

## Tumour necrosis factor- $\alpha$ production during cytomegalovirus infection in immunosuppressed rats

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The production of tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 and IL-6, all proinflammatory cytokines, was investigated in radiation-immunosuppressed rats infected with rat cytomegalovirus (RCMV). At day 7 post-infection, when the animals showed disease signs, high TNF- $\alpha$  levels were detected in the serum and in homogenates of various organ tissues. In contrast, IL-1 and IL-6 levels were not significantly elevated. Moreover, replication of RCMV induced TNF- $\alpha$  expression

in different types of cells grown *in vitro*. When frozen tissue sections were examined by immunohistology, TNF- $\alpha$ -producing cells were found in areas with extensive pathology in the lungs, spleen and liver. Both lymphocytes and RCMV-infected cells were identified as the sources of TNF- $\alpha$ . Its abundance in RCMV-infected rats suggests an important role for TNF- $\alpha$  in CMV pathogenesis.

### Introduction

Cytomegalovirus (CMV) infections are a major cause of death in persons receiving immunosuppressive treatment (Ho, 1982). Interstitial pneumonitis, acceleration of the graft-versus-host disease observed in bone marrow transplant patients and inhibition of haematopoietic stem cell differentiation are major causes of death in CMV disease (Ho, 1982; Meyers *et al.*, 1986). However, only a few infected cells are detectable per organ, even in generalized CMV disease; therefore mechanisms other than viral cytolysis must contribute to organ dysfunction (Ho, 1982).

Cytokines play an important role in the pathogenesis of infectious diseases. The production of proinflammatory cytokines, such as tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 and IL-6, is often associated with the observed pathology (Beutler & Cerami, 1987; Dinarello, 1989; Grau *et al.*, 1987). The ability of IL-1 to induce fever led to its discovery; but it possesses a wide range of other activities, e.g. the induction of hepatic acute phase protein synthesis and neutrophilia, and the enhancement of T cell responses (Dinarello, 1989). Its non-immunological functions in particular resemble those of TNF (Beutler & Cerami, 1987; Vassali, 1992). Like IL-1 and TNF, IL-6 is an endogenous pyrogen and an inducer of acute phase responses (Van Snick, 1990). In addition, it regulates haematopoiesis and acts as a

differentiation factor for B cells and cytotoxic T cells. These three proinflammatory cytokines are produced by a wide variety of cells, and coordinated expression has been observed during virus infection. They possess antiviral activity (Billiau, 1987; Wong & Goeddel, 1986) and determine the course of infections, as shown by blocking experiments (Beutler & Cerami, 1985; Dinarello, 1985; Grau *et al.*, 1987).

As the first step towards defining their role in CMV pathogenesis we have analysed the production of IL-1, IL-6 and TNF- $\alpha$  in a rat CMV (RCMV) infection model. RCMV resembles its human counterpart in that it replicates only poorly in the immunocompetent host, whereas it reaches high titres in certain organs of immunosuppressed subjects (Stals *et al.*, 1990). In this study, we demonstrate the preferential expression of TNF- $\alpha$  during RCMV infection and propose an important role for this cytokine in the pathogenesis of CMV disease.

### Methods

*Animals.* Inbred, specific pathogen-free, male Brown Norway (BN) and Wistar rats were obtained from Harlan. The animals were kept in filter top cages. The experimental protocols were approved by the institution's Animal Welfare Committee.

*Cells.* Rat embryo fibroblast (REF) cells were prepared from 17-day-old rat embryos and grown in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum (DMEM-FCS), penicillin

(100 U/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml). Cells were grown in plastic culture flasks (Nunc) at 37 °C in a humidified CO<sub>2</sub> incubator and used at the third passage.

Glial cell cultures were prepared as previously described (Koper *et al.*, 1984). Briefly, cerebra of 7-day-old Wistar rats were minced and incubated with trypsin (45 min at 37 °C). The tissue was then triturated in the presence of soybean trypsin inhibitor and sieved through a nylon screen to remove debris. The cell suspension was plated on poly-L-lysine-coated glass coverslips and cultured for 24 h in DMEM-FCS. Under these conditions the glial cells developed into glial fibrillary acidic protein-positive (GFAP<sup>+</sup>) astrocyte-enriched (> 98%) cultures.

For the isolation of spleen macrophages (spleen adherent cells), rats were sacrificed, their spleens were minced, the erythrocytes were lysed and the cells seeded into the 16 mm dishes of 24-well plates. After 1 h, non-adherent cells were removed and the remaining cells were cultured for the indicated times in DMEM-FCS. Adherent cells were identified as macrophages by their morphology and their non-specific esterase activity (> 95% positive).

**Virus.** Stocks of RCMV were prepared as 10% (w/v) homogenates of salivary gland tissue taken from BN rats that had been inoculated 3 weeks previously with 10<sup>4</sup> p.f.u. of RCMV via the intraperitoneal (i.p.) route. RCMV was passaged in REF cells. At maximal c.p.e. supernatants were harvested and centrifuged at 900 g for 10 min. Virus was plaque-titrated (Bruggeman *et al.*, 1982) and stored in aliquots at -70 °C until use. Only cell-culture derived virus was used in this study.

**Experimental design.** Whole-body irradiation-immunosuppressed rats (5 Gy at 350 cGy/min on day -1) were infected via the i.p. route with 5 × 10<sup>5</sup> p.f.u. RCMV on day 0. Blood samples obtained after orbital puncture were allowed to clot at 4 °C for 1 h, centrifuged and the serum was kept at -20 °C until use. Organs were removed on day 7 p.i. and homogenized (10% w/v) in DMEM containing 10% FCS; after low-speed centrifugation supernatants were assayed for infectious virus and cytokines. Cytokines in sera and organ homogenates were tested starting at a 1:10 and 1:100 dilution, respectively.

**TNF induction.** Cells of the different types were resuspended in DMEM-FCS and plated at a concentration of 1 × 10<sup>6</sup>/well into six-well plates (Nunc). The plates were incubated until the cells had reached subconfluence and treated with either 1 µg/ml lipopolysaccharide (LPS; from *Escherichia coli* serotype O128:B12, Sigma) or RCMV at an m.o.i. of 3. Virus preparations and culture media were found to contain < 60 pg/ml of contaminating endotoxin as tested by the COA-test (Chromogenix). RCMV was u.v.-irradiated (10 min), preincubated with normal rat serum or RCMV-neutralizing rat serum (1:20 dilution, 30 min, 37 °C). As a negative control, cells were mock-infected and cultured in medium alone. At various time points after inoculation, culture fluid was collected, centrifuged and stored at -20 °C until use. Supernatants were u.v.-irradiated before their use in the bioassay.

**Assay for TNF.** TNF activity in sera, tissue homogenates and tissue culture supernatant was determined in a biological assay using WEHI-164 mouse fibrosarcoma cells (Espevik & Nissen-Meyer, 1986). Cells were collected during their logarithmic growth phase and suspended at a concentration of 4 × 10<sup>5</sup>/ml in RPMI 1640 medium containing 10% FCS. Fifty µl of the suspension was added in triplicate to 50 µl volumes of the test sample in flat-bottom 96-well plates (Nunc) and incubated at 37 °C for 18 h. TNF specificity was established by incubation of the sample for 1 h at 37 °C with either an excess of affinity-purified (recombinant murine TNF-α-Sepharose) goat anti-TNF-α antibodies, or a hamster monoclonal antibody (MAb) directed against murine TNF-α (Sanbio) able to neutralize rat TNF-α (Sheenan *et al.*, 1989). Cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma). After addition of 20 µl of MTT (5 mg/ml in PBS) to each well and incubation at 37 °C for 4 h,

100 µl of lysis buffer (20% w/v SDS in 50% dimethylformamide) was added; incubation was continued overnight at 37 °C after which time absorbance was read at 578 nm. Concentrations of TNF are given in pg/ml using recombinant murine (rm) TNF-α as a standard (sp. act. 75 × 10<sup>6</sup> U/mg protein, as determined in L929 cells).

**TNF ELISA.** TNF was quantified using a rat TNF-α-specific ELISA. Wells of a 96-well microtitre plate (Flow) were coated for 16 h at 4 °C with 100 µl of purified goat anti-TNF-α antibodies at 10 µg/ml in PBS. The wells were washed 10 times with Tris-buffered saline (20 mM-Tris-HCl, 150 mM-NaCl pH 7.4) containing 0.05% Tween 20 (TBS buffer). After blocking with 200 µl of a 2% BSA solution in PBS the plates were emptied and incubated with the samples. As a positive control recombinant rat TNF-α (sp. act. 7 × 10<sup>6</sup> U/mg) was used. Thereafter the wells were extensively washed and incubated with 100 µl of biotinylated rabbit anti-TNF-α antibodies at 2 µg/ml in PBS. After 10 washing cycles, 100 µl of anti-biotin-alkaline phosphatase conjugate (1 µg/ml diluted in 1% BSA) was added to each well. Again the plate was incubated and subsequently washed 10 times with TBS. Then 100 µl of a solution of 0.5 mg/ml *p*-nitrophenyl phosphate (Sigma) in 0.1 M-glycine-NaOH pH 10.4 containing 1 mM-MgCl<sub>2</sub> and 1 mM-ZnCl<sub>2</sub> was added to each well. The substrate conversion was stopped by the addition of 30 µl 3 M-NaOH and the absorbance was read at 405 nm. All incubations were for 1 h at 37 °C.

**IL-1 assay.** A murine T cell line clone (D10) was used to determine IL-1 bioactivity as described by Helle *et al.* (1988). Briefly, 10<sup>4</sup> cells were incubated in flat-bottom well plates in the presence of 50 U/ml rIL-2 with the samples to be tested and 1 µCi [<sup>3</sup>H]thymidine (Amersham; sp. act. 0.7 × 10<sup>8</sup> to 1.1 × 10<sup>8</sup> MBq/mmol) was added between 64 and 68 h later. IL-1 concentrations were calculated from a standard curve obtained with rIL-1α, showing the relationship between incorporation and cytokine concentration. The detection limit of the assay was 0.05 to 0.09 U/ml.

**IL-6 assay.** The murine hybridoma cell line B9 was used to detect IL-6 bioactivity as described by Helle *et al.* (1988). Briefly, cells (5000/200 µl) were incubated in the presence of samples to be tested in flat-bottom well plates. Proliferation was measured by labelling from 64 to 68 h using 1 µCi [<sup>3</sup>H]thymidine (Amersham). IL-6 concentrations were calculated from a standard curve obtained with rIL-6, showing the relationship between incorporation and cytokine concentration. One unit was defined as the amount of IL-6 that gives rise to 50% incorporation.

**Immunocytochemistry.** Cells grown on coverslips were washed twice, fixed for 5 min at room temperature in 4% paraformaldehyde and rinsed twice with PBS. Before staining they were permeabilized using 0.1% saponin (Sigma) in PBS containing 2% normal goat serum for 20 min. After rinsing (all washes were in PBS-saponin), they were incubated overnight at 4 °C with the primary antibody. Mouse antibody against GFAP (Dakopatts) was used to identify astrocytes. Goat anti-TNF-α antibodies were used to detect TNF-α expression. After rinsing, they were reacted with rabbit anti-goat antibodies (Sigma) and biotinylated goat anti-rabbit IgG antibody (Sigma) for 30 min followed by three additional rinses. Avidin-coated tetramethylrhodamine isothiocyanate complexes (Nordic) were used at a 1:100 dilution for 30 min. Coverslips treated with normal rabbit serum instead of the anti-TNF serum, or preincubated with rmTNF-α to neutralize the anti-TNF antibodies, served as negative controls. After incubation, the preparations were washed, mounted in PBS-glycerol containing 2% 1,4-diazabicyclo(2.2.2)octane and examined in an epifluorescence microscope.

**Histology.** Organ samples were frozen in liquid nitrogen immediately after removal and kept at -70 °C until use or fixed in para-

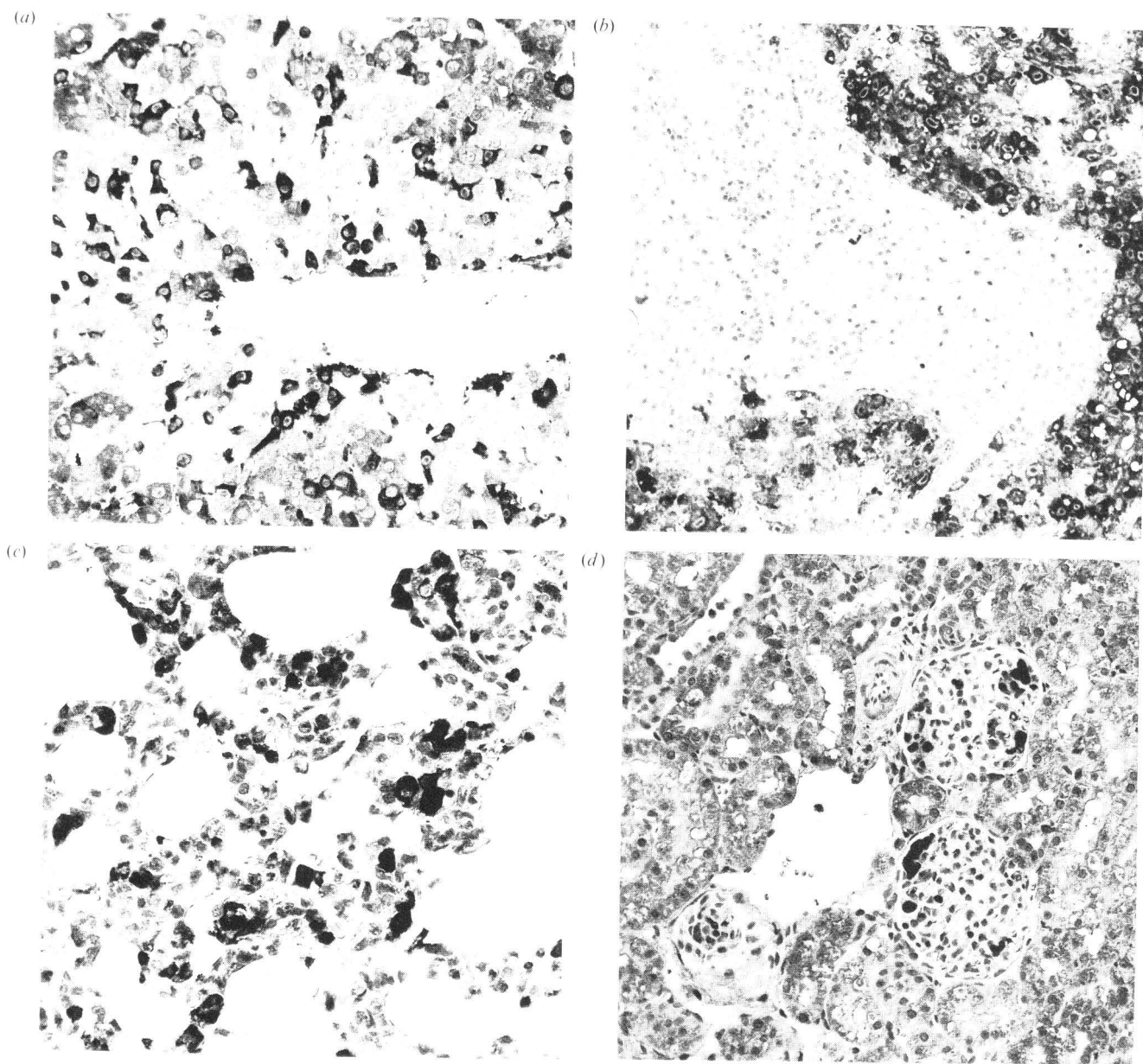


Fig. 1. Immunohistochemical detection of RCMV antigen-containing cells in the liver (*a*), spleen (*b*), lungs (*c*) and kidney (*d*) of infected rats at day 7 p.i. Note the pneumonitis (*c*) and the absence of RCMV antigen in the follicles of the spleen (*b*). No specific staining was observed in sections incubated with irrelevant antibodies (not shown, see also Fig. 4*d*). Bar marker under (*d*) represents 100  $\mu$ m for (*a*), (*b*) and (*d*); marker under (*c*) represents 50  $\mu$ m.

formaldehyde-lysine periodate and embedded in paraffin wax. Paraffin sections (5  $\mu$ m) were dewaxed and rehydrated in Tris-buffered saline (0.05 M-Tris HCl pH 7.6) containing 0.1% Triton X-100, which was used for all washings. The sections were incubated with 1% H<sub>2</sub>O<sub>2</sub> in methanol for 15 min to block endogenous peroxidase activity and preincubated for 20 min at room temperature with normal goat serum (diluted 1/40) to reduce non-specific binding. Cryostat sections (8  $\mu$ m, cut at -30 °C) were fixed for 10 min in acetone containing 0.02% H<sub>2</sub>O<sub>2</sub>. Sections were examined for the presence of viral antigen and TNF- $\alpha$  expression as described previously (Van den Eertwegh *et al.*, 1991). Briefly, the slides were washed and incubated overnight at 4 °C with a mixture of MAbs (8 and 35; Stals *et al.*, 1990) directed against

nuclear and cytoplasmic RCMV antigens, respectively, or goat anti-TNF- $\alpha$  antibody. All reagents were diluted in PBS containing 0.1% BSA and titrated to obtain optimal results. Subsequently, slides were washed three times for 5 min and incubated for 30 min at room temperature with the diluted secondary antibody conjugates (rabbit anti-mouse peroxidase or rabbit anti-goat peroxidase; Sigma) containing 1% normal rat serum. Thereafter, slides were washed in PBS and histochemical peroxidase reactions were performed as previously described (Van den Eertwegh *et al.*, 1991). Peroxidase activity was detected using 0.003% H<sub>2</sub>O<sub>2</sub> and 0.5% 3,3'-diaminobenzidine in 0.05 M-Tris-HCl buffer pH 8.3. The preparations were counterstained with haematoxylin, dehydrated and mounted.

**Results**

*Cytokine production in RCMV-infected radiation-immunosuppressed rats*

Radiation-immunosuppressed rats infected with  $2 \times 10^5$  p.f.u. of RCMV succumbed 8 to 10 days p.i.; no mortality was observed over a period of 30 days in animals that had received whole-body irradiation only. At day 7 p.i. the infected rats showed extensive pneumonitis, hepatitis and disseminated intravascular coagulation; they also exhibited a severe anaemia, hypotension and hypothermia (data not shown). Most viral antigen-expressing cells found in the lungs, spleen, liver, bone marrow and kidney were identified morphologically as macrophages and hepatocytes (Fig. 1). No evidence was found of infection of lymphocytes. Sera obtained at day 7 p.i. were analysed for the presence of the macrophage-derived cytokines IL-1, IL-6 and TNF. No IL-1 activity was detected ( $< 0.5$  U/ml,  $n = 6$ ) and IL-6 serum levels were only moderately elevated: 40 to 60 U/ml in infected compared to 24 to 29 U/ml in immunosuppressed uninfected rats. In contrast, high levels of bioactive TNF- $\alpha$  were detected in the infected rats (Fig. 2). Pre-incubation of the sera with goat antibodies or a hamster MAb directed against murine TNF- $\alpha$  neutralized  $> 90\%$  of the TNF bioactivity. Similar levels were found using a rat TNF- $\alpha$ -specific

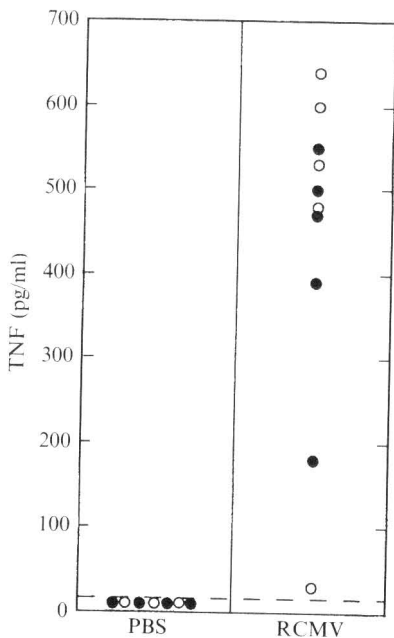


Fig. 2. Detection of TNF activity levels in sera of individual RCMV-infected rats. Radiation-immunosuppressed rats were injected with PBS or infected with  $1 \times 10^6$  p.f.u. of RCMV and their sera obtained at day 7 p.i. were analysed in a bioassay (closed circles) or a rat TNF- $\alpha$ -specific ELISA (open circles). The detection limit (broken line) for both assays was 20 pg/ml.

ELISA (Fig. 2). Radiation-immunosuppressed uninfected rats had  $< 20$  pg TNF/ml in their serum. Thus, of the three cytokines tested, only TNF- $\alpha$  is over-expressed at day 7 p.i.

*Tissue-specific cytokine expression*

Tissue homogenates of the spleen, liver and lungs were found to contain high levels of TNF- $\alpha$  whereas organs of the uninfected control rats were devoid of detectable TNF activity (Fig. 3). High TNF levels in organs coincided with high RCMV titres, with the notable exception of the lungs: these contained much lower virus titres accompanied by the highest TNF levels measured and the most pronounced pathology. IL-1 and IL-6 bioactivities in the liver and spleen of RCMV-infected

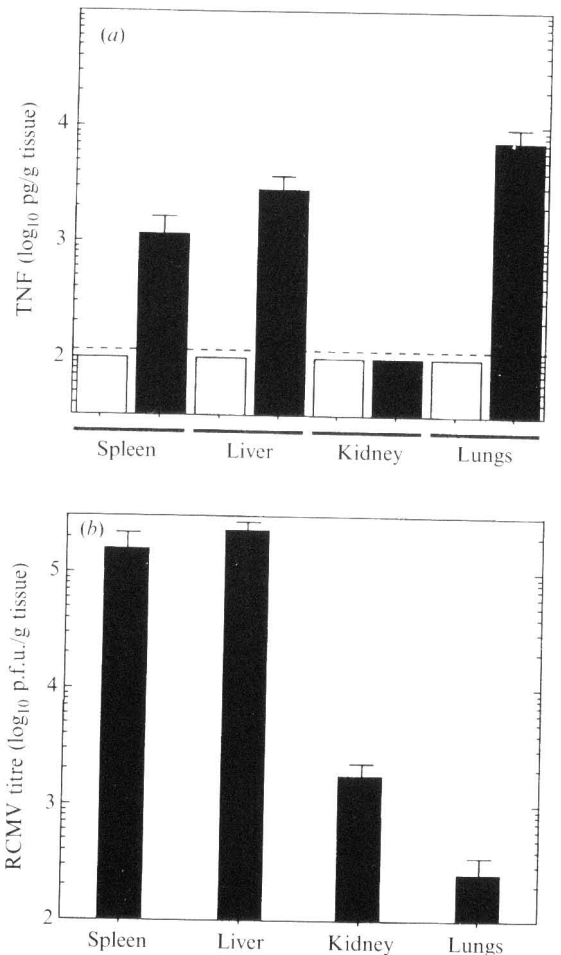


Fig. 3. TNF bioactivity (a) and RCMV infectivity titres (b) in tissue homogenates of infected immunosuppressed BN rats ( $n = 4$ ). Animals-immunosuppressed by whole-body irradiation were injected 1 day later with  $2 \times 10^5$  p.f.u. RCMV (closed bars) or PBS (open bars) via the i.p. route. Spleen, liver, kidneys and lungs were removed on day 7 p.i., and tissue homogenates were analysed for the presence of TNF (a) and infectivity (b). The detection limit (broken line) for the TNF assay was 125 pg/g tissue. Data are expressed as mean titre  $\pm$  S.E.M.

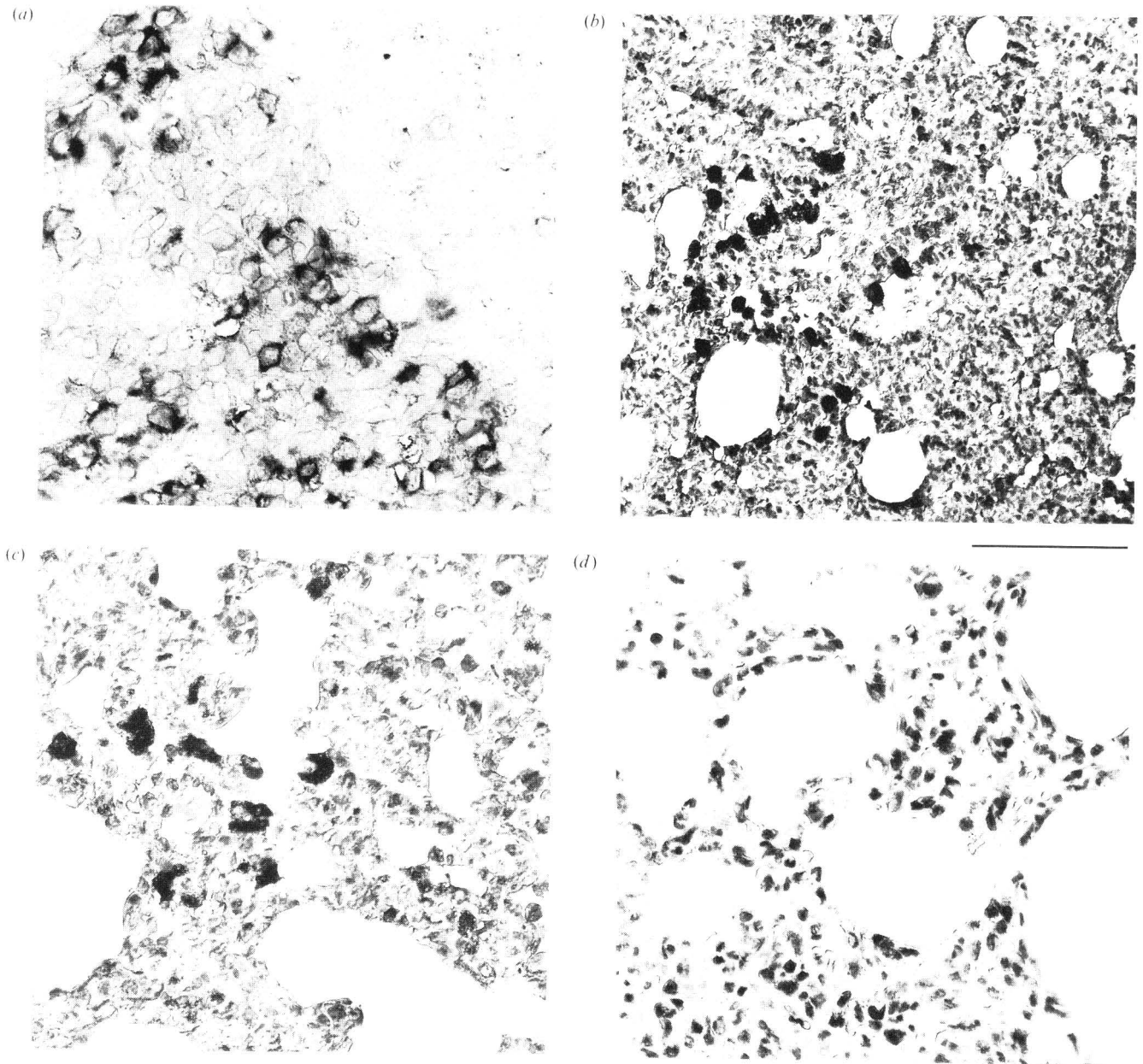


Fig. 4. Immunohistochemical detection of TNF- $\alpha$  in sections of spleen (a) and lungs (b to d) of RCMV-infected rats at day 7 p.i. Shown are stainings with goat anti-TNF- $\alpha$  (a to c) and normal goat serum (d). Bar marker under (d) represents 50  $\mu$ m for (a), (c) and (d); marker under (b) represents 100  $\mu$ m.

rats did not differ significantly from those in control animals, whereas low levels of both were detected in the lungs (not shown). Notably, IL-6 expression was elevated in the kidneys (155 U/g tissue compared to < 50 in control animals), whereas neither TNF nor IL-1 bioactivity could be detected in these organs. Of these three cytokines examined, again only TNF- $\alpha$  levels were significantly raised in tissue homogenates. Therefore subsequent studies were focused on this cytokine.

#### Cellular localization of TNF- $\alpha$ production

The cellular source of TNF- $\alpha$  was identified by immunohistology. Large numbers of TNF- $\alpha$ -producing cells (TNF- $\alpha$ -PC) were observed in the spleen, lungs and liver. In the spleen, they were found both in the red pulp and the periarterial lymphocyte sheets, areas which never showed RCMV antigen staining (Fig. 1 and 4). Lymphoid cells had considerably higher levels of

expression of TNF- $\alpha$  than other TNF- $\alpha$ -PC, e.g. macrophages. In the liver, TNF- $\alpha$ -PC were morphologically identified as Kupffer cells; in the lungs both macrophages and infiltrating lymphocytes were TNF- $\alpha$ -positive. Organs obtained at earlier time points (days 1 and 4 p.i.) showed less or no staining. At 1 day after irradiation, however, a few TNF- $\alpha$ -PC were observed in irradiated control rats. No TNF- $\alpha$  staining was observed in the kidneys, in sections treated with an irrelevant control antiserum as primary antibody or in irradiated control rats at days -1, 4 and 7 p.i. (data not shown).

#### Effect of RCMV on TNF- $\alpha$ expression in vitro

We next determined whether RCMV infection can induce TNF production in rat embryo fibroblasts, astrocytes and spleen macrophages (Table 1). Mock infection of REF cells and macrophages resulted in the release of low amounts of TNF; upon exposure to LPS all cell types secreted TNF. RCMV infection induced significant TNF secretion from REF cells and astrocytes. These cells were permissive for RCMV infection as confirmed by antigen expression and c.p.e. The TNF activity could be neutralized by a goat antiserum specific for TNF- $\alpha$ . In contrast, freshly isolated macrophages, which supported RCMV replication only to a limited extent (0.01% RCMV-positive cells), also failed to secrete increased amounts of TNF after RCMV infection. However, when the macrophages had been cultured for 5 days before infection ('aged macrophages'; Table 1), they supported RCMV replication which coincided with increased

Table 1. In vitro TNF production after RCMV infection\*

Cell type	Cell treatment				
	Mock	LPS	RCMV	RCMV+ $\alpha$ -TNF	RCMV antigen†
REF	20	2720	370	< 10	> 95
Astrocyte	< 10	1260	780	< 10	> 95
Macrophage	120	1850	125	NT‡	0.01
Aged macrophage	230	2250	820	< 10	40

\* TNF levels are expressed in pg/ml. The detection limit for TNF was 10 pg/ml. Supernatants of RCMV-infected cells were preincubated with goat anti-TNF- $\alpha$  antibodies (anti-TNF) and tested for residual TNF activity.

† Cells were fixed 24 h p.i. and stained with a MAbs against RCMV. Indicated is the percentage of cells expressing RCMV antigen/well.

‡ NT, Not tested.

release of TNF. These data suggest that replication of RCMV is required for TNF secretion.

#### RCMV specificity of TNF production

We further investigated the RCMV specificity of TNF- $\alpha$  secretion in astrocytes. Our glial cell cultures consisted of > 98% GFAP<sup>+</sup> astrocytes. RCMV c.p.e. became visible 48 h p.i., at which time infectious virus could be recovered from the supernatant. Double staining revealed that only GFAP<sup>+</sup> astrocytes were also RCMV-positive (data not shown). Immunostaining at 5 h p.i. using goat anti-TNF- $\alpha$  antibodies demonstrated the

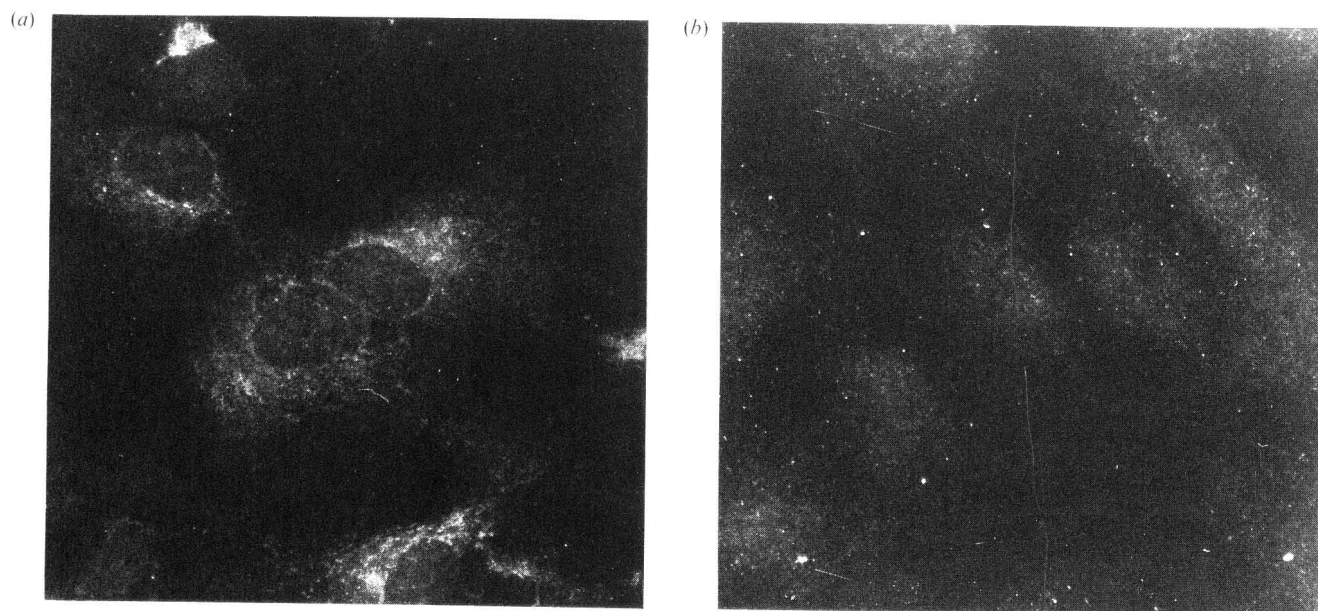


Fig. 5. Immunofluorescence staining of TNF- $\alpha$ -PC. Glial cell cultures were infected with RCMV (a) or mock-infected (b). At 5 h p.i. cultures were fixed and stained with anti-TNF antibodies. Bar marker represents 25  $\mu$ m.

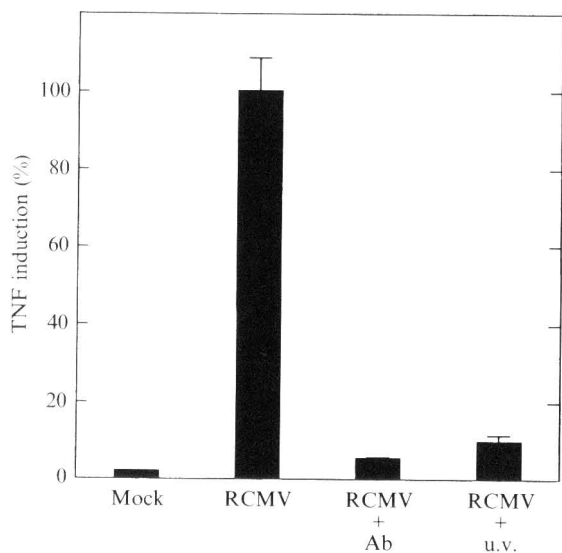


Fig. 6. RCMV-specific induction of TNF production. Astrocyte cultures were mock-treated (mock), RCMV-infected (RCMV), or inoculated with antibody-neutralized RCMV (RCMV + Ab) or u.v.-irradiated virus (RCMV + u.v.). TNF bioactivity was determined at 24 h p.i. 100% TNF induction corresponds to 240 pg/ml.

presence of cytoplasmic TNF- $\alpha$  (Fig. 5). The intensity of TNF staining was comparable to that found in LPS-stimulated cells (not shown). The RCMV specificity of TNF production was shown by incubation of the inoculum with a rat immune serum for 1 h prior to infection of astrocytes. This measure reduced RCMV infectivity by  $10^5$ -fold and TNF production by  $> 95\%$  (Fig. 6). Similar results were obtained with purified virus (not shown). We next determined whether interaction of the virus particle with its receptor is sufficient for TNF- $\alpha$  production or whether viral replication is required. U.v. irradiation reduced RCMV infectivity by  $10^4$ -fold and its TNF inducing capacity by 90% (Fig. 6), indicating that replication is required to induce TNF production in the target cell.

## Discussion

Evidence is accumulating that TNF is important in many pathophysiological conditions (Beutler & Cerami, 1987; Vassali, 1992). During septic shock LPS triggers the release of large amounts of TNF- $\alpha$ , which can be life-threatening (Beutler & Cerami, 1987). Over-expression of this cytokine has also been reported in parasitic infections (Grau *et al.*, 1987). *In vitro*, infection with either RNA or DNA viruses, such as Sendai virus, human immunodeficiency virus, and human CMV can trigger the production of TNF (Aderka *et al.*, 1986; Merrill *et al.*, 1989; Dudding *et al.*, 1989). However, little is known about its production and role during viral

infections *in vivo*. In RCMV-infected rats we identified the tissue and cellular sources of TNF and assessed their relative contribution to total TNF production; activity was found in various organs and in the serum. In contrast, in immunocompetent RCMV-infected rats, which showed minimal viral replication and no overt clinical signs, only transient TNF expression was noted (Haagmans *et al.*, 1994). Induction of TNF by adenovirus infection has been demonstrated at the mRNA level in mice (Ginsberg *et al.*, 1991). However, since TNF biosynthesis is largely controlled at the translational level (Han *et al.*, 1990), this may not reflect protein synthesis. Recently, increased serum TNF concentrations were observed in liver transplant patients suffering from CMV disease (Tilg *et al.*, 1991). Moreover, TNF- $\alpha$  mRNA was shown to be abundant in the colonic mucosa from AIDS patients with CMV colitis (Smith *et al.*, 1992).

Several cell types including macrophages, mast cells, T and B lymphocytes, natural killer cells, fibroblasts and astrocytes produce TNF (Vassali *et al.*, 1992). Immunohistochemical examination of frozen tissues from infected rats revealed that lymphoid and several types of virus-infected cells are TNF- $\alpha$ -PC. Moreover, in accordance with the capacity of irradiation to induce TNF- $\alpha$  (Hallahan *et al.*, 1989), we also found low levels of TNF- $\alpha$ -PC 1 day after irradiation. *In vitro* experiments revealed that fibroblasts and astrocytes were permissive for RCMV infection and produced TNF levels comparable with those observed after stimulation with LPS. In contrast, spleen macrophages, highly resistant to *in vitro* RCMV infection, also failed to produce TNF, which suggests that CMV permissiveness correlates with TNF synthesis. Indeed, our *in vitro* data indicate that RCMV replication is essential for TNF induction. Similarly, HCMV replication induces the expression of TNF- $\alpha$  mRNA (Dudding *et al.*, 1989).

TNF expression may explain some of the observed pathological features of CMV disease such as pneumonia, anaemia, thrombocytopenia and blood coagulation disorders. Recombinant TNF administered to rats also induces anaemia and activation of both the coagulation and fibrinolytic system (Tracey *et al.*, 1986). Pneumonia is often observed in fatal CMV disease and may be linked to the pathogenic action of TNF- $\alpha$ . Cytotoxic mediators produced by infiltrating T cells upon recognition of virus-infected cells in the lung have been postulated to cause CMV-associated interstitial pneumonitis (Grundy *et al.*, 1987). Protective effects on the other hand may be exerted by its antiviral activity, although they are rather selective and less potent than those of interferons (Wong & Goeddel, 1986). However, in conjunction with interferon- $\gamma$  it can exert synergistic antiviral activity (Schijns *et al.*, 1991; Wong & Goeddel, 1986).

In contrast to TNF, IL-1 and IL-6 production was not significantly elevated. Preliminary experiments indicate that IL-1 expression is down-regulated by the action of transforming growth factor- $\beta$  whereas IL-6 induction is directly inhibited by RCMV (Haagmans *et al.*, 1994). Down-regulation of IL-1 and IL-6 may explain some of the immunosuppressive phenomena observed in CMV infection. IL-1 and IL-6 both act as haemopoietin-1 on bone marrow cultures (Moore & Warren, 1987) and as co-mitogens for thymocytes and T cells (Dinarello, 1989). The molar concentration of TNF required to stimulate immunocompetent cells is much higher than that of IL-1.

Our observations may contribute to the understanding of CMV pathogenesis. The capacity of CMV to induce TNF may be of vital importance for AIDS patients, for example, for whom this virus constitutes the most important opportunistic viral pathogen; in addition, CMV-induced TNF is known to enhance HIV expression (Duh *et al.*, 1989; Macher *et al.*, 1983; Fiala *et al.*, 1986). Apart from cytolysis CMV may induce direct or indirect overexpression of TNF- $\alpha$  which may in turn contribute to pathology. Our recent data suggest that neutralization of TNF activity may represent a therapeutic tool to modulate CMV disease (Haagmans *et al.*, 1994).

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