The murine IL-2-dependent cytotoxic T-cell line CTLL can be preserved by freezing

SIR,
Continuous passaging of several different cell lines is an expensive and tedious task, especially when certain cells are needed only rarely. Consequently, techniques have been developed for storing animal cells frozen. Freezing should arrest cell growth in a reversible manner and it is common to freeze animal cells in culture medium supplemented with 8% dimethylsulfoxide (DMSO) chilling the samples stepwise to - 196°C in liquid nitrogen. To our knowledge there are few data on the general applicability of this method to cell lines. In particular, there is a general view among investigators active in interleukin-2 (IL2) research that the murine IL2-dependent cytotoxic T-cell line CTLL should not be frozen because the cells lose viability. The CTLL line is widely used as target cells to assay human and murine IL2 by measuring the dilution limit at which the lymphokine still exerts its growth stimulating effect on the target cell. Preservation by freezing would avoid the necessity of continuously maintaining the cells in active growth, so we have investigated the effect of freezing and thawing on CTLL cells under different conditions. They were frozen in HEPES-buffered RPMI 1640 medium supplemented with 1% glutamine, 20% foetal bovine serum (Flow Laboratories) and appropriate concentrations of either DMSO or glycerol. Storage was at - 20°C, - 70°C or - 196°C (liquid nitrogen). In our hands, freezing in 8% glycerol in liquid nitrogen gave the best recovery of CTLL cell growth upon thawing, although freezing in 16% glycerol still allowed some recovery of viable cells. In contrast to what was generally expected, cells could also be revived after freezing in 8% DMSO.

Much of the success of these manoevres depends on the freezing and thawing procedures. To freeze cells in liquid nitrogen, cells were put into straws (30 000 cells each) which were sealed. After the straws had been chilled at 4°C for 30 min, they were transferred to a container with liquid nitrogen and kept for 45 min hanging a few centimetres above the surface of the liquid before being submerged in the nitrogen. To recover the cells, straws were quickly thawed at 37°C, fresh medium was added dropwise and the cells were collected by a single centri-fugation step (10 min, 500 g). By this procedure, 95% of the cells were viable as determined by trypan blue exclusion. CTLL cells have been stored frozen for up to 4 months without loss of viability.

CTLL cells have also been successfully stored at - 70°C. For this purpose, the cell suspension (in either 8% glycerol or 8% DMSO) was transferred into 1 ml plastic vials with screw caps and moved directly into a - 70°C freezer. After 4 months of storage, the viable cell count was lower than in samples stored in liquid nitrogen but cultures could be grown, even at a low cell dose of 30 000 cells per vial. Preservation of CTLL cells at - 20°C was not possible under any conditions tested, although this temperature was suitable for other cell types, e.g. human B-lymphoblastoid cell lines.

In conclusion, in contrast to what seems generally to be believed, we have shown that the murine cell line CTLL can be preserved frozen, either in liquid nitrogen or at - 70°C. The latter method may be especially useful to overlap short breaks in the laboratory work or whenever suitable equipment for liquid nitrogen manipulation and storage is not available. In addition, it is an extremely rapid and safe method to keep an aliquet of the cell line as a duplicate sample in case contamination of the culture or another laboratory accident has occurred. For long-term preservation we advise freezing in liquid nitrogen in the presence of 8% glycerol.

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Is tumor necrosis factor a physiological mediator?

SIR,
As authors of various papers on the role of tumor necrosis factor (TNF) in the induction of tumor necrosis and regression, we read with great interest the article of Playfair *et al.* (*Immunol. Today,* 1984, 5, 165–166) on the role of TNF in the host defense against malaria. We would like to contribute to the discussion, especially on the issue whether TNF, defined by its *in-vivo* capacity to necrotize and occasionally to make tumors regress completely, is a relevant mediator of host defense against tumors.

The production of serum with measurable amounts of TNF in several animal species (tumor necrosis serum, TNS) by endotoxin requires extreme macrophage activation[1,2] and is invariably lethal to the donor within 24 h (Refs 2 and 3). Nevertheless a non-lethal dose of endotoxin can induce tumor necrosis and complete regression in tumor-bearing animals[4]. We have been unable to demonstrate TNF in such mice, while its absence could not be attributed to absorption to the tumor target cells[4]. So far as we know the presence of TNF in tumor-bearing animals undergoing tumor necrosis and regression has not been reported by others.

Besides endotoxin and endotoxin-induced TNS, various non-endotoxin agents are able to induce tumor necrosis and regression in Meth A tumor-bearing mice, such as concanavalin A, and the synthetic polyribonucleotides poly I:C and poly A:U (Refs 5 and 6). Other agents such as histamine, serotonin, adrenalin and isoproterenol can induce tumor necrosis without subsequent regression[7,8]. In contrast to endotoxin none of these agents are able to provoke the release of TNF in the serum of mice pretreated with a macrophage-activating agent, despite the fact that the majority of these sera have sometimes very remarkable cytostatic activity against the same tumor target cells *in vitro*[5,8]. Although highly purified TNF was shown to have *in-vivo* as well as *in-vitro* antitumor action[9], the above data indicate that *in-vitro* activity is not a reliable parameter for the presence of TNF. Our recent observations[10] confirm this. Chemically detoxified endotoxin from *Salmonella typhimurium Re* (Ribi Immunochem Inc.) was shown to be a very weak inducer of necrosis and regression of Meth A tumors even at high doses, while the parent endotoxin is very active. Addition of a small amount of muramyl dipeptide (MDP; with no significant antitumor action of its own) to both endotoxins resulted in equal, dramatically enhanced, antitumor activity: very extensive necrosis and a high incidence of complete cures. Toxic side effects, however, diverged largely. MDP combined with the toxic preparation induced severe diarrhoea, lethargy and a high incidence of lethality, but the other combination was effective without these side effects[10]. The divergence of toxicity seems to correlate very well with the different capacity of the two agents to induce TNF (Bloksma, N., Hofhuis, F. M. A. and Willers, J. M. N. unpublished observations). The toxic preparation induces TNS with high levels of

TNF, and addition of MDP results in a moderate increase. The detoxified preparation alone or combined with MDP, however, fails to induce TNF in sera of mice, although the *in-vitro* cytostatic activity of the latter sera is as high as in TNS elicited with the toxic preparation.

In conclusion, data presented above indicate that TNF cannot be regarded as a general mediator of induced tumor necrosis and regression. As the factor seems to be only produced under extreme, life-threatening, conditions, it is questionable whether this factor has been selected for during evolution of host defense against tumors or malaria. ▯

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*** This letter was shown to Professor Playfair and colleagues, who reply as follows:

SIR,
Dr Bloksma and her colleagues have drawn attention to a very important aspect of work on TNF – namely the confused state of the terminology. There is no doubt that, as they have clearly shown, the necrosis of tumours *in vivo* and the killing of tumour cells *in vitro* are separable, if overlapping, phenomena and it is certainly a pity that the factors under study in many laboratories for their *in-vitro* effects are still loosely referred to as TNF and the serum from which they come as TNS. We would be the first to agree that these factors (of which at least three can be identified in the serum of suitably treated animals) deserve another name. Indeed this was one purpose of our original article. It will probably not be long before cloned material is available and a logical nomenclature for these and related cytotoxins can be devised.

Meanwhile we wish to re-emphasise that effective levels of the factors causing death of L929 tumour cells and of blood-stage malaria parasites, which we believe to be largely the same, are by no means restricted to extreme or life-threatening conditions. For example, mice whose lethal *Plasmodium yoelii* infection has been cured by repeated injections of 'TNS' appear perfectly healthy, and it is still our contention that local levels of some cytotoxin may play a part in controlling blood-stage malaria. We can in fact detect low levels of cytotoxic activity in the serum of infected mice. The same argument can be applied to other cytotoxic factors such as reactive oxygen intermediates, which are indubitably effective against many microorganisms but are not found in serum because of their short half life.

It seems highly unlikely to us that Nature has allowed such potent molecules to evolve without a purpose. ▯

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Autoimmune induction by epithelial cells bearing class II MHC antigens

SIR,
I should like to comment on the interesting and plausible hypothesis of autoimmunity by Franco Bottazzo and his colleagues (*Immunol. Today* 1984, 5, 23). They suggest that HLA-DR antigen is expressed on endocrine epithelial cells, possibly induced by γ interferon which is elicited by a trivial viral infection in genetically predisposed subjects. A reversal in polarity in DR expression on the external thyroid epithelial cell wall enables the DR-positive cells to present self-antigens to auto-reactive T cells and induce an autoimmune response[1]. In view of their demonstration that DR antigen is found in thyrotoxic or Hashimoto thyroid follicular cells but not normal cells[2] and that DR expression could be induced in normal thyroid cells by mitogenic stimulation[3], probably mediated by γ interferon, the hypothesis is well substantiated. However, in order to switch on the helper T cell to induce antibodies or cell-mediated immunity to the endocrine antigens, the T suppressor cell activity has to be depressed or by-passed[4]. Their hypothesis does not account for this requirement. Furthermore, autoimmunity can develop in the face of suppression, as in malaria.

Gershon and his colleagues postulated that suppression can be overcome by contrasuppressor cells[5], as was found in thymectomised mice which develop anti-DNA antibodies and become Coomb's positive when stimulated by polyclonal B-cell activators[6]. We suggested that the DR+ epithelial cells might induce both the T helper and contrasuppressor circuits, thereby preventing suppressor cells from inhibiting the helper function which is required to generate autoantibodies. However, this introduces another level of complexity. The situation would be greatly simplified and cellular economy maintained by postulating that the DR+ endocrine epithelial cells can both present the self-antigen to T helper cells and prevent any T suppressor cells from inhibiting the helper cells. The hypothesis is thereby extended to suggest that the DR+ endocrine epithelial cells have a dual function: they present antigen to activate T helper cells and function as contrasuppressor cells. This would by-pass the necessity to account for a decrease in suppression or indeed, autoimmunity in the face of suppressor activity.

This extended hypothesis of autoimmunity is based on our findings that a small subset of T8+ cells can bind antigen[7], present the antigen to T4+ helper cells to induce helper function[8,9] and prevent the major subset of T8+ suppressor cells from inhibiting T4 helper cells[10]. Preliminary characterization of this subset of T cells suggests that it is T8+, T3+, T4−, Ia+, antigen binding and *Vicia villosa* binding cell (Lehner, T., Avery, J. and Jones T., unpublished observations). Thus, a T8+ cell subset fulfills the criteria postulated to be necessary for endocrine autoimmunity: that the cells express Ia, that they bind antigen, present it to T4 helper cells and prevent T8 suppressor cell activity. This extended hypothesis of autoimmunity