Participation of endogenous tumour necrosis factor α in host resistance to cytomegalovirus infection

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Interferon gamma (IFNγ) represents an essential cytokine involved in murine cytomegalovirus (MCMV) clearance from the salivary gland and the control of horizontal transmission. Because IFNγ cannot be responsible for all cytokine effects during recovery from MCMV infection we have now tested the potential participation of tumour necrosis factor alpha (TNFα) in the antiviral defence. Neutralization of endogenous TNFα abolished the antiviral activity of CD4 T cells in immunocompetent as well as in CD8 subset-deficient mice. These data suggest that the antiviral effect of the CD4 subset requires the presence of at least two cytokines, namely IFNγ and TNFα. Depletion of endogenous TNFα in adoptive cell transfer recipients diminished the antiviral function of CD8 T lymphocytes suggesting that TNFα also participates in CD8 T cell effector functions. Furthermore, endogenous cytokines were found to be required for survival after infection with lethal doses of MCMV, whereas immunotherapy with recombinant TNFα and IFNγ could not limit virus replication in vivo. The results suggest that, similar to IFNγ, TNFα is an integral part of the protective mechanisms involved in cytomegalovirus clearance.

Introduction

Recovery following a primary infection with cytomegalovirus (CMV) requires the function of T lymphocytes that limit the pathological manifestations and eliminate the cells supporting virus growth. However, it is commonly accepted that T lymphocytes limit virus replication not only by direct cytolytic activity against infected cells but also by producing various cytokines at the site of infection (for reviews, see Doherty et al., 1992 and Ramshaw et al., 1992). The medical interest in human CMV (HCMV) is due to its propensity to cause severe diseases in congenitally infected infants and immunocompromised patients (Meyers, 1984; Stagno et al., 1986). HCMV is the most frequent viral cause of death in patients with AIDS (Drew, 1988), in spite of the presence of functional virus-specific CD8 T lymphocytes (Walker et al., 1987). The inability of T lymphocytes in AIDS patients to control virus infection has been associated with the diminished capability of mononuclear cells in the production of cytokines, namely interleukin (IL)-2 (Kirkpatrick et al., 1985), tumour necrosis factor alpha (TNFα) (Ammann et al., 1987) and interferon gamma (IFNγ) (Murray et al., 1984). Thus, the role of cytokines in recovery from CMV infection under normal physiological conditions, as well as in various pathological conditions, needs to be addressed.

Our own studies with the mouse model for CMV infection concentrated on the characterization of the protective principles (for reviews, see Koszinowski et al., 1990, 1992), definition of major antigens (Reddehase & Koszinowski, 1989), identification of antigenic peptides presented by the major histocompatibility complex (Reddehase et al., 1989), principles of variable antigen presentation during viral replication (Del Val et al., 1989) and the construction of experimental recombinant vaccines (Volkmer et al., 1987; Jonjić et al., 1988; Del Val et al., 1991a, b). It has been shown that CD8 T lymphocytes are the major protective principle involved in murine CMV (MCMV) clearance (Reddehase et al., 1985, 1987). In otherwise healthy hosts, CD8 T cells do not even require the presence of CD4 helper T lymphocytes for virus elimination (Reddehase et al., 1988). The only exception is the salivary glands, where MCMV clearance requires cooperation with the CD4 subset (Jonjić et al., 1989). However, under specific experimental circumstances, such as depletion of the CD8 T cell subset, CD4 T lymphocytes fully compensate for the CD8 deficiency. Mice retaining the CD4 T cell subset can therefore clear CMV in all tissues including the salivary glands with clearance kinetics similar to those of normal mice (Jonjić et al., 1990).

Although essential for this compensatory antiviral activity, CD4 T lymphocytes are not protective on their
own. These findings strongly point to the role of cytokines in the control of CMV infection. Experiments using in vivo neutralization of IFNγ provided evidence for the role of this cytokine in T lymphocyte-mediated virus clearance (Lučin et al., 1992). However, its direct anti-CMV effect is questionable, since the effect requires high concentrations of the cytokine in vitro. In the present study we provide evidence that TNFα produced endogenously during MCMV infection also can play an important role in restricting CMV replication.

**Methods**

**Mice.** Six- to 8-week-old BALB/c mice were obtained from our breeding colony at the Faculty of Medicine, University of Rijeka. They were bred and housed under barrier conditions and were specific pathogen-free.

**Virus, virus titration and infection conditions.** Sucrose gradient-purified mouse embryo fibroblast culture-propagated MCMV (Smith strain, code VR-194, ATCC) was used. The infective virus in tissues was quantified by a plaque assay (Reddehase et al., 1985). The detection limit was 100 p.f.u. of MCMV per organ homogenate. Virus titres (x and y) were regarded as significantly different for P (x versus y) < 0.05 (one-sided), where P is the observed probability value and z is a selected significance level (Wilcoxon-Mann-Whitney exact detection limit was 100 p.f.u, of MCMV per organ homogenate. Virus titres (x and y) were regarded as significantly different for P (x versus y) < 0.05 (one-sided), where P is the observed probability value and z is a selected significance level (Wilcoxon-Mann-Whitney exact

**Reagents and antibodies.** Recombinant (r) murine TNFα and rIFNγ were generously provided by G. R. Adolf, Bender Vienna, Vienna, Austria. The specific activity of rIFNγ stock (lot no. H3. RD48) was 107 units (U)/mg, as determined by the L cell encephalomyocarditis virus bioassay. Specific activity of rTNFα stock was 5 x 107 U/mg, as determined by cytotoxic assay on L929 cells. Monoclonal antibodies (MAbs) and antisera included YTS 191.1.2 (rat IgG2b) which recognize the murine CD8 antigen, YTS 169.4.2 (rat IgG2b) which is specific for murine CD8 antigen (Cobbeld et al., 1984), R4.6A2 (rat IgGl) which neutralizes murine IFNγ (Spatzalny & Havell, 1984), and 11B11 (rat IgGl) which neutralizes murine IL-4 (Ohara & Paul, 1985). MAbs were partially purified from ascitic fluid by ammonium sulphate precipitation followed by dialysis in PBS (pH 7.6) and stored at −30 °C.

Natural mouse TNF was produced according to the procedure described by Silva & Faccioli (1992). In brief, peritoneal cells (5 x 106) were stimulated with lipopolysaccharide (10 μg/ml) overnight and supernatant was tested in the L929 cell bioassay.

The anti-TNFα antibody used was polyclonal rabbit antiserum raised against pure rTNFα and was capable of neutralizing both natural and recombinant TNFα (Fig. 1). The antiserum was produced according to the protocol described by Nauciel & Espinasse-Maes (1992). Rabbits were immunized by subcutaneous injections of 10 μg of TNFα in complete Freund’s adjuvant. Two weeks later animals were boosted with the same amount of TNFα in incomplete Freund’s adjuvant and again, with 5 μg rTNFα in saline, 3 days before bleeding. The antiserum was extensively dialysed against PBS and stored at −30 °C before use.

**L929 TNF bioassay.** The assay was carried out as described elsewhere (Wang et al., 1985). Neutralization of rTNFα with various anti-TNFα serum dilutions was performed at 4 °C over 1 h. After the neutralization, 100 μl of each sample was added to L929 cells. The test was done in the presence of 4 μg/ml actinomycin D. After overnight incubation at 37 °C with 5% CO2 in air, TNF-mediated cytotoxic effects on L929 cells were visualized by crystal violet staining. The neutralizing capacity of anti-TNFα serum was defined as the reciprocal value of the highest serum dilution that, when reacted with an equal volume of test sample containing 1 ng/ml rTNFα, neutralized 50% or more of the cytotoxic activity. According to this assay 1 ml of anti-TNFα serum was able to neutralize approx. 64 μg of rTNFα (3 x 106 U).

**In vivo treatment of mice.** In vivo depletion of CD4+ and CD8+ T lymphocyte subsets was carried out as described previously (Jonjic et al., 1989, 1990). Immunocompetent mice, either euthymic or thymectomized as well as adoptive transfer recipients, were injected intraperitoneally with 1 mg of anti-CD4 and anti-CD8 MAbs every fourth day, starting 1 h after infection and cell transfer. For in vivo neutralization of IFNγ and IL-4, mice were injected with 200 μg of R4.6A2 and 11B11 MAbs, respectively, every other day starting 1 h after infection. If not stated otherwise, the neutralization of endogenous TNFα was accomplished by intravenous injection of 100 μl of rabbit anti-TNFα serum and this treatment was repeated every other day for 2 weeks. A control group of mice received the same amount of normal rabbit serum.

The prophylactic adoptive cell transfer was performed as described previously (Reddehase et al., 1985). In brief, recipient mice were immunodepleted by total-body γ-irradiation, with 6 Gy delivered as a single dose. Spleen cells from donor mice were infused into the tail vein 2 h after irradiation and infection. Virus titres in tissues of recipients were determined 2 weeks later.

**Results**

**Failure of immunotherapy with exogenous TNFα alone or combined with IFNγ to limit MCMV replication in vivo**

It has been shown that rIFNγ on its own has only a moderate direct antiviral effect in vitro and when given in vivo fails to prevent MCMV replication in immuno-
therapy with rTNFα and rIFNγ could not limit virus
replication in vivo and could not enhance the antiviral
effect of transferred lymphocytes. Compatible results
were obtained in three independent experiments, one of
which is shown.

In addition, we tested whether rTNFα can limit virus
replication in salivary glands of CD4 subset-depleted
mice persistently infected with MCMV. Two weeks after
MCMV infection and CD4 subset depletion, when
persistent MCMV infection in salivary glands was
established, mice were treated with rTNFα or with
rIFNγ for 2 weeks, after which virus titres in salivary
glands were determined. As shown in Table 1(d),
treatment with rTNFα had no effect on virus titres in
salivary glands. Therapy, with rIFNγ was also ineffective,
in agreement with previously published data (Lućin et

**Evidence for the role of endogenous TNFα in limiting
MCMV replication in vivo**

Attempts to treat viral infections by the administration
of recombinant cytokines have been largely unsuccessful
(Klavinskis et al., 1989; Soike et al., 1989), probably
because of the short half-life of recombinant cytokines
in vivo and the need for their localization at the sites of viral
infection. Therefore, the inability of rTNFα to mount
anti-MCMV activity and to synergize with rIFNγ in vivo
does not rule out the possible physiological functions of
this cytokine. To determine whether TNFα is involved in
the response to MCMV, endogenous TNFα was neutral-
ized in vivo. Groups of normal immunocompetent
BALB/c mice were injected either with rabbit antiserum
against mouse TNFα or, as a control, with normal rabbit
serum (NRS). Antibody treatment was repeated every
other day for 2 weeks. The effect of TNFα neutralization
was compared with the effect of deletion of the CD4 or
the CD8 T cell subset. Two weeks after infection the
virus titres in organs were determined. The results shown
in Fig. 2 reveal that the neutralization of endogenous
TNFα in immunocompetent mice resulted in higher virus
yield from salivary glands in comparison with controls
and the group of mice that were depleted of CD8 T
lymphocytes. As expected from previous work (Jonjić et
al., 1989), depletion of the CD4 subset resulted in high
virus titres in salivary glands. Remarkably, and similar
to previously published data for IFNγ depletion (Lućin
et al., 1992), neutralization of endogenous TNFα in
otherwise immunocompetent animals did not have a
significant effect upon virus clearance in tissues other
than the salivary glands (data not shown).

The failure of TNFα neutralization to compromise
virus clearance in other tissues could be due to the
amounts of antibody being insufficient to neutralize all
endogenous TNFα. To investigate this possibility a
Fig. 2. Effect of TNFα neutralization on the course of MCMV infection in the salivary gland. BALB/c mice were compared with respect to virus production in the salivary glands 2 weeks post-infection and treatment with antibodies to TNFα (immune rabbit serum), CD4 or CD8 according to schedule described in Methods. Control group received NRS. Titres of individual mice (●) and median values (—) are shown. There is a significant difference in virus titres of anti-TNFα serum-treated and CD4-depleted mice in comparison with control group (P < 0.008 and P < 0.001, respectively).

Fig. 3. Kinetics of TNFα neutralizing antibody titre after transfer. MCMV-primed mice received a single dose (100 μl) of rabbit anti-TNFα serum or NRS. Mice were bled at 0 h, and 1, 2 and 3 days after injection, and their pooled sera were tested for TNFα neutralizing capacity. The neutralizing capacity of sera was defined as the reciprocal value of the highest serum dilution that, when reacted with an equal volume of test sample containing 1 ng/ml TNFα, neutralized 50% of the cytotoxic activity in the L929 cell bioassay. Results represent the mean value of four replicate cultures and the bars represent the S.E.M.

was due to the administration of insufficient amounts of neutralizing antibodies.

TNFα is an integral part of CD4 T lymphocyte function

The fact that TNFα neutralization abolishes or delays MCMV clearance only in the salivary glands of the immunocompetent host suggests that TNFα may be primarily involved in CD4 subset-dependent antiviral functions, which only in this organ cannot be compensated by CD8 T lymphocytes. According to the distinct cytokine pattern that they secrete, CD4 T cells can be functionally subdivided into T helper type 1 (TH1) and TH2 subsets (Mosmann & Moore, 1991). We have shown previously that the antiviral effectors belong to the TH1 subset because neutralization of endogenous IFNγ prevents CD4-dependent antiviral function in salivary glands (Lučin et al., 1992). Therefore, the effect of TNFα neutralization on virus clearance in animals that rely entirely on CD4 subset could affect the virus clearance in tissues other than the salivary glands. To test
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Antibody treatment

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<th>NRS</th>
<th>αCD4</th>
<th>αTNFα</th>
<th>αL-4</th>
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Lungs

Salivary glands

Spleen

Virus titre (log_{10} p.f.u.)

DL, Detection limit. There is a significant difference in virus titres between mice treated with anti-TNFα serum and the control serum for lungs (P < 0.008) and salivary glands (P < 0.005).

this, CD8-depleted mice were used as a model for the assessment of CD4 T cell functions. The mice were MCMV-infected and treated with neutralizing antibodies to TNFα and IL-4. As expected from the lack of function of CD8 lymphocytes in salivary glands, CD8 subset depletion did not potentiate the effect of TNFα neutralization in this organ (Fig. 4). The situation was different, however, when virus titres were studied in the lungs. In contrast to the effect of TNFα neutralization in immunocompetent mice, lungs of CD8-depleted mice treated with anti-TNFα contained significantly more virus. This indicates that, in the absence of CD8 T lymphocytes, TNFα is an essential cytokine for virus clearance in the lungs, whereas virus control in the spleen was affected only after depletion of the CD4 subset. These results suggest that TNFα plays an important role in CD4 subset-dependent antiviral function. In contrast with the effect of anti-TNFα serum, neutralization of IL-4 did not show any influence on MCMV replication, confirming the predominant role of the TH1 subset in MCMV clearance.

Requirement of TNFα for antiviral activity of MCMV-primed splenocytes

In addition, the involvement of endogenous TNFα in the antiviral function of MCMV-primed splenocytes was also tested in an adoptive cell transfer system (Fig. 5). Cell donors were fully immunocompetent mice, either MCMV-primed or non-primed. Irradiated and MCMV-infected cell transfer recipients received, on the day of cell transfer, either control serum or antibodies to TNFα, to IFNγ, to IL-4 or to CD8. With the exception of anti-CD8 MAb that was injected every 4 days, other antibodies were injected every other day for 2 weeks until the mice were killed and virus titres in tissues were
are not only marginally involved in virus clearance but the results presented in Table 2 show that both cytokines are essential for resistance to a lethal dose of MCMV.

The effect of the neutralization of endogenous TNFα and IFNγ on survival of mice after infection with salivary gland isolate of MCMV

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Anti-IFNγ</th>
<th>Anti-TNFα</th>
<th>Survivors/total number of mice</th>
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<tr>
<td></td>
<td>− −</td>
<td>10/20 (50%)</td>
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<td>− +</td>
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<td>1/8 (12%)</td>
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<td>− −</td>
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<td>2/8 (25%)</td>
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<td>− −</td>
<td>+ +</td>
<td>0/8 (0%)</td>
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* Neutralization of endogenous TNFα and IFNγ was accomplished by in vivo administration of MAb R4.6A2 and rabbit anti-TNFα polyclonal serum, respectively, at the day of infection with 1 LD50 (1 × 10⁵) of salivary gland isolate of MCMV. Antibody treatment was repeated 48 h after infection. The percentage of survivors is shown in parenthesis.

Depletion of a single cytokine reduced the number of survivors after lethal infection, whereas neutralization of both resulted in a fatal outcome.

**Discussion**

Although it is generally accepted that the resistance to many viral infections relies on the activation of T cells, the final effector mechanisms by which infected cells are eliminated and virus replication is inhibited are not understood in detail. Both CD4 and CD8 T cells respond to primary MCMV infection in immunocompetent mice, but the antiviral protective activity can usually be transferred only by CD8 T lymphocytes (Reddehase et al., 1985, 1987, 1988). However, the CD4 subset can compensate for a complete lack of CD8 T lymphocytes. CD8-depleted mice recover from acute MCMV infection and the virus establishes latency (Jonjic et al., 1990). This points to a hierarchical role of effector functions and demonstrates at the same time the flexibility of the immune system.

Since CD4 T cells are not protective by themselves, even if derived from the CD8-depleted host, it is reasonable to postulate that CD4-dependent effector functions are, at least in part, cytokine-mediated. Several cytokines show antiviral effects in general, but there is no doubt that a central role is played by IFNγ, a factor that has been implicated in virtually every viral infection (Quinnin & Manischewitz, 1987; Leist et al., 1989; Karupiah et al., 1990; Kohonen-Corish et al., 1990). We have recently demonstrated that IFNγ is a key factor for MCMV clearance from salivary glands and, therefore, for the prevention of horizontal spread of CMV (Lučin et al., 1992). However, our studies also indicated that IFNγ is not the only cytokine involved in virus clearance from salivary glands. The search for such a cytokine focused on TNFα, which has been implicated in a number of parasitic (Havell, 1987; Titus et al., 1989; Chen et al., 1992; Johnson, 1992; Silva & Faccioli, 1992), viral (Rossol-Voth et al., 1991; Sambhi et al., 1991) and bacterial infections (Nauciel & Espinase-Maes, 1992). TNFα is a pluripotent cytokine with a variety of biological properties and is produced by many cell types, but mostly by activated macrophages (Larrick & Wright, 1990).

The present report provides evidence suggesting that TNFα contributes to the recovery of mice from primary infection with MCMV and also has a role in antiviral clearance mediated by primed T lymphocytes. Similar to the effect of the CD4 subset depletion or of IFNγ neutralization, the neutralization of TNFα abolished or delayed virus clearance from salivary glands. Further, in CD8-depleted mice TNFα appeared to be critical for virus clearance not only in salivary glands but also in...
other organs, such as lungs, indicating that TNFα contributes mainly to the CD4 subset-dependent antiviral effector mechanism. The negative effect of IL-4 neutralization suggests that this mechanism is dependent on the TH1 subset.

Our results do not exclude a role of TNFα in the CD8 subset-mediated control of MCMV infection since anti-TNFα treatment affected the protective capacity of adoptively transferred cells, which is predominantly mediated by CD8 T lymphocytes. Nevertheless, it appears that the effect of anti-TNFα treatment has a less dramatic consequence on functions that rely on the CD8 subset. The present results have also shown that depletion of TNFα even alters the survival rate of mice infected with 1 LD₅₀ of MCMV. Since this early host defence against a high virus dose requires the active participation of natural killer cells and macrophages (Bukowski et al., 1985), both TNFα and IFNγ probably play an important role in the T cell-independent arm of the antiviral response. However, it should be noted that, owing to the technical conditions of repeated antibody injection, a short half-life of antibody and the uncertainty considering the distribution of the antibodies in the tissues, our data probably underestimate the role of local TNFα in anti-CMV defence.

The neutralization of endogenous TNFα indicated its important physiological role in the enhancement of host resistance to herpesviruses. The nature of the mechanisms involved is not clear. TNFα may enhance host resistance against various intracellular parasites and viruses by modulating a cascade of specific and non-specific defence mechanisms. Such mechanisms include the local recruitment of inflammatory cells, triggering microbicidal action by activating macrophages to release reactive oxygen metabolites, regulation of production of other cytokines and the expression of their receptors, promotion of proliferation and differentiation of B lymphocytes, and augmentation of humoral immunity (for review, see Titus et al., 1991).

Endogenously produced TNFα has been shown to be an important mediator in infection by vaccinia virus. TNFα expressed by recombinant virus demonstrated that localized production of this cytokine during viral infection leads to the rapid and efficient clearance of vaccinia virus in normal and in immunodeficient mice suggesting that, once provided in sufficient concentration, TNFα can act antivirally in a T cell-independent manner (Sambhi et al., 1991). In certain tumour systems, however, the protective activity of TNFα has been found to rely on T cell-dependent mechanisms (Asher et al., 1991). This is illustrated by the finding that the ability of tumour cells to regress in vivo after transfection with the TNFα gene can be prevented by depletion of the CD4 or the CD8 subset. Another possibility is that anti-TNFα serum exhibits its effect by acting directly on CD4 cells that display TNFα on their surface. It has been shown that membrane-associated TNFα is involved in the activation of anti-leishmanial defence (Sypek & Wyler, 1991), and that it may be a mechanism of targeting activation signals to macrophages in an antigen-specific and genetically restricted manner. Given the fact that TNFα is synthesized by many cell types and is involved in so many defence strategies, the question arises whether, for the given infection, major and typical producers of the cytokine can be identified. This is entirely open for CMV and further studies must show whether the inhomogeneous organ distribution of cells related to the monocyte and macrophage lineage could offer an explanation for the variable organ manifestations of HCMV disease.

In the present study we could not demonstrate protective effects of rTNFα alone nor synergistic effects in combination with rIFNγ. This is not entirely unexpected. Although different recombinant cytokines are active in vitro, studies in vivo have been hampered either because of short half-life or difficulties encountered in targeting the molecules to the site of infection or immune reaction (Kohonen-Corish et al., 1990; Sambhi et al., 1991; Ramshaw et al., 1992). Our studies confirmed that exogenous TNFα has a very short half-life in vivo: as early as 2 h after intravenous injection of 400 ng of rTNFα no residual activity in the serum of treated mice could be demonstrated by the cytotoxic assay on L929 cells (B. Polić, unpublished data).

In conclusion, our studies in a model system for CMV disease are the first to demonstrate that endogenous TNFα is required in vivo for the efficacious control of CMV infection. Neutralization of endogenously produced TNFα affects the protective capacity of MCMV-primed adoptively transferred lymphocytes and prevents the clearance of virus from salivary glands of acutely infected immunocompetent mice. In addition, during CD4 subset control of virus clearance anti-TNFα treatment compromises virus clearance not only in salivary glands but also in the lungs. To assess the potential role of cytokines during the different clinical manifestations of human CMV disease further studies are required to define the conditions under which the CD4 subset control gains importance.

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References

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