Tumour necrosis factor-α production during cytomegalovirus infection in immunosuppressed rats

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The production of tumour necrosis factor (TNF)-α, 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-α 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-α expression in different types of cells grown in vitro. When frozen tissue sections were examined by immunohistology, TNF-α-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-α. Its abundance in RCMV-infected rats suggests an important role for TNF-α 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)-α, 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-α 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-α 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 CO2 incubator and used at the third passage.

Glial cell cultures were prepared as previously described (Koper et
al., 1984). Briefly, cerebella 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.

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% (v/v) homogenates of
salivary gland tissue taken from BN rats that had been inoculated
3 weeks previously with 10^4 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-purified (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 x 10^5 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 dilution. Virus preparations and culture media were found to
contain < 60 pg/ml of contaminating endotoxin as tested by the
COA-test (Chromomenix). RCMV was used u.v.-irradiated (10 min).

**TNF induction.** Cells of the different types were resuspended in
DMEM–FCS and plated at a concentration of 1 x 10^5/well into six-
well plates (Nunc). The plates were incubated until the cells had
reached confluence 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 (Chromomenix). RCMV was used u.v.-irradiated (10 min).

**IL-1 assay.** A murine T cell line clone (D10) was used to detect IL-1
bioactivity as described by Helle et al. (1988). Briefly, 10^6 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 [3H]thymidine (Amersham; sp.
act. 0.7 x 10^8 to 1.1 x 10^8 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 [3H]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, they were reacted with rabbit anti-goat antibodies
instead of the anti-TNF serum, or preincubated with rIL-1α( to
neutralize rat 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-diazobicyclo(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-
formaldehyde-lysine periodate and embedded in paraffin wax. Paraffin sections (5 μm) 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% H₂O₂ 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 μm, cut at −30°C) were fixed for 10 min in acetone containing 0.02% H₂O₂. Sections were examined for the presence of viral antigen and TNF-α 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-α 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% H₂O₂ 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-α were detected in the infected rats (Fig. 2). Pre-incubation of the sera with goat antibodies or a hamster MAb directed against murine TNF-α neutralized > 90% of the TNF bioactivity. Similar levels were found using a rat TNF-α-specific ELISA (Fig. 2). Radiation-immunosuppressed uninfected rats had < 20 pg TNF/ml in their serum. Thus, of the three cytokines tested, only TNF-α 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-α 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. 2. Detection of TNF activity levels in sera of individual RCMV-infected rats. Radiation-immunosuppressed rats were injected with PBS or infected with $1 \times 10^5$ 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-α-specific ELISA (open circles). The detection limit (broken line) for both assays was 20 pg/ml.](image)

![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 ± S.E.M.](image)
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-α levels were significantly raised in tissue homogenates. Therefore subsequent studies were focused on this cytokine.

Cellular localization of TNF-α production

The cellular source of TNF-α was identified by immunohistology. Large numbers of TNF-α-producing cells (TNF-α-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

Fig. 4. Immunohistochemical detection of TNF-α 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-α (a to c) and normal goat serum (d). Bar marker under (d) represents 50 μm for (a), (c) and (d); marker under (b) represents 100 μm.
expression of TNF-α than other TNF-α-PC, e.g. macrophages. In the liver, TNF-α-PC were morphologically identified as Kupffer cells; in the lungs both macrophages and infiltrating lymphocytes were TNF-α-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-α-PC were observed in irradiated control rats. No TNF-α 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-α 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-α. 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>
<thead>
<tr>
<th>Cell type</th>
<th>Mock</th>
<th>LPS</th>
<th>RCMV</th>
<th>RCMV + α-TNF</th>
<th>RCMV antigen†</th>
</tr>
</thead>
<tbody>
<tr>
<td>REF</td>
<td>20</td>
<td>2720</td>
<td>370</td>
<td>&lt; 10</td>
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<td>1260</td>
<td>780</td>
<td>&lt; 10</td>
<td>&gt; 95</td>
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<tr>
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<td>1850</td>
<td>125</td>
<td>NT‡</td>
<td>0.01</td>
</tr>
<tr>
<td>Aged macrophage</td>
<td>230</td>
<td>2250</td>
<td>820</td>
<td>&lt; 10</td>
<td>40</td>
</tr>
</tbody>
</table>

* 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-α antibodies (anti-TNF) and tested for residual TNF activity.
† Cells were fixed 24 h p.i. and stained with a MAb 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-α secretion in astrocytes. Our glial cell cultures consisted of > 98% GFAP⁺ 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⁺ astrocytes were also RCMV-positive (data not shown). Immunostaining at 5 h p.i. using goat anti-TNF-α antibodies demonstrated the

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Fig. 5. Immunofluorescence staining of TNF-α-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 μm.
is known about its production and role during viral infections. 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-α 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-α-PC. Moreover, in accordance with the capacity of irradiation to induce TNF-α (Hallahan et al., 1989), we also found low levels of TNF-α-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-α 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-α. 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-γ 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-β 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-α 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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References


MERRILL, J. E., KUYANAGI, Y. & CHEN, I. S. Y. (1989). Interleukin-1 and tumor necrosis factor α can be induced by mononuclear phagocytes by human immunodeficiency virus type 1 binding to the CD4 receptor. Journal of Virology 63, 4404–4409.


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