pathogenesis of idiosyncratic drug reactions. *Drug Metab.Rev. 24*: 299-366.

4. **Yung, R. L., K. J. Johnson, and B. C. Richardson**. 1995. Biology of disease: New concepts in the pathogenesis of drug-induced lupus. *Lab.Invest. 73*: 746-759.

5. **Kretz-Rommel, A., S. R. Duncan, and R. L. Rubin**. 1997. Autoimmunity caused by disruption of central T cell tolerance. *J.Clin.Invest. 99*: 1888-1896.

6. **Dean, J. H. and M. J. Murray**. 1991. Toxic Responses to the Immune System. In *Casarett and Doull's Toxicology*. 4 ed. M.O. Amdur, J. Doull and C.D. Klaassen, eds. Pergamon Press, New York, p. 282-333.

7. **Uetrecht, J. P.** 1985. Reactivity and possible significance of hydroxylamine and nitroso metabolites of procainamide. *J.Pharmacol.Exp.Ther. 232*: 420-425.

8. **Hein, D. W. and W. W. Weber**. 1989. Metabolism of procainamide, hydralazine, and isoniazid in relation to autoimmune(-like) reactions. In *Autoimmunity and Toxicology. Immune disregulation induced by drugs and chemicals.* M.E. Kammüller, N. Bloksma and W. Seinen, eds. Elsevier Science Publishers B.V., Amsterdam, New York, Oxford, p. 239-265.

9. **Rubin, R. L. and J. T. Curnutte** . 1989. Metabolism of procainamide to the cytotoxic hydroxylamine by neutrophils activated in vitro. *J.Clin.Invest. 83*: 1336-1343.

10. **Freeman, R. W., J. P. Uetrecht, R. L. Woosley, J. A. Oates, and R. D. Harbison**. 1981. Covalent binding of procainamide in vitro and in vivo to hepatic protein in mice. *Drug Metab.Dispos. 9*: 188-192.

11. **Kubicka-Muranyi, M., R. Goebels, C. Goebel, J. P. Uetrecht, and E. Gleichmann**. 1993. T lymphocytes ignore procainamide, but respond to its reactive metabolites in peritoneal cells: demonstration by the adoptive transfer popliteal lymph node assay. *Toxicol.Appl.Pharmacol. 122*: 88-94.

12. **Lessard, E., A. Fortin, P. M. Bélanger, P. Beaune, B. A. Hamelin, and J. Turgeon**. 1997. Role of CYP2D6 in the N-hydroxylation of procainamide. *Pharmacogenetics 7*: 381-390.

13. **Masubuchi, Y., T. Iwasa, S. Hosokawa, T. Suzuki, T. Horie, S. Imaoka, Y. Funae, and S. Narimatutsu**. 1997. Selective deficiency of debrisoquine 4-hydroxylase activity in mouse liver microsomes. *J.Pharmacol.Exp.Ther. 282* : 1435-1441.

14. **Daly, A. K., S. Cholerton, M. Armstrong, and J. R. Idle**. 1994. Genotyping for polymorphisms in xenobiotic metabolism as a predictor of disease susceptibility. *Environ.Health Perspect. 102*: 55-61.

15. **Henschler, R. and H. R. Glatt**. 1995. Induction of cytochrome P4501A1 in haemopoietic stem cells by hydroxylated metabolites of benzene. *Toxic.in Vitro 9*: 453-457.

16. **Germolec, D. R., N. H. Adams, and M. I. Luster**. 1995. Comparative assessment of metabolic enzyme levels in macrophage populations of the F344Rat. *Biochem.Pharmacol. 50*: 1495-1504.

17. **Uetrecht, J. P., N. Zahid, and R. Rubin**. 1988. Metabolism of procainamide to a hydroxylamine by human neutrophils and mononuclear leukocytes. *Chem.Res.Toxicol. 1*: 74-78.

18. **Jiang, X., G. Khursigara, and R. L. Rubin**. 1994. Transformation of lupus-inducing drugs to cytotoxic products by activated neutrophils. *Science 266*: 810-813.

19. **Herschman, H. R.** 1996. Review - Prostaglandin synthase 2. *Biochem.Biophys.Acta 1299*: 125-140.

20. **Golly, I. and P. Hlavica**. 1985. *N*-Oxidation of 4-chloroaniline by prostaglandin synthase. *Biochem.J. 260*: 803-809.

21. **Levy, G. N., K. J. Martell, J. H. DeLeon, and W. W. Weber**. 1992. Metabolic, molecular genetic and toxicological aspects of the acetylation polymorphism in inbred mice. *Pharmacogenetics 2*: 197-206.

22. **Wolkenstein, P., V. Carriere, D. Charue, S. Bastuji-Garin, J. Revuz, J. C. Roujeau, P. H. Beaune, and M. Bagot** . 1995. A slow acetylator genotype is a risk factor for sulphonamide-induced toxic epidermal necrolysis and Stevens-Johnson syndrome. *Pharmacogenetics 5*: 255-258.

23. **Woosley, R. L., D. E. Drayer, M. M. Reidenberg, A. S. Nies, K. Carr, and J. A. Oates**. 1978. Effect of acetylator phenotype on the rate at which procainamide induces antinuclear antibodies and the lupus syndrome. *New Engl.J.Med. 298*: 1157-1159.

24. **Bloksma, N., M. Kubicka-Muranyi, H.-C. Schuppe, E. Gleichmann, and H. Gleichmann**. 1995. A predictive immunotoxicological test system: suitability of the popliteal lymph node assay in rats and mice. *Crit.Rev.Toxicol. 25* : 369-396.

25. **Martell, K. J., G. N. Levy, and W. W. Weber**. 1992. Cloned mouse N-acetyltransferases: enzymatic properties of expressed Nat-1 and Nat-2 gene products. *Mol.Pharmacol. 42*: 265-272.

26. **Suzuki, K., H. Ota, S. Sasagawa, T. Sakatani, and T. Fujikura**. 1983. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal.Biochem. 132*: 345-352.

27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*: 680-685.

28. **Katsutani, N. and H. Shionoya**. 1992. Popliteal lymph node enlargement induced by procainamide. *J.Immunopharmacol. 14*: 681-686.

29. **Martell, K. J., K. P. Vatsis, and W. W. Weber**. 1991. Molecular genetic basis of rapid and slow acetylation in mice. *Mol.Pharmacol. 40*: 218-227.

30. **Uetrecht, J. P., B. J. Sweetman, R. L. Woosley, and J. A. Oates**. 1984. Metabolism of procainamide to a hydroxylamine by rat and human hepatic microsomes. *Drug Metab.Dispos. 12*: 77-81.

31. **Raederstorff, D., M. Pantze, H. Bachmann, and U. Moser**. 1996. Anti-inflammatory properties of docosahexaenoic and eicosapentaenoic acids in phorbol-ester-induced mouse ear inflammation. *Int.Arch.Allergy Immunol. 111*: 284-290.

32. **Eling, T. E., D. C. Thompson, G. L. Foureman, J. F. Curtis, and M. F. Hughes**. 1990. Prostaglandin H synthase and xenobiotic oxidation. *Annu.Rev.Pharmacol.Toxicol. 30*: 1-45.

33. **Degen, G. H.** 1993. Prostaglandin H synthase containing cell lines as tools for studying metabolism and toxicity of xenobiotics. *Toxicology 82*: 243-256.

34. **Vogel, C., U. S. Schuhmacher, G. H. Degen, H. M. Bolt, T. Pineau, and J. Abel**. 1998. Modulation of prostaglandin H synthase-2 mRNA expression by 2,3,7,8-Tetrachlorodibenzo-p-dioxin in mice. *Arch.Biochem.Biophys. 351*: 265-271.

35. **Butler, M. A., M. Iwasaki, P. Guengerich, and F. F. Kadlubar**. 1989. Human cytochrome P-450PA (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic acylamines. *Proc.Natl.Acad.Sci. 86*: 7696-7700.

36. **Stahl, N., T. J. Farruggella, T. G. Boulton, Z. Zhong, J. E. Darnell, and G. D. Yancopoulos**. 1995. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science 267*: 1349-1353.

37. **Whitlock, J. P., Jr., M. S. Denison, J. M. Fisher, and E. S. Shen**. 1989. Induction of hepatic cytochrome P450 gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Mol.Biol.Med. 6*: 169-178.

38. **van Furth, R.** 1992. Development and distribution of mononuclear phagocytes. In *Inflammation: Principles and clinical correlates*. 2 ed. J.I. Gallin, I.M. Goldstein and R. Snyderman, eds. Raven Press, New York, p. 325-339.

39. **Levy, G. N., J. G. Chung, and W. W. Weber**. 1994. 2-Aminofluorene metabolism and DNA adduct formation by mononuclear leukocytes from rapid and slow acetylator mouse strains. *Carcinogenesis 15*: 353-357.

40. **Ten Veen, J. H. and T. E. Feltkamp**. 1972. Studies on drug induced lupus erythematosus in mice. I. Drug induced antinuclear antibodies. *Clin.Exp.Immunol. 11*: 265-276.

41. **Sonnhag, C., E. Karlsson, and J. Hed**. 1979. Procainamide-induced Lupus erythematosus-like syndrome in relation to acetylator phenotype and plasma levels of procainamide. *Acta Med.Scand. 206*: 245-251.

42. **Goppelt-Struebe, M. and A. Moeller**. 1990. Differential effect of diacylglycerols and phorbol ester on arachidonic acid metabolism in bone marrow-derived macrophages. *Biol.Chem.Hoppe-Seyler 371*: 369-374.

43. **Raunio, H. and O. Pelkonen**. 1983. Effect of polycyclic aromatic compounds and phorbol esters on ornithine decarboxylase and aryl hydrocarbon hydroxylase activities in mouse liver. *Cancer Research 43*: 782-786.

44. **Berghard, A., K. Gradin, I. Pongratz, M. Whitelaw, and L. Poellinger**. 1993. Cross-coupling of signal transduction pathways: the dioxin receptor mediates induction of cytochrome P-4501A1 expression via a protein kinase C-dependent mechanism. *Mol.Cell.Biol. 13*: 677-689.

45. **Hoff, T., D. DeWitt, V. Kaever, K. Resch, and M. Goppelt-Struebe**. 1993. Differentiation-associated expression of prostaglandin G/H synthase in monocytic cells. *Fed.Eur.Biochem.Soc. 320*: 38-42.

46. **Hamasaki, Y. and T. E. Eling**. 1995. EGF and TPA stimulate de novo synthesis of PGHS-1 and PGHS-2 through different signal transduction pathways. *Prostagl.Leuko.Essent.Acids 53*: 225-229.

47. **Gleichmann, E., S. T. Pals, A. G. Rolink, T. Radaszkiewicz, and H. Gleichmann**. 1984. Graft-versus-host reactions: clues to the etiopathology of a spectrum of immunological diseases. *Immunol.Today 5*: 324-332.

48. **Goldman, M., P. Druet, and E. Gleichmann**. 1991. TH2 cells in systemic autoimmunity: insights from allogeneic diseases and chemically-induced autoimmunity. *Immunol.Today 12*: 223-227.

49. **Hilkens, C. M. U., A. Snijders, H. Vermeulen, P. H. Vandermeide, E. A. Wierenga, and M. L. Kapsenberg**. 1996. Accessory cell-derived IL-12 and prostaglandin E_2 determine the IFN-(gamma) level of activated human $CD4^+$ T cells. *J.Immunol. 156*: 1722-1727.

50. **Richaud-Patin, Y., J. Alcocer-Varela, and L. Llorente**. 1995. High levels of TH2 cytokine gene expression in systemic lupus erythematosus. *Rev.Invest.Clin. 47*: 267-272.

51. **De Wit, D., M. Van Mechelen, C. Zanin, J.-M. Doutrelepont, T. Velu, C. Gerad, D. Abramowicz, J. P. Scheerlinck, P. De Baetselier, J. Urbain, O. Leo, M. Goldman, and M. Moser**. 1993. Preferential activation of Th2 cells in chronic graft-versus-host reaction. *J.Immunol. 150*: 361-366.

52. **Abel, J., W. Li, O. Döhr, C. Vogel, and S. Donath**. 1996. Dose-response relationship of cytochrome P4501B1 mRNA induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in livers of C57BL/6J and DBA/2J mice. *Arch.Toxicol. 70*: 510-513.

53. **Kimura, A., F. J. Gonzales, and D. W. Nebert**. 1984. Comparison of the complete cytochrome P_1-450 and P_3-450 cDNA nucleotide and amino acid sequences. *J.Biol.Chem. 259*: 10705-10713.

54. **DeWitt, D. L., E. A. El-Harith, S. A. Kraemer, M. J. Andrews, E. F. Yao, R. L. Armstrong, and W. L. Smith**. 1990. The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J.Biol.Chem. 265*: 5192-5198.

55. **O'Banion, M. K., V. D. Winn, and D. A. Young**. 1992. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc.Natl.Acad.Sci. 89*: 4888-4892.

CHAPTER 5

T Cells Ignore Aniline, a Prohapten, but Respond to its Reactive Metabolites Generated by Phagocytes: Possible Implications for the Pathogenesis of Toxic Oil Syndrome

Marty Wulferink, José González, Carsten Goebel, and Ernst Gleichmann

The most basic arylamine, aniline, belongs to a class of compound notorious for inducing allergic and autoimmune reactions. In 1981 in Spain, many people succumbed to the toxic oil syndrome (TOS), a disease caused by ingestion of cooking oil contaminated with aniline. Indirect evidence points towards an immune pathogenesis of TOS driven by T lymphocytes, but it is unclear to which antigens these cells could react. Here, using the popliteal lymph node (PLN) assay in mice, we analyzed the sensitizing potential of aniline, its metabolites, and some of the aniline-coupled lipids detected in the contaminated cooking oil. Whereas aniline itself and its non-protein-reactive metabolites nitrobenzene, p-aminophenol and N-acetyl-p-aminophenol, failed to elicit PLN responses, its reactive metabolites nitrosobenzene and N-hydroxylaniline did. The aniline-coupled lipids, namely linoleic anilide and linolenic anilide, and a mixture of fatty acid esters of 3-(N-phenylamino)-1,2-propanediol, all implicated in TOS, induced significant PLN responses, whereas the respective aniline-free lipids, linoleic acid, linolenic acid, and triolein did not. Hence, the aniline moiety plays a crucial role in the immunogenicity of the aniline-coupled lipids of TOS. PLN responses to the reactive aniline metabolites and the one aniline-coupled lipid tested, linolenic anilide, were T cell-dependent. Secondary PLN responses to nitrosobenzene were detectable not only after priming with

nitrosobenzene, but, in some experiments, also after priming with linolenic anilide. This suggests that the aniline moiety was cleaved from the aniline-coupled lipid and metabolized to the intermediate nitrosobenzene that generated the prospective neoantigens. Consistent with this, in lymphocyte proliferation tests in vitro, T cells primed to nitrosobenzene reacted in anamnestic fashion to white bone marrow cells (WBMCs) pulsed with aniline. Hence, we propose that aniline is a prohapten that can be metabolized by WBMCs, which form neo-antigens that are recognized by T cells. The possible significance of these findings for the pathogenesis of TOS is discussed.

Introduction

Aniline is industrially synthesized on a large scale as the parent compound for a variety of different arylamines including various drugs and dyes. Occupational poisoning by aniline was common in the past (1), and exposure to aniline still occurs today. Both acute toxicity, characterized by methemoglobinemia and hemolytic anemia, and carcinogenic effects due to chronic aniline exposure have been studied in detail (2-4). With respect to the immune system, aniline itself is not known to induce adverse immune reactions in humans. In contrast, aniline derivatives, which possess a functional group in *para*-position to the amine group and, hence, are called *para*-compounds, are notorious for inducing allergic and autoimmune reactions (5,6). Examples of such *para*-substituted aniline derivatives are *p*-phenylenediamine, sulfonamides, and procainamide, which can induce allergic contact dermatitis (7), agranulocytosis (8), delayed-type hypersensitivity associated with multiorgan toxicity (9)**,** and drug-induced lupus (10), respectively. For two reasons the sensitizing potential of aniline deserves to be studied in detail: first, the well-known sensitizing potential of the above-mentioned arylamines, and second, the occurrence of the toxic oil syndrome (TOS), a mass poisoning occurring in Spain that affected more than 20,000 people after consuming rapeseed oil contaminated with aniline (11).

Fatty acid anilides and fatty acid esters of phenylamino-propanediol (PAP), detected as abnormal compounds in case-related samples of toxic oil, have been incriminated as etiologic agents of TOS (12,13). Clinical features such as eosinophilia, elevated serum IgE, and signs and symptoms of systemic autoimmune disease point towards an immune pathogenesis of the syndrome (14). This was further corroborated by the detection of T cell infiltrates in the affected tissues and of increased levels of soluble interleukin-2 receptor in the

serum of TOS patients (14). It has been proposed (15) that graft-versus-host-like reactions of T lymphocytes were involved in the pathogenesis of TOS. This concept implies that neoantigens induced by the aniline-contaminated oil were displayed by antigen-presenting cells (APC) and recognized by T cells. Classical T cells, bearing an αβ-T cell receptor, are unable to recognize small molecular weight compounds per se, but are able to react to them when bound to proteins, more exactly peptides presented by molecules of the major histocompatibility complex (16). Therefore, it is conceivable that in TOS patients T cells reacted to self-peptides which were haptenated by the etiologic agent of the disease. Considering that the aniline-coupled lipids implicated in TOS are neither proteins nor protein-reactive and that their metabolic pathway is unknown, it is a difficult task to test whether the derivatives of the aniline-coupled lipids can haptenate self-proteins so that they are recognized by T cells. Therefore, we decided first to study the aniline moiety per se for its potential to act as a hapten. More specifically, we asked whether aniline can be considered a prohapten that can be metabolized by phagocytic cells into a protein-reactive hapten capable of sensitizing T cells. The knowledge gained from this approach was then applied to probe the T cell-sensitizing potential of synthetic aniline-coupled lipids implicated in TOS.

Materials and Methods

Mice Female C57BL/6J, B10.S, BALB/c, BALB/c nu/+, and BALB/c nu/nu mice were purchased from Harlan Winkelmann GmbH (Borchen, Germany). Animals were kept under specific pathogen-free conditions and had free access to a standard diet (Ssniff Spezialdiäten GmbH, Soest, Germany) and tap water. They were six to ten weeks of age at the onset of the experiments. C57BL/6J mice were used, unless stated otherwise.

Chemicals Aniline, nitrosobenzene, nitrobenzene, *p*-benzoquinone, *p*-aminophenol, and *N*-acetyl-*p*-aminophenol (acetaminophen) were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). *N*-hydroxylaniline was prepared by hydroxylation of aniline in the laboratory of Dr. Robert L. Rubin (La Jolla, California, USA) and kindly provided to us. ***Caution:*** *aniline, nitrosobenzene, nitrobenzene, and N-hydroxylaniline can induce methemoglobinemia and are teratogenic; p-aminophenol and p-benzoquinone are possible contact-sensitizers. Substances were handled accordingly.*

Linoleic anilide, linolenic anilide, and a mixture of fatty acid esters of PAP containing

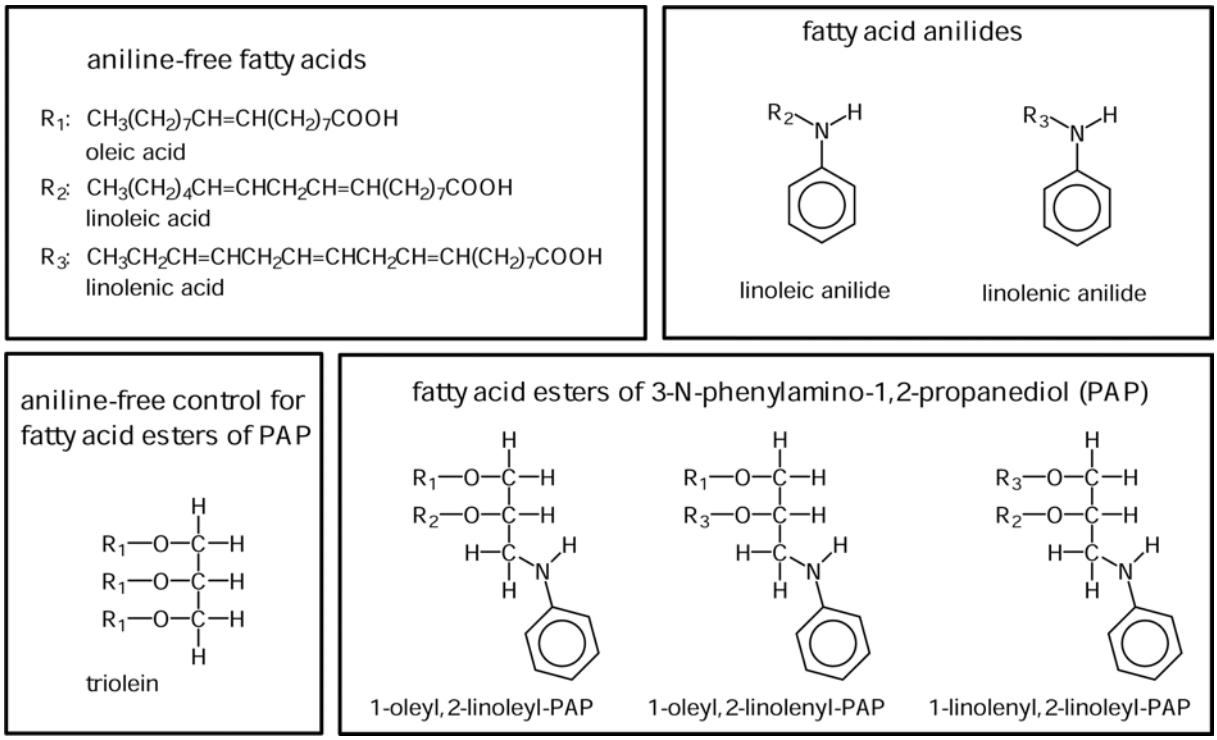
equimolar amounts of 1-oleyl,2-linoleyl-PAP; 1-oleyl,2-linolenyl-PAP; and 1-linolenyl,2-linoleyl-PAP, were synthesized by Dr. A. Meseguer (Consejo de Superior de Investigaciones Científicas, Barcelona, Spain) as described (17). Chemical structures of the aniline-coupled lipids tested are shown in Scheme 1. The aniline-coupled lipids mentioned above and their respective aniline-free control compounds linoleic acid, linolenic acid, and triolein were provided in blind-coded fashion by Dr. M. Posada, general coordinator for research projects on TOS (Instituto Carlos III, Madrid, Spain).

Sterile, pyrogen-free RPMI 1640 medium, fetal calf serum, penicillin, streptomycin, L-glutamine, pyruvate, and non-essential amino acids were obtained from Life Technologies GmbH (Eggenstein, Germany). ^{3}H-dThd (248 GBq/mmol) was obtained from ICN Biomedicals GmbH (Eschwege, Germany). Sterile filtered phosphate-buffered saline (PBS) contained NaCl (138 mM), KCl (2.7 mM), Na_2HPO_4 (6.5 mM), and KH_2PO_4 (1.5 mM); its pH was adjusted to 7.4.

Popliteal lymph node (PLN) assay *Primary PLN reaction.* The assay detects the immunostimulatory capacity of low molecular weight substances and was performed as described before (18). In short, the test compounds aniline, *N*-hydroxylaniline, nitrosobenzene, nitrobenzene, *p*-aminophenol, and *N*-acetyl-*p*-aminophenol, respectively, were dissolved in ethanol and diluted in PBS to a final ethanol concentration of 0.1 %; the solvent is referred to as ethanol/PBS. Linoleic anilide, linolenic anilide, the mixture of PAP esters, and the respective control substances linoleic acid, linolenic acid, and triolein, were emulsified in PBS by repeated ultrasonication on ice.

Homogenates of white bone marrow cells (WBMCs) were prepared in PBS (see below). Sheep red blood cells were washed three times, and the cell number was adjusted in PBS. On day 0, animals received a single sc injection (50 µL) of the test compound into the left hind footpad. On day six, PLN of treated and untreated sides were removed, and cell numbers of individual PLN were counted using a Casy1 automatic cell counter (Schärfe Systems GmbH, Reutlingen, Germany). The PLN cell count index from each mouse was calculated by dividing the cell count of the treated side by that of the control side.

Secondary PLN reaction. The assay was performed as described (18). Mice were primed by a single sc injection (50 µL), containing the compounds indicated, into the left hind footpad. Thirteen weeks later, the time period required for the enlarged PLNs to revert to their normal size and cellularity following the injection of aniline-coupled lipids, groups of mice were challenged by a second sc injection (50 µL) into the same foot pad. The doses of nitrosobenzene, *p*-benzoquinone, and linolenic anilide used for recall were suboptimal, that



Scheme 1. Chemical structures of the aniline-coupled lipids and their aniline-free controls used. In the structural formulas of the anilides, the PAP esters and triolein, R_1, R_2, and R_3 have to be replaced by oleic acid, linoleic acid, and linolenic acid, respectively.

means they were just too low to induce a primary PLN response in unprimed mice. Four days later, PLNs of treated and untreated sides were removed and cell count indices determined.

Flow cytometric analysis of PLN cells Six days after injection, PLNs from the treated side were removed and cells from each individual mouse were double-stained with either FITC-labeled anti-αβ-T cell receptor and PE-labeled anti-B220, or FITC-labeled anti-CD4 and PE-labeled anti-CD8 (all obtained from Pharmingen, Hamburg, Germany). Percentage of T, B, CD4 and CD8 cells were determined using a FACScan flow cytometer and Cellquest Software (Becton Dickinson, Heidelberg, Germany).

Isolation of WBMCs Mice were sacrificed and their femora and tibiae removed. Both ends of the bones were cut off, and the marrow was flushed with PBS, using a 25-gauge needle. Mature red blood cells were removed by osmotic lysis, and remaining cells were resuspended in PBS. These cells are referred to as WBMCs.

Cell culture conditions In all experiments performed in vitro, RPMI 1640 was supplemented with 10% fetal calf serum, L-glutamine (2 mM), pyruvate (1 mM), non-essential amino acids, penicillin (10 U/mL), and streptomycin (10 µg/mL); this is referred to as medium. Cells were cultured at 37°C in a humidified atmosphere containing 7% CO_2.

Treatment of WBMCs for experiments *in vivo* Isolated WBMCs were cultured in medium and incubated with either 0.1 % ethanol or 1 mM aniline in 0.1 % ethanol. After two days of culturing, WBMCs were harvested with a cell scraper, washed, resuspended in PBS, counted, and homogenized by freeze-thawing and ultrasonication (5 x 10 s at 30 kHz) using a Labsonic V 200 (B. Braun Melsungen AG, Melsungen, Germany). After homogenization, the number of WBMC equivalents was adjusted to the desired concentration by dilution in PBS and injected as described above for the PLN assay.

Treatment of WBMCs for experiments *in vitro* Isolated WBMCs were incubated in medium containing either 0.1 % ethanol or 1 mM aniline in 0.1 % ethanol. After one day of culture, cells were harvested with a cell scraper, washed, resuspended in medium, irradiated with 20 Gy using a Gammacell 2000 (Molsgaard, Copenhagen, Denmark), and used in the T-cell proliferation test *in vitro*.

Treatment of prospective T-cell donors Mice received two sc injections (on day 0 and day seven) at the base of tail, each containing 100 nmol nitrosobenzene in 50 µL ethanol/PBS. Control mice received ethanol/PBS only. On day 14 spleens were removed, splenic T cells were enriched (see below) and used in the T-cell proliferation test *in vitro*.

Enrichment of T cells Spleens of donor animals were removed and splenocytes were pooled in PBS. $B220^+$ cells were removed using a magnetic cell separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), as described (19). In short, 10^8 spleen cells were incubated (15 min at 4 °C) in 1 mL PBS containing 50 µL anti-mouse-B220 monoclonal antibodies coupled with magnetic microbeads (Miltenyi Biotec GmbH). Stained cells were withdrawn from the cell suspension in a high gradient magnetic field. After separation, the cell fractions were tested for T-cell purity with FITC labeled anti-Thy1.2 (Pharmingen) using a FACScan flow cytometer. Cells in the unstained fraction after separation contained 85 to 95% $Thy1.2^+$ cells and are referred to as enriched T cells. Enriched T cells were washed, resuspended in medium and used in the T-cell proliferation test *in vitro*.

Preparation of APC Mice were sacrificed, their spleens removed and a single-cell suspension was prepared. Red blood cells were removed by osmotic lysis, and remaining cells were resuspended in PBS, irradiated with 20 Gy, and used as APCs.

T-cell proliferation test *in vitro* Enriched T cells from treated or untreated spleen cell donors were co-cultured for 96 h in 96-well round-bottom plates (1 x 10^5 T cells/well) with 5 x 10^3 WBMCs and with or without 1 x 10^5 APCs, as indicated. ^{3}H-dThd (1µCi) was added to each well 18 h before harvesting. Cells were harvested using a cell harvester on filter coated with a solid scintillator (Ready filter with Xtalscint, Beckman Instruments, Fullerton, California, USA). ^{3}H-dThd incorporation was measured in a beta-scintillation counter (LS 6000 IC, Beckman Instruments).

Statistical analysis Values of PLN cell count indices, expressed as arithmetic means ± SD, were obtained from six to twelve animals per group. All experiments were performed at least twice to assess reproducibility of the data. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, California, USA). PLN cell count indices were compared using ANOVA with Bonferroni comparison.

Results

Differential capacity of aniline, its metabolites, and aniline-coupled lipids for induction of primary PLN reactions

Structural formulas of the test compounds used are shown in Scheme 1. In order to assess the capacity of aniline and its metabolites as well as that of fatty acid anilides and PAP esters (Scheme 1) to induce an immune reaction, mice received an sc injection of one of these compounds into a hind footpad. After six days, the cell numbers of the draining and the contralateral lymph node were determined. As shown in Figure 1A, aniline and its non-reactive metabolites nitrobenzene, *p*-aminphenol and *N*-acetyl-*p*-aminophenol failed to induce a PLN response. By contrast, nitrosobenzene proved to be a potent and *N*-hydroxylaniline a weak inducer of PLN responses. Linoleic anilide, linolenic anilide, and the indicated mixture of PAP esters respectively, induced significantly higher PLN responses when compared to equimolar doses of the respective aniline-free lipids (Figure 1B). Note that nitrosobenzene had already induced a significant PLN response at a 140-fold lower dose compared to the aniline-coupled lipids (Figure 1A). At doses higher than 0.2 µmol/mouse, nitrosobenzene could not be tested because it was insoluble. Kinetics of the primary PLN responses to nitrosobenzene and linolenic anilide showed that

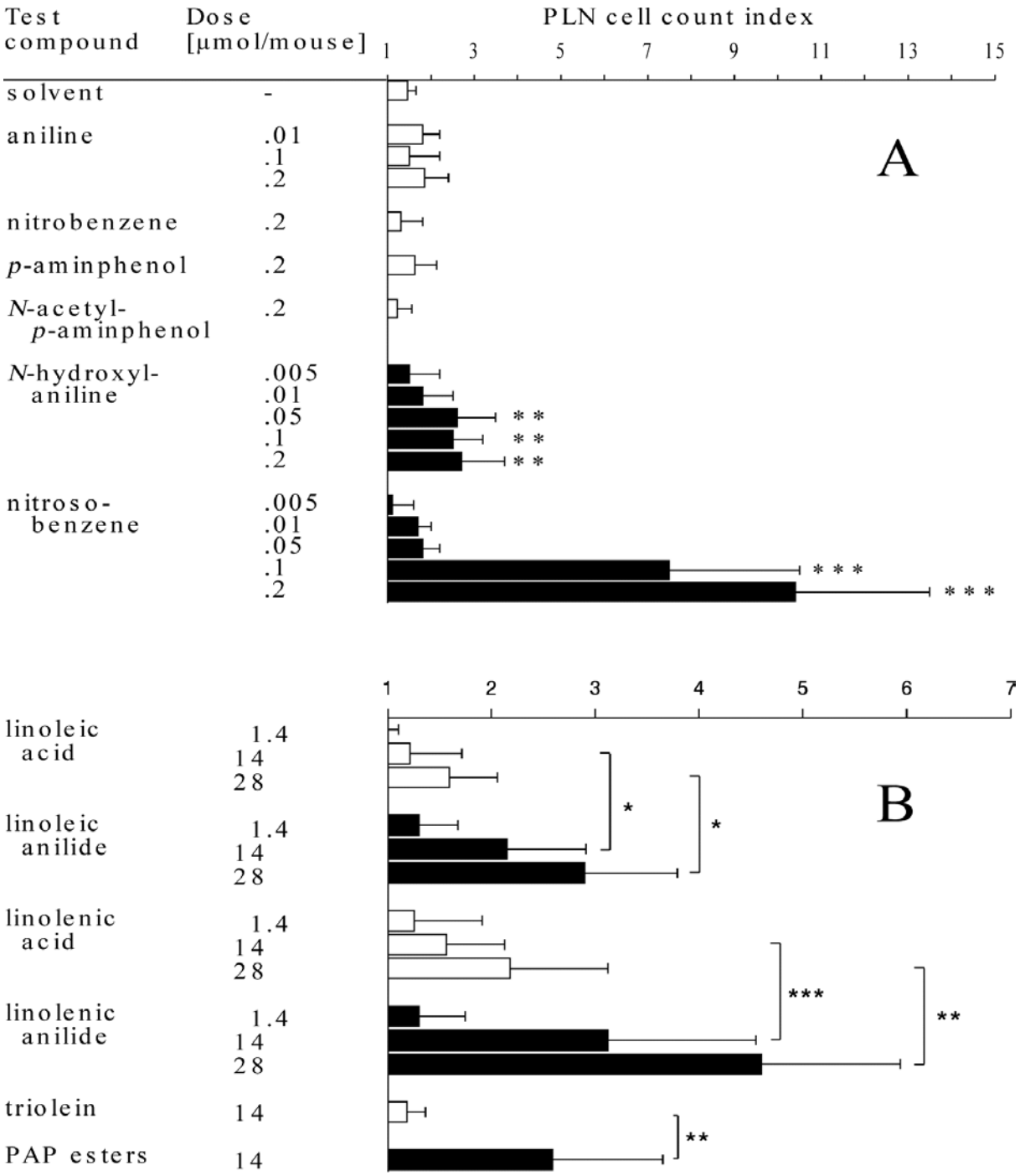


Figure 1. Primary PLN responses to reactive aniline metabolites and the aniline-coupled lipids of TOS. On day 0, groups of mice received an sc injection of the indicated test compound at the dose specified. PLN cell count indices were determined on day six. **Panel A**: Showing PLN reactions to aniline and its non reactive (open bars) and reactive metabolites (closed bars), respectively. The solvent used was ethanol/PBS. **Panel B**: Showing PLN reactions to the fatty acid anilides indicated and the mixture of PAP esters specified (closed bars). Control groups received the respective aniline-free lipids, i.e., the fatty acid indicated or triolein (open bars). Bars indicate arithmetic means + SD of two pooled experiments with six animals each. Asterisks indicate a significant difference (*$p<0.05$;**$p<0.01$; ***$p<0.001$) between the group indicated and the solvent control (**A**) or the control group indicated by brackets (**B**).

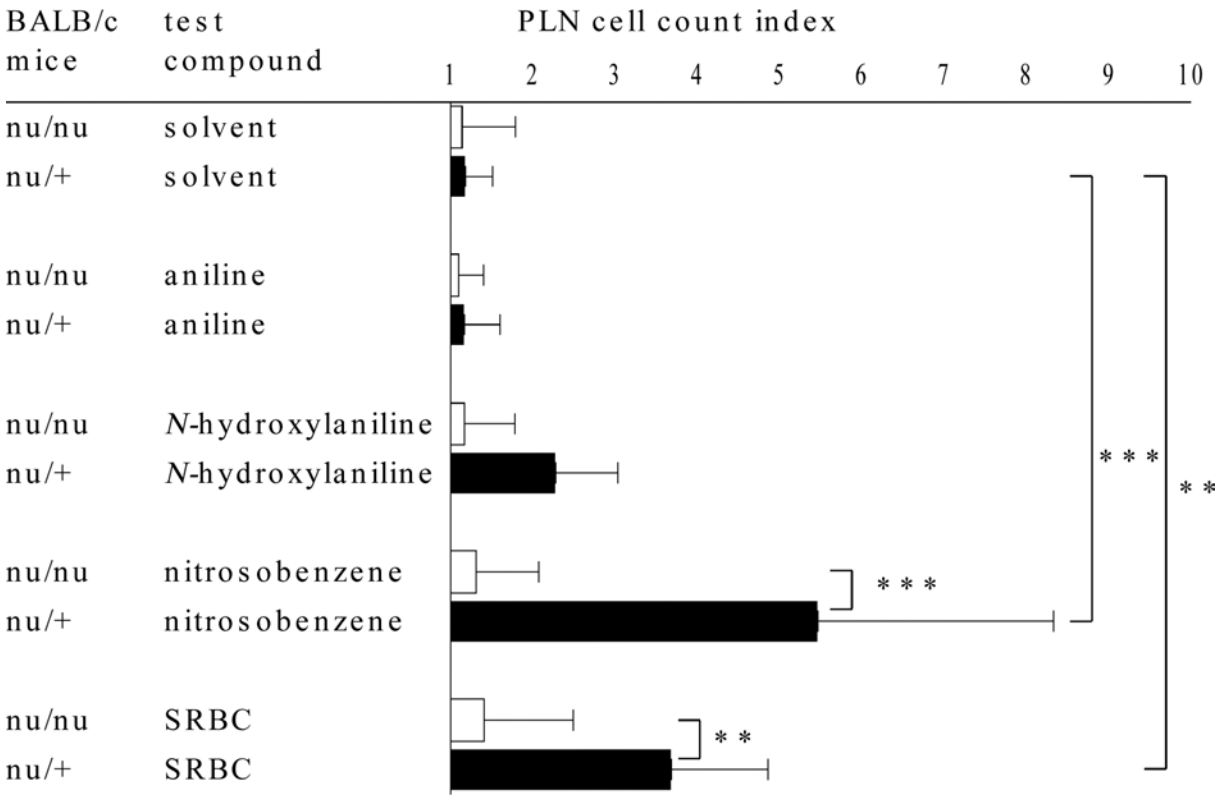


Figure 2. Showing T cell dependence of the primary PLN response to nitrosobenzene, *N*-hydroxylaniline, and linolenic anilide, respectively. On day 0, groups of athymic (nu/nu) mice (open bars) and euthymic (nu/+) mice (closed bars) on BALB/c genetic background received an sc injection containing 0.1 µmol of either aniline, nitrosobenzene, *N*-hydroxylaniline, 14 µmol of linolenic anilide, 14 µmol of linolenic acid, 1 x 10^7 sheep red blood cells, or ethanol/PBS only. PLN responses were measured on day six. Bars indicate arithmetic means + SD of two pooled experiments with five animals each. Asterisks indicate a significant difference (**$p<0.01$; ***$p<0.001$) between the groups compared by brackets.

their responses were maximal on day six after injection and then steadily declined until reaching normal values by day 90 and day 30, respectively (data not shown).

T-cell dependence of the PLN response to nitrosobenzene, *N*-hydroxylaniline, and linolenic anilide

For assessment of T-cell involvement in the PLN reaction, T cell-deficient BALB/c nu/nu mice or T cell-containing littermates (nu/+) received an sc injection, containing 0.1 µmol of either nitrosobenzene, aniline, or *N*-hydroxylaniline, 14 µmol of linolenic anilide or linolenic acid, or solvent only into a hind footpad; 1 x 10^7 sheep red blood cells were used as positive control because they are known to induce a T cell-dependent PLN response in mice

(20). In nu/+ mice, nitrosobenzene, linolenic anilide, and sheep red blood cells were found to induce significant PLN responses; and *N*-hydroxylaniline likewise evoked a response, albeit not statistically significant. By contrast, none of the test compounds elicited a significant PLN response in nu/nu mice (Figure 2).

B cell involvement in the PLN response to nitrosobenzene and linolenic anilide, respectively

For assessing the role of B cells in the PLN reaction, PLN cells were stained for T cell receptor, B220, CD4 and CD8 expression six days after injection of the test compounds. Although the PLN response to nitrosobenzene and linolenic anilide, respectively, is T cell-dependent (Figure 2), the PLN enlargement was mainly due to an increase in B cells (Table 1). No significant differences in percentage of $CD4^+$ and $CD8^+$ cells between the different groups could be detected.

Table 1. Flow cytometry results of PLN cells after injection of aniline and its derivatives

Compound tested	% T cells[a]	% B cells[b]	%$CD4^+$ of T cells[c]	%$CD8^+$ of T cells[d]
solvent	72.3 ± 4.1[e]	25.3 ± 4.4	53.4 6.5	37.3 4.4
aniline	72.8 ± 7.4	24.6 ± 7.6	52.2 3.6	38.4 6.6
nitrosobenzene	45.9 ± 2.8***[f]	51.6 ± 2.6***	50.4 3.7	41.4 3.0
linolenic acid	72.3 ± 6.0	24.3 ± 7.2	50.5 4.1	43.3 3.8
linolenic anilide	57.5 ± 7.7***	38.1 ± 8.5***	56.8 6.4	40.8 4.0
triolein	75.3 ± 5.5	22.1 ± 5.8	53.8 3.6	37.0 3.4
PAP esters	72.1 ± 7.3	25.8 ± 7.6	49.1 3.5	36.0 5.8

[a] % of T cells was determined as % of total PLN cells expressing αβ-T cell receptor; [b] % of B cells was determined as % of total PLN cells expressing CD45R (B220) without expressing T cell receptor; [c] % $CD4^+$ of T cells was determined as % of T cell receptor bearing cells expressing CD4; [d] % $CD8^+$ of T cells was determined as % of T cell receptor bearing cells expressing CD8; [e] shown are mean and SD of two pooled experiments with each four animals (n=8); [f] asterisks indicate a significant difference (***$p<0.001$) between the group indicated and its respective control group (aniline and linolenic acid, respectively).

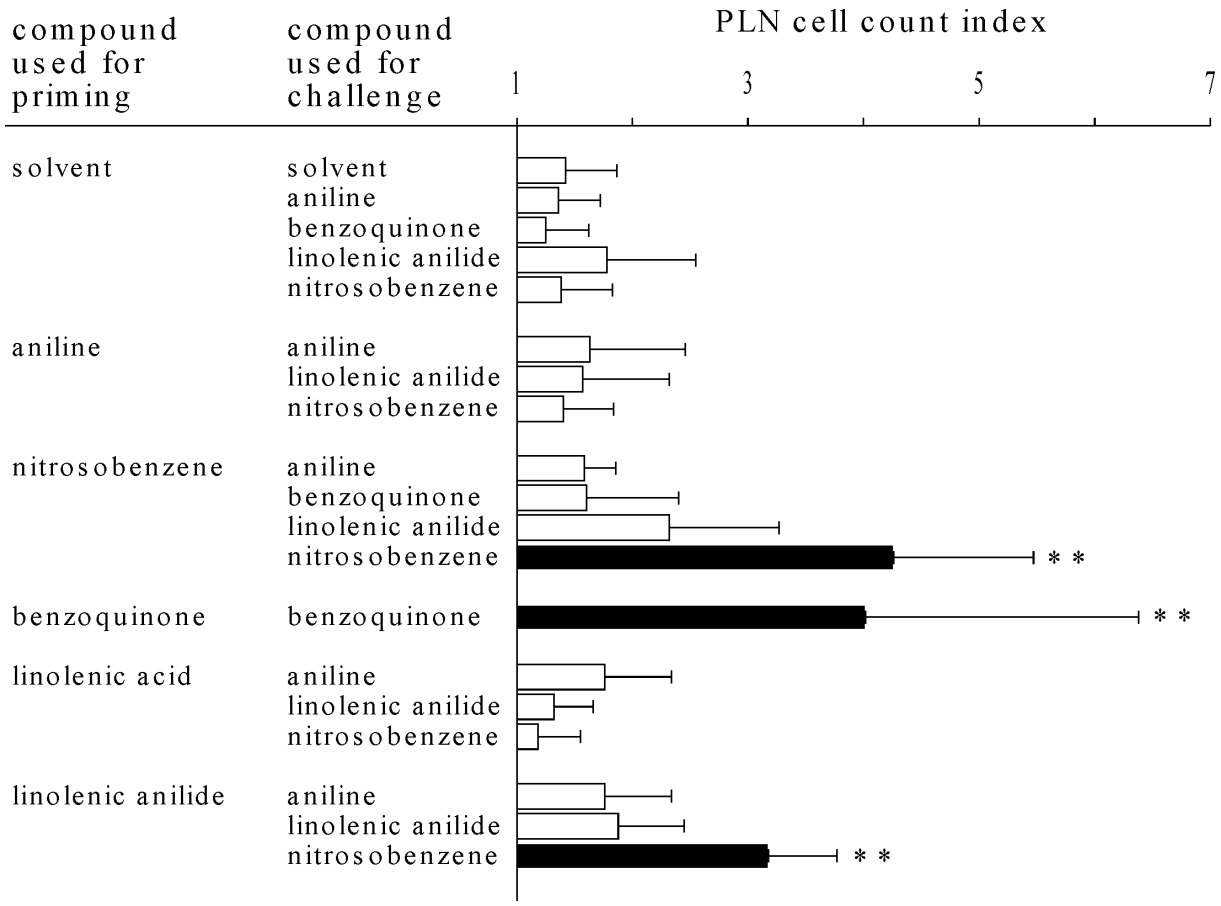


Figure 3. Secondary PLN responses against nitrosobenzene. Groups of mice received an sc injection containing 0.1 µmol of either aniline, nitrosobenzene, or *p*-benzoquinone, 28 µmol of linolenic anilide or linolenic acid, or ethanol/PBS only. After 13 weeks, mice were challenged by a second sc injection containing 0.005 µmol of either nitrosobenzene or aniline, 0.0001 µmol of *p*-benzoquinone, 1.4 µmol of linolenic anilide, or solvent only. Four days later, PLN cell count indices were determined. Bars indicate arithmetic means + SD of one experiment with six animals per group. Asterisks indicate a significant difference (**$p<0.01$) between the closed bars and each of the open bars.

Secondary PLN responses to nitrosobenzene

When performed after a single injection of a test compound, the PLN assay is unable to differentiate between an antigen-specific and a non-specific PLN reaction. To discriminate between these two possibilities, we investigated whether a secondary PLN response to nitrosobenzene could be induced in animals primed to this compound. For control of specificity, *p*-benzoquinone was used. *p*-Benzoquinone is a contact sensitizer whose ability to induce specific secondary PLN responses has been established (21). For this purpose, mice were primed by injecting either ethanol/PBS, 0.1 µmol aniline, nitrosobenzene, or *p*-benzoquinone dissolved in ethanol/PBS into a hind

footpad. After 13 weeks, groups of mice were challenged by a second sc injection into the same foot pad of either ethanol/PBS, 0.005 μmol aniline or nitrosobenzene, or 0.0001 μmol *p*-benzoquinone dissolved in ethanol/PBS. As shown in Figure 3, a recall response was only detectable in those mice which had been both primed and challenged with nitrosobenzene or *p*-benzoquinone, respectively.

To investigate secondary PLN responses against one of the aniline-coupled lipids, mice were primed with linolenic anilide (28 μmol /mouse). After size and cellularity of the draining PLNs had reverted back to normal, the mice received recall injections consisting of suboptimal doses of nitrosobenzene (0.005 μmol/mouse), linolenic anilide (1.4 μmol/mouse), and aniline (0.005 μmol/mouse), respectively. As shown in Figure 3, animals which had been primed with linolenic anilide responded only upon recall with nitrosobenzene, not upon recall with linolenic anilide. In contrast, mice which had received linolenic acid for priming, failed to mount a PLN response upon recall with nitrosobenzene, and the same was true when free aniline was used for priming. In another experiment, in which mice were primed with a dose of 14 μmol/mouse of linolenic anilide, we also detected a secondary response to nitrosobenzene (data not shown). However, the secondary response to nitrosobenzene in mice primed to linolenic anilide was not reproducible. For both priming doses mentioned above (28 and 14 μmol / mouse), the experiment was performed three times, but for each priming dose, secondary responses to nitrosobenzene were obtained only once.

Primary PLN response to WBMCs pulsed with aniline

It has been reported that neutrophils, monocytes, and macrophages are able to metabolize arylamines (22). Therefore we asked whether WBMCs, which are rich in precursors of granulocytes and macrophages (23), are able to metabolize aniline to a protein-reactive metabolite that, in turn, would be able to haptenate self-proteins and elicit a PLN response. To answer this question, WBMCs were cultured in the presence of aniline, homogenized in order to make cellular proteins available for uptake by APCs, and used in the PLN assay. Figure 4 shows that up to 5 x 10^6 homogenized WBMCs cultured with solvent only, induced background responses in the draining PLN, whereas a dose-response

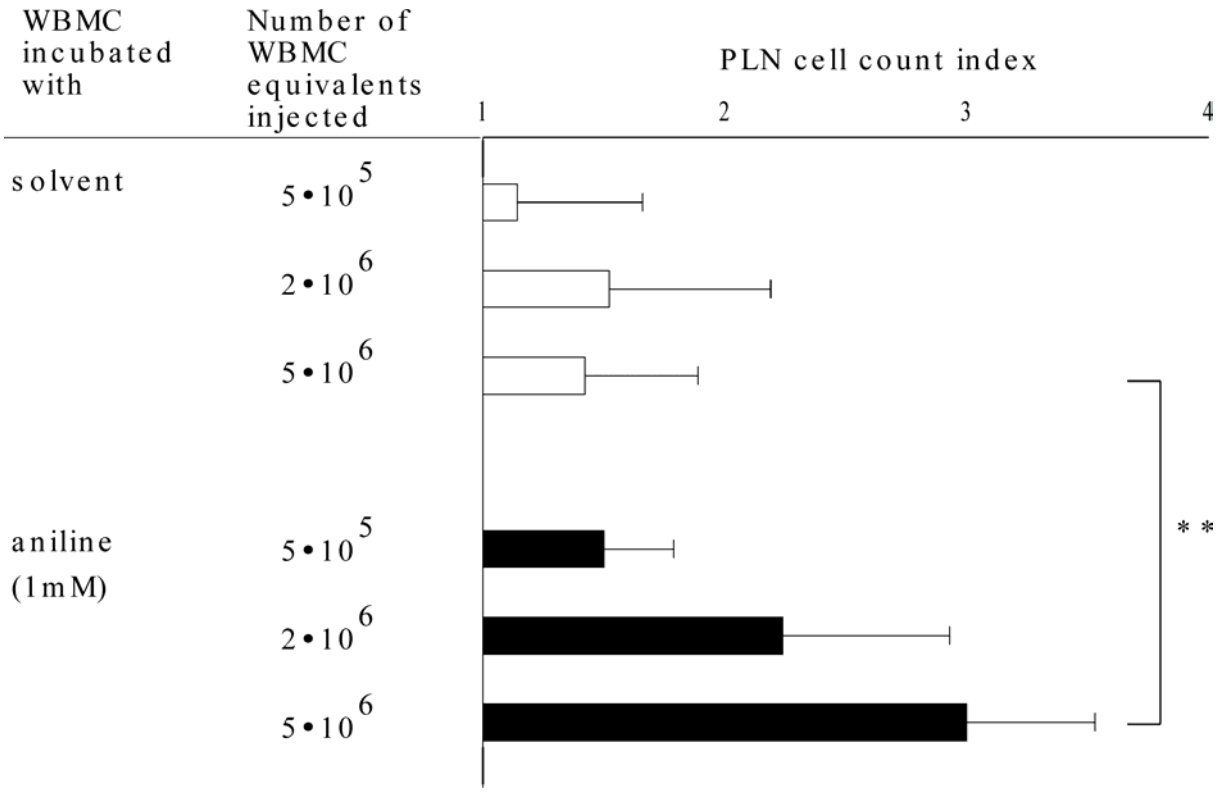


Figure 4. Primary PLN response against aniline-pulsed WBMCs. WBMCs were incubated in medium containing either 1 mM aniline dissolved in 0.1 % ethanol or ethanol only (solvent). WBMCs were washed, resuspended in PBS, and homogenized. On day 0, groups of mice received an sc injection of 50 µL PBS containing the indicated cell equivalents of homogenized WBMCs. PLN cell count indices were determined on day six. Bars indicate arithmetic means + SD of two pooled experiments with five animals each. Asterisks indicate a significant difference (**$p<0.01$) between the group which received aniline-treated WBMCs and the respective control group.

relationship was seen in response to homogenized WBMCs cultured with aniline. At the highest dose tested (5 x 10^6 cells per mouse), there was a significantly higher PLN reaction against the aniline-pretreated WBMCs compared to the control WBMCs pretreated with solvent only.

Nitrosobenzene-primed T cells show a recall response when cultured with APCs and WBMCs pulsed with aniline

We then asked whether the PLN reaction to aniline-pulsed WBMCs shown in Figure 4 was antigen-specific or not. To test this, the lymphocyte proliferation test *in vitro* was used. WBMCs from BALB/c mice were preincubated in the presence or absence of aniline and subsequently cocultured with syngeneic

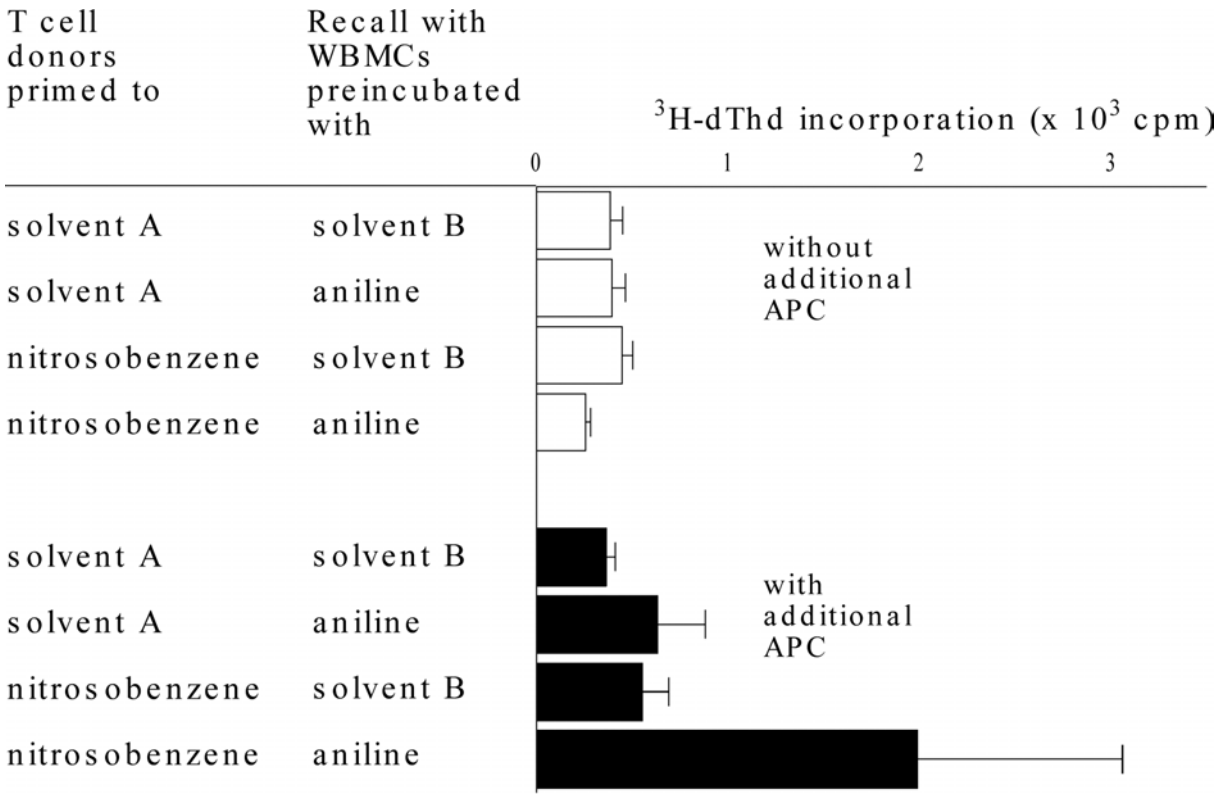


Figure 5. *In vitro* secondary response of nitrosobenzene-primed T cells against WBMCs pulsed with aniline. Groups of BALB/c mice served as prospective T cell donors, they were primed by two sc injections containing either an immunogenic dose of nitrosobenzene or ethanol/PBS only (solvent A). For recall *in vitro*, WBMCs were preincubated in medium containing either 1 mM aniline dissolved in 0.1 % ethanol or 0.1 % ethanol (solvent B) only. Washed WBMCs were cocultured with T cells from donor mice primed as indicated. Cultures were performed in the absence (upper panel) or presence (lower panel) of irradiated spleen cells from untreated mice, which served as APCs. Bars indicate arithmetic means + SD of six replicates.

T cells, primed with either nitrosobenzene or solvent. Figure 5 shows the results of a representative experiment. Provided additional APCs were supplied, nitrosobenzene-primed T cells reacted against aniline-pulsed WBMCs, but not against control WBMCs pulsed with ethanol. In four out of six experiments of this type, nitrosobenzene-primed T cells mounted a secondary response against WBMCs pulsed with aniline. In none of these six experiments, T cells from PBS-treated animals reacted to WBMCs pulsed with aniline. These results show that nitrosobenzene-primed T cells can react in an anamnestic fashion towards a neo-antigen, which is induced in aniline-pulsed WBMCs.

Discussion

Adverse immune reactions to chemicals are thought to arise due to T cell reactions to chemical-induced neoantigens (10,24,25). In the past years, pioneering studies investigating the nature of such neoantigens and conditions of how they are generated have been performed (reviewed in 26-30). Thus, in order to be recognizable by T cells, electrophilic organic haptens, such as trinitrophenyl and 3-pentadecyl-catechol, were found to require covalent bonding to a nucleophilic amino-acid side-chain of protein. More exactly, for T cell recognition, the hapten must be bound to protein fragments fitting into the peptide-binding groove of molecules of the major histocompatibility complex (27,31,32). Chemicals which themselves, due to their lack of protein reactivity, are unable to act as haptens, but whose reactive intermediates are able to do so, are termed prohaptens (33,34). Their reactive intermediates are able to bond in covalent fashion to self proteins, and it is assumed that these are processed and their neoantigens presented to T cells (24). If the T cells recogni-zing neoantigens simultaneously receive costimulatory signals from APCs, they are primed and will start an immune response. Individuals whose T cells were primed to a given antigen harbor memory T cells and, hence, respond faster and require less antigen upon specific recall than non-sensitized individuals. With respect to neoantigens induced by reactive intermediates, the specificity of the immune response can be used to determine whether or not administration of a given parent compound has generated neoantigens identical with those induced by the reactive metabolite under study (35-38). Questions raised in the present paper concern the T cell-sensitizing potential of aniline, its metabolites, and of the aniline-coupled lipids implicated in TOS. Table 2 gives an overview of the results obtained with the different compounds tested in the PLN assay.

In the case of aniline, which is unable to covalently bond to protein (1), our results are in line with the prohapten-hapten concept described above. Injection of free aniline failed to induce a PLN reaction, whereas injection of its reactive metabolites, *N*-hydroxylaniline and nitrosobenzene, succeeded to do so. Mice primed to nitrosobenzene, but not those injected with solvent or aniline, showed an enhanced PLN response to a small dose of nitrosobenzene, indicating a specific anamnestic response to this compound. Our results obtained with aniline-pulsed WBMCs support the general notion (8,36) that cells of the immune system, especially phagocytic cells, can metabolize

Table 2. Overview of the results of experimental sensitization to aniline and its derivatives

	PLN assay			
Compound tested	Primary PLN response	T cell dependence	Secondary PLN response	% B cells increased
aniline	no			
nitrobenzene	no			
p-aminophenol	no			
N-acetyl-*p*-aminphenol	no			
N-hydroxylaniline	yes	yes	n.d.[a]	n.d.
nitrosobenzene	yes	yes	yes [b,c]	yes
linolenic acid	no			
linolenic anilide	yes	yes	no	yes
linoleic acid	no			
linoleic anilide	yes	n.d.	n.d.	n.d.
triolein	no			
PAP esters	yes	n.d.	n.d.	no

[a] n.d.: not determined; [b] In two out of six experiments, secondary PLN responses to nitrosobenzene also were detectable in mice primed to linolenic anilide. [c] *In vitro*, nitrosobenzene-primed T cells responded in anamnestic fashion to aniline-pulsed WBMCs as well.

prohaptens, such as aniline, into protein-reactive haptens, such as nitrosobenzene, and thus give rise to immunologically relevant neoantigens.

Unlike exposure to aniline, exposure to its *para*-substituted derivatives,

such as *para*-phenylenediamine and procainamide, is known to induce adverse immune reactions in humans as well as experimental animals (10,39-41). We propose that the difference may be explained by the different toxicokinetics of aniline and its *para*-substituted derivatives. Aniline is known to be primarily metabolized in the liver (3,42,43). The metabolite thus formed, *N*-hydroxylaniline, diffuses into the blood and, after oxidation to nitrosobenzene with concomitant formation of methemoglobin, it covalently bonds to hemoglobin and accumulates in erythrocytes and the spleen (44). Whether hemoglobin thus haptenated spontaneously elicits immune reactions is not known. As most of the adverse immune reactions to the *para*-substituted derivatives of aniline occur extrahepatically, e.g., in the skin or the immune system, it is conceivable that the reactive intermediates involved are formed in the affected tissues themselves (24). This assumption is corroborated by the fact that the addition of chemical groups in *para*-position to the amine group of aniline can prevent hepatic metabolism of aniline through aniline-4-hydroxylase, i.e. CYP2E1, activity (45), thus favoring extrahepatic metabolism of the respective arylamine and increasing the risk of adverse immune reactions at extrahepatic sites. Two such para-substituted metabolites are *p*-aminophenol and *N*-acetyl-*p*-aminophenol, which are major aniline metabolites formed during hepatic aniline metabolites (45). Both can be further oxidized to their respective quinones, which are highly protein-reactive. The oxidation to the reactive quinones mainly takes place in the liver, where they are detoxified by the high levels of GSH present in this organ, or, in case detoxification fails, induce liver damage (46). Both *p*-aminophenol and *N*-acetyl-*p*-aminophenol failed to induce a primary PLN reaction, suggesting that the quinones are not formed locally following injection of the para-hydroxylated aniline metabolites.

As the lack of sensitizing capacity of free aniline seems to be related to the high degree of its hepatic metabolism and detoxification (47), we enhanced its extrahepatic metabolism by deliberately targeting aniline to the extrahepatic metabolic system. This was achieved by pulsing WBMCs with aniline *in vitro* and using them as antigen. In the PLN assay, WBMCs pulsed with aniline, but not those pulsed with solvent, were able to induce a significant response. Moreover, we observed a recall response of nitrosobenzene-primed T cells to such aniline-pulsed WBMCs in the lymphocyte proliferation test; WBMCs are rich in neutrophil precursors, and these are known to have a high metabolizing capacity for arylamines (8,22). In this test system, three cell types apparently

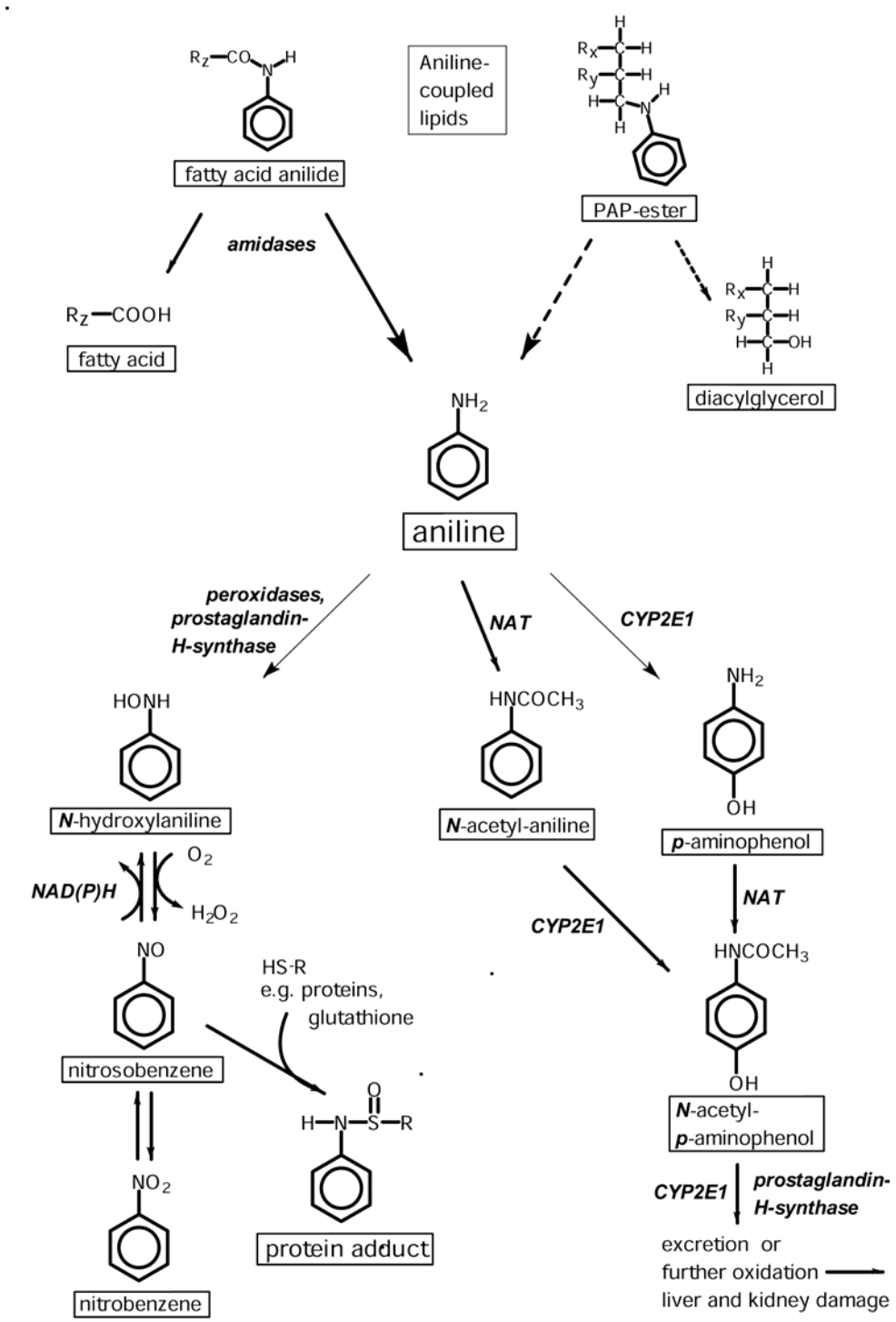
had to cooperate: first, WBMCs that converted the prohapten aniline into the hapten nitrosobenzene; second, APCs that picked up proteins haptenated by nitrosobenzene and presented the relevant neoantigen to the primed T cells; and third, T cells primed to the hapten-induced neoantigen and responding to it upon recall.

As far as the aniline-coupled lipids implicated in TOS are concerned, we found that the immunostimulatory capacity of aniline-coupled lipids clearly exceeded that of equimolar concentrations of lipids devoid of aniline. This distinction could not be made in the study of Bell et al. (48), who reproduced some of the pathological signs of TOS in mice; these authors only investigated the aniline-coupled lipid oleic anilide, but did not study the respective aniline-free control oleic acid. Another clear-cut result obtained in the present study is the T cell-dependence and B cell involvement of the immune response to linolenic anilide. Beyond this, however, our results obtained with aniline-coupled lipids are less conclusive. The inability of free aniline to induce a PLN reaction conforms with previous findings made with other non-reactive, prohaptenic chemicals, such as procainamide, propylthiouracil, and benzene, all of which failed to induce primary PLN reactions (18,21,26,37,49). Given that both aniline-coupled lipids and aniline failed to be protein-reactive, why then did aniline-coupled lipids, but not aniline, induce primary PLN responses? There are two different, mutually not exclusive explanations to account for this and both of them focus on the lipid moiety, as this distinguishes the aniline-coupled lipids from aniline and the other prohaptens mentioned above.

Due to their lipid moiety the aniline-coupled lipids probably were retained at the site of injection. The prolonged retention time would lead to a higher local concentration of the respective compound, enabling the local metabolizing system to cleave aniline extrahepatically from the parent compound and form sufficient reactive metabolite (*N*-oxidation of aniline to nitrosobenzene) to induce an immune response. More specifically, through their fatty acid moiety the aniline-coupled lipids probably can be taken up by lipid receptors present on monocytes, macrophages, and, perhaps, dendritic cells (50), where they might be metabolized into haptenic metabolites, such as nitrosobenzene. Covalent bonding of nitrosobenzene to unidentified self-proteins (Scheme 2) creates neoantigens that are recognized by T cells. The initial step in this hypothetical chain of events is supported by the results of Bioque *et al*. (51,52) who showed that polymorphonuclear cells and macrophages, by an amidase-like activity, can

cleave the aniline moiety from fatty acid anilides. Macrophages, notably, can also act as APC and thus interact with T cells. The second and the third step in the hypothetical pathogenesis of TOS, namely *N*-oxidation of aniline and nitrosobenzene-induced neoantigen formation in phagocytes, are supported by our finding that T cells primed to nitrosobenzene reacted in anamnestic fashion when WBMCs pulsed with aniline were used as recall antigen in vitro. Disappointingly, however, results of those experiments that were designed to assess the validity of the entire chain of events postulated (Scheme 2, left pathway) failed to give a conclusive answer. While in two out of six experiments performed animals primed to linolenic anilide, indeed, mounted an anamnestic response to nitrosobenzene, as expected according to our hypothesis, this result could not be reproduced in the other four experiments. This might suggest that the immune response seen after priming with a single dose of linolenic anilide was not only directed against nitrosobenzene, but against other types of antigen as well. It should be noted in this context that mice primed with linolenic anilide did not react upon recall to suboptimal doses of linolenic anilide itself. Conceivably, the combination of both the low dose of linolenic anilide used for recall and the short period of time after the recall injection induced an amount of nitrosobenzene that was too low for local formation of the relevant neoantigen.

It might also be possible that the lipid moiety of fatty acid anilides provide signal 2 by their intrinsic adjuvant effect on APC, including macrophages, directing the immune response towards the aniline moiety. The adjuvant effect of lipids is well known, and this is especially so when they are covalently bound to the immunizing antigen (53,54); notably, this is just the way lipids are linked to aniline in fatty acid anilides and PAP esters. Furthermore, aniline injected without the lipid moiety would rapidly diffuse through the injection site to be detoxified in the liver, as discussed above. Hence, after injection of free aniline, the concentration of reactive metabolite formed locally probably was too low for induction of an immune response. A somewhat different hypothesis to account for the immunostimulatory capacity of aniline-coupled lipids and the lack of this capacity in aniline administered alone could be that the aniline-coupled lipids were able to activate T cells via non-classical pathways. In this regard, it has been reported that CD1 molecules, a newly described family of antigen-presenting molecules not encoded by the major histocompatibility complex, are able to present antigenic



Scheme 2. Pathway of oxidative aniline metabolism and postulated metabolism of aniline-coupled lipids implicated in TOS. Aniline is *N*-oxidized to nitrosobenzene, which can bind to sulphydryl groups (HS-R) of proteins or glutathione (lower left). Para-hydroxylation and subsequent *N*-acetylation of aniline results in aminophenol and *N*-acetly-*p*-aminophenol, respectively. These metabolites can be excreted or further oxidized to quinone imines which can induce liver and kidney damage (lower right). Aniline can be cleaved from fatty acid anilides, presumably by intracellular amidases (see text). A similar cleavage of aniline from PAP esters has not yet been demonstrated. Rx, Ry, and Rz stand for aliphatic chains of fatty acids (see Scheme 1).

lipids to T cells (55), thus raising the question of whether the aniline-coupled lipids of TOS can induce CD1-restricted T cell responses. The original publications on CD1-restricted T cell responses to lipids were performed with $CD8^+$ T cells using their αβ-T cell receptor, but CD1-restricted responses have been also described for $CD4^+$ and $CD4^- CD8^-$ T cells, respectively, as well as for NK-T cells and T cells using their γδ receptors (56,57). Evidence accumulates that cells of the innate immune system rather than classical T or B cells initiate adverse immune reactions to chemicals (58). As cells of the innate immune system do not mount anamnestic responses upon a second encounter with a small amount of the same antigen, this could explain our inability to detect an anamnestic response to linolenic anilide in mice primed to this compound.

In conclusion, the results presented here show that aniline can be considered a prohapten. Depending on mode of external or internal exposure, its site of metabolism into the hapten nitrosobenzene may shift from the liver to extrahepatic tissue, such as WBMCs. After haptenating unidentified self-proteins or -peptides, nitrosobenzene apparently creates neoantigens and thus can act as a T cell sensitizer. This mechanism can be invoked for the pathogenesis of TOS. However, a sequence of multiple biochemical and cellular events is involved in the anamnestic anti-hapten responses following priming with a complex, unusual prohapten, such as linolenic anilide, and challenge with the hapten. This complexity might explain why the results of the evoked anamnestic responses to nitrosobenzene after priming with linolenic anilide were so poorly reproducible, unlike the results of those experiments where both recall and priming were carried out with nitrosobenzene. Therefore, as an additional explanation for the immunostimulatory effect of aniline-coupled lipids we proposed antigen presentation by CD1 and recognition by non-classical T cells, and experiments are now under way to test this.

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References

1. **Neumann, H.-G. and W. Albrecht**. 1985. Biomonitoring of aniline and nitrobenzene. *Arch.Toxicol. 57*: 1-5.

2. **Harrison, J. H. J. and D. J. Jollow**. 1987. Contribution of aniline metabolites to aniline-induced methemoglobinemia. *Mol.Pharmacol. 32*: 423-431.

3. **Harrison, J. H. J. and D. J. Jollow**. 1986. Role of aniline metabolites in aniline-induced hemolytic anemia. *J.Pharmacol.Exp.Ther. 238*: 1045-1054.

4. **Malejka-Giganti, D. and C. L. Ritter**. 1994. Peroxidative metabolism of carcinogenic N-arylhydroxamic acids: implications for tumorigenesis. *Env.Health Perspect. 102(suppl 6)*: 75-81.

5. **Seidenari, S., L. Mantovani, B. M. Manzini, and M. Pignatti**. 1997. Cross-sensitizations between azo dyes and para-amino compound. A study of 236 azo-dye-sensitive subjects. *Contact Dermatitis 36*: 91-96.

6. **Estlander, T.** 1988. Allergic dermatoses and respiratory diseases from reactive dyes. *Contact Dermatitis 18*: 290-297.

7. **Krasteva, M., J.-F. Nicolas, G. Chabeau, J.-L. Garrigue, H. Bour, J. Thivolet, and D. Schmitt**. 1993. Dissociation of allergenic and immunogenic functions in contact sensitivity to para-phenylenediamine. *Int.Arch.Allergy Immunol. 102*: 200-204.

8. **Uetrecht, J. P.** 1992. The role of leukocyte-generated reactive metabolites in the pathogenesis of idiosyncratic drug reactions. *Drug Metab.Rev. 24*: 299-366.

9. **Basketter, D. A., G. F. Gerberick, I. Kimber, and S. E. Loveless**. 1996. The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. *Food Chem.Toxicol. 34*: 985-997.

10. **Uetrecht, J. P.** 1988. Mechanism of drug-induced lupus. *Chem.Res.Toxicol. 1*: 133-143.

11. **Tabuenca, J. M.** 1981. Toxic-allergic syndrome caused by ingestion of rapeseed oil denatured with aniline. *Lancet 12*: 567-568.

12. **Kilbourne, E. M., J. T. Bernet, M. Posada de la Paz, R. H. Hill, I. Abaitua Borda,**

and M. M. Zack. 1988. Chemical correlates of pathogenicity of oils related to the toxic oil syndrome epidemic in Spain. *Am.J.Epidemiol. 127*: 1210-1227.

13. **Hill, R. H. J., H. H. Schurz, M. Posada de la Paz, I. A. Borda, R. M. Philen, E. M. Kilbourne, S. L. Head, S. L. Bailey, W. J. Driskell, J. R. Barr, and L. L. Needham**. 1995. Possible etiologic agents for toxic oil syndrome: Fatty acid esters of 3-(N-phenylamino)-1,2-propanediol. *Arch.Env.Contam.Toxicol. 28*: 259-264.

14. **Merk, H. F., D. Niederau, M. Hertl, and F. Jugert**. 1991. Drug metabolism and drug allergy. In *New trends in allergy III*. J. Ring and B. Przybilla, eds. Springer-Verlag, Berlin, p. 269-279.

15. **Gleichmann, H. and E. Gleichmann**. 1984. GVHD, a model for Spanish toxic oil syndrome? *Lancet 1*: 1474.

16. **Martin, S. and H. U. Weltzien**. 1994. T cell recognition of haptens, a molecular view. *Int.Arch.Allergy Immunol. 104*: 10-16.

17. **Ferrer, M., M. Galceran, F. Sanchez-Baeza, J. Casas, and A. Messeguer**. 1993. Synthesis of aniline derivatives with potential toxicological implications to the spanish toxic oil syndrome. *Liebigs Ann.Chem.* 507-511.

18. **Bloksma, N., M. Kubicka-Muranyi, H.-C. Schuppe, E. Gleichmann, and H. Gleichmann**. 1995. Predictive immunotoxicological test systems: Suitability of the popliteal lymph node assay in mice and rats. *Crit.Rev.Toxicol. 25*: 369-396.

19. **Miltenyi, S., W. Müller, W. Weichel, and A. Radbruch**. 1990. High gradient magnetic cell separation with MACS. *Cytometry 11*: 231-238.

20. **Kubicka-Muranyi, M., R. Goebels, C. Goebel, J. Uetrecht, and E. Gleichmann**. 1993. T lymphocytes ignore procainamide, but respond to its reactive metabolites in peritoneal cells: demonstration by the adoptive transfer popliteal lymph node assay. *Toxicol.Appl.Pharmacol. 122*: 88-94.

21. **Gollhausen, R., B. Przybilla, and J. Ring**. 1989. Reproducibility of patch tests. *J.Am.Acad.Dermatol. 21*: 1196-1202.

22. **Uetrecht, J., N. Zahid, and R. Rubin**. 1988. Metabolism of procainamide to a hydroxylamine by human neutrophils and mononuclear leukocytes. *Chem.Res.Toxicol. 1*: 74-78.

23. **Padovan, E., D. Mauri-Hellweg, W. J. Pichler, and H. U. Weltzien**. 1996. T cell recognition of penicillin G: structural features determining antigenic specificity. *Eur.J.Immunol. 26*: 42-48.

24. **Griem, P., M. Wulferink, B. Sachs, J. B. Gonzalez, and E. Gleichmann**. 1998. Allergic and autoimmune reactions to xenobiotics: how do they arise? *Immunol.Today 19*: 133-141.

25. **Mauri-Hellweg, D., F. Bettens, D. Mauri, C. Brander, T. Hunziker, and W. J. Pichler**. 1995. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazipine. *J.Immunol. 155*: 462-472.

26. **Griem, P., M. Wulferink, B. Sachs, J. B. Gonzales, and E. Gleichmann**. 1998. Allergic and autoimmune reactions to xenobiotics: how do they arise? *Immunol.Today 19*: 133-141.

27. **Cavani, A., C. J. Hackett, K. J. Wilson, J. B. Rothbard, and S. I. Katz**. 1995. Characterization of epitopes recognized by hapten-specific CD4+ T cells. *J.Immunol. 154*: 1232-1238.

28. **Gelber, C., L. Gemmell, D. McAteer, M. Homola, P. Swain, A. Liu, J. Wilson, and M. Gefter**. 1997. Down-regulation of poison ivy/oak-induced contact sensitivity by treatment with a class II MHC binding peptide:hapten conjugate. *J.Immunol. 158*: 2425-2434.

29. **Padovan, E., T. Bauer, M. M. Tongio, H. Kalbacher, and H. U. Weltzien**. 1997. Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy. *Eur.J.Immunol. 27*: 1303-1307.

30. **Martin, S. and H. U. Weltzien**. 1994. T cell recognition of haptens, a molecular view. *Int.Arch.Allergy Immunol. 104*: 10-16.

31. **Weltzien, H. U., C. Moulon, S. Martin, E. Pardovan, U. Hartmann, and J. Kohler**. 1996. T cell immune responses to haptens. Structural models for allergic and autoimmune reactions. *Toxicology 107*: 141-151.

32. **Cohen, S. D., N. R. Pumford, E. A. Khairallah, K. Boekelheide, L. R. Pohl, H. R. Amouzadeh, and J. A. Hinson**. 1997. Selective protein covalent binding and target organ toxicity. *Toxicol.Appl.Pharmacol. 143*: 1-12.

33. **Landsteiner, K. and J. Jacobs**. 1936. Studies on the sensitization of animals with simple chemical compounds. *J.Exp.Med. 64*: 625-639.

34. **Dupuis, G. and C. Benezra**. 1982. Allergic contact dermatitis to simple chemicals. In *Dermatology*. 1 ed. C.D. Calnan and H.I. Maibach, eds. Dekker,M., New York/Basel, p. 66-69.

35. **Griem, P., K. Takahashi, H. Kalbacher, and E. Gleichmann**. 1995. The

antirheumatic drug disodium aurothiomalate inhibits CD4+ T cell recognition of peptides containing two or more cysteine residues. *J.Immunol. 155*: 1575-1587.

36. **von Schmiedeberg, S., U. Hanten, C. Goebel, H.-C. Schuppe, J. Uetrecht, and E. Gleichmann**. 1996. T cells ignore the parent drug propylthiouracil but are sensitized to a reactive metabolite generated in vivo. *Clin.Immunol.Immunopathol. 80*: 162-170.

37. **Gollhausen, R., B. Przybilla, and J. Ring**. 1989. Reproducibility of patch test results: Comparison of TRUE test and Fin chamber test results. *J.Am.Acad.Dermatol. 21*: 843-846.

38. **Lachapelle, J.-M. and J.-L. Antoine**. 1989. Problems raised by the simultaneous reproducibility of positive allergic patch test reactions in man. *J.Am.Acad.Dermatol. 21*: 850-854.

39. **Kalish, R. S. and J. A. Wood**. 1995. Sensitization of mice to paraphenylenediamine and strucurally-related compounds: adjuvant effects of vitamin A supplementation. *Contact Dermatitis 33*: 407-413.

40. **Goldberg, B. J., F. F. Herman, and I. Hirata**. 1987. Systemic anaphylaxis due to an oxidation product of p-phenylenediamine in a hair dye. *Ann.Allergy 58*: 205-208.

41. **Katsutani, N. and H. Shionoya**. 1992. Popliteal lymph node enlargement induced by procainamide. *J.Immunopharmacol. 14*: 681-686.

42. **Khan, M. F., B. S. Kaphalia, and G. A. S. Ansari**. 1995. Erythrocyte-aniline interaction leads to their accumulation and iron deposition in rat spleen. *J.Toxicol.Environ.Health 44*: 415-421.

43. **Kiese, M. and K. Taeger**. 1976. The fate of phenylhydroxylamine in human red cells. *Naunyn-Schmiedebergs Arch.Pharmacol. 292*: 59-66.

44. **Eyer, P.** 1988. Detoxication of N-oxygenated arylamines in erythrocytes. An overview. *Xenobiotica 18*: 1327-1333.

45. **Parkinson, A.** 1996. Biotransformation of xenobiotics. In *Casarett & Doull's toxicology*. 5th ed. C.D. Klaassen, ed. McGraw-Hill, New York, p. 113-186.

46. **Lee, S. S. T., J. T. M. Buters, T. Pineau, P. Fernandez-Salguero, and F. J. Gonzalez**. 1996. Role of CYP2E1 in the hepatotoxicity of acetaminophen. *J.Biol.Chem. 271*: 12063-12067.

47. **Maples, K. R., P. Eyer, and R. P. Mason**. 1989. Aniline-, phenylhydroxylamine-, nitrosobenzene-, and nitrobenzene-induced hemoglobin thiyl free radical formation in

vivo and in vitro. *Mol.Pharmacol. 37*: 311-318.

48. **Bell, S., M. V. Hobbs, and R. L. Rubin**. 1992. Isotype-restricted hyperimmunity in a murine model of the toxic oil syndrome. *J.Immunol. 148*: 3369-3376.

49. **Schmiedeberg, S. v., U. Hanten, C. Goebel, H. C. Schuppe, J. Uetrecht, and E. Gleichmann**. 1996. T cells ignore the parent drug propylthiouracil but are sensitized to a reactive metabolite generated in vivo. *Clin.Immunol.Immunopathol. 80*: 162-170.

50. **Terpstra, V., D. A. Bird, and D. Steinberg**. 1998. Evidence that the lipid moiety of oxidized low density lipoprotein plays a role in its interaction with macrophage receptors. *Proc.Natl.Acad.Sci.USA 95*: 1806-1811.

51. **Bioque, G., J. Abian, O. Bulbena, J. Rosello-Catafau, and E. Gelpi**. 1994. N-phenyllinoleamide metabolism by human polymorphonuclear leukocytes. *Xenobiotica 24*: 613-621.

52. **Bioque, G., J. Abian, O. Bulbena, J. Rosello-Catafau, and E. Gelpi**. 1995. Mass spectrometric identification of N-phenyllinoleamide metabolites in mouse peritoneal macrophages. *Rapid Commun.Mass Spectrum. 9*: 753-760.

53. **Hopp, T. P.** 1984. Immunogenicity of a synthetic HBsAg peptide: enhancement by conjugation to fatty acid carrier. *Mol.Immunol. 21*: 13-16.

54. **Benmohamed, R., H. Grass-Masse, A. Tartar, P. Daubersies, K. Brahimini, M. Bossus, A. Thomas, and P. Druilhe**. 1997. Lipopeptide immunization without adjuvant induces potent and long-lasting B, T helper and cytotoxic T lymphocytes against malaria liver stage antigen in mice and chimpanzees. *Eur.J.Immunol. 27*: 1242-1253.

55. **Zeng, Z. H., A. R. Castano, B. W. Segelke, E. A. Stura, P. A. Peterson, and I. A. Wilson**. 1997. Crystal structure of mouse CD1: an MHC-like fold with a large hydrophobic binding groove. *Science 277*: 339-345.

56. **Bell, S. J. D. and W. J. Pichler**. 1989. Penicillin-allergic patients react to penicillin-modified "self". *Allergy 44*: 199-203.

57. **Sabbe, L. J. M., L. de Bode, and J. J. van Rood**. 1983. Analysis of variability in lymphocyte transformation tests. *J.Immunol.Methods 57*: 21-32.

58. **Uetrecht, J. P.** 1999. New concepts in immunology relevant to idiosyncratic drug reactions: the "danger hypothesis" and innate immune system. *Chem.Res.Toxicol. 12*: 387-395.

CHAPTER 6

Are NKT cells involved in the pathology of the Spanish toxic oil syndrome? A pilot study

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The toxic oil syndrome (TOS), an epidemic-like food poisoning that occurred in 1981 in Spain, was caused by ingestion of cooking oil contaminated with aniline. Indirect evidence points towards an immune pathogenesis of TOS driven by T lymphocytes, but it is unclear to which antigens these cells could react. In a previous paper, we showed that aniline-coupled lipids, namely linoleic anilide and linolenic anilide, and a mixture of fatty acid esters of 3-(N-phenylamino)-1,2-propanediol, all implicated in TOS, induced significant popliteal lymph node responses, whereas the respective aniline-free lipids, linoleic acid, linolenic acid, and triolein did not. Secondary immune reactions towards the oil anilides tested, however, could not be obtained. This and the fact that the etiologic agents are lipids, which cannot be presented by MHC molecules but can be presented by CD1 molecules, led to the hypothesis that CD1-reactive T cells, or NKT cells, may be involved in the pathogenesis of TOS. Here, using Jα281$^{-/-}$ mice deficient in invariant-NKT (^{inv}NKT) cells, we investigated this hypothesis. Injection of PAP ester in the hind footpads of either ^{inv}NKT cell deficient or wild-type mice showed comparable enlargement of the draining popliteal lymph nodes. Flow cytometric analysis of lymph node subpopulations (NK cells, NKT cells, T cells, B cells, and CD4$^+$ and CD8$^+$ T cells) revealed no hint as to the role of ^{inv}NKT cells in the pathogenesis of TOS. We therefore conclude that ^{inv}NKT cells are not involved in the lymph node enlargement seen after injection of PAP ester in the hind footpad of mice. The possible role of non-invariant NKT cells in the pathogenesis of TOS and the differences between humans and mice in NKT cell recognition are discussed.

Introduction

In 1981 a mass poisoning occurred in Spain that affected more than 20,000 people after they had consumed rape seed oil contaminated with aniline (1). The illness, which came to be called the toxic oil syndrome (TOS) presented itself in an acute, an intermediate, and a chronic phase (2). The acute phase was dominated by eosinophilia, pulmonary oedema, myalgias, fever, and rash. It was followed by an intermediate phase consisting of myalgias, weight loss, skin oedema, hepatopathy and sicca syndrome. The chronic phase was characterized by peripheral neuropathy, hepatopathy, scleroderma and pulmonary hypertension.

Fatty acid anilides and fatty acid esters of 3-(*N*-phenylamino)-1,2-propanediol (PAP), detected as abnormal compounds in case-related samples of toxic oil, have been incriminated as the etiologic agents of TOS (3,4). The clinical features outlined above point towards an autoimmune pathogenesis of the syndrome (5). This was further corroborated by the detection of T cell infiltrates in the affected tissues and of increased levels of soluble interleukin-2 receptor in the serum of TOS patients (5). It has been proposed (6) that graft-versus-host-like reactions of T lymphocytes were involved in the pathogenesis of TOS. This concept implies that neoantigens induced by the aniline-contaminated oil were displayed by antigen-presenting cells (APC) and recognized by T cells. Classical T cells, bearing an αβ-T cell receptor, are unable to recognize small molecular weight compounds per se, but are able to react to them when bound to proteins, more exactly, when bound to peptides presented by molecules of the major histocompatibility complex (7). In a previous paper (8) we investigated the possibility of neoantigen formation by protein-reactive intermediates of fatty acid anilides and PAP esters. We showed that C57BL/6J mice that were primed with fatty acid anilides could be recalled in vivo with nitrosobenzene, a protein-reactive metabolite of aniline. However, we could not show anamnestic responses to the aniline coupled lipids themselves.

Publications on a recently discovered subpopulation of T cells, termed NKT cells (9-11), led to a new hypothesis on the induction of TOS. It involves direct presentation of the aniline coupled lipids on CD1, an MHC-like molecule. Lipids presented by CD1 can be recognized by NKT cells that upon recognition do not clonally expand but secrete large amounts of IFN-γ and/or IL-4 (Fig. 1).

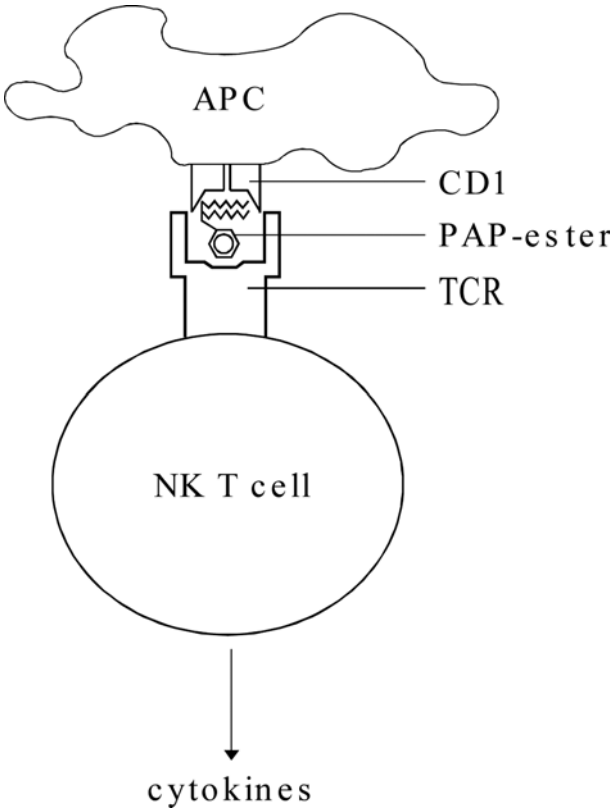

Figure 1. Hypothesis: Recognition of PAP-ester on CD1 by NKT cells. In analogy to other lipid antigens (12), PAP esters may be presented by CD1 molecules on the surface of APC. This CD1-lipid complex can be recognized by the NKT cell, which then rapidly secretes large amounts of IFN-γ and IL-4.

These cytokines can stimulate potential autoreactive T cells to become harmful autoaggressive T cells. Our previous findings and the fact that NKT cells react to lipids presented to them on CD1 molecules, but do not mount secondary responses make it likely, that NKT cells play a role in the pathogenesis of TOS.

A substantial fraction of murine NKT cells use an invariant TCR α-chain, namely Vα14-Jα281 (13); in this paper they are referred to as ^{inv}NKT cells. Here, we investigated if NKT cells play a role in the pathology of TOS by injecting PAP esters in the hind footpad of both wild-type (WT) and Jα281$^{-/-}$ mice which are deficient in NKT cells expressing the invariant Vα14-Jα281 TCR chain. We then evaluated the immune response in the draining lymph node through cell count and flow cytometric analysis.

Materials and Methods

Mice Male C57BL/6J mice were purchased from Harlan Winkelmann GmbH (Borchen, Germany). Male Jα281$^{-/-}$ mice were generated at Chiba University (Chiba, Japan) and backcrossed nine times to C57BL/6 mice (14,15); they were obtained from Harvard Medical School (Boston, USA) and will be referred to as ^{inv}NKT$^{-/-}$. Animals were kept under specific pathogen-free conditions and had free access to a standard diet (Ssniff Spezialdiäten GmbH, Soest, Germany) and tap water. They were 12 weeks of age at the onset of the experiments.

Chemicals A mixture of PAP esters containing equimolar amounts of 1-oleyl,2-linoleyl-PAP; 1-oleyl,2-linolenyl-PAP; and 1-linolenyl,2-linoleyl-PAP, were synthesized by Dr. A. Meseguer (Consejo de Superior de Investigaciones Científicas, Barcelona, Spain) as described (16). The aniline-free control compound triolein was also provided by Dr. A. Meseguer.

Popliteal lymph node (PLN) assay The assay detects the immunostimulatory capacity of low molecular weight substances and was performed as described before (17). In short, the mixture of PAP esters, and the control substance triolein, were emulsified in PBS containing 1% ethanol by repeated ultrasonication on ice. On day 0, animals received a single sc injection (50 μL) of the test compound into the left hind footpad. On day six, PLN of treated and untreated sides were removed, and cell numbers of individual PLNs were counted using a Casy1 automatic cell counter (Schärfe Systems GmbH, Reutlingen, Germany). The PLN cell count index from each mouse was calculated by dividing the cell count of the treated side by that of the control side.

Flow cytometric analysis of PLN cells Six days after injection, PLNs from the treated side were removed and cells from each individual mouse were triple-stained with either FITC-labeled anti-αβ-T cell receptor, PE-labeled anti-NK1.1 and PerCP-labeled anti-CD3, or FITC-labeled anti-CD19, PE-labeled anti-CD8 and PerCP-labeled anti-CD4 (all obtained from Pharmingen, Hamburg, Germany). Percentage of NK, NKT, T, B, CD4 and CD8 cells were determined using a FACScalibur flow cytometer and Cellquest Software (Becton Dickinson, Heidelberg, Germany).

Statistical analysis Values of PLN cell count indices, expressed as arithmetic means ± SD, were obtained from three to four animals per group. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, California, USA). PLN cell count indices and percentages of subpopulations were compared using ANOVA with Bonferroni comparison.

Results

The PLN response to PAP ester is not dependent on ^{inv}NKT cells

To assess NKT-cell involvement in the PLN reaction, ^{inv}NKT cell deficient or WT mice received an sc injection, containing 14 μmol of PAP ester or triolein into a hind footpad. In both strains, PAP ester induced a significant PLN response in comparison with the aniline free control oil triolein (Fig. 2). The PLN response did not differ between the two strains compared, implicating that ^{inv}NKT cells are not involved in the PLN response to PAP ester.

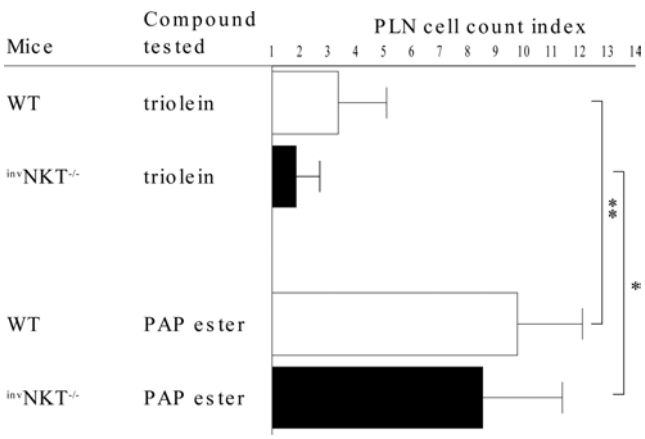

Figure 2. The primary PLN response to PAP ester is NKT cell independent. On day 0, groups of WT mice and ^{inv}NKT cell deficient mice received an sc injection containing 14 µmol of either PAP ester or triolein. PLN responses were measured on day six. Bars indicate arithmetic means + SD of groups of three ($^{inv}NKT^{-/-}$) or four (WT) mice. Asterisks indicate a significant difference (*p<0.05; **p<0.01) between the groups compared by brackets.

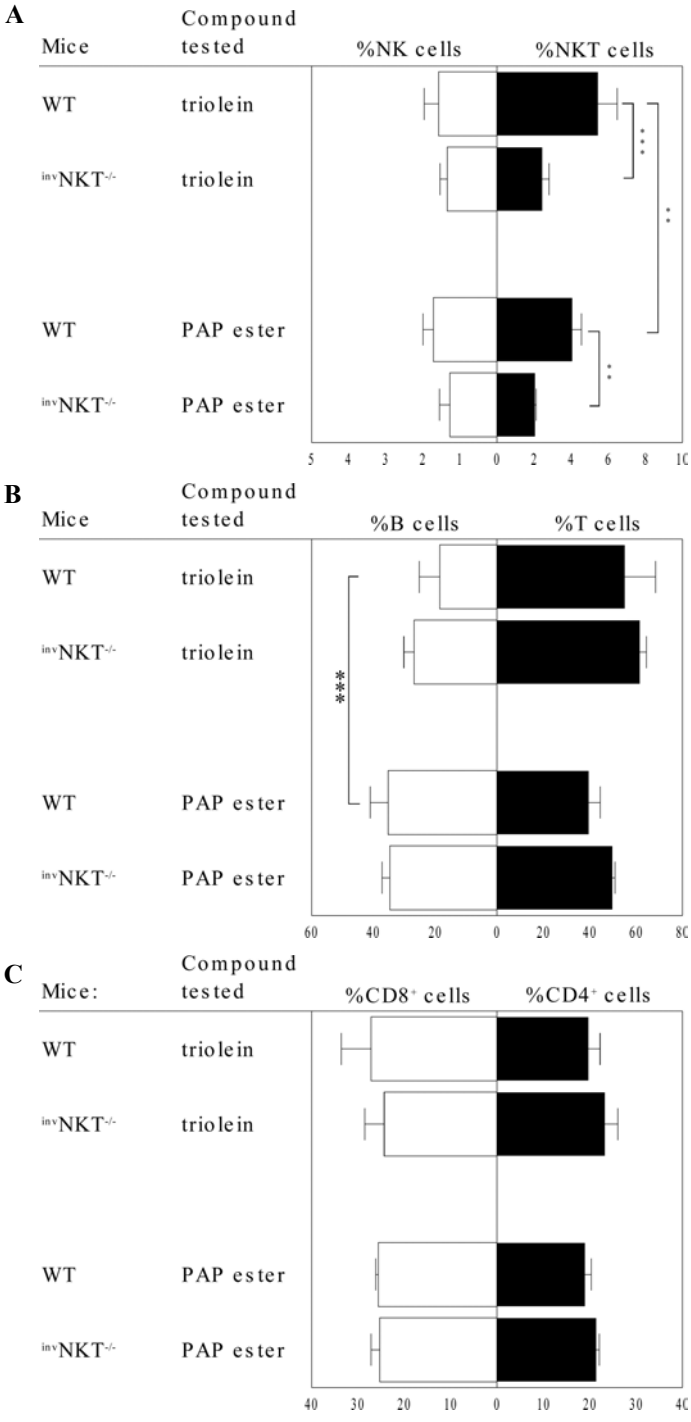

Figure 3. Flow cytometric analysis of PLN cells after injection of either PAP ester or triolein. Percentage of NK and NKT cells was determined as % of PLN cells that are $NK1.1^{+}\beta TCR^{-}CD3^{-}$ or $NK1.1^{+}\beta TCR^{+}CD3^{+}$, respectively (A). Percentage of T cells and B cells is defined as % of PLN cells that are $\alpha\beta\text{-}TCR^{+}NK1.1^{-}$, respectively $CD19^{+}$ (B). Percentage of $CD4^{+}$ and $CD8^{+}$ T cells is defined as % of PLN cells expressing CD4, respectively CD8 (C). Bars indicate arithmetic means + SD of three ($^{inv}NKT^{-/-}$) or four (WT) mice. Asterisks indicate a significant difference (**p < 0.01; ***p < 0.001) between the groups compared by brackets.

Influence of PAP ester injection on lymphocyte subpopulations in the PLN

For assessing the role of NK cells, NKT cells, B cells, as well as $CD4^+$ and $CD8^+$ T cells in the PLN enlargement seen after injection of PAP ester, PLN cells were stained for NK1.1, CD19, αβ-TCR, CD3, CD4 and CD8 expression six days after injection of the test compounds. Fig. 3 shows the percentages of NK and NKT cells (A), B and T cells (B), and $CD4^+$ and $CD8^+$ cells (C) in the draining lymph node. Compared with injection of triolein, injection of PAP ester induced only in WT mice a significant increase in percentage of B cells (Fig. 3B) together with a low, but significant decrease in percentage of NKT cells (Fig. 3A). In $^{inv}NKT^{-/-}$ mice, this relative increase in B cells and decrease in NKT cells was also seen but it was not statistically significant. However, as the data were obtained from one experiment using only 3 ($^{inv}NKT^{-/-}$), respectively 4 (WT) mice per group, the statistical difference between the two different strains may disappear when more mice per group are used. In both strains, there was no significant change in percentage of $CD4^+$ or $CD8^+$ T cells after injection of PAP ester (Fig. 3C).

Discussion

Recently PAP esters were identified, based upon epidemiological evidence, as the most likely causative agent in TOS (18). In a recent paper, we showed that a single injection of 14 μmol of PAP ester in the hind footpad of C57BL/6 mice increased the cell count of the draining popliteal lymph node threefold in comparison to aniline-free triolein. PAP esters as such are not protein reactive and can therefore not directly bind to proteins. As PAP ester are lipids and therefore could be presented by CD1, we hypothesized that PAP esters exert their immunotoxic effect through activation of NKT cells. To investigate this hypothesis, we used $J\alpha281^{-/-}$ mice which cannot express the invariant Vα14-Jα281 TCR α-chain, which is expressed by the majority of murine NKT cells (13). In the experiment described above, mice deficient in ^{inv}NKT cells did not show any difference in the PLN response to PAP ester compared with WT mice. As expected, the percentage of NKT cells was lower in $^{inv}NKT^{-/-}$ mice compared

with WT mice. There was only a marginal reduction in percentage of NKT cells after injection of PAP ester compared with injection of triolein in both strains, which might be due to the increase in percentage of B cells. In conclusion, ^{inv}NKT cells seem not to be involved in the local immune reaction seen after injection of PAP ester in the hind footpad.

Although ^{inv}NKT cells do not play a role in the primary PLN reaction after injection of PAP ester in C57BL/6 mice, they may have played a role in the human pathogenesis. Since no group has successfully established an animal model of TOS up to now, the situation in humans might be different to that in mice. The following differences in the murine model and the human situation might be responsible for this: (i) There are different CD1 (CD1a, b, c, d and e) molecules in humans, whereas there is only CD1d in mice (19). If CD1d were not capable of presenting PAP ester, one of the other CD1 molecules in human might do so, whereas in mice, PAP ester cannot be presented to CD1-reactive T cells. (ii) The invariant chain of human NKT cells (Vα24-JαQ) is different from the invariant chain of murine NKT cells (Vα14-Jα281) and may therefore have different specificity. (iii) The route of exposure may play a role. Whereas in 1981, the toxic oil was ingested orally and first delivered to the liver where 24% of all lymphocytes are NKT cells (19), injection in the footpad delivers the oil straight into the draining lymph node, where NKT cells comprise only 3% of all lymphocytes.

Furthermore, although Jα281$^{-/-}$ mice are called NKT-cell deficient, they are deficient only in ^{inv}NKT cells. Recently, Exley *et al* (20) showed that α-galactosylceramide, a potent ^{inv}NKT cell stimulator, protects mice against an acute cytopathic virus. They also showed that without stimulation of ^{inv}NKT cells, Jα281$^{-/-}$ mice were protected equally well against the virus when compared with WT mice, whereas protection was lost in CD1d$^{-/-}$ mice. This indicates equivalent roles for CD1d-reactive invariant and "non-invariant" NKT cells in resistance to acute virus infection. In other words, if NKT cells do play a role in TOS, the non-invariant NKT cells might have taken over this role in ^{inv}NKT cell deficient mice and thus might be responsible for the PLN enlargement seen after injection of PAP ester. Further experiments with blocking CD1d antibodies and CD1d$^{-/-}$ mice will elucidate this hypothesis.

Acknowledgement

The authors would like to thank Masaru Taniguchi (Chiba, Japan) for the permission to use Jα281$^{-/-}$ mice and Mark Exley (Boston, USA) for the transfer of these mice. The help of Angel Messeguer (Barcelona, Spain) in synthesizing PAP ester is greatly appreciated. We thank Willem Seinen, Andrea Rönnau, and Laura Layland for critically reading the manuscript.

References

1. **Tabuenca, J. M.** 1981. Toxic-allergic syndrome caused by ingestion of rapeseed oil denatured with aniline. *Lancet 12*: 567-568.

2. **Grandjean, E. C.** 1992. Introduction. In *Toxic oil syndrome*. J. Nadal and S. Tarkowski, eds. WHO, Copenhagen, p. 1-5.

3. **Kilbourne, E. M., J. T. Bernet, M. Posada de la Paz, R. H. Hill, I. Abaitua Borda, and M. M. Zack**. 1988. Chemical correlates of pathogenicity of oils related to the toxic oil syndrome epidemic in Spain. *Am.J.Epidemiol. 127*: 1210-1227.

4. **Hill, R. H. J., H. H. Schurz, M. Posada de la Paz, I. A. Borda, R. M. Philen, E. M. Kilbourne, S. L. Head, S. L. Bailey, W. J. Driskell, J. R. Barr, and L. L. Needham**. 1995. Possible etiologic agents for toxic oil syndrome: Fatty acid esters of 3-(N-phenylamino)-1,2-propanediol. *Arch.Env.Contam.Toxicol. 28*: 259-264.

5. **Merk, H. F., D. Niederau, M. Hertl, and F. Jugert**. 1991. Drug metabolism and drug allergy. In *New trends in allergy III*. J. Ring and B. Przybilla, eds. Springer-Verlag, Berlin, p. 269-279.

6. **Gleichmann, H. and E. Gleichmann**. 1984. GVHD, a model for Spanish toxic oil syndrome? *Lancet 1*: 1474.

7. **Martin, S. and H. U. Weltzien**. 1994. T cell recognition of haptens, a molecular view. *Int.Arch.Allergy Immunol. 104*: 10-16.

8. **Wulferink, M., J. Gonzalez, C. Goebel, and E. Gleichmann**. 2001. T cells ignore aniline, a prohapten, but respond to its reactive metabolites generated by phagocytes: possible implications for the pathogenesis of toxic oil syndrome. *Chem.Res.Toxicol. 14*: 389-397.

9. **Bendelac, A.** 1995. Mouse NK1^{+} T cells. *Curr.Opin.Immunol. 7*: 367-374.

10. **Bix, M. and R. M. Locksley**. 1995. Natural T cells. Cells that co-express NKRP-1 and TCR. *J.Immunol. 155*: 1020-1022.

11. **MacDonald, H. R.** 1995. NK1.1+ T cell receptor a/b cell: new clues to their origin, specificity, and function. *J.Exp.Med. 1892*: 633-638.

12. **Prigozy, T. I. and M. Kronenberg**. 1998. Presentation of bacterial lipid antigens by CD1 molecules. *Trends.Microbiol. 6*: 454-458.

13. **Lantz, O. and A. Bendelac**. 1994. An invariant T cell receptor α chain is used by a unique subset of MHC class I specific $CD4^+$ and $CD4^-8^-$ T cells in mice and humans. *J.Exp.Med. 180*: 1097-1106.

14. **Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi**. 1997. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science 278*: 1623-1629.

15. **Sonoda, K.-H., D. E. Faunce, M. Taniguchi, M. Exley, S. Balk, and J. Stein-Streilein**. 2001. NK T Cell-derived IL-10 is essential for the differetiation of antigen-specific T regulatory cells in systemic tolerance. *J.Immunol. 166*: 42-50.

16. **Ferrer, M., M. Galceran, F. Sanchez-Baeza, J. Casas, and A. Messeguer**. 1993. Synthesis of aniline derivatives with potential toxicological implications to the spanish toxic oil syndrome. *Liebigs Ann.Chem.* 507-511.

17. **Bloksma, N., M. Kubicka-Muranyi, H.-C. Schuppe, E. Gleichmann, and H. Gleichmann**. 1995. Predictive immunotoxicological test systems: Suitability of the popliteal lymph node assay in mice and rats. *Crit.Rev.Toxicol. 25*: 369-396.

18. **Posada de la Paz, M., R. M. Philen, H. H. Schurz, R. H. Hill, O. Gimenez-Ribota, A. Gómez de la Cámera, E. M. Kilbourne, and I. Abaitua Borda**. 1999. Epidemiologic evidence of a new class of compounds associated with toxic oil syndrome. *Epidemiology 10*: 130-134.

19. **Bendelac, A., M. N. Rivera, S.-H. Park, and J. H. Roark**. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu.Rev.Immunol. 15*: 535-562.

20. **Exley, M. A., N. J. Bigley, O. Cheng, S. M. A. Tahir, S. T. Smiley, Q. L. Carter, H. F. Stills, M. J. Grusby, Y. Koezuka, M. Taniguchi, and S. P. Balk**. 2001. CD1d-reactive T-cell activation lead to ameloriation of disease caused by diabetogenic encephalomyocarditis virus. *J.Leukoc.Biol. 69*: 713-718.

CHAPTER 7

Cross-Sensitization to Haptens Can Be Due to Different Mechanisms: Formation of Common Haptenic Metabolites, T Cell recognition of Cryptic Peptides, and True Cross-Reactivity

Marty Wulferink, Sabine Dierkes, and Ernst Gleichmann

Benzene derivatives which contain functional groups in the para-position are notorious for inducing adverse immune effects; in this study they are referred to as para-compounds. They can covalently bind to self-proteins generating neoantigens which can be recognized by T cells. Once sensitized to a given para-compound, most people react to more than this one para-compound. To analyze T cell cross-reactivity to para-compounds at the clonal level, we established $CD4^+$ T-cell hybridomas from mice immunized with adducts of self-globin and one of three different para-compounds, namely p-aminophenol, p-phenylenediamine, or Bandrowski's base. Some of the obtained hybridomas reacted not only to the immunizing antigen but also to metabolically related para-compounds bound to the same protein, thus suggesting formation of common metabolites. One of the hybridomas reacted against a non-haptenated native peptide of hemoglobin but not to the full length globin; such a peptide fulfills the definition of a cryptic peptide. Other hybridomas cross-reacted to globin adducts of metabolically unrelated para-compounds, but failed to recognize native peptides, which denotes them as truly cross-reactive cells whose TCRs failed to distinguish among the different haptens. Two even showed a heteroclitic reaction. To summarize, we found evidence that cross-sensitization to para-compounds can be due to any one of three different mechanisms: (i) metabolic transformation of a variety of

different parent compounds to a common reactive metabolite acting as the hapten, (ii) recognition of identical cryptic self-peptides generated after haptenation of the respective self-protein, and (iii) true cross-reactivity where different haptens are recognized by the same TCR.

Introduction

Benzene derivatives that possess two functional groups in the para-position are notorious for inducing allergic and autoimmune reactions in humans (1,2). Worldwide, these so-called para-compounds are still in use as drugs, color developing agents, hair and textile dyes. Examples here are the hair dye *p*-phenylenediamine (pPD), the photographic developer *p*-hydroquinone, sulphonamide drugs, and procainamide, an anti-arrhythmic drug. These compounds can induce contact hypersensitivity (3,4), agranulocytosis (5), delayed-type hypersensitivity associated with multiorgan toxicity (6), and drug-induced lupus (7,8), respectively. Because of their widespread use, humans come into contact with a variety of different para-compounds. Cross-sensitization to chemically different para-compounds has been frequently observed (1,9-15); the term denotes the fact that an individual who is sensitized to a given chemical, here a para-compound, can be challenged with a chemically related compound without prior sensitization to it.

Although T cell reactions to haptens can be highly specific (16,17), discriminating even between two stereoisomers of a hapten (18), T cell cross-reactivity to different para-compounds has also been frequently observed (19). The mechanism of this cross-reactivity has not been analyzed experimentally, but three possible mechanisms have been envisaged to account for it (4,9,12). First, during metabolism of different prohaptenic para-compounds, a common reactive metabolite could be generated that acts as a hapten; as a consequence, identical neoantigens would be seen by T cells. Second, stereotype binding of chemically related chemicals to self-proteins may lead to presentation of identical cryptic peptides. Third, the TCR of a given T cell could be incapable of distinguishing among the peptide adducts formed by different haptens and thus would truly cross-react to two or more chemicals.

The first possibility, a common reactive metabolite, has been studied for different para-compounds by Basketter and Goodwin (4) using the

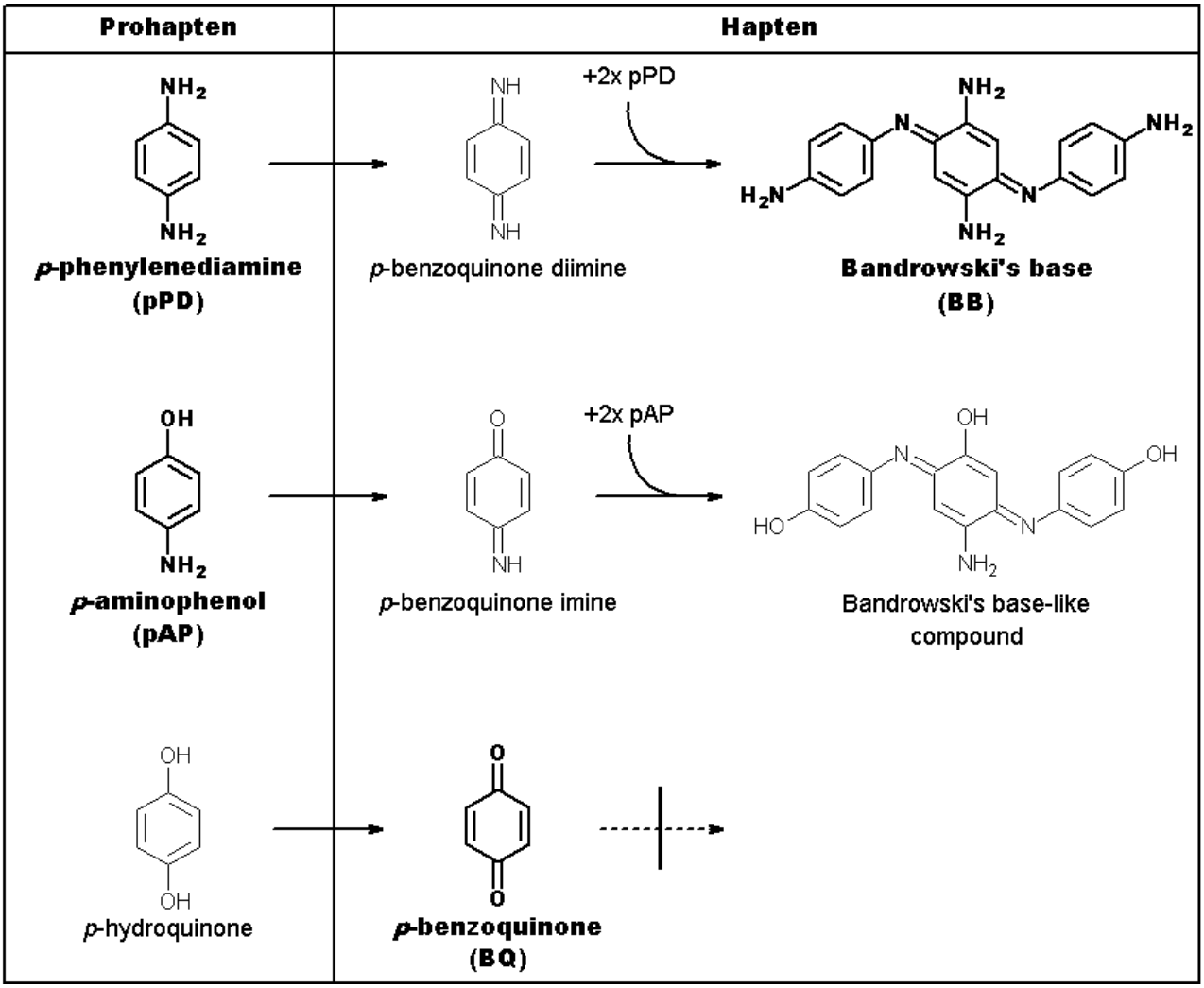

Figure 1. Chemical structures of the different para-compounds studied. pPD and BB are metabolically related and were used, together with pAP and BQ, to study cross-reactions towards para-compounds. Bold characters indicate the compounds used for coupling and immunization. The haptens shown, preferentially bind to cysteine residues (23,24) in proteins.

maximization test in guinea pigs. Although their results did not rule out cross-sensitization to a common haptenic metabolite derived from the different prohaptenic para-compounds tested, they considered it unlikely that this was the only mechanism involved. However, conclusive results could not be obtained with the in vivo test they used.

The second mechanism, presentation of identical cryptic peptides, has been implicated as an explanation for the frequently observed cross-sensitization to different heavy metal ions. Griem and colleagues (20) demonstrated that murine $CD4^+$ T cell hybridomas raised against Au(III)-treated bovine RNase A recognized a cryptic peptide of the RNase. Cryptic peptides are peptides that are not normally presented (21) and fail to carry the causative agent. The same cryptic peptide was presented when RNase A was denatured by S-sulfonation of

its cysteine residues or when it was treated with either Ni(III), Pd(II), Pd(IV), or Pt(IV) (20). Most classical haptens, even though they differ in structure, are electrophiles and, as such, preferentially bind to nucleophilic amino acids, such as cysteine and lysine (22). The metabolites of the para-compounds studied here, the respective quinones (Fig. 1), preferentially bind to cysteine residues (23,24). Comparable to Au(III), binding of different para-compounds to self-proteins may hinder enzymatic cleavage in stereotype fashion and lead to presentation of identical cryptic peptides.

The third possibility, true cross-reactivity, has to be considered when cross-sensitization to different chemicals cannot be explained by either a common metabolite or a cryptic peptide.

The animal models that were used for studying cross-sensitization to para-compounds (25), cannot distinguish between the three possible explanations outlined above. Both the variety of different T cell clones and of potential candidate self-proteins that can be haptenated is too large for analysis in vivo or an analysis solely based on reactions of bulk T cells in vitro. To circumvent these difficulties, we have established $CD4^+$ T cell hybridomas from mice immunized with murine hemoglobin adducts of para-phenylenediamine, Bandrowski's base (BB) (3), and para-aminophenol (pAP), respectively. Although it is unknown whether hemoglobin is relevant as a carrier protein for para-compounds in vivo, for the following reasons it is a suitable model protein: (i) it has six free cysteine residues for coupling the studied para-compounds, (ii) its amino acid sequence has been elucidated, (iii) it has an active center that can oxidize prohaptenic, i.e., non-protein-reactive para-compounds, such as pPD and pAP, into their reactive quinones (26,27), and (iv) it is easy to obtain. Here, using defined neoantigens and $CD4^+$ T cell hybridomas, we found evidence for all three possible mechanisms for cross-sensitivity mentioned above, that is, (i) formation of a common metabolite, (ii) reactivity to cryptic peptides, and (iii) true cross-reactivity.

Materials and Methods

Mice Specific pathogen-free female BALB/c mice, expressing both $I\text{-}A^d$ and $I\text{-}E^d$, were obtained from Harlan-Winkelmann GmbH (Borchen, Germany). Animals received a standard diet and tap water ad libitum, and were used at 9-20 wks of age.

Culture media TC medium consists of RPMI 1640, supplemented with 50 µg/ml gentamycin, essential and non-essential amino acids, 0.5 mM sodium pyruvate, 71.5 µM β-mercapto-ethanol, and 5% FCS. HAT medium was prepared by adding 110 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine to TC medium. HT medium was prepared by adding 110 µM hypoxanthine and 16 µM thymidine to TC medium. Supernatant from the T cell line EXC-5 was used for T cell expansion prior to fusion. Amongst other cytokines, it contained IL-2, IL-4, and IFN-γ (unpublished results).

Chemicals BB was obtained from ICN Biomedicals GmbH (Eschwege, Germany); pPD, pAP, and benzoquinone (BQ) were obtained from Sigma-Aldrich GmbH (Deisenhofen, Germany). Chemical structures of the compounds used (bold) and their intermediates are shown in Fig. 1.

Peptides On the basis of both their I-E^d binding motifs and presence of a cysteine for coupling the para-compounds to be studied, four different peptides from the α- and β-chains of mouse hemoglobin (sequence listed in Swissprot, accession nos.: P01942, P02088, and P02089) were obtained from Jerini Biotools GmbH (Berlin, Germany). These peptides, shown in Fig. 2, were haptenated and their adducts purified as described below.
In other experiments, 67 non-haptenated peptides (15 aa long, 10 aa overlap between two adjacent peptides), spanning the entire sequence of mouse hemoglobin, were used, they were obtained from Jerini biotools GmbH. The peptides were supplied lyophilized on nitrocellulose membranes. Before use, all 67 peptides were dissolved in saline at a concentration of 200 µM, aliquoted and stored at -20° C.

Anchor positions for I-E^d:					1			4				9			
α(96-108):			V	N	*F*	K	L	*L*	S	H	**C**	L	*L*	V	T
β(84-96):	T	F	A	S	*L*	S	E	*L*	H	**C**	D	K	*L*		
β(6-19)^{dmajor}:			A	E	K	*A*	A	V	*S*	**C**	L	W	G	*K*	V	N
β(6-19)^{dminor}:			A	E	K	*S*	A	V	*S*	**C**	L	W	A	*K*	V	N

Figure 2. Selected peptides from the α- and β-chain of mouse hemoglobin. Peptides were selected for two qualities, their binding motifs to I-E^d (italics) and presence of the nucleophilic amino acid cysteine (bold) to which the electrophilic haptens studied are known to bind in covalent fashion (23,24). The peptides β(6-19)^{dmajor} and β(6-19)^{dminor} are from the two different β-chains expressed in BALB/c mice, dmajor and dminor, respectively, which differ at one aa at position nine (SWISSPROT, see also under Material and Methods). All peptides indicated were coupled to BB, pPD, pAP, and BQ, respectively, and used to restimulate hybridomas.

Cell lines Thymoma line BW5147 (TCR$\alpha^-\beta^-$) was kindly provided by H.-G. Burgert (Freiburg, Germany).

Antigens *Isolation of hemoglobin:* Heparinized blood was obtained from untreated BALB/c mice and washed three times with saline. Erythrocytes were lysed in 17 mM Tris-HCl, 160 mM NH_4Cl, pH 7.2 (10 min, 37° C). After centrifugation at 15,000 g the soluble proteins were fractionated on a Sephadex-G25 (Pharmacia, Uppsala, Sweden) column. The red-colored hemoglobin fraction was collected and haptenated as described below.
Haptenation of hemoglobin: Freshly isolated hemoglobin was diluted to 50 mg/ml in 5 ml of saline and 0.5 ml of a 0.15 M solution of either BB, pPD, pAP or BQ in absolute ethanol, or ethanol only, was added to the hemoglobin. After 1 h incubation at 37° C with occasional stirring, 45 ml of -20° C cold acetone containing 0.1% HCl was added. Acetone precipitation was performed for two reasons: (i) the hemoglobin is freed of heme and breaks into 2 α-chains and 2 β-chains, and (ii) the precipitating chains are washed to eliminate free hapten. The precipitated globin chains were spun down at 1,600 g and the pellet washed twice with cold acetone (-20° C) containing 0.1% HCl. The pellet was dried overnight at 50° C and resuspended in distilled water to a final concentration of 10 mg/ml and sterile filtered through 0.2 µm Supor® Acrodisc® filters (Gelman Sciences, Ann Arbor, MI, USA) before use. The resulting BB globin, pPD globin, and pAP globin adducts were used for immunization and, together with BQ globin, for restimulation of T cells.
Haptenation of peptides and purification of peptide adducts: Cysteine-containing peptides (13, respectively 14 aa long) from the α- and β-chain of hemoglobin, shown in Fig. 2, were used. Peptides were coupled to BB, pPD, pAP, and BQ, respectively, by incubating the peptide and the chemical to which it would be coupled for 2 hr at 37° C. After incubation, the adducts and the native peptide, respectively, were purified using RP-HPLC on a LaChrom® HPLC System (Merck, Darmstadt, Germany). The HPLC column was a Lichrospher® WP300 RP-18 (5 µm) column and the mobile phase a mixture of water, acetonitril, and trifluoric acid, starting at 90:10:0.05 and continuously changing to 10:90:0.05 over 40 minutes at a flow rate of 1 ml/min. The fractions containing native or haptenated peptide, respectively, were vacuum-dried using an Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany) and solubilized in saline at a concentration of 200 µM.

Immunization 100 µg of protein adduct (BB, pPD, or pAP globin) in 25 µl saline was mixed with 25 µl IFA and injected into the hind footpads of BALB/c mice. In one experiment, 100 ng of pure BB, pPD or pAP in 1% ethanol/saline (50 µl) without adjuvant was used.

T cell proliferation assay Ten days after immunization, popliteal lymph nodes (PLNs) were removed and single-cell suspensions prepared. Cells were plated in tissue-type Petri dishes for 2 h at 37° C for depletion of adherent cells to decrease unspecific stimulation caused by

macrophages and dendritic cells containing the Ag used for immunization. Non-adherent cells were mixed 1:5 with irradiated (20 Gy) syngeneic spleen cells from non-immunized mice and plated in a 96-well plate at a densitiy of $5x10^5$ cells per well. As control, PLN cells from non-immunized mice were used. The cells were incubated for 4 days with different concentrations of the respective antigen at 37° C. Cell proliferation was measured by adding 18.5 kBq [^{3}H]thymidine for the last 16 h. Cells were harvested onto Ready Filters with Xtalscint (Beckman Instruments, Fullerton, CA), and the amount of radioactive thymidine incorporated was measured in a LS6000 β-counter (Beckman). Experiments were done using six replicate wells and performed twice to show reproducibility.

Generation of T cell hybridomas Ten days after immunization, PLNs were removed and single-cell suspensions prepared. Cells ($2x10^6$/ml) were restimulated in TC medium with the appropriate antigen for 2 days. The activated T cells were propagated for 2 more days using EXC-5 supernatant. T cell blasts isolated by Ficoll-gradient centrifugation were fused with BW5147 thymoma cells using polyethylene glycol 1500 (Boehringer, Mannheim, Germany), as described by the manufacturer, and plated in 96 well-plates. After selection of hybridomas in HAT medium for 2 weeks, cells were cultured for 1 week in HT medium, which was then gradually exchanged with TC medium. Hybridomas reacting to one of the used antigens in T cell hybridoma stimulation assays were subcloned twice by limiting dilution.

Preparation of ConA blasts Spleen cells of untreated BALB/c mice were cultured for 24 h with Concanavalin A (ConA, 1.25 µg/ml). Cells were washed twice and cultured for another 24 h in TC medium. After washing, they were used in the IL-2 bioassay for detection of IL-2 secreted by Ag-specific hybridomas.

T cell hybridoma stimulation assay (IL-2 bioassay) Hybridomas ($1x10^5$) were cocultured with syngeneic spleen cells acting as APC ($5x10^5$) in the presence or absence of antigen or native globin. After 24 h, culture supernatants (50 µl) were transferred to a new 96-well plate, frozen at -70°C, and after thawing tested for the presence of IL-2 by adding IL-2-dependent ConA blasts ($2x10^4$ in 50 µl). After 18 h, 18.5 kBq [^{3}H]thymidine was added. Six hours later, cells were harvested and [^{3}H]thymidine incorporation was measured. Experiments were done in triplicate cultures and performed at least twice to ensure reproducibility.

MHC restriction analysis T-cell hybridoma stimulation assay was performed as described above. Before adding the antigen, anti-I-A^d, anti-I-A^d/E^d, or the respective isotype control mAb (40 µg/ml) were added. All antibodies were obtained from Pharmingen (Hamburg, Germany). Experiments were done in triplicate cultures and performed twice.

TCR analysis T hybridoma cells ($2x10^5$ per well) were stained with FITC-coupled mAb to Vα2, Vα3.2b, Vα8, Vα11^{b,d}, Vβ2, Vβ3, Vβ4, Vβ5.1,5.2, Vβ6, Vβ7, Vβ8.3, Vβ9, Vβ10^b,

Vβ12, Vβ13, Vβ14, and Vβ17[a], respectively. FITC-coupled anti-βTCR, directed to the invariable part of the β-chain, was used as positive control for expression of αβTCR. All antibodies were obtained form Pharmingen and used at 0.02 μg/ml.

Statistical analysis Values of ^{3}[H]-thymidine (Thd) incorporation, expressed as arithmetic means ± SD, were obtained from two to six independent cultures. All experiments were performed at least twice to assess reproducibility of the data. The data were statistically analyzed with GraphPad Prism software (GraphPad Software, Inc., San Diego, California, USA) using ANOVA with Bonferroni comparison.

Results

Bulk T cells from mice immunized to a given para-compound coupled to self-globin reacted to the adduct, but not to free para-compound

To characterize the specificity of bulk T cells reacting to the adducts of self-globin and para-compounds used in this study, we first asked whether this reaction was carrier-dependent. Therefore, mice were immunized by an s.c. injection of either haptenated globin in IFA or the respective free hapten alone. Cells from the draining lymph node and irradiated spleen cells as APC were incubated with either saline, haptenated globin (25 μg/ml), native globin (25 μg/ml), or free hapten (100 μM), and their proliferation was determined. Upon recall in vitro, lymph node cells from mice primed with the globin adduct of BB showed carrier dependence in that they reacted only to the adduct used for immunization, i.e., BB globin, not to free BB or native globin (Fig. 3). When the mice had been immunized with free BB, however, their lymph node cells not only showed an anamnestic response to free BB, as could be expected, but they reacted weakly to globin-bound BB as well. Conceivably, after priming with free BB in vivo, a spectrum of different self-proteins, one of them being globin, were haptenated, creating different neoantigens to which T cells were primed. In vitro, the addition of free BB to the culture of APC and T cells may have haptenated the same or a similar spectrum of self-proteins, leading to recall of BB-primed T cells. In contrast, BB globin-primed T cells could not be recalled with free BB (Fig. 3), as there was no globin or hemoglobin present in the culture. T cells from mice immunized with pAP and pPD globin adducts were tested correspondingly in that they were restimulated in vitro using the

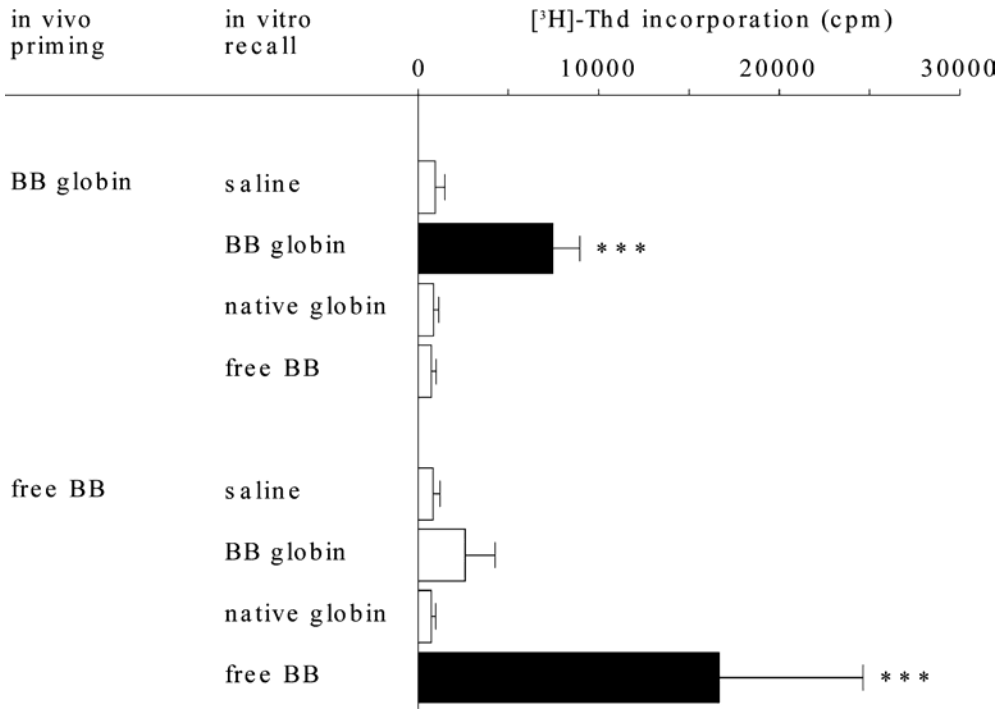

Figure 3. Bulk T cells obtained from mice primed to BB-coupled self-globin show carrier dependence. Mice were primed by injection into both hind footpads of either BB globin in IFA or free BB in saline. After 10 days, draining lymph nodes were removed and cell suspensions prepared. For recall in vitro, lymph node cells were coincubated with either saline, BB globin (25 µg/ml), native globin (25 µg/ml), or free BB (100 µM). After four days, proliferation was measured. Bars represent mean + SD of six separate cultures. Asterisks indicate a significant difference [(***) $p < 0.001$] between the black bars and all other bars.

adduct used for immunization, the respective free hapten, or native globin. T cells obtained from the immunized mice could be restimulated only with the adduct, not with native globin or free hapten (data not shown). BQ could not be tested in this assay, because, as a free hapten, it is toxic in concentrations above 0.01 µM (T. Pape, unpublished data).

T cell hybridomas immunized to a single para-compound bound to self-globin cross-reacted to other para-compounds bound to the same protein

To characterize the specificity of the T cells at single-cell level, $CD4^+$ T cell hybridomas were established. Mice were primed by injection of either pPD globin, pAP globin, or BB globin in IFA. Lymph node cells were fused with BW5147 thymoma cells to obtain immortalized T cells. After subcloning twice, T cell hybridomas specific for the hapten-globin adduct used for immunization were tested for their cross-reactivity towards other para-compounds bound to the same protein. Fig. 4 shows that individual T cell hybridomas reacted differently

to the different hapten-globin adducts used for recall. Although the first two hybridomas shown, 2A3 and 7C3 (Fig. 4A and 4B), were both obtained from a mouse immunized with pPD globin, they reacted differently: both reacted to pPD globin and BB globin, but only 2A3 reacted to preparations of pAP globin and BQ globin as well. Similar results were obtained with hybridomas 1B4 and 1A10 (Fig. 4C and 4D): although both were primed to pAP globin and could be challenged with this Ag, only 1B4 reacted to all four preparations of self-globin adducts. These results and those obtained with another pAP globin and two BB globin-primed hybridomas, 2B2, 3H9, and 4G11, are summarized in Table 1. The results of restimulation of hybridoma 2B2, which was generated after priming with pAP globin, are shown in Fig. 5. Not only did 2B2 cross-react to pPD globin and BB globin, but its reaction to the latter was even stronger than that to pAP globin, the adduct used for priming.

MHC restriction analysis of hybridomas recognizing haptenated globin

In order to analyze MHC restriction of the hybridomas used in this study, APC were blocked with mAb against I-A^d or against I-A^d/I-E^d before performing a hybridoma specificity test. Hybridomas were classified as I-A^d restricted when the reaction against the haptenated globin could be blocked with either mAb used, whereas I-E^d restricted hybridomas can only be blocked by the I-A^d/I-E^d mAb. Out of the seven hybridomas studied in detail, five were restricted to I-E^d and two to I-A^d (Table 1).

Peptide specificity of I-E^d restricted hybridomas recognizing haptenated globin

To characterize the peptide specificity of the I-E^d restricted hybridomas specific for haptenated globin (Table 1), binding motifs of I-E^d molecules (28) were taken into account. Four different peptides from the α- and β-chain of mouse hemoglobin were selected (Fig. 2), each containing amino acids necessary for binding to I-E^d and a cysteine as nucleophilic binding partner for the electrophilic haptens used (23,24). Hybridomas were restimulated with the

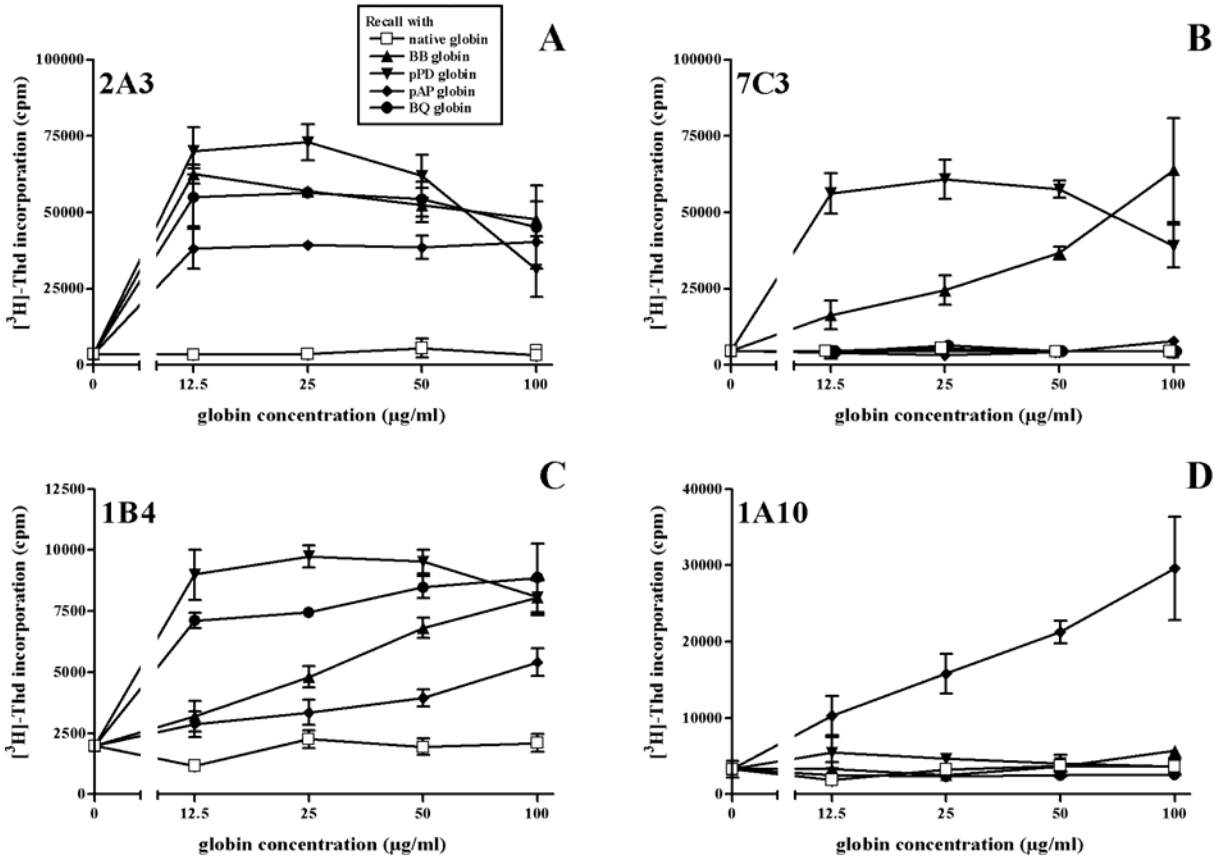

Figure 4. Following immunization of mice to a given para-compound coupled to self-globin, some T cell hybridomas cross-reacted to the same protein haptenated with different para-compounds. Four different $CD4^+$ T cell hybridomas, established from mice primed with either pPD globin (A and B) or pAP globin in IFA (C and D) were tested for their cross-reactivity using adducts of self-globin with a variety of different para-compounds, as indicated in the key, for recall. After 24h of culture, supernatants were analyzed for their IL-2 content through proliferation of IL-2 dependent ConA blasts. Symbols represent mean ± SD of three separate cultures.

different haptenated and native peptides, respectively, and their IL-2 production was measured. As shown in Table 1, four out of the five I-E^d restricted hybridomas analyzed in detail recognized the haptenated peptide 96 to 108 from the globin α-chain (α(96-108)), whereas hybridoma 4G11 reacted to haptenated peptide β(6-19). No reaction was observed with any of the five I-E^d restricted hybridomas shown in Table 1 when the peptides specified in Fig. 2 were used in their non-haptenated form (data not shown).

Table 1. Overview of results obtained with seven $CD4^+$ T cell hybridomas that reacted to the para-compounds indicated and were studied in detail

Clone	Obtained from mouse primed against	MHC restriction[1]	Cross-reactivity to globin haptenated with	Haptenated I-E^d peptide recognized[2]	I-A^d peptide recognized	TCR phenotype[3]
2A3	pPD globin	I-E^d	pAP, BB, BQ	α(96-108)		n.i.[4]
7C3	pPD globin	I-E^d	BB	α(96-108)		n.i.
1B4	pAP globin	I-A^d	pPD[6], BB[6], BQ[6]		α(96-110)[5]	Vα2/Vβ10b
1A10	pAP globin	I-E^d	no cross-reactivity observed	α(96-108)		Vα2/Vβ10b
2B2	pAP globin	I-E^d	BB[6], pPD	α(96-108)		n.i./Vβ6
3H9	BB globin	I-A^d	pPD		α(96-110)[7]	n.i./Vβ13
4G11	BB globin	I-E^d	pPD	$\beta(6\text{-}19)^{dminor}$		Vα8/Vβ6

[1] MHC restriction was determined using anti-I-E^d and anti -I-E^d/A^d antibodies in blocking experiments.

[2] Peptide recognition was determined using 4 different peptides from the α- and β-chain of hemoglobin, each of which contained a binding motif for I-E^d and a cysteine-residue. They were used either native or haptenated with the same para-compound as that used for immunization.

[3] TCR analysis was determined using the Vα and Vβ antibodies listed under Materials and Methods.

[4] n.i. = not identified.

[5] Peptide recognition was determined using the peptide a(96-110), either native or haptenated with BB or BQ. In addition to the BB-haptenated and BQ-haptenated peptide, the native peptide was also recognized (cryptic peptide).

[6] Heteroclitic reaction (see Discussion).

[7] Peptide recognition was determined using the peptide a(96-110), either native or haptenated with the same para-compound as that used for immunization. BB-haptenated peptide, but not the native peptide was recognized.

Peptide specificity of I-A^d restricted hybridomas recognizing haptenated globin

Unlike the I-E^d restricted hybridomas, the two I-A^d restricted hybridomas, 1B4 and 3H9, failed to react to any of the four peptides selected on their ability to bind to I-E^d, irrespective of whether they were haptenated or not (data not shown). Two possibilities may account for this: (i) the two I-A^d restricted

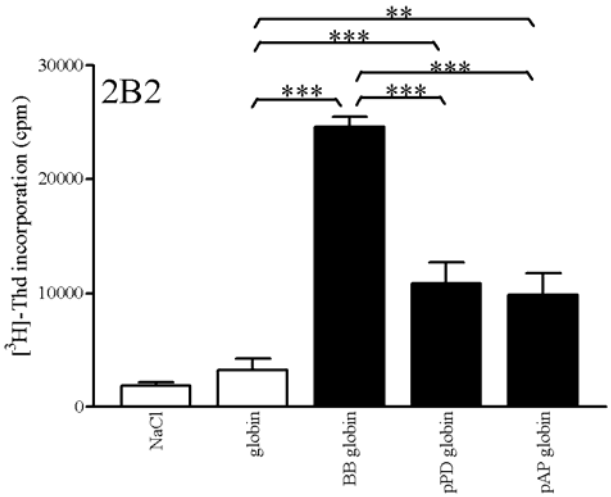

Figure 5. Heteroclitic reactivity of a T cell hybridoma to a hapten-protein adduct other than to which it was primed. $CD4^+$ T cell hybridoma 2B2, obtained from a mouse immunized to pAP globin, was tested for its cross-reactivity using adducts of self-globin with a variety of different para-compounds, as indicated in the key, for recall. After 24h of culture, supernatants were analyzed for their IL-2 content through proliferation of IL-2 dependent ConA blasts. Bars represent mean ± SD of three separate cultures. Asterisks indicate a significant difference (**=p<0.01 and ***= p<0.001) between groups compared by brackets.

hybridomas recognized cryptic, i.e., non-haptenated peptides, not necessarily containing a cysteine residue, or (ii) they recognized cysteine-containing, haptenated peptides with a binding motif for $I\text{-}A^d$. The different binding motifs for $I\text{-}E^d$ and $I\text{-}A^d$ might not allow the four cysteine-containing peptides previously selected on their motif for $I\text{-}E^d$ and shown in Fig. 2 to be presented by $I\text{-}A^d$ molecules.

T cell hybridoma 1B4 reacted to native peptides from the α-chain of hemoglobin

Unfortunately, the knowledge about peptide-binding motifs for $I\text{-}A^d$ (28) was not sufficient to select peptides from hemoglobin according to their binding capacity to this MHC molecule. Therefore, we started with investigating the possibility that 1B4 and 3H9 recognized a cryptic peptide, i.e., a non-haptenated peptide, from the α- or β-chain of hemoglobin. Therefore, hybridomas 1B4 and 3H9 were tested for their reactivity towards 67 partially overlapping peptides spanning the whole sequence of the α- and β-chain of mouse hemoglobin. Fig. 6 shows the reaction of hybridoma 1B4 against the 67 overlapping native peptides. Reactions were detected against the two adjacent, partially overlapping non-haptenated peptides, nos. 20 and 21; these match peptides α(96-110) and α(101-115), respectively. Hence, 1B4, which was generated after priming with pAP globin and showed broad cross-reactivity to all other globin adducts tested (Fig. 4C), reacted also to non-haptenated globin peptides. Hybridoma 3H9, as well as two other, $I\text{-}E^d$ restricted hybridomas that showed broad cross-reactivity, 2A3 and 2B2, failed to react to any of the 67 peptides used (data not shown).

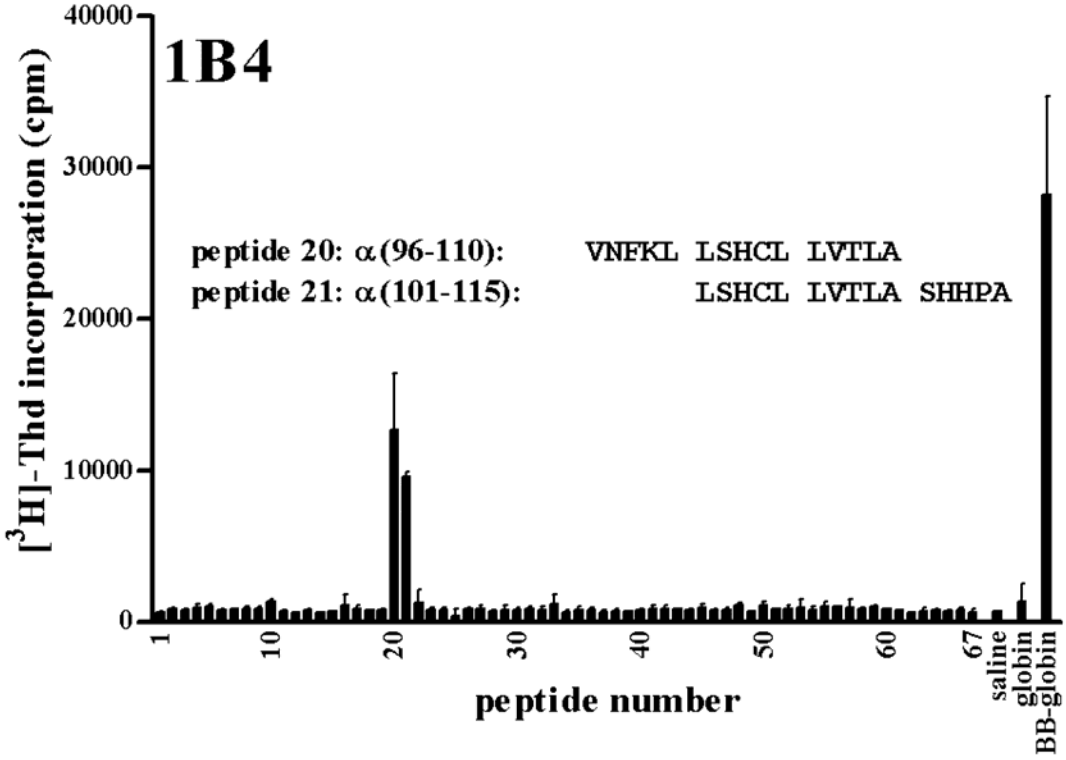

Figure 6. Immunization of mice with haptenated globin allowed for generation of a T cell hybridoma reacting to non-haptenated, cryptic peptides of this self-protein. Hybridoma 1B4, generated after priming with pAP globin, was tested with 67 native peptides (20 μM) spanning the whole sequence of hemoglobin. After 24 h culture, supernatants were analyzed for their IL-2 content through proliferation of IL-2 dependent ConA blasts. Bars represent mean + SD of two separate cultures.

T cell hybridoma 1B4 also recognized haptenated peptide α(96-110)

As shown in Fig. 6, the peptides α(96-110) and α(101-115) recognized by hybridoma 1B4 contain a cysteine located at position 104. To test whether this hybridoma would react against the native peptides only or against the haptenated peptides as well, peptide α(96-110) was haptenated with BB and BQ, respectively, and tested in the restimulation assay. Fig. 7 shows that hybridoma 1B4 reacted better to the haptenated peptide than to the native peptide. It should be noted that peptide α(96-110) is only two aa longer than peptide α(96-108) which was selected on its ability to be presented by I-E^d (Fig. 2). Hybridoma 1B4, which is I-A^d restricted (Table 1), however, reacted only to peptides α(96-110) and α(101-115), not to α(96-108), neither haptenated nor in its native form (data not shown). Conceivably, the aa at positions 109 or 110 are necessary either for binding to I-A^d, or for recognition by hybridoma 1B4.

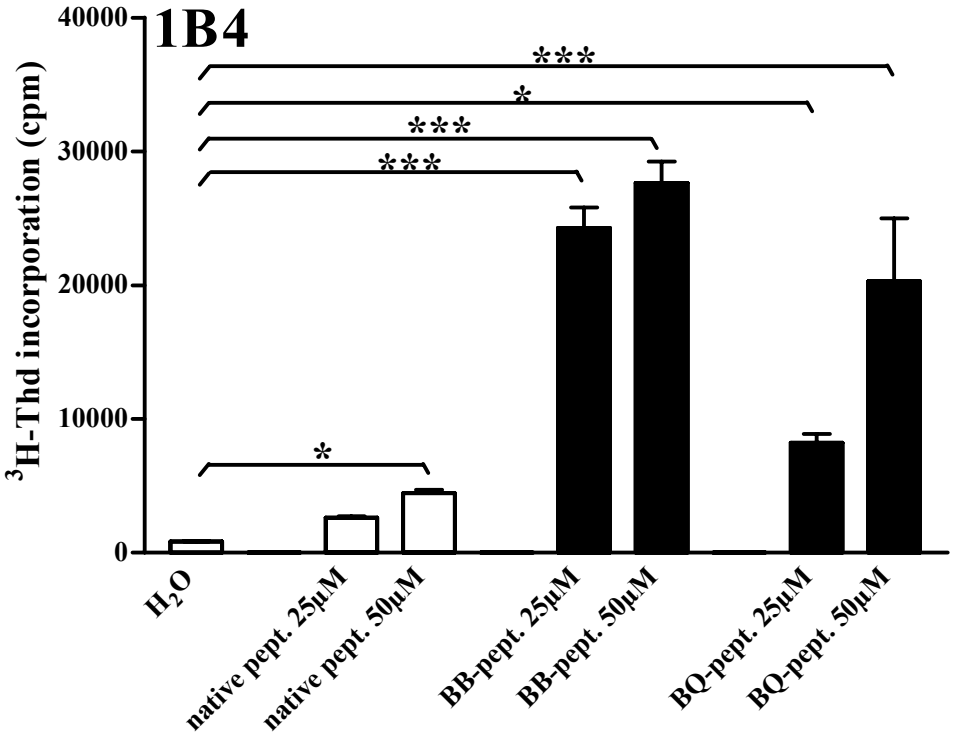

Figure 7. Reactivity of T cell hybridoma 1B4 to the haptenated as well as to the native self-peptide α(96-110). This hybridoma, obtained from a mouse immunized with haptenated globin, was tested for its reactivity against peptide α(96-110) as well as this self-peptide haptenated with BQ and BB, respectively. After 24 h incubation of the hybridoma cells, APC, and the peptide or globin indicated, supernatants were analyzed for IL-2 content through proliferation of IL-2 dependent ConA blasts. Bars represent mean + SD of three separate cultures. Asterisks indicate a significant difference [(*) $p < 0.05$ and (***) $p < 0.001$] between groups compared by brackets.

Analysis of variable segments of the α- and β-chain of the TCR on different hybridomas

To determine the Vα- and Vβ-TCR expression on the hybridomas used in this study, antibodies against different variable parts of the TCR's α- and β-chains were used. Each hybridoma tested was stained with the different antibodies commercially available (see under Materials and Methods) and analyzed by immune flow cytometry. Results are summarized in Table 1. In the case of hybridomas 2A3 and 7C3, the α- and β-TCR variable chains could not be identified, although these hybridomas did express αβTCR, as determined by mAbs against the invariable part of the β-chain. Conceivably, 2A3 and 7C3 may express variable α- and β-chains other than those to which mAbs were available and tested. The same is true for the α-chains of hybridomas 2B2 and 3H9, which

could not be identified either. Table 1 shows that, although hybridomas 1B4 and 1A10, both obtained from a mouse immunized with pAP globin, express the same Vα and Vβ elements, they differ in both their MHC restriction and their cross-reactivity pattern: 1B4 was I-A^d restricted and showed extensive cross-reactivity, whereas 1A10 was I-E^d restricted and showed highly specific responses to pAP globin. Furthermore, no preferential usage of the α- and β-chain was observed after immunization with para-compounds bound to globin.

Discussion

Although it is evident for most sensitizing chemicals that protein adducts are involved in their recognition by T cells (reviewed in Ref. 29), a difficulty in trying to study T cell reactions to sensitizing chemicals is the fact that the ultimate neoantigens formed by these compounds are not known; the only exception from this rule is the model hapten trinitrophenol (TNP) (16,30,31). In order to study the molecular mechanism of T-cell cross-reactivity to para-compounds, in the present investigation we synthesized four defined hapten-globin conjugates. Three of them, namely BB globin, pPD globin, and pAP globin were used for priming and these, together with BQ globin, were used for restimulation of T cells. We found, as expected, that bulk T cells of mice immunized with one of the three adducts mentioned above were only restimulated by the respective adduct and not by native globin alone. T cells from these immunized mice were used to establish a total of 94 $CD4^+$ T cell hybridomas which recognized the respective adduct used for immunization. These hybridomas enabled us to analyze cross-reactivity of single T cell clones, instead of using heterogeneous T cell populations present in vivo or in assays with bulk T cells. The data obtained with seven of these hybridomas, which were studied in greater detail, is summarized in Table 1.

Indeed, some T cell clones, such as 1B4, 2A3, 2B2, and 7C3, showed cross-reactivity in that they reacted to hapten-protein conjugates to which they had not been primed. One possible explanation is the formation of an identical metabolite derived from the different para-compounds. Conceivably, by coupling pPD to hemoglobin, an oxidizing protein, BB was formed through oxidation of pPD and subsequent trimerization with two other pPD molecules (3,32). For instance, the reaction to BB globin of hybridoma 7C3, originally primed to pPD globin, might be explained by this. Hybridomas 1B4, 2A3, and

2B2, however, cross-reacted to different para-compounds, like BB and pAP, that, as shown in Fig. 1, are not metabolically related. Their cross-reactivity, therefore, cannot be explained by formation of a common metabolite so that formation of cryptic peptides or true cross-reactivity must be considered.

Analysis of the specificity of hybridoma 1B4 showed that it reacted to two partially overlapping, native peptides from the α-chain of globin, α(96-110) and α(101-115), even though it failed to react to the native globin. Such peptides, to which T cells are raised that can only be recalled by the peptide itself, but not by the respective native protein, have been termed cryptic peptides (21). Previously, presentation of cryptic peptides and recognition by $CD4^+$ T cell hybridomas have been shown after immunization with bovine RNase denatured by Au(III) (20), but up to now no cryptic peptides have been identified after coupling a self-protein with a classical hapten, i.e., a covalently binding non-metal compound. Presentation of cryptic self-peptides caused by haptenation of the respective self-proteins may be relevant for the development of autoimmunity because, by definition, T cell tolerance to cryptic self-peptides could not be established as long as these were not presented (21). Hence, it has been proposed that presentation of cryptic self-peptides by APCs is the initial step for the development of drug-induced autoimmunity (29).

No reaction to cryptic peptides was observed with hybridomas 2A3 and 2B2. As their cross-reactivity pattern could not be explained by common metabolites either, true cross-reactivity might be an explanation for their reactivity to hapten-protein adducts against which they had not been immunized. True cross-reactivity of T cell hybridomas is not in line with the concept of 'one clonotype, one specificity'. As already mentioned, this paradigm has been undermined by theoretical considerations (33), and these are supported by a number of experimental observations (34-38). According to Mason (33), for optimal function of T cell recognition, three conflicting conditions have to be met. First, a large number of foreign peptides must be recognized for defense against microbes. Second, the specificity of the TCR must be high enough to respond to the foreign peptides, but not to self-peptides. Third, the frequency of T cells retained after negative selection and responding to a given foreign peptide must be high enough to ensure a rapid response. The first two conditions maybe best met by a highly specific, but diverse TCR repertoire. However, the more specific and diverse the TCR repertoire, the lower the frequency of T cells responding to a single foreign peptide will be. Because there are many more

different foreign peptides than there are T cells in a mouse (33), a considerable number of foreign peptides would go unrecognized if there were no cross-reactivity. To enable the immune system to react fast and efficiently to pathogenic peptides, Mason estimates that a single naive $CD4^+$ T cell should be able to react to almost half a million different 11-mer peptides. Hence, by necessity, extensive cross-reactivity to peptides should be an intrinsic feature of the TCR. For classical haptens however, cross-reactivity at the single T cell level has not been reported before. Up to now, only highly specific clones were described that are capable of discriminating amongst haptens with minor differences in side-chains (18,39). In contrast, our results show both a good degree of specificity, comparable with that reported for other haptens (18,39), and extensive cross-reactivity. Specificity was manifested, for instance, by hybridoma 1A10, while hybridoma 2A3 and 1B4 displayed extensive cross-reactivity. An extreme manifestation of the intrinsic cross-reactivity of T cells was observed with hybridoma 2B2 which reacted even more strongly to an adduct not used for immunization, namely BB globin, than to the pAP globin used for immunization. The term heteroclitic reaction (40-42) denotes this phenomenon, it means that the immune response to an Ag not used for immunization is stronger than that to the immunizing Ag. Heterocliticity was also observed with hybridoma 1B4: it reacted to the immunizing pAP globin, but reacted even more strongly to the three globin adducts which were not used for immunization.

A concept which can be used to explain T cell cross-reactivity is the 'avidity-pit model' proposed by Sandberg *et al*. (43). Avidity is defined here as the mathematical product of the TCRs' affinity towards a given peptide-MHC complex and the number of such complexes recognized by the T cell on the surface of an APC. According to that concept, immediately after thymocytes were positively selected on their ability to recognize self-MHC complexes with low avidity, their activation threshold is set higher than their level of self-recognition. In order to reach their activation or avidity threshold and react, peripheral T cells have to recognize a given peptide with higher avidity than the original self-peptide by which they were positively selected. This means that either their TCR recognizes the new peptide with higher affinity or that the epitope density of this particular peptide on the surface of APCs is increased. If, indeed, enhancement of avidity is the only prerequisite for T-cell recognition, this would imply that a given T cell cross-reacts to all peptides, including those

altered by haptens, which it recognizes with higher avidity than the self-peptide by which it was positively selected in the thymus. The avidity-pit model also implies that, even after activation by a foreign peptide, the T cell keeps its low avidity for the original self peptide; hence, it can be activated if APCs present that peptide at a higher density than normal.

The extensive cross-reactivity seen with hybridoma 1B4 may therefore be explained by the avidity-pit model. Hybridoma 1B4 cross-reacted with all four hapten-globin preparations offered for recall, and at the peptide level it was found to react to the haptenated peptide α(96-110). Unexpectedly, however, the same peptide was also recognized in its native, unhaptenated form, albeit that here the IL-2 production was lower, presumably due to lower affinity of 1B4 for the native peptide than for the haptenated one. The following hypothesis may explain these results. As can be deduced from our results with peptide α(96-108) that was recognized by hybridomas 2A3, 7C3, 1A10, and 2B2 in its haptenated, but not in its native form, haptenation of hemoglobin apparently leads to binding of the hapten to the cysteine at position 104 of the α-chain. After immunization with haptenated hemoglobin in vivo, a given T cell, conceivably positively selected on its ability to recognize the native peptide α(96-110) with low avidity in the thymus, recognized the haptenated peptide with higher avidity and reacts against it. After fusion, the hybridoma generated was found to react to haptenated globin and haptenated peptide α(96-110), implying that the T cell from which it was derived had been activated by the haptenated peptide α(96-110) *in vivo*. By adding the native self-peptide to APC *in vitro*, we increased the number of peptide-MHC complexes on the surface of the APC, thereby raising the avidity for binding of 1B4 and crossing the avidity threshold for its activation. Nonetheless, the haptenated peptide was recognized with higher affinity by the TCR of 1B4 and, therefore, a more vigorous reaction to the haptenated than the native peptide ensued.

In conclusion, there are three aspects of our analysis concerning the fine specificity of T cell reactivity to different haptens. First, with regard to allergology, our results show that T cell cross-reactivity to para-compounds indeed exists at the clonal level and, depending on the clone tested, can be due to any one of the three mechanisms discussed above. Second, the alteration of self-protein by covalently bound hapten may raise T cells reacting to cryptic self-peptides and thus be relevant for drug-induced autoimmunity. Third, with regard to T cell physiology our findings of true cross-reactivity and heterocliticity

provide experimental support for the theories of Mason (33) and Sandberg et al (43) which describe a limited discriminatory capacity of T cells carrying an αβTCR.

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References

1. **Seidenari, S., L. Mantovani, B. M. Manzini, and M. Pignatti**. 1997. Cross-sensitizations between azo dyes and para-amino compound. A study of 236 azo-dye-sensitive subjects. *Contact Dermatitis 36*: 91-96.

2. **Estlander, T.** 1988. Allergic dermatoses and respiratory diseases from reactive dyes. *Contact Dermatitis 18*: 290-297.

3. **Krasteva, M., J.-F. Nicolas, G. Chabeau, J.-L. Garrigue, H. Bour, J. Thivolet, and D. Schmitt**. 1993. Dissociation of allergenic and immunogenic functions in contact sensitivity to para-phenylenediamine. *Int.Arch.Allergy Immunol. 102*: 200-204.

4. **Basketter, D. A. and B. F. J. Goodwin**. 1988. Investigation of the prohapten concept: Cross reactions between 1,4-substituted benzene derivatives in the guinea pig. *Contact Dermatitis 19*: 248-253.

5. **Uetrecht, J. P.** 1992. The role of leukocyte-generated reactive metabolites in the pathogenesis of idiosyncratic drug reactions. *Drug Met.Rev. 24*: 299-366.

6. **Basketter, D. A., G. F. Gerberick, I. Kimber, and S. E. Loveless**. 1996. The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. *Food Chem.Toxicol. 34*: 985-997.

7. **Uetrecht, J. P.** 1988. Mechanism of drug-induced lupus. *Chem.Res.Toxicol. 1*: 133-143.

8. **Uetrecht, J. and B. Sokoluk**. 1992. Comparative metabolism and covalent binding of procainamide by human leukocytes. *Drug Metab.Dispos. 20*: 120-123.

9. **Basketter, D. A., A. Dooms-Goossens, A.-T. Karlberg, and J.-P. Lepoittevin**. 1995. The chemistry of contact allergy: why is a molecule allergenic? *Contact Dermatitis 32*: 65-73.

10. **Benezra, C. and H. Maibach**. 1984. True cross-sensitization, false cross-sensitization and otherwise. *Contact Dermatitis 11*: 65-69.

11. **Li, Q., H. Inagaki, and M. Minami**. 1996. Evaluation of cross-sensitization among dye-intermediate agents using a modified lymphocyte transformation test. *Arch.Toxicol. 70*: 414-419.

12. **Lidén, C. and A. Boman**. 1988. Contact allergy to colour developing agents in the guinea pig. *Contact Dermatitis 19*: 290-295.

13. **Lisi, P. and K. Hansel**. 1998. Is benzoquinone the prohapten in cross-sensitivity among aminobenzene compounds. *Contact Dermatitis 39*: 304.(Abstract)

14. **Rudzki, E.** 1977. Cross reactions in occupational contact dermatitis. I. Aromatic amines. *Berufsdermatosen 25*: 236-245.

15. **Shigematsu, T., N. Ozawa, and H. Nakayama**. 1988. In vitro study of the cross-sensitivity of hair dye using hapten-specific lymphocytes. *Contact Dermatitis 19*: 30-35.

16. **Kohler, J., U. Hartmann, R. Grimm, U. Pflugfelder, and H. U. Weltzien**. 1997. Carrier-independent hapten recognition and promiscuous MHC restriction by CD4 T cells induced by trinitrophenylated peptides. *J.Immunol. 158*: 591-597.

17. **Schnyder, B., C. Burkhart, K. Schnyder-Frutig, S. von Greyerz, D. J. Naisbitt, M. Pirmohamed, B. K. Park, and W. J. Pichler**. 2000. Recognition of sulfamethoxazole and its reactive metabolites by drug specific $CD4^+$ T cells from allergic individuals. *J.Immunol. 164*: 6647-6654.

18. **Nagata, N., U. Hurtenbach, and E. Gleichmann**. 1986. Specific sensitization of LYT-1^+2^- T cells to spleen cells modified by the drug D-penicillamine or a stereoisomer. *J.Immunol. 136*: 136-142.

19. **Edman, B.** 1991. Computerized analysis of concominant contact allergens. *Contact Dermatitis 24*: 110-113.

20. **Griem, P., K. Panthel, H. Kalbacher, and E. Gleichmann**. 1996. Alteration of a model antigen by Au(III) leads to T cell sensitization to cryptic peptides. *Eur.J.Immunol. 26*: 279-287.

21. **Sercarz, E. E., P. V. Lehman, A. Ametani, G. Benichou, A. Miller, and K. Moudgil**. 1993. Dominance and crypticity of T cell antigenic determinants. *Annu.Rev.Immunol. 11*: 729-766.

22. **Cohen, S. D., N. R. Pumford, E. A. Khairallah, K. Boekelheide, L. R. Pohl, H. R. Amouzadeh, and J. A. Hinson**. 1997. Selective protein covalent binding and target organ toxicity. *Toxicol.Appl.Pharmacol. 143*: 1-12.

23. **Klos, C., M. Koob, C. Kramer, and W. Dekant**. 1992. p-Aminophenol nephrotoxicity: biosynthesis of toxic glutathione conjugates. *Toxicol.Appl.Pharmacol. 115*: 98-106.

24. **Schlosser, M. J., R. D. Shurina, and G. F. Kalf**. 1990. Prostaglandin H synthase catalyzed oxidation of hydroquinone to a sulfhydril-binding and DNA-damaging metabolite. *Chem.Res.Toxicol. 3*: 333-339.

25. **Rudzki, E., K. Ostaszewski, Z. Grzywa, and A. Kozlowska**. 1976. Sensitivity to some rubber additives. *Contact Dermatitis 2*: 24-27.

26. **Stolze, K. and H. Nohl**. 1999. Free radical formation and erythrocyte membrane alterations during MetHb formation induced by the BHA metabolite, tert-butylhydroquinone. *Free Radic.Res. 30*: 295-303.

27. **Winterbourn, C. C., J. K. French, and R. F. Claridge**. 1979. The reaction of menadione with haemoglobin. Mechanism and effect of superoxide dismutase. *Biochem.J. 179*: 665-673.

28. **Rammensee, H. G., J. Bachmann, and S. Stevanovic**. 1997. *MHC ligands and peptide motifs*. Springer Verlag, New York.

29. **Griem, P., M. Wulferink, B. Sachs, J. B. Gonzalez, and E. Gleichmann**. 1998. Allergic and autoimmune reactions to xenobiotics: how do they arise? *Immunol.Today 19*: 133-141.

30. **Cavani, A., C. J. Hackett, K. J. Wilson, J. B. Rothbard, and S. I. Katz**. 1995. Characterization of epitopes recognized by hapten-specific CD4+ T cells. *J.Immunol. 154*: 1232-1238.

31. **Kohler, J., S. Martin, U. Pflugfelder, H. Ruh, J. Vollmer, and H. U. Weltzien**. 1995. Cross-reactive trinitrophenylated peptides as antigens for class II major histocompatibility complex-restricted T cells and inducers of contact sensitivity in mice. Limited T cell receptor repertoire. *Eur.J.Immunol. 25*: 92-101.

32. **Blake, A. J., P. Hubberstey, and D. J. Quinlan**. 1996. Bandrowski's Base. *Acta Cryst. C52*: 1774-1776.

33. **Mason, D.** 1998. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol.Today 19*: 395-404.

34. **Bhardwaj, V., V. Kumar, H. M. Geysen, and E. E. Sercarz**. 1993. Degenerate recognition of a dissimilar antigenic peptide by myelin basic protein-reactive T cells. Implications for thymic education and autoimmunity. *J.Immunol. 151*: 5000-5010.

35. **Kersh, G. J. and P. M. Allen**. 1996. Structural basis for T cell recognition of altered peptide ligands: a single T cell receptor can productively recognize a large continuum of related ligands. *J.Exp.Med. 184*: 1259-1268.

36. **Kaliyaperumal, A., C. Mohan, W. Wu, and S. K. Datta**. 1996. Nucleosomal peptide epitopes for nephritis-inducing T helper cells of murine lupus. *J.Exp.Med. 183*: 2459-2469.

37. **Wucherpfennig, K. W. and J. L. Strominger**. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell 80*: 695-705.

38. **Reay, P. A., R. M. Kantor, and M. M. Davis**. 1994. Use of global amino acid replacement to define the requirements for MHC binding and T cell recognition of moth cytochrome c (93-103). *J.Immunol. 152*: 3946-3957.

39. **Padovan, E., T. Bauer, M. M. Tongio, H. Kalbacher, and H. U. Weltzien**. 1997. Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy. *Eur.J.Immunol. 27*: 1303-1307.

40. **Mäkelä, O.** 1965. Single lymph node cells producing heteroclitic bacteriophage antibody. *J.Immunol. 95*: 378-386.

41. **Zügel, U., R. Wang, G. Shih, A. Sette, J. Alexander, and H. M. Grey**. 1998. Termination of peripheral tolerance to a T cell epitope by heteroclitic antigen analogues. *J.Immunol. 161*: 1705-1709.

42. **Rayfield, L. S., K. K. Sundaram, and T. A. Smith**. 1991. Erythrocyte-reactive T-cell lines and clones from normal mice recognize serum-derived antigens. *Immunology 73*: 438-443.

43. **Sandberg, J. K., L. Franksson, J. Sundbäck, J. Michaelsson, M. Petersson, A. Achour, R. P. A. Wallin, N. E. Sherman, T. Bergman, H. Jörnvall, D. F. Hunt, R. Kiessling, and K. Kärre**. 2000. T cell tolerance based on avidity thresholds rather than

complete deletion allows maintenance of maximal repertoire diversity. *J.Immunol. 165*: 25-33.

CHAPTER 8

The CellELISA: a rapid method for measuring specific T-cell hybridoma reactions

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We have adapted the sandwich ELISA to rapidly screen a large number of T-cell hybridomas. The method, called CellELISA is similar to the sandwich ELISA, but instead of transferring the supernatants in cytokine-coated 96-well plates, the cells are cultured directly in sterile, cytokine-coated wells. After 24 hours, the plates are washed, incubated with the detection antibody and developed using ortho-phenyldiamine substrate. Compared with the cytokine bioassay, where supernatants are tested on cytokine-dependent ConA blasts, the CellELISA is as sensitive, but does not require radioactivity and takes one day less. Compared with the sandwich ELISA, the CellELISA is more sensitive, especially in the lower range of cytokine secretion. We conclude, that the CellELISA is a suitable method for rapid screening and measuring specific reactions of T-cell hybridomas.

Introduction

Chemical induced allergy and autoimmunity is often observed after administration of certain drugs (1-5) or after intoxication with occupational or environmental chemicals (6-8). A major difficulty in studying the mechanisms of chemical-induced adverse immune effects is the fact that the ultimate neoantigens are unknown (9). While there are different approaches to investigate the molecular structure of these antigens, most investigations rely on establishing monoclonal T cell populations: either T-cell clones (10,11) or T-cell hybridomas (12,13).

The most widely used technique to measure activation of T-cell clones is detection of proliferation upon challenge with the appropriate antigen. Two techniques can be used in order to measure proliferation: (i) incorporation of labeled nucleotides into DNA (^{3}H-thymidine, bromodeoxyuridine) and (ii) cell number dependent transformation of tetrazolium dyes (MTT, WST, etc). Recently, measurement of proliferation has been extended by the measurement of cytokines, either by sandwich ELISA or flow cytometry. With these techniques one can differentiate between a Th1 and a Th2 responses (14,15).

For T-cell hybridomas it is not possible to measure direct proliferation upon challenge, as hybridomas proliferate independent of recognition of the appropriate antigen. In contrast to T-cell clones, activation of T-cell hybridomas can only be measured through cytokine secretion. This can be done in the cytokine bioassay by using IL2/IL4 dependent cells or by direct measurement of cytokines in an ELISA (16).

Initial screening of T-cell hybridomas is time consuming and laborious. Generally only a few hundred hybridomas can be tested at one time for their specificity to the antigen used for immunization. Conceivably, a time-saving test combined with an early endpoint would be optimal for a first screening of newly established hybridomas. We have therefore adapted the sandwich ELISA technique to rapidly screen large numbers of T-cell hybridomas for their specificity. Instead of transferring the supernatant from the culture plate into the sandwich-ELISA plate or the cytokine bioassay plate, the CellELISA uses only one plate for both culture and detection of cytokines, reducing costs and workload, and enhancing sensitivity. The CellELISA has the additional advantages that it takes one day less than the cytokine bioassay and does not require radioactive incorporation.

Materials & Methods

Mice Specific pathogen-free female BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were obtained from Harlan-Winkelmann GmbH (Borchen, Germany). Animals were kept on a standard diet and tap water ad libitum, and used at 8-10 wks of age.

Materials *p*-Aminophenol (pAP) was obtained from Sigma-Aldrich GmbH (Deisenhofen, Germany). Purified anti-CD3 (clone 145-2C11), anti-CD28 (clone 37.51, NA/LE), anti-IL-2 (clone JES6-1A12) and anti-IL-4 (clone BVD4-1D11), biotinylated anti-IL-2 (clone JES6-5H4) and anti-IL-4 (clone BVD6-24G2), and FITC-labeled anti-I-A^b (clone AF6-120.1) were obtained from BD Biosciences (Heidelberg, Germany). Streptavidin-coupled horse radish peroxidase was obtained from Amersham Pharmacia Biotech (Freiburg, Germany). Magnetic beads coupled to anti-CD8a (clone 53-6.7) and anti-I-A (clone M5/114.15.2) were obtained from Miltenyi GmbH (Mönchen-Gladbach, Germany). Tumor cocktail (TC) medium consisting of RPMI 1640 (PAA laboratories GmbH, Cölbe, Germany), supplemented with 50 µg/ml gentamycin, essential and non-essential amino acids, 1 mM sodium pyruvate (all from PAA laboratories GmbH), 50 µM β-mercapto-ethanol (Sigma Aldrich GmbH), and 5% FCS (Sigma Aldrich GmbH) was used throughout unless stated otherwise. HAT medium was prepared by adding 110 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine (all from Life Technologies GmbH, Eggenstein, Germany) to TC medium. HT medium was prepared by adding 110 µM hypoxanthine and 16 µM thymidine to TC medium. Supernatant harvested from the T cell line EXC-5 was used for T cell expansion prior to fusion. Amongst other cytokines, it contains IL-2, IL-4, and IFN-γ (unpublished results). 96-Well plates (type Microlon) were obtained from Greiner GmbH (Frickenhausen, Germany) and used throughout the experiments.

Hemoglobin isolation Heparinized blood was obtained from untreated BALB/c mice and washed three times with saline. Erythrocytes were lysed in 17 mM Tris-HCl, 160 mM NH_4Cl, pH 7.2 (10 min, 37° C). After centrifugation at 15,000 g the soluble proteins were fractionated on a Sephadex-G25 (Pharmacia, Uppsala, Sweden) column. The red-colored hemoglobin fraction was collected and haptenated as described below.

Haptenation of hemoglobin Freshly isolated hemoglobin was diluted to 50 mg/ml in 5 ml of saline and 0.5 ml of a 0.15 M solution of pAP in absolute ethanol was added to the hemoglobin. After 1 h incubation with occasional stirring, 45 ml of -20° C cold acetone

containing 0.1% HCl was added. The precipitated globin chains were spun down at 1,600 g and the pellet washed twice with -20° C cold acetone containing 0.1% HCl. The pellet was dried overnight at 50° C and resuspended in distilled water to a final concentration of 10 mg/ml and sterile filtered through 0.2 µm Supor® Acrodisc® filters (Gelman Sciences, Ann Arbor, MI, USA) before use.

Immunization 100 µg of pAP globin in 25 µl saline was mixed with 25 µl incomplete Freund's adjuvant (Sigma-Aldrich GmbH) and injected into the hind footpads of BALB/c mice.

Generation of T-cell hybridomas T-cell hybridomas were generated as described previously (**Chapter 7**, Wulferink *et al*, submitted). In short, lymph node cells from immunized mice were restimulated in TC medium with the appropriate antigen for 2 days. The activated T cells were expanded for a further two days using EXC5 supernatant. The resulting T-cell blasts were fused with BW5147 thymoma cells using polyethylene glycol 1500 (Boehringer, Mannheim, Germany), as described by the manufacturer. Hybridomas reacting to pAP globin in T-cell hybridoma stimulation assays were subcloned twice by limiting dilution.

Preparation of ConA blasts Spleens cells of untreated BALB/c mice were cultured for 24 h with Concanavalin A (ConA, 1.25 µg/ml). Cells were washed twice and cultured for another 24 h in TC medium. After washing, they were used in the T-cell hybridoma stimulation assay for detection of IL-2 secreted by Ag-specific hybridomas.

T-cell hybridoma stimulation assay using IL-2 dependent ConA blasts (Fig. 1) Hybridomas ($1x10^5$) were cocultured with syngeneic spleen cells acting as APCs ($5x10^5$) in the presence or absence of antigen. After 24 h, culture supernatants (50 µl) were transferred to a new 96-well plate, frozen at -70°C, and after thawing tested for the presence of IL-2 by adding IL-2-dependent ConA blasts ($2x10^4$ in 50 µl). After 18 h, 18.5 kBq [^{3}H]thymidine was added. Six hours later, cells were harvested and [^{3}H]thymidine incorporation was measured. Experiments were done in triplicate cultures and performed twice to ensure reproducibility.

T-cell hybridoma stimulation assay using the CellELISA (Fig. 1) 96-Well plates were coated with either purified anti-IL-2 (2 µg/ml) or anti-IL-4 (2 µg/ml) mAb overnight at 4° C. They were blocked with 1% BSA in PBS for 2 h at RT and washed three times with PBS. All

solutions were sterile filtered before use. Hybridomas ($1x10^5$) were cocultured with syngeneic spleen cells acting as APCs ($5x10^5$) in the presence or absence of antigen or native globin. After 24 h plates were washed three times with wash solution (PBS containing 0.5 % Tween-20) and incubated for 2 hours at RT with biotinylated anti-IL-2 (1 µg/ml) or anti-IL-4 (1 µg/ml) mAb, respectively. Plates were washed three times with wash solution, incubated with streptavidin-horseradish-peroxidase (1:2000 in PBS) for 30 minutes at RT and washed 5 times with wash solution. Plates were developed using OPD tablets (Sigma-Aldrich GmbH) as described by the manufacturer and measured using a 96-well-plate reader (Dynex Technologies, Denkendorf, Germany).

Purification of $CD4^+$ T cells Spleen cells of untreated C57BL/6 mice were passed over a nylon wool column to enrich T cells. Enriched T cells were stained with magnetically labeled anti-$I\text{-}A^d$ mAbs, washed and passed through an AutoMACS (Miltenyi GmbH) according to the manufacturers Protocol (Protocol: DepleteS). The negative fraction, depleted of APC was stained with magnetically labeled anti-CD8a mAbs, washed and passed through an AutoMACS (Protocol: DepleteS). The resulting enriched $CD4^+$ T cells had less than 2% contaminating APC as determined by flow cytometry using FITC-labeled anti-$I\text{-}A^b$ mAbs.

T cell stimulation assay 96-Well plates were coated overnight at 4°C with 50 µl binding solution (0.1 M Na_2HPO_4, adjusted to pH 9.0 with 0.1 M NaH_2PO_4) containing 5µg/ml anti-CD3 and 1 µg/ml of either anti-IL-2, anti-IL-4, or isotype control. Plates were blocked with 1% BSA in PBS for 2h at RT and washed three times with PBS containing 0.5% Tween. Purified $CD4^+$ splenic T cells from untreated C57BL/6 mice were plated at a densitiy of $2x10^5$ cells per well and incubated with different concentrations of anti-CD28. After 2 days, the plates coated with anti-IL-2 and anti-IL-4 were developed as described above (CellELISA). From the plates coated with isotype control, supernatant was taken for a sandwich-ELISA and the IL-2 bioassay, respectively.

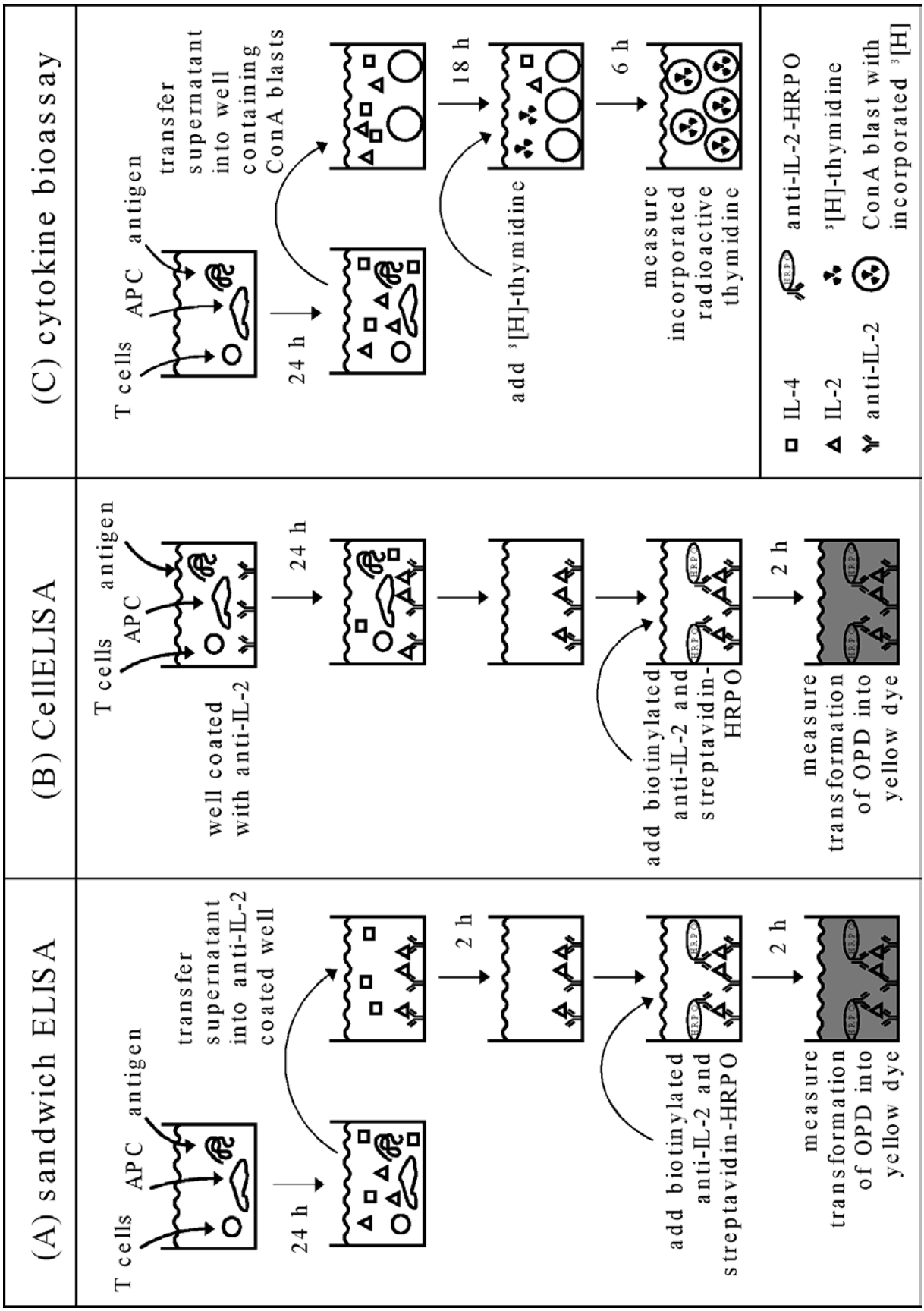

Figure 1. Schematic overview of the three different methods used for measuring cytokine secretion of T-cells: (A) sandwich ELISA, (B) CellELISA, and (C) cytokine bioassay. For detailed description, see Materials and Methods.

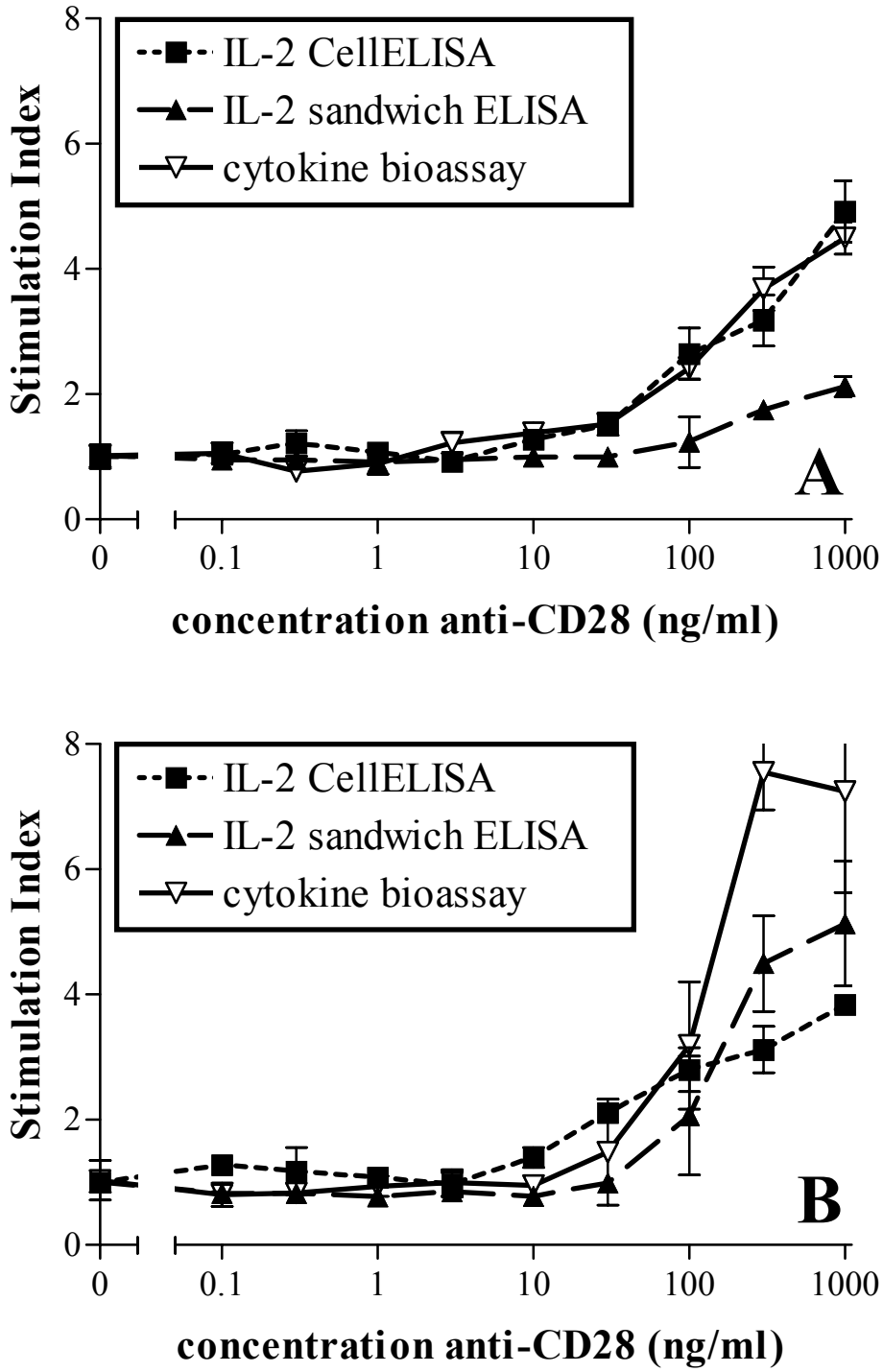

Figure 2. Sensitivity of the CellELISA in comparison to the sandwich-ELISA and the cytokine bioassay. Splenic $CD4^+$ T cells were stimulated in 96-well plates coated with anti-CD3 in combination with either anti-IL-2 or anti-IL-4 for CellELISA, or isotype control mAb for sandwich-ELISA and cytokine bioassay. Soluble anti-CD28 was added in different concentrations and after 48 h, cytokine production was measured by either directly developing the CellELISA plates or transferring supernatants to the sandwich-ELISA or cytokine bioassay plates (see Materials and Methods and Fig. 1). Results of two independent experiments are shown (A and B, respectively).

Results and Discussion

Sensitivity of the different T cell activation assays

We first determined the sensitivity of the conventional sandwich ELISA, the CellELISA, and the cytokine bioassay. For this we stimulated purified splenic $CD4^+$ T cells of C57BL/6 mice with plate-bound anti-CD3 and different concentrations of anti-CD28. Fig. 2 shows stimulation indices in relation to anti-CD28 concentration for the different T cell activation assays tested in two independent experiments (Fig. 2A and 2B, respectively). In the first test (Fig. 2A), sensitivity of the IL-2 CellELISA was comparable with that of the cytokine bioassay. In the second test (Fig. 2B), the CellELISA was more sensitive in the lower range of T cell activation (10-30 ng/ml anti-CD28) but with higher anti-CD28 concentrations, the stimulation indices of the cytokine bioassay exceeded that of the IL-2 CellELISA. Compared with the sandwich ELISA, sensitivity in the lower range was higher in the CellELISA in both experiments. In the second experiment, the stimulation indices obtained with higher concentrations of anti-CD28 in the sandwich ELISA also exceed that obtained in the CellELISA. A comparison of experiment 1 and 2 shows less interexperimental variation with CellELISA compared to the sandwich ELISA and the cytokine bioassay over the whole range of T cell stimulation.

Comparison of the CellELISA and bioassay for the screening of newly generated T-cell hybridomas

To compare the suitability of the CellELISA for screening newly generated T-cell hybridomas, we compared the IL-2 CellELISA and the IL-4 CellELISA with the cytokine bioassay, used by many investigators for detection of antigen-specific hybridomas. Table 1 shows a comparison of the CellELISA and the cytokine bioassay with regard to identification of specific T-cell hybridomas. A total of 128 hybridomas were tested for recognition of pAP globin, the antigen used for immunization. 42 of these 128 hybridomas were positive (SI > 2) in at least one of the tests used; these are shown in Table 1. Of these 42 hybridomas, 29 could be identified as specific in all three tests. From the 13 hybridomas negative in at least one of the tests, six were negative in the cytokine bioassay but positive in either the IL-4 (1 hybridoma) or the IL-2 and the IL-4

(5 hybridomas) CellELISA. Another six of these hybridomas were negative in the IL-4 CellELISA but positive in both IL-2 CellELISA and bioassay. From the

Table 1. Stimulation indices of specific T-cell hybridomas using the IL-2 and IL-4 CellELISA, and the cytokine bioassay

Hybridoma	IL-2[a]	IL-4[b]	^{3}[H][c]
Hybridomas positive in all three tests			
1A1	**16**	**3**	**4**
1A2	**32**	**5**	**7**
1A4	**32**	**8**	**4**
1B2	**13**	**5**	**2**
1B3	**18**	**10**	**5**
1B5	**14**	**10**	**5**
1C1	**15**	**5**	**4**
1C2	**22**	**15**	**5**
1C3	**25**	**5**	**4**
1C5	**11**	**4**	**5**
1C6	**24**	**3**	**7**
1D1	**26**	**4**	**4**
1D3	**18**	**6**	**8**
1D4	**22**	**3**	**5**
1D5	**23**	**2**	**7**
1D6	**4**	**3**	**5**
2A2	**12**	**3**	**5**
2A3	**21**	**4**	**4**
2A5	**17**	**12**	**4**
2B1	**10**	**2**	**5**
2B2	**9**	**4**	**9**
2B3	**12**	**11**	**6**
2B5	**22**	**2**	**9**
2C1	**42**	**9**	**5**
2C2	**3**	**11**	**10**
2C3	**11**	**14**	**6**
2C5	**37**	**20**	**2**
2C6	**34**	**15**	**7**
2D1	**41**	**8**	**7**

Hybridoma	IL-2	IL-4	^{3}[H]
Hybridomas negative in all three tests			
1B6	*1*	*1*	*<1*
1C4	*1*	*1*	*1*
+ 84 hybridomas with similar results			
Hybridomas negative in the cytokine bioassay			
1A3	**32**	**5**	*1*
1A6	**17**	**3**	*<1*
2A4	**54**	**11**	*1*
2B4[d]	*1*	**3**	*1*
2C4	**3**	**4**	*<1*
2D2	**50**	**27**	*<1*
Hybridomas negative in the IL-4 CellELISA			
1A5	**6**	*1*	**2**
1B1	**4**	*<1*	**3**
1B4	**18**	*1*	**4**
1D2	**8**	*<1*	**5**
2A1	**4**	*<1*	**3**
2A6	**11**	*1*	**8**
Hybridomas negative in the IL-2 CellELISA			
2B4[d]	*1*	**3**	*1*
2B6	*1*	**2**	**6**

Average index of positive hybridomas:
IL-2[a]: 19.8 ± 12.8
IL-4[b]: 7.3 ± 5.7
^{3}H[c]: 5.3 ± 2.0

[a] Stimulation index obtained with IL-2 CellELISA

[b] Stimulation index obtained with IL-4 CellELISA

[c] Stimulation index obtained with cytokine bioassay

[d] Hybridoma 2B4 was negative in the cytokine bioassay as well as in the IL-2 CellELISA

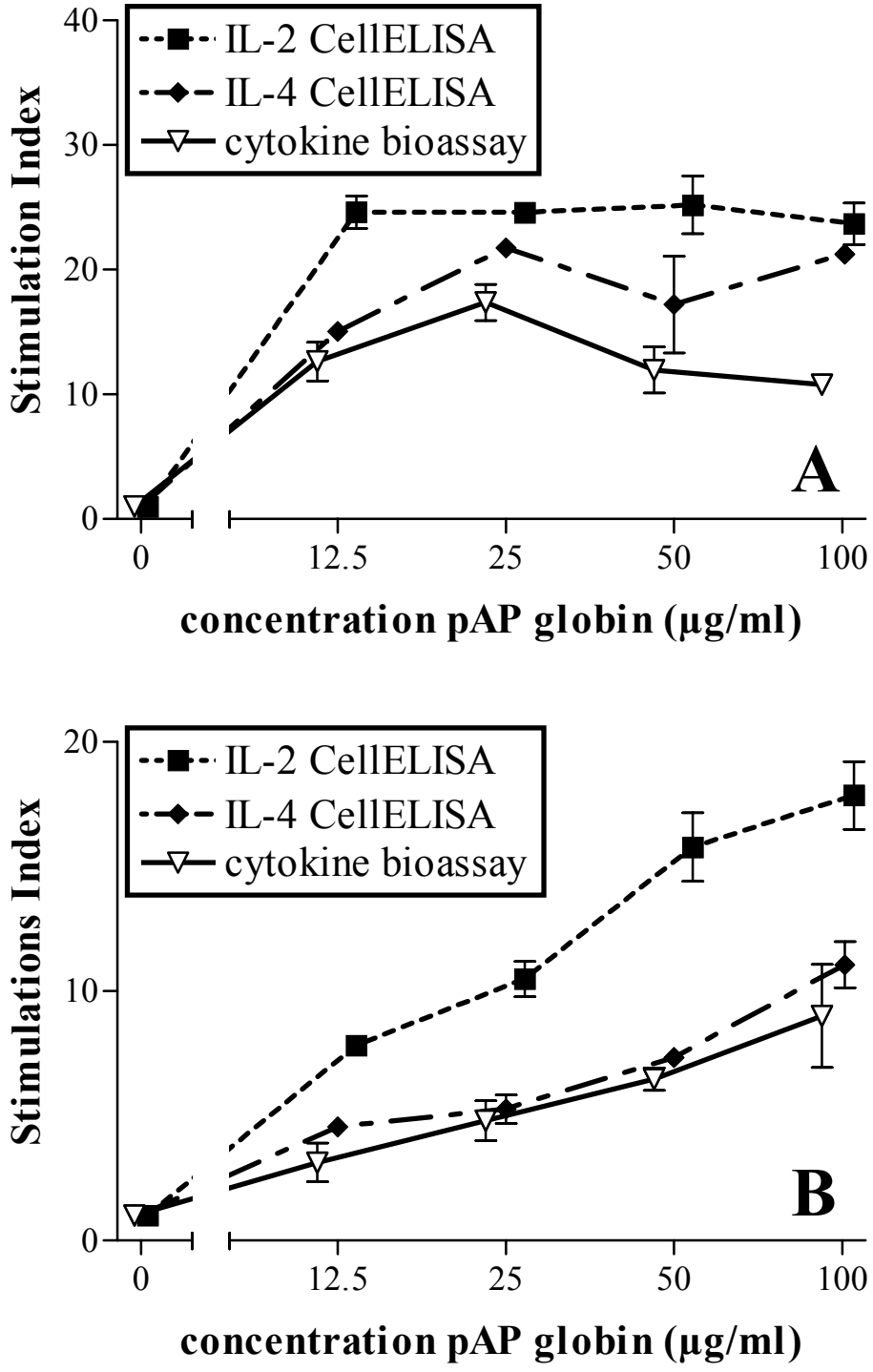

Figure 3. Comparison of CellELISA and cytokine bioassay using two established pAP globin-specific T-cell hybridomas (A and B, respectively). APCs, T-cell hybridomas and different concentrations of antigen were cocultured in a plate coated with either anti-IL-2 or anti-IL-4 for CellELISA, or non-coated for the cytokine bioassay. After 24 hours, cytokine production was measured by either directly developing the CellELISA plates or transferring supernatants to cytokine bioassay plates (see Materials and Methods). SIs were calculated by dividing the values of cultures with antigen by the values of cultures without antigen. Shown are SI ± SD of six replicate cultures.

two hybridomas that were rejected by the IL-2 CellELISA both were identified by the IL-4 CellELISA and one of them also by the cytokine bioassay. All hybridomas were retested to assure that no false positive reactions were obtained. In conclusion, none of the tests used were able to identify all of the specific T-cell hybridomas. The IL-2 CellELISA however, showed the least frequency of false negative test results (4.5%). Both the IL-4 CellELISA and the cytokine bioassay demonstrated the same frequency of false negative results (13.6%). If average indices of positive hybridomas were compared, IL-2 CellELISA showed the highest indices, followed by the IL-4 CellELISA (Table 1). This means that discrimination of positive and negative T cell-hybridomas is easier in both CellELISAs than it is in the cytokine bioassay.

Comparison of CellELISA and cytokine bioassay for testing established T-cell hybridomas

After initial screening and subsequent subcloning of specific T-cell hybridomas, the next step will generally be to show dose-dependency, fine specificity, and/or MHC-dependency. To test whether the CellELISA is adequate for this purpose, we used a total of 7 established and subcloned T-cell hybridomas to compare the CellELISA with the cytokine bioassay. Fig. 3 shows the dose dependency reaction of two representative hybridomas. Hybridoma 1A10 (Fig. 3A) showed comparable reactions in IL-2 CellELISA and IL-4 CellELISA. The cytokine bioassay was less sensitive with this hybridoma. Hybridoma 1B4 produced a higher SI in the IL-2 CellELISA than both the IL-4 CellELISA and the cytokine bioassay. In conclusion, all three assays are useful for further studying established and subcloned hybridomas. However, these results and those obtained with 5 other hybridomas (data not shown) show that the IL-2 CellELISA is more sensitive than both the IL-4 CellELISA and the cytokine bioassay. This makes the IL-2 CellELISA more suitable for inhibition experiments where an initially high stimulation index is required to show (relative) inhibition by antibodies or chemicals.

In conclusion, the CellELISA is suitable for rapid screening of newly generated hybridomas as well as for the characterization of established hybridomas. The advantage of the CellELISA in comparison to the normal sanwich ELISA is its lower workload and higher sensitivity, especially in the lower range of cytokine secretion. A recent publication depicted that measuring

cytokine secretion by sandwich ELISA is influenced by the consumption of cytokines by the cells (17). Consequently, if a limited amount of cytokines is secreted, it may be consumed almost completely and therefore leave no cytokines to be detected by the sandwich ELISA. In contrast, in the CellELISA this effect is negligible, as the cytokines are rapidly bound to the plate after being secreted. Consumption of cytokines by cells can explain both the lower sensitivity of the sandwich ELISA in the range of 10-30 ng/ml anti-CD28, where all cytokines may be consumed by the cells, as well as the higher stimulation indices in the range of 100-1000 ng/ml. In this range, the cytokines consumed by the cells induce cell proliferation and activation with subsequent cytokine production, which are consumed by the cells, and so on, and so forth. This "snowball effect" is absent in the CellELISA, as the cytokines needed for proliferation are caught and held by the antibodies coated to the well.

Compared with the cytokine bioassay, the CellELISA has similar sensitivity, but the interexperimental variance is lower in the latter. This might be due to the use of ConA blasts in the cytokine bioassay, which brings an extra variable into the test system. The use of non-radioactive materials in the CellELISA may also be seen as an advantage.

References

1. **Bell, S. J. D. and W. J. Pichler**. 1989. Penicillin-allergic patients react to penicillin-modified "self". *Allergy 44*: 199-203.

2. **Schnyder, B., C. Burkhart, K. Schnyder-Frutig, S. von Greyerz, D. J. Naisbitt, M. Pirmohamed, B. K. Park, and W. J. Pichler**. 2000. Recognition of sulfamethoxazole and its reactive metabolites by drug specific $CD4^+$ T cells from allergic individuals. *J.Immunol. 164*: 6647-6654.

3. **Goebel, C., C. Vogel, M. Wulferink, S. Mittmann, B. Sachs, S. Schraa, J. Abel, G. Degen, J. Uetrecht, and E. Gleichmann**. 1999. Procainamide, a drug causing lupus, induces prostaglandin H synthase-2 and formation of T cell-sensitizing drug metabolites in mouse macrophages. *Chem.Res.Toxicol. 12*: 500.

4. **Kubicka-Muranyi, M., C. Goebel, P. Griem, H.-C. Schuppe, J. Uetrecht, and E. Gleichmann**. 1998. Adverse immune reactions to drugs (gold, procainamide) and environmental chemicals (mercury, platinum): the role of phagocytic cells in generating immunogenic metabolites. In *Symposium in Immunology*. Eibl, M.M., Huber, C., Decker, H.A., Wahn, U., eds. Springer Verlag, Berlin, Heidelberg, p. 189-210.

5. **Uetrecht, J. P.** 1999. New concepts in immunology relevant to idiosyncratic drug reactions: The "danger hypothesis" and innate immune system. *Chem.Res.Toxicol. 12*: 387-395.

6. **Gelber, C., L. Gemmell, D. McAteer, M. Homola, P. Swain, A. Liu, J. Wilson, and M. Gefter**. 1997. Down-regulation of poison ivy/oak-induced contact sensitivity by treatment with a class II MHC binding peptide:hapten conjugate. *J.Immunol. 158*: 2425-2434.

7. **Schuppe, H.-C., A. C. Rönnau, S. von Schmiedeberg, T. Ruzicka, E. Gleichmann, and P. Griem**. 1998. Immunomodulation by heavy metal compounds. *Clin.Dermatol. 16*: 149-157.

8. **Griem, P. and E. Gleichmann**. 1995. Metal ion induced autoimmunity. *Curr.Opin.Immunol. 7*: 831-838.

9. **Griem, P., M. Wulferink, B. Sachs, J. B. Gonzalez, and E. Gleichmann**. 1998. Allergic and autoimmune reactions to xenobiotics: how do they arise? *Immunol.Today 19*: 133-141.

10. **von Greyerz, S., M. P. Zanni, K. Frutig, B. Schnyder, C. Burkhart, and W. J. Pichler**. 2001. Interaction of sulfonamide derivatives with the TCR of sulfamethoxazole-specific human alpha beta+ T cell clones. *J.Immunol. 162*: 595-602.

11. **Hemmer, B., T. Kondo, B. Gran, C. Pinilla, I. Cortese, J. Pascal, A. Tzou, H. F. McFarland, R. Houghton, and R. Martin**. 2000. Minimal peptide length requirements for CD4(+) T cell clones: implications for molecular mimicry and T cell survival. *Int.Immunol. 12*: 375-383.

12. **Schneider, S. C. and N. A. Mitchison**. 1995. Self-reactive T-cell hybridomas and tolerance. *J.Immunol. 154*: 3796-3805.

13. **von Vultée, C., P. Griem, and E. Gleichmann**. 1997. Generation of murine $CD4^+$ T cell hybridomas specific for nickel. *Immunobiol. 197*: 297.(Abstract)

14. **van Zijverden, M., A. van der Pijl, M. Bol, F. A. van Pinxteren, C. de Haar, A. H. Penninks, H. van Looveren, and R. Pieters**. 2000. Diesel exhaust, carbon black, and silica particles display distinct Th1/Th2 modulating activity. *Toxicol.Appl.Pharmacol. 168*: 131-139.

15. **Cavani, A., D. Mei, E. Guerra, S. Corinti, M. Giani, L. Pirrotta, P. Puddu, and G. Girolomoni**. 1998. Patients with allergic contact dermatitis to nickel and nonallergic

individuals display different nickel-specific T cell responses. Evidence for the presence of effector $CD8^+$ and regulatory $CD4^+$ T cells. *J.Invest.Dermatol. 111*: 621-628.

16. **Varma, C. K., S.-W. Li, L. E. Hood, W. Ladiges, and G. E. Osman**. 1997. Rapid detection of bovine type II collagen-specific T-cell hybridomas. *Hybridoma 16*: 287-290.

17. **Ewen, C. and M. E. Baca-Estrada**. 2001. Evaluation of interleukin-4 concentration by ELISA is influenced by the consumption of IL-4 by cultured cells. *J.Interferon Cytokine Res. 21*: 39-43.

CHAPTER 9

General discussion

Introduction

In this thesis, mechanisms involved in chemical-induced allergy and autoimmunity were investigated. A major difficulty in studying the mechanisms of chemical-induced adverse immune effects is the fact that the ultimate neoantigens are unknown. In **Chapter 2** we discussed some of the steps possibly involved in the formation of the neoantigens and subsequent T cell recognition. We could substantiate the hypothesis that extrahepatic metabolism plays an important role in the formation of neoantigen with the prohaptens procainamide and aniline in **Chapters 4 and 5**, respectively. In **Chapter 7**, the processing of protein adducts, presentation of neoantigens and subsequent recognition of either haptenated peptides or cryptic peptides by T cells was shown. Furthermore, **Chapter 7** elucidates some of the mechanisms involved in cross-sensitization and discusses the possible implications of T-cell cross-reactivity in chemical induced allergy and autoimmunity.

Metabolism

Model haptens like trinitrophenol (TNP), dinitrochlorobenzene (DNCB) or dinitrofluorobenzene (DNFB) can directly bind to proteins (1-3). However, most chemicals that humans come into contact with in daily life, like drugs and dyes,

are not protein-reactive perse but can be converted into reactive metabolites in the human body. In **Chapters 3**, **4 and 5** we showed for benzene, procainamide and aniline, respectively, that formation of protein-reactive metabolites is a prerequisite for the induction of chemical-induced allergy and autoimmunity.

Hepatic metabolism of chemicals. The main organ for metabolic conversion of chemicals is the liver. In comparison with its high metabolic activity however, adverse immune reactions in the liver are relatively rare. This might be due to the fact, that protein-reactive metabolites formed in the liver are detoxified by glutathion-conjugation, glucuronidation, or other detoxifying mechanisms which render them unavailable for conjugation with self-proteins (4). Furthermore, the environment in the liver (cytokines and specialized cells) seems to have a tolerogenic rather than activating effect on T cells (5,6).

Extrahepatic metabolism. Quantitatively, extrahepatic metabolic conversion of chemicals is less important than hepatic metabolism. Nonetheless, extrahepatic metabolism of chemicals, especially by cells of the immune system, appears to play a crucial role in the induction of chemical-induced allergy or autoimmunity (**Chapter 2**). An antigen presenting cell (APC) can, on its own, convert a prohapten into a protein-reactive hapten and, after adduct formation with a cytoplasmic self-protein, process and present the newly formed adduct to T cells. In **Chapters 4 and 5** we showed extrahepatic formation of T cell sensitizing metabolites after pulsing phagocytic cells with procainamide and aniline, respectively.

Processing and Presentation

After adduct formation, protein-hapten adducts are processed by the APCs' processing machinery. The resulting peptides can then be presented by MHC molecules on the cell surface (7). Only few of the hapten-protein conjugates formed after exposure to chemicals are processed in such a way, that fragments are presented as neoantigens on MHC molecules. In **Chapter 7**, we showed that such neoantigens can either be hapten-peptide conjugates, or cryptic peptides (see also **Chapter 1**). Presentation of hapten-peptide conjugates and subsequent recognition by T cells leads to allergic reactions, whereas presentation of cryptic peptides, i.e. native self-peptides that are normally not presented, might lead to autoimmunity.

T cell recognition

A key feature in the induction of chemical-induced allergy and autoimmunity seems to be activation of T cells specific for the respective chemical (**Chapters 2-4, 6**). In many cases of drug-induced allergy, the existence of specific T cells could be confirmed by in vitro restimulation of patient T cells with the causal drug (8) or one of its reactive metabolites (9, reviewed in 10). Although drug-specific T cells could be identified in allergic patients, the specificity was not always perfect as cross-reactions to chemicals that were not causal for the induction of the allergy, were frequently observed (11). In **Chapter 7** we investigated the mechanisms of such cross-sensitization. We observed conversion of different prohaptens into identical haptens as well as presentation of identical cryptic peptides after APCs were pulsed with different hapten-protein adducts. However, these mechanisms may both be interpreted as false T cell cross-reactivity, as the T cell itself does not cross-react to different antigens but instead, different chemicals lead to an identical neoantigen. In addition, we demonstrated true cross-reactivity, i.e., a single TCR cannot distinguish among different haptens. At first glance, T cell cross-reactivity may not seem to be consistent with the dogma of "one clone - one specificity". However, in a theoretical review, Mason (12) argues that given a relatively small number of T cells and an enormous numerical excess of viral and microbial peptides, T cells must be able to cross-react with several thousands of different peptides in order to mount a rapid immune response. Additional theoretical support for true cross-reactivity of the TCR comes from the "avidity pit" model of Sandberg *et al* (13). Their model postulates that TCR signaling can be set in motion by enhancing the avidity of TCR-MHC-complex interactions rather than by absolute recognition of a given antigen. They argue that in the thymus, T cells are positively selected by their ability to recognize self-peptide-MHC complexes with low avidity. In the periphery, these T cells would be activated by peptides possessing higher avidity for the TCR than those by which they were originally selected. By altering a self-peptide, the avidity of the T cell towards the peptide could be enhanced. When a certain threshold, the 'avidity threshold' is reached, the T cell would be activated. Both Mason's and Sandberg's theory are discussed in more detail in **Chapter 7**. While Mason (12) and Sandberg *et al* (13) provide theoretical evidence for true cross-reactivity to different peptides, Grogan *et al.* (14) experimentally showed that, indeed, T cells specific for one peptide of

myelin basic protein cross-react with a dozen different microbial peptides. Similarly, with respect to haptens, we showed in **Chapter 7** that T cells specific for a given para-compound truly cross-react to at least three other para-compounds that were metabolically not related.

Role of NKT cells in chemical induced allergy and autoimmunity

A few years ago three independent sets of studies led to the discovery of a new kind of T cell that bears markers, normally expressed on NK cells (15-17). These so called NKT cells are thought to play a major role in the maintenance of (self-)tolerance and induction of autoimmunity (18-21). They express a restricted TCR repertoire made of an invariant TCR α chain, Vα14-Jα281,

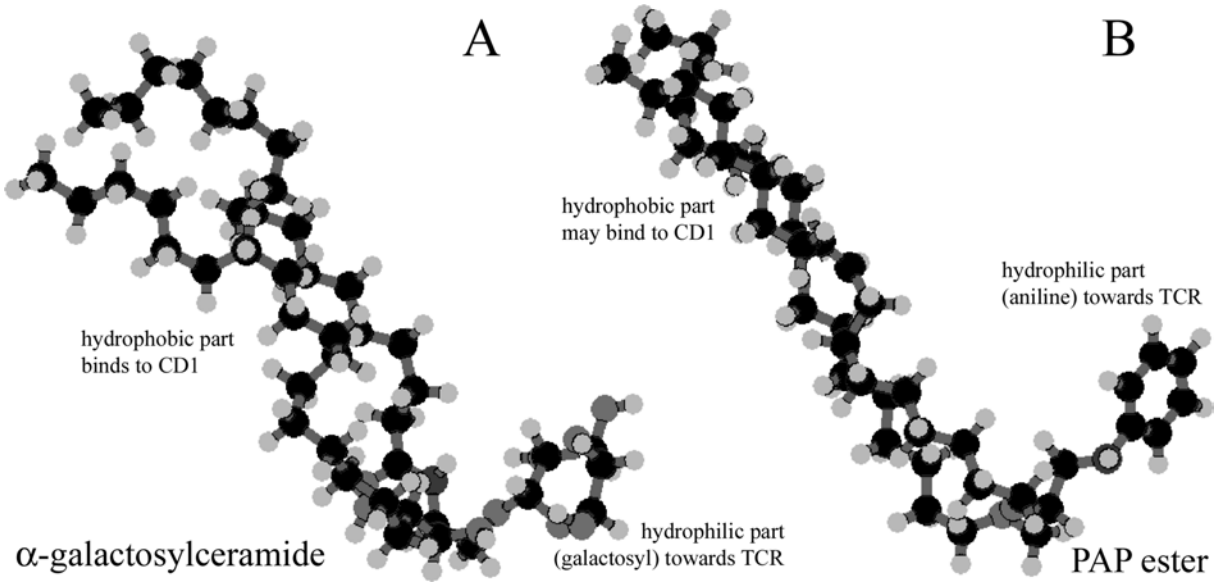

Figure 1. Three dimensional view of α-galactosylceramide (A) and PAP ester (B). α-Galactosylceramide, which is isolated from a marine sponge, is known to activate NKT cells (22). It is conceivable that, considering the similarity of the hydrophobic parts, PAP ester can likewise be presented by CD1. The hydrophilic part is then directed away from the CD1 molecule to be recognized by the NKT cells' TCR.

associated with polyclonal Vβ8, Vβ7 and Vβ2 TCR β chains (reviewed in Ref. 21). In contrast to classical T cells, NKT cells do not recognize antigen on the MHC, but on CD1, a highly conserved, MHC-like molecule on the surface of APCs (23) and intestinal epithelial cells (24). CD1 molecules can present lipids and other hydrophobic antigens to NKT cells, which can secrete high levels of

IFN-γ and IL-4 upon first encounter of these antigens (25,26). This led us to believe that NKT cells might play a role in the induction of the toxic oil syndrome (TOS), which was elicited after the ingestion of rape seed oil contaminated with aniline (**Chapter 5**). Esters of fatty acids with 3-(*N*-phenylamino)-1,2-propandiol (PAP ester) were found in case oils of TOS (27) and are at present considered to be responsible for induction of the syndrome (28). Considering the similarity of PAP ester with α-galactosylceramide (Fig. 1), which is known to be presented by CD1 and can activate NKT cells (22), it is conceivable that PAP esters can be presented by CD1 and directly activate NKT cells. Upon activation, NKT cells do not proliferate but directly produce large amounts of IL-4 and IFN-γ. Consequently they do not acquire memory and do not mount secondary responses. These new theories involving NKT cells and the difficulty in inducing secondary immune responses against the aniline coupled lipids in our previous investigations stimulated us to investigate the role of NKT cells in TOS. For this purpose a pilot experiment was performed using NKT cell deficient Jα281$^{-/-}$ mice (**Chapter 6**). The results of these experiments show that NKT cells, at least invariant Vα14-Jα281^{+}, do not play a role in the lymph node enlargement seen after injection of PAP ester. Studies whether "non-invariant" NKT cell may have played a role in the human pathogenesis are underway.

Outlook

During drug development, the time-point that adverse immune effects are discovered is mostly in the late clinical phase, when a great number of volunteers or patients are being treated with the drug. Sometimes, adverse immune effects are not seen before the drug has been officially released to the market. Thus, although only few people suffer from drug-induced adverse immune effects, the costs (illness, withdrawal of the drug, etc.) can be enormous. The risk that a certain individual suffers from chemical-induced adverse immune effects is dependent on numerous factors, including the panel of metabolizing enzymes, MHC alleles, chemical dose and route of exposure, etc. These factors and their role in chemical induced adverse immune effects are discussed in **Chapter 2**. They shape the environment in which a T cell encounters its antigen and, in line with the avidity thresholds theory of Sandberg *et al* (13), determine if the avidity of T-cell-antigen interactions reaches the avidity threshold needed for T cell activation. All these factors concern only

signal 1, i.e., recognition of the antigen by the TCR. Other factors, e.g., simultaneous virus or bacterial infections, oxidative stress produced by chemicals, etc., can induce costimulation, which is necessary for activation of T cells and consequent initiation of unwanted immune reactions.

More information on the mechanisms involved and a suitable animal test model for chemical induced allergy and autoimmunity might help to identify "unsafe" drugs at an early stage and thus save quality of human life and reduce health costs. Recent developments in genomics may result in the development of more personalized drug therapy (29). By using, e.g., cDNA microarray technology (30), variances in an individuals' drug-detoxifying enzyme panel, or certain HLA alleles known to predispose for allergic reactions can be identified. With this knowledge, choice of drug and drug-dose schemes can be fine-tuned accordingly, thereby reducing the risk of adverse drug-reactions, including those mediated by the immune system. For example, the higher incidence of procainamide-induced lupus in slow acetylators, which have a lower activity of *N*-acetyltransferase-2 (31), can be neutralized if the drug dose is corrected accordingly (32). Slower detoxification of procainamide by *N*-acetyl-transferase-2 enzyme in the liver leads to higher levels of the drug in the periphery, where neutrophils and mononuclear leukocytes can convert the drug into its protein-reactive metabolite *N*-hydroxylamino-procainamide (33) leading to accumulation of neoantigen and higher incidence of procainamide-induced lupus (34). Furthermore, awareness of the need for metabolic conversion and knowledge of the metabolic pathway of drugs may help in drug-development and drug-testing. This knowledge may help to develop animal models that represent a human population which is most sensitive to adverse immune effects of a certain drug-group. Genomics may be helpful in this respect too: a special tool can analyze changes in human gene transcription in response to environmental agents. This so called ToxChip enables quick identification of changes in the expression of enzymes, such as induction of PGHS-2 after procainamide exposure (**Chapter 4**), cytokines, costimulatory molecules on APC, etc. (35). As the ToxChip shows possible adverse immune effects before the effector phase starts and damage is inflicted to the individual, it can be used in drug-screening as well as in individual drug treatment. However, at the moment the costs for such a screening are tremendous and it will take some years before this can be done routinely.

In summary, this thesis aims towards a better understanding of the mechanisms that lead to chemical-induced adverse immune effects. We focussed thereby on the initial induction stage of the immune reaction that consists of three major steps: (i) formation of neoantigen, (ii) processing and presentation of the neoantigen, and (iii) recognition of peptide-MHC-complex by the T cell.

The first step, formation of neoantigen, i.e., extrahepatic metabolism of a prohaptenic chemical into a haptenic, protein reactive intermediate and binding of this hapten to self-proteins was shown in **Chapters 4, 5 and 7**. Although other groups showed recognition of non-covalently bound, non-protein reactive drugs (36,37) we found that the prohaptens benzene, procainamide and aniline could not induce specific T cell reactions, whereas *p*-benzoquinone, *N*-hydroxyl-procainamide, and nitrosobenzene could.

The second step, processing of the formed neoantigen and presentation of haptenated self-peptides or cryptic peptides on MHC molecules of APCs was implicitly shown in **Chapters 3, 4, and 5** and explicitly shown in **Chapter 7.** Here we blocked the MHCII molecules of APCs pulsed with whole protein adduct, thereby completely inhibiting T cell recognition of the formed neoantigen.

This leads us to the third step, recognition of the peptide-MHC-complex by T cells, specific for the neoantigen. In **Chapter 7**, we showed that T cells not only specifically recognize the hapten-peptide adduct, but cross-react to similar haptens coupled to the same peptide, as well as to cryptic peptides. The fact that there is true cross-reactivity to haptens may on the one hand be favorable with regard to measures taken to desensitize individuals allergic to chemicals. On the other hand, cross-reactivity should be taken into consideration when, after drug-induced adverse immune reactions have occurred, an alternative drug has to be chosen. The fact that haptenation of self-proteins raises T cells that react to cryptic self-peptides might be relevant for drug-induced autoimmunity, as here the immune reaction, though induced by a foreign chemical, is now directed towards a self-antigen.

References

1. **de Weck, A. L., K. Vogler, J. R. Frey, and H. Geleick**. 1966. The induction of contact hypersensitivity to dinitrochlorobenzene (DNCB) in guinea-pigs by dinitrophenyl-amino acids. *Int.Arch.Allergy Appl.Immunol. 29*: 174-184.

2. **Hirsh, D. C., A. A. Amkraut, and J. P. Steward**. 1972. Effect of carrier modification on the cellular and serologic responses of the rat to the TNP hapten. I. Dependence of the immunogenicity of the antigen upon processing by macrophages, as well as upon the distribution and retention of the antigen. *J.Immunol. 108*: 765-775.

3. **Friend, J. V. and M. Lane**. 1973. In vitro studies of contact hypersensitivity. The effect of the haptens 2,4-dinitrochlorobenzene (DNCB) and 2,4-dinitrofluorobenzene (DNFB) and of hapten-protein conjugates on the migration of guinea-pig peritoneal exudate cells. *Immunology 25*: 869-874.

4. **Parkinson, A.** 1996. Biotransformation of xenobiotics. In *Casarett & Doull's toxicology*. 5th ed. C.D. Klaassen, ed. McGraw-Hill, New York, p. 113-186.

5. **Trop, S., D. Samsonov, I. Gotsman, R. Alper, J. Diment, and Y. Ilan**. 1999. Liver-associated lymphocytes expressing NK 1.1 are essential for oral immune tolerance induction in a murine model. *Hepatology 29*: 746-755.

6. **Meyer, D., C. Otto, C. Rummel, H. J. Gassel, W. Timmermann, K. Ulrichs, and A. Thiede**. 2000. "Tolerogenic effect" of the liver for a small bowel allograft. *Transplant.Int. 13 Suppl 1*: S123-S126.

7. **Allen, P. M. and E. R. Unanue**. 1984. Antigen processing and presentation by macrophages. *Am.J.Anat. 170*: 483-490.

8. **Nyfeler, B. and W. J. Pichler**. 1997. The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity. *Clin.Exp.Allergy 27*: 175-181.

9. **Rönnau, A. C., B. Sachs, S. von Schmiedeberg, H. Hunzelmann, T. Ruzicka, E. Gleichmann, and H.-C. Schuppe**. 1997. Cutaneous adverse reaction to ciprofloxacin: Demonstration of specific lymphocyte proliferation and cross-reactivity to ofloxacin in vitro. *Acta Derm.Venerol. 77*: 285-288.

10. **Sachs, B., A. C. Rönnau, and E. Gleichmann**. 1997. Stellenwert des Lymphocytentransformationstests (LTT) in der allergologischen Diagnostik. *Deutsche Medizinische Wochenschrift 122*: 949-950.

11. **Seidenari, S., L. Mantovani, B. M. Manzini, and M. Pignatti**. 1997. Cross-sensitizations between azo dyes and para-amino compound. A study of 236 azo-dye-sensitive subjects. *Contact Dermatitis 36*: 91-96.

12. **Mason, D.** 1998. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol.Today 19*: 395-404.

13. **Sandberg, J. K., L. Franksson, J. Sundbäck, J. Michaelsson, M. Petersson, A. Achour, R. P. A. Wallin, N. E. Sherman, T. Bergman, H. Jörnvall, D. F. Hunt, R. Kiessling, and K. Kärre**. 2000. T cell tolerance based on avidity thresholds rather than complete deletion allows maintenance of maximal repertoire diversity. *J.Immunol. 165*: 25-33.

14. **Grogan, J. L., A. Kramer, A. Nogai, L. Dong, M. Ohde, J. Schneider-Mergener, and T. Kamradt**. 1999. Cross-reactivity of myelin basic protein-specific T cells with multiple microbial peptides: experimental autoimmune encephalomyelitis induction in TCR transgenic mice. *J.Immunol. 163*: 3764-3770.

15. **Bendelac, A.** 1995. Mouse $NK1^+$ T cells. *Curr.Opin.Immunol. 7*: 367-374.

16. **Bix, M. and R. M. Locksley**. 1995. Natural T cells. Cells that co-express NKRP-1 and TCR. *J.Immunol. 155*: 1020-1022.

17. **MacDonald, H. R.** 1995. NK1.1+ T cell receptor a/b cell: new clues to their origin, specificity, and function. *J.Exp.Med. 1892*: 633-638.

18. **Sonoda, K.-H., M. Exley, S. Snapper, S. P. Balk, and J. Stein-Streilein**. 1999. CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune priviliged site. *J.Exp.Med. 190*: 1215-1225.

19. **Hong, S. and L. van Kaer**. 1999. Immune privilege: keeping an eye on natural killer T cells. *J.Exp.Med. 190*: 1197-1200.

20. **Zeng, D., M. Dick, L. Cheng, M. Amano, S. Dejbakhsh-Jones, P. Huie, R. Sibley, and S. Strober**. 1998. Subsets of transgenic T cells that recognize CD1 induce or prevent murine lupus: role of cytokines. *J.Exp.Med. 187*: 525-536.

21. **Bendelac, A., M. N. Rivera, S.-H. Park, and J. H. Roark**. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu.Rev.Immunol. 15*: 535-562.

22. **Kitamura, H., K. Iwakabe, T. Yahata, S. Nishimura, A. Ohta, Y. Ohmi, M. Sato, K. Takeda, K. Okumura, L. van Kaer, T. Kawano, M. Taniguchi, and T. Nishimura**. 1999. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J.Exp.Med. 189*: 1121-1128.

23. **Beckmann, E. B. and B. B. Brenner**. 1995. MHC class I-like, class II-like and CD1 molecules: distinct roles in immunity. *Immunol.Today 16* : 349-352.

24. **Bleicher, P. A., S. P. Balk, S. J. Hagen, R. S. Blumberg, T. J. Flotte, and C. Terhorst**. 1990. Expression of murine CD1 on gastrointestinal epithelium. *Science 250*: 679-682.

25. **Matsuda, J. L. and M. Kronenberg**. 2001. Presentation of self and microbial lipids by CD1 molecules. *Curr.Opin.Immunol. 13*: 19-25.

26. **Prigozy, T. I. and M. Kronenberg**. 1998. Presentation of bacterial lipid antigens by CD1 molecules. *Trends.Microbiol. 6*: 454-458.

27. **Hill, R. H. J., H. H. Schurz, M. Posada de la Paz, I. A. Borda, R. M. Philen, E. M. Kilbourne, S. L. Head, S. L. Bailey, W. J. Driskell, J. R. Barr, and L. L. Needham**. 1995. Possible etiologic agents for toxic oil syndrome: Fatty acid esters of 3-(N-phenylamino)-1,2-propanediol. *Arch.Env.Contam.Toxicol. 28*: 259-264.

28. **Posada de la Paz, M., R. M. Philen, H. H. Schurz, R. H. Hill, O. Gimenez-Ribota, A. Gómez de la Cámera, E. M. Kilbourne, and I. Abaitua Borda**. 1999. Epidemiologic evidence of a new class of compounds associated with toxic oil syndrome. *Epidemiology 10*: 130-134.

29. **Kennedy, G. C.** 2000. The impact of genomics on therapeutic drug development. *EXS 89*: 1-10.

30. **Afshari, C. A., E. F. Nuwaysir, and J. C. Barrett**. 1999. Application of complementary DNA microarray technology to carcinogen identification, toxicology, and drug safety evalutation. *Cancer Res. 59*: 4759-4760.

31. **Drayer, D. E. and M. M. Reidenberg**. 1977. Clinical consequences of polymorphic acetylation of basic drugs. *Clin.Pharmacol.Ther. 22*: 251-258.

32. **Reidenberg, M. M., D. E. Drayer, M. Levy, and H. Warner**. 1975. Polymorphic acetylation of procainamide in man. *Clin.Pharmacol.Ther. 17*: 722-730.

33. **Rubin, R. L. and J. T. Curnutte**. 1989. Metabolism of procainamide to the cytotoxic hydroxylamine by neutrophils activated in vitro. *J.Clin.Invest. 83*: 1336-1343.

34. **Uetrecht, J. and B. Sokoluk**. 1992. Comparative metabolism and covalent binding of procainamide by human leukocytes. *Drug Metab.Dispos. 20*: 120-123.

35. **Medlin, J. F.** 1999. Timely toxicology. *Env.Health Perspect. 107*: A256-A258.

36. **Schnyder, B., C. Burkhart, K. Schnyder-Frutig, S. von Greyerz, D. J. Naisbitt, M. Pirmohamed, B. K. Park, and W. J. Pichler**. 2000. Recognition of sulfamethoxazole

and its reactive metabolites by drug specific $CD4^+$ T cells from allergic individuals. *J.Immunol. 164*: 6647-6654.

37. **Zanni, M. P., S. von Greyerz, B. Schnyder, K. A. Branker, K. Frutig, Y. Hari, S. Valitutti, and W. J. Pichler**. 1998. HLA-restricted, processing- and metabolism-independent pathway of drug recognition by human alpha beta T lymphocytes. *J.Clin.Invest. 102*: 1591-1598.

Korte samenvatting

De belangrijkste opgave van het immuunsysteem is ervoor te zorgen dat ziekteverwekkers (virussen, bacteriën, schimmels en parasieten) worden herkend, aangevallen en afgebroken. Naast *macrofagen* spelen *T cellen* en *B cellen* een belangrijke rol in de afweerreacties tegen indringers. De macrofagen kunnen indringers opsporen en delen ervan aan de T cellen presenteren. T cellen kunnen dan op verschillende manieren reageren: Ze kunnen óf de cellen die het herkende antigeen presenteren vernietigen (cytotoxische T cellen), óf B cellen helpen tegen de indringers antilichamen te vormen (T helper cellen).

Bepaalde chemische verbindingen (bijv. geneesmiddelen) kunnen er echter voor zorgen dat het immuunsysteem op een ongewenste manier reageert. Ongewenste reacties van het immuunsysteem tegen chemische verbindingen worden ook wel allergische reacties genoemd. Reageert het immuunsysteem onder invloed van chemische verbindingen tegen lichaamseigen structuren spreken we van auto-immuunziekten. Om beter te begrijpen hoe chemische verbindingen het immuunsysteem kunnen beïnvloeden en een allergische of auto-immuunreactie kunnen veroorzaken, is in **hoofdstuk 1** beschreven hoe het lichaam ervoor zorgt, dat gereageerd wordt tegen indringers (vreemd) maar niet tegen het eigen lichaam (zelf). Een aantal factoren spelen hierbij een rol: herkenning van de vreemde structuur (antigeen), herkenning van gevaar (co-stimulatie) en, indien het immuunsysteem het antigeen reeds eerder is tegengekomen, hoe deze primaire immuunreactie verlopen is (specifiek immunologisch geheugen). Om te kunnen voorspellen hoe bepaalde chemische stoffen het immunologische evenwicht verstoren, is het noodzakelijk om de antigenen waar tegen de immuunreactie is gericht te kennen. Dit is voor de meeste chemisch-geïnduceerde ongewenste immuunreacties nog niet voldoende bekend. In dit proefschrift is een aanzet gemaakt, deze antigenen beter te karakteriseren en de mechanismen die ertoe leiden dat allergische of auto-immuunreacties ontstaan beter te begrijpen.

In **hoofdstuk 2** wordt ingegaan op de verschillende factoren die leiden tot het ontstaan van nieuwe, gemodificeerde zelfantigenen (neo-antigenen). Een belangrijke rol hierbij spelen enzymen die chemische stoffen omzetten in metabolieten, die óf onschadelijk zijn en worden uitgescheiden, óf schade aanrichten door aan lichaamseigen eiwitten (zelfproteïnen) te binden. Deze schadelijke, proteïn-reactieve metabolieten worden ook wel *haptenen* genoemd,

de stoffen waaruit ze zijn ontstaan, *prohaptenen*. Omdat niet elke mens dezelfde enzymen bezit, zijn sommige mensen beter in staat chemische stoffen onschadelijk te maken dan andere mensen. Dit is o.a. een reden waarom sommige mensen allergisch op bepaalde geneesmiddelen reageren en anderen deze meerdere jaren achtereen zonder problemen in kunnen nemen. Om te kunnen voorspellen of een bepaald geneesmiddel goed wordt verdragen, is het noodzakelijk de enzymen die voor de ontgifting én voor de vorming van reactieve metabolieten verantwoordelijk zijn te kennen. Belangrijk hierbij is dat de cellen van het immuunsysteem zelf, en niet de lever, bij het ontstaan van immunologisch relevante reactieve chemische stoffen de hoofdrol spelen. Een ander belangrijk aspect in het ontstaan van immuunreacties tegen chemische stoffen is aanwezigheid van co-stimulerende signalen. Deze kunnen bijv. ontstaan door schade die de metabolieten in weefsels aanrichten, of door gelijktijdige infecties met virussen of bacteriën.

In **hoofdstukken 3, 4 en 5** kon worden aangetoond dat alleen haptenen, (proteïn-reactieve chemische stoffen) een immuunreacties kunnen veroorzaken. Dit werd gedaan m.b.v. de popliteale lymfeklier test, waarbij de te onderzoeken stof in de achterpoot van muizen wordt geïnjecteerd en daarna naar de zwelling van de lymfeklier die in direct contact staat met de plaats van injectie (drainerende lymfeklier) wordt gekeken. Hierbij kon worden aangetoond dat macrofagen, prohaptenen in haptenen kunnen omzetten. Ook kon een adduct bestaande uit hapteen en een 35 kD groot zelfproteïn zichtbaar gemaakt worden. De vraag of dit neo-antigeen daadwerkelijk voor de ongewenste immuunreacties tegen het hier onderzochte geneesmiddel (procainamide) verantwoordelijk is, wordt op het moment onderzocht.

Een aantal jaren geleden is er een nieuw soort immuun-cel ontdekt, de zogenaamde *natuurlijke killer T cel (NKT cel)*. Deze cel kan relatief snel, maar niet zeer specifiek bepaalde antigenen herkennen en hier tegen reageren. Het bijzondere aan deze cellen is, dat ze niet, zoals T cellen, tegen eiwit-brokstukken reageren, maar tegen vetten. Ook blijken ze een belangrijke rol te spelen bij het ontstaan, maar ook bij het verhinderen van auto-immuunziekten. Een auto-immuunziekte waarbij NKT cellen mogelijk een rol zouden kunnen spelen, is het Spaanse toxische olie syndroom, waarbij duizenden mensen ziek werden na het eten van olie, besmet met aniline. In **hoofdstuk 5** kon m.b.v. de popliteale lymfeklier test worden aangetoond dat vetzuur-aniline verbindingen, die aanwezig waren in de besmette olie een immuunreactie konden veroorzaken. In

hoofdstuk 6 konden wij echter m.b.v. gentechnisch gemodificeerde muizen aantonen, dat de kwantitatief grootste subgroep van NKT cellen hierbij geen rol speelt. Extra onderzoek is echter nodig om de rol van andere soorten van NKT cellen in het ontstaan van het toxische olie syndroom te kunnen onderzoeken.

Naast de tot nu toe beschreven onderzoeken, die erop gericht waren de cellen van het immuunsysteem die verantwoordelijk zijn voor de inductiefase van ongewenste immuunreacties te identificeren, is er ook een poging gedaan om op het niveau van neo-antigeen herkenning door T cellen een beter beeld te krijgen van wat er zich hier afspeelt. Hiervoor zijn muizen tegen bepaalde chemische stoffen allergisch gemaakt. De T cellen van deze muizen zijn daarna gefuseerd met tumorcellen, waardoor ze oneindig doorgroeien. Deze zijn zo verdunt, dat elke hieruit resulterende populatie uit slechts één cel ontstaan is (kloneren). Deze T celklonen zijn onderzocht op herkenning van verschillende chemische stoffen die aan één en hetzelfde zelfproteïne zijn gekoppeld. Uit de resultaten, die beschreven staan in **hoofdstuk 7**, kan geconcludeerd worden, dat T cellen niet zo specifiek op een bepaalde chemische stof reageren als vroeger werd gedacht. Eén en dezelfde T cel kan verschillende chemische stoffen herkennen en hiertegen reageren. Gezien de taak van het immuunsysteem is dit ook niet erg verwonderlijk. Het immuunsysteem moet namenlijk zeer veel verschillende antigenen herkennen en hiertegen reageren. Het immuunsysteem weet tijdens zijn ontwikkeling echter niet welke antigenen er allemaal op de wereld bestaan (en zal de meeste ook nooit tegen komen). Het moet dus op alles voorbereid zijn. Alhoewel een mens meerdere miljarden T cellen bezit, zouden deze, wanneer elke T cel slechts één theoretisch mogelijk antigeen zou herkennen, niet in staat zijn alle mogelijke indringers te herkennen en tijdig te vernietigen. Kruisreactiviteit is dus niet zoals vroeger werd gedacht een uitzondering, maar een noodzakelijkheid van T cellen.

Uit dit proefschrift blijkt, dat vele factoren bij het ontstaan van chemisch-geïnduceerde allergiën en auto-immuunziekten een rol spelen. De belangrijkste zijn: het ontstaan van chemisch reactieve metabolieten die met zelfproteïnen reageren en zo neo-antigenen vormen, co-stimulatie, en tenslotte activering van T cellen die (kruis)reageren met de ontstane neo-antigenen. De resultaten uit dit proefschrift kunnen helpen immunologische risico's van bijv. geneesmiddelen beter in te schatten en eventueel de dosis zo aan te passen, dat het risico op ongewenste immunologische bijwerkingen gering blijft. Nieuwe technieken uit

andere disciplines (bijv. genomics) kunnen hierbij helpen, indien snel en accuraat een analyse van verschillende enzymen en weefselantigenen (histocompatibiliteits-complexen) wordt gemaakt. Op basis van deze analyse en de chemische eigenschappen van verschillende geneesmiddelen kan dan worden bepaald welk geneesmiddel in welke dosering zal worden gegeven.

Kurze Zusammenfassung

Eine der wesentlichsten Aufgaben des Immunsystems ist die Abwehr von eindringenden Krankheitserregern wie Viren, Bakterien, Pilzen und Parasiten. Dem unspezifischen und spezifischen Immunsystem stehen eine Anzahl unterschiedlicher Zellsorten zur Verfügung: für die unspezifische Abwehr insbesondere die Makrophagen, für die spezifische Abwehr T- und B-Zellen. Makrophagen können die Krankheitserreger aufnehmen (daher auch als "Freßzellen" bezeichnet) und präsentieren anschließend Teile dieser Erreger den T-Zellen. Die T-Zellen können dann auf verschiedene Weise reagieren: Entweder zerstören sie jene Zellen, die das erkannte Antigen präsentieren (zytotoxische T-Zellen), oder sie helfen den B-Zellen Antikörper gegen die Krankheitserreger zu bilden (Helfer-T-Zellen).

Bestimmte Stoffe (z.B. Medikamente) können jedoch unerwünschte Reaktionen des Immunsystems verursachen. Eine dieser unerwünschten Antworten ist die allergische Reaktion. Reagiert das Immunsystem unter dem Einfluß von chemischen Verbindungen gegen körpereigene Strukturen, spricht man von Autoimmunerkrankungen. Um besser verstehen zu können, wie chemische Verbindungen das Immunsystem beeinflussen und allergische- bzw. Autoimmunreaktionen hervorrufen, wird in **Kapitel 1** beschrieben, wie das Immunsystem zwischen "Eindringlingen" (Nicht-Selbst) und körpereigenen Strukturen (Selbst) zu unterscheiden vermag. Hierbei spielen folgende Faktoren eine Rolle: Erkennung der fremden Struktur (Antigen), Erkennung von Gefahr (Co-Stimulation) und für den Fall, daß das Immunsystem bereits früher Kontakt mit dem Antigen hatte, die Erinnerung an die damalige Primärantwort (spezifisch immunologisches Gedächtnis: Zweitantwort, Toleranz). Um vorhersagen zu können, wie bestimmte Chemikalien das immunologische Gleichgewicht stören, ist die genaue Kenntnis der antigenen Struktur Voraussetzung. Diese Strukturen sind leider bis heute noch wenig bekannt. Ziel dieser Arbeit war es, diese Antigene besser zu charakterisieren und die Mechanismen, die zur Entstehung von allergischen- bzw. Autoimmunreaktionen führen, besser zu verstehen.

In **Kapitel 2** wird auf die verschiedenen Faktoren, die zur Entstehung von neuen, veränderten Selbstantigenen (Neo-Antigenen) führen, eingegangen. Hierbei spielen Enzyme welche die Substanzen metabolisieren eine wichtige Rolle. Neben stabilen Verbindungen entstehen hierbei auch protein-reaktive

Metabolite, die an körpereigene Eiweiße (Selbstproteine) binden können und so Neo-Antigene formen. Da nicht jeder Mensch die gleiche Enzymausstattung besitzt, sind manche Menschen eher in der Lage bestimmte Stoffe zu "entgiften" als andere. Daher reagieren manche Personen allergisch auf ein bestimmtes Medikament, wohingegen andere dasselbe Medikament jahrelang ohne Probleme einnehmen können. Die Vorhersage ob ein bestimmtes Medikament vertragen wird, ist jedoch dadurch erschwert, daß mehrere Enzymketten ineinandergreifen, wobei die Enzymkombination die zur Eliminierung eines Stoffes günstig ist, zur Eliminierung eines anderen Stoffes hingegen von Nachteil sein kann. Interessanterweise spielen bei der Bildung von immunologisch relevanten reaktiven Chemikalien nicht die Leber, sondern Zellen des Immunsystems eine entscheidende Rolle. Eine weitere Voraussetzung für die Entstehung von Immunreaktionen gegen Chemikalien sind co-stimulierende Signale (Signal 2). Diese entstehen beispielsweise durch Gewebsschäden, verursacht durch die chemischen Verbindungen selbst oder durch gleichzeitige Infektion mit Viren oder Bakterien.

In den **Kapiteln 3, 4 und 5** konnte gezeigt werden, daß nur chemisch reaktive d.h. chemische Verbindungen, die an Eiweiße binden, Immunreaktionen hervorrufen können. Der Nachweis erfolgte unter anderem mit dem poplitealen Lymphknotentest. Hierbei wird die zu untersuchende chemische Verbindung in eine der beiden Hinterpfoten einer Maus injiziert und die Schwellung des drainierenden Lymphknotens mit der unbehandelten Seite verglichen. Des weiteren konnte gezeigt werden, daß Zellen des Immunsystems selbst, insbesondere die sogenannten Makrophagen, unreaktive Substanzen in reaktive, d.h. an Proteine bindende Metabolite umwandeln können. In diesem Zusammenhang gelang in Makrophagen der Nachweis eines Adduktes bestehend aus einem Metabolit und einem 35 kD großen Selbstprotein. Die Frage, ob dieses Addukt tatsächlich für die unerwünschten Immunreaktionen gegen das untersuchte Medikament (Procainamid) verantwortlich ist, wird zur Zeit noch untersucht.

Vor einigen Jahren wurde eine neue Art von Immunzellen entdeckt: die sogenannte natürliche Killer T-Zelle (NKT-Zelle). Diese kann relativ schnell, jedoch nicht sehr spezifisch, bestimmte Antigene erkennen und dagegen reagieren. Das Besondere an diesen Zellen ist, daß sie nicht wie klassische T-Zellen gegen Eiweißbruchstücke reagieren, sondern gegen Fette. Ferner scheinen sie eine wichtige Rolle sowohl bei der Entstehung als auch bei der

Verhinderung von Autoimmunerkrankungen zu spielen. Eine Autoimmunerkrankung bei der NKT-Zellen möglicherweise eine Rolle gespielt haben, ist das Toxische-Öl-Syndrom, an welchem in den 80er Jahren in Spanien Tausende nach dem Genuß von mit Anilin verunreinigtem Speiseöl erkrankten. In **Kapitel 5** konnte gezeigt werden, daß Verbindungen welche in jenem Öl vorkamen im poplitealen Lymphknotentest eine Immunreaktion hervorrufen. In **Kapitel 6** haben wir mit Hilfe von gentechnisch veränderten Mäusen jedoch gezeigt, daß die quantitativ größte Subgruppe von NKT-Zellen hierbei keine Rolle spielt. Weitere Untersuchungen sind nötig, um die Rolle anderer Arten von NKT-Zellen in der Entstehung des Toxischen-Öl-Syndroms zu untersuchen.

Neben den bisher beschriebenen Untersuchungen, deren Ziel es war die für die Induktionsphase einer Immunantwort verantwortlichen Zellen zu identifizieren, wurde auch ein Versuch unternommen auf der Ebene von Neo-Antigenerkennung durch T-Zellen ein tieferes Verständnis zu erlangen. Hierfür wurden Mäuse gegen bestimmte chemische Stoffe sensibilisiert. Die sensibilisierten T-Zellen dieser Mäuse wurden danach immortalisiert und so verdünnt, daß jede nachfolgend gewachsene Population von einer einzigen Mutterzelle abstammt (Klonierung). Anschließend wurde die Feinspezifität einzelner Klone untersucht. Die in **Kapitel 7** beschriebenen Ergebnisse zeigen, daß T-Zellen nicht so spezifisch reagieren, wie bisher gedacht wurde. Ein und dieselbe T-Zelle kann verschiedene Stoffe erkennen und hiergegen reagieren. Bedenkt man die Aufgabe des Immunsystems ist dies auch nicht verwunderlich. Das Immunsystem muß nämlich eine Vielzahl unterschiedlicher Antigene erkennen. Das Immunsystem weiß zum Zeitpunkt seiner Entwicklung jedoch nicht, welche Antigene überhaupt auf der Erde bestehen (und wird den meisten auch nie begegnen). Dennoch muß es theoretisch in der Lage sein gegen all diese Antigene eine Abwehr aufzubauen. Obwohl der Mensch mehrere Milliarden T-Zellen besitzt wären diese, insofern eine T-Zelle nur jeweils ein Antigen erkennt, nicht in der Lage alle Krankheitserreger zu erkennen und rechtzeitig zu vernichten. T-Zellkreuzreaktivität ist somit nicht wie früher gedacht wurde eine Ausnahme, sondern eine Notwendigkeit.

Somit spielen viele Faktoren bei der Entstehung von chemisch induzierten Allergien und Autoimmunerkrankungen eine Rolle. Die Wichtigsten hierbei sind: die Bildung reaktiver Metabolite, welche mit Selbstproteinen reagieren und so Neo-Antigene formen, Co-Stimulation und schließlich Aktivierung von

T-Zellen, die mit dem Neo-Antigen (kreuz)reagieren. Die Ergebnisse dieser Arbeit können dazu beitragen immunologische Risiken unter anderem von Medikamenten besser einzuschätzen und gegebenenfalls Dosierungen anzupassen, um die Risiken von unerwünschten Arzneimittelnebenwirkungen zu minimieren. Neue Techniken aus anderen Fachgebieten (z.B. Genomics) können hierzu durch Analyse des Enzympanels und der Gewebsantigene (Histokompatibilitätskomplexe) beitragen. Auf der Basis einer solchen Analyse und den chemischen Eigenschaften der Arzneimittel kann dann bestimmt werden, welches Arzneimittel welchem Patienten in welcher Dosierung gegeben werden kann.

Levensloop

Marty Wulferink werd geboren op 8 februari 1971 te Almelo. In 1989 behaalde hij het VWO diploma aan het Pius X college te Almelo. Aansluitend begon hij met de studie geneeskunde aan de Katholieke Universiteit Nijmegen. Hij behaalde het propedeutisch examen geneeskunde in 1990. Zijn overstap naar de studie gezondheidswetenschappen aan dezelfde universiteit in 1991 werd afgesloten met het doctoraal examen in de vakrichting toxicologie in 1995. Tijdens zijn eerste stage in de vakgroep celbiologie en histologie aan de medische faculteit Nijmegen onder leiding van Dr. L.G. Poels werden de eerste contacten gelegd met Prof. E. Gleichmann, hoofd van de afdeling immunologie aan het medisch instituut voor milieuhygiëne te Dusseldorf, alwaar de tweede stage en het in dit proefschrift beschreven promotieonderzoek werden verricht.

List of publications and manuscripts in preparation

Griem, P., M. Wulferink, B. Sachs, J. B. Gonzalez, and E. Gleichmann. 1998. Allergic and autoimmune reactions to xenobiotics: how do they arise? *Immunol.Today 19*: 133-141.

Ewens, S., M. Wulferink, C. Goebel, E. Gleichmann. 1999. T-cell dependent immune reactions to reactive benzene metabolites in mice. *Arch.Toxicol. 73*: 159-167

Goebel, C., C. Vogel and M. Wulferink, S. Mittmann, B. Sachs, S. Schraa, J. Abel, G. Degen, J. Uetrecht, and E. Gleichmann. 1999. Procainamide, a drug causing lupus, induces prostaglandin H synthase-2 and formation of T cell-sensitizing drug metabolites in mouse macrophages. *Chem.Res.Toxicol. 12*: 488-500.

Rönnau, A.C., M. Wulferink, E. Gleichmann, E. Unver, T. Ruzicka, J. Krutmann, M. Grewe. 2000. Anaphylaxis to polyvinylpyrrolidone in analgesic preparation. *Br.J.Dermatol. 143*: 1055-1058

Wulferink, M., J. González, C. Goebel, and E. Gleichmann. 2001. T cells ignore aniline, a prohapten, but respond to its reactive metabolites generated by phagocytes: possible implications for the pathogenesis of toxic oil syndrome. *Chem.Res.Toxicol. 14*: 389-397.

Wulferink, M., S. Dierkes, and E. Gleichmann. 2001. T Cell cross-sensitivity to haptens: T cell recognition of common haptenic metabolites, recognition of identical cryptic self-peptides and true cross-reactivity. *(Submitted)*

von Schmiedeberg, S., T.K. Hoffmann, M. Wulferink, R. Thier, H. Bier, T. Ruzicka, P. Lehmann. 2001 Dapson-induzierte Agranulazytose: die Rolle Fremdstoff-metabolisierende Enzyme am Beispiel einer Kasuistik. *(Submitted)*

Wulferink, M., S. Dierkes, J. González, and E. Gleichmann. Are NKT cells involved in the pathology of the Spanish toxic oil syndrome? A pilot study. *(manuscript in preparation)*

Wulferink, M., S. Dierkes, and E. Gleichmann. The CellELISA: a rapid method for measuring specific T-cell hybridoma reactions. *(manuscript in preparation)*

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