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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 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.*, 1991*a*, *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) $< \alpha = 0{\cdot}05$ (one-sided), where P is the observed probability value and α is a selected significance level (Wilcoxon-Mann-Whitney exact rank sum test). Mice were infected by injection of 2×10^5 p.f.u. of MCMV into a hind footpad. The third passage of salivary gland isolate of MCMV was prepared and used in challenge experiments, according to the protocol described elsewhere (Jonjić *et al.*, 1988).

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 10^7 units (U)/mg, as determined by the L cell encephalomyocarditis virus bioassay. Specific activity of rTNFα stock was 5×10^7 U/mg, as determined by cytotoxic assay on L929 cells.

Monoclonal antibodies (MAbs) and antisera included YTS 191.1.2 (rat IgG2b) which recognizes the murine CD4 antigen, YTS 169.4.2 (rat IgG2b) which is specific for murine CD8 antigen (Cobbold *et al.*, 1984), R4.6A2 (rat IgG1) which neutralizes murine IFNγ (Spitalny & Havell, 1984), and 11B11 (rat IgG1) 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.

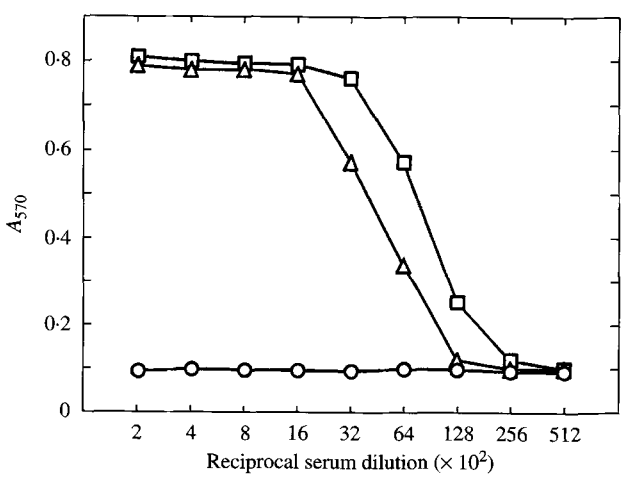

Fig. 1. Specificity and neutralizing capacity of polyclonal rabbit anti-TNFα serum. The ability of the rabbit antiserum to neutralize 50 U/ml of murine natural (□) or recombinant (△) and human recombinant (○) TNF was tested in an L929 cell bioassay. Results represent the mean value of triplicate cultures.

Natural mouse TNF was produced according to the procedure described by Silva & Faccioli (1992). In brief, peritoneal cells (5×10^6) 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 boostered 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% CO_2 in air, TNF-mediated cytopathic 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. 6·4 μg of rTNFα ($3{\cdot}2 \times 10^5$ U).

In vivo *treatment of mice. In vivo* depletion of $CD4^+$ and $CD8^+$ T lymphocyte subsets was carried out as described previously (Jonjić *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 MAb 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-TNF 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-

Table 1. *Therapy with rTNFα either alone or in combination with rIFNγ cannot replace antiviral function of T cells* in vivo

Protocol*	Treatment	Range of virus titres in salivary glands (log_{10} p.f.u.)†
(*a*)	Control‡	6·1–6·8
	rTNFα	6·2–6·7
	rIFNγ	6·1–6·3
	rTNFα + rIFNγ	6·4–6·9
(*b*)	Control	6·0–6·5
	rTNFα	6·0–6·8
	rIFNγ	6·1–6·5
	rTNFα + rIFNγ	6·2–6·4
(*c*)	Control	5·5–6·0
	rTNFα	5·3–6·1
	rIFNγ	5·2–5·6
	rTNFα + rIFNγ	ND§
(*d*)	Control	5·6–5·9
	rTNFα	5·7–6·9
	rIFNγ	5·5–6·1
	rTNFα + rIFNγ	ND

*(*a*) Irradiated (6 Gy) and MCMV-infected BALB/c mice were injected either with 400 ng of rTNFα, 2 μg of rIFNγ or a combination of both cytokines, every other day for 2 weeks. (*b*) Irradiated (6 Gy) and MCMV-infected mice were transferred with $2·5 \times 10^5$ non-primed spleen cells and treated with recombinant cytokines as above. (*c*) Irradiated (6 Gy) and MCMV-infected mice were transferred with $2·5 \times 10^5$ MCMV-primed spleen cells and treated with recombinant cytokines as above. (*d*) Thymectomized mice were depleted of CD4 T lymphocytes and infected with MCMV. Treatment with cytokines was initiated after 2 weeks as above.

† Six mice were used per group. The data represent the range of titres found in individual mice.

‡ The control groups of mice were injected with PBS alone.

§ ND, Not determined.

deficient mice (Lučin *et al.*, 1992). To investigate the effect of TNFα and its possible synergy with IFNγ *in vivo*, mice were immunodepleted by 6 Gy total-body γ-irradiation and infected with MCMV. Following this treatment animals were transferred either with normal syngeneic lymphocytes or with immune spleen cells derived from mice latently infected with MCMV, or were left untransferred. Each group was divided into subgroups that were treated with recombinant cytokines as indicated in Table 1. Treatment with cytokines was repeated every other day for 2 weeks when the mice were sacrificed and virus titres in tissues were determined. Neither rTNFα alone, nor in combination with rIFNγ, had a significant effect on virus spread *in vivo*, whether given to nontransferred immunodepleted mice (Table 1*a*) or to mice transferred with nonprimed lymphocytes (Table 1*b*). In accordance with our previous findings for rIFNγ, rTNFα also failed to enhance antiviral capacity of MCMV-primed spleen cells transferred to immunodepleted recipients (Table 1*c*). Taken together, immunotherapy 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 (Lučin *et al.*, 1992).

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 neutralized *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 depletion 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 (Lučin *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

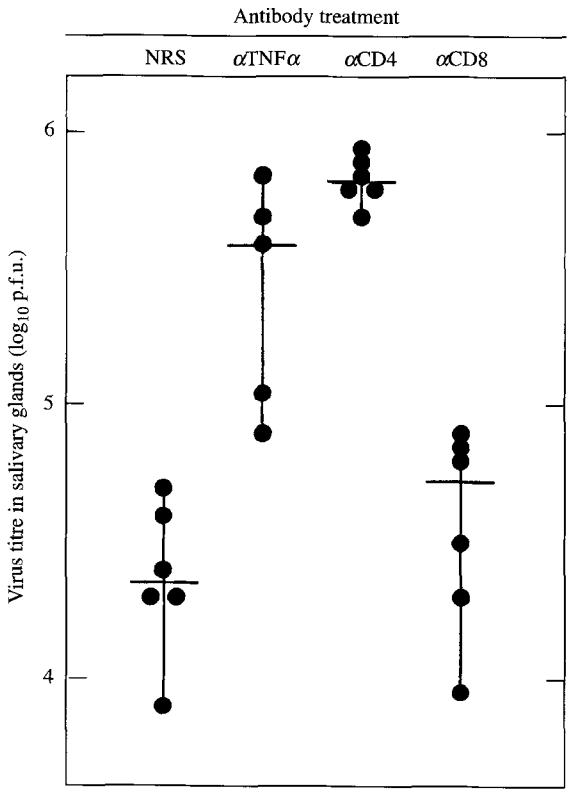

Fig. 2. Effect of TNFα neutralization on the course of MCMV infection in the salivary gland. BALB/c mice were compared with respect to virus production in the salivary glands 2 weeks post-infection and treatment with antibodies to TNFα (immune rabbit serum), CD4 or CD8 according to schedule described 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 ($P < 0{\cdot}008$ and $P < 0{\cdot}001$, respectively).

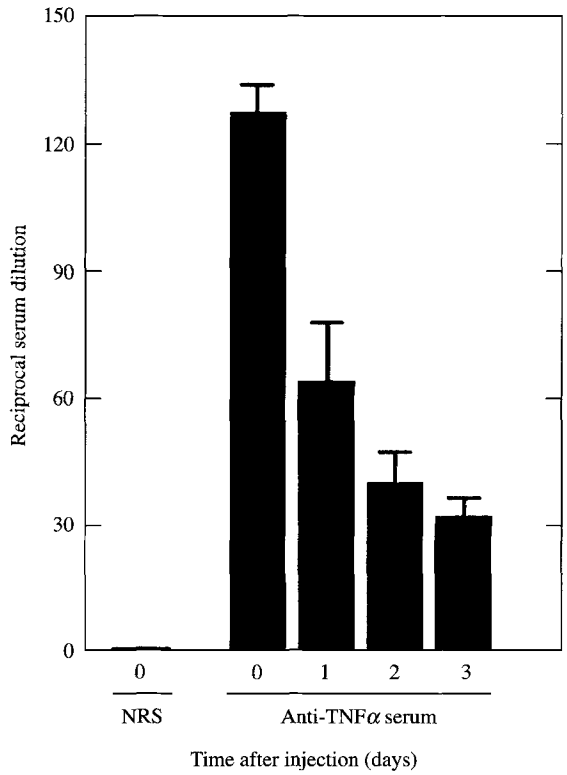

Fig. 3. Kinetics of TNFα neutralizing antibody titre after transfer. MCMV-primed mice received a single dose (100 μl) of rabbit anti-TNFα serum or NRS. Mice were bled at 2 h, and 1, 2 and 3 days after injection, and their pooled sera were tested for TNFα neutralizing capacity. The neutralizing capacity 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.

group of mice was given anti-TNFα serum and clearance kinetics of the antibodies were determined. MCMV-infected mice were injected with 100 μl of rabbit anti-TNFα serum, or NRS in controls, and were bled 0, 1, 2 and 3 days after injection. The neutralizing titres of antibodies persisting in mouse sera were determined using the L929 TNF bioassay. As expected, the excess of TNFα neutralizing titres in the sera of the injected mice declined with time after injection but levels were still significant 3 days after treatment (Fig. 3). This result confirmed that anti-TNFα serum used in the experiments was appropriate for prolonged neutralization of TNFα *in vivo*. Note that in all our experiments the *in vivo* administration of 100 μl of anti-TNFα serum was performed every other day during the course of the experiment. Anti-TNFα activity was absent from the sera of mice treated with NRS. It therefore appeared unlikely that the failure of anti-TNFα treatment to affect virus replication in tissues other than the salivary glands 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

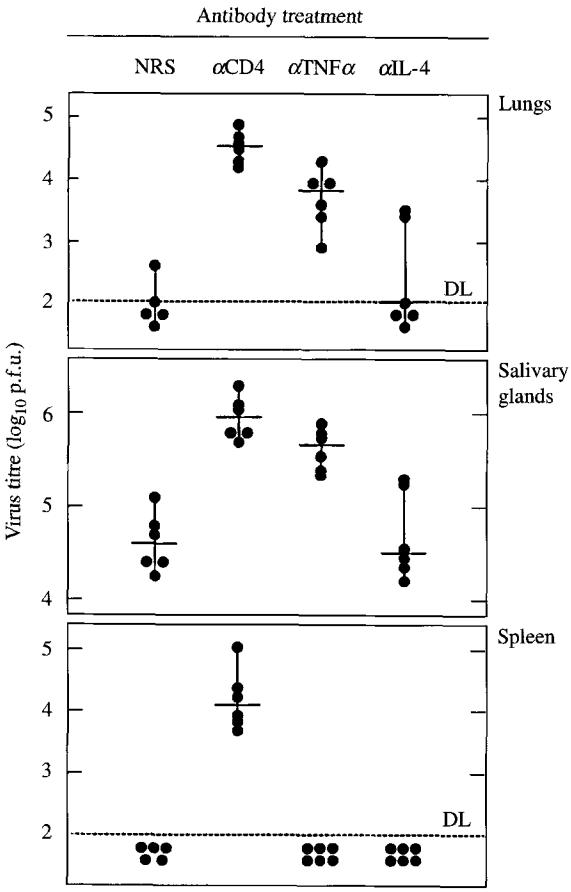

Fig. 4. Effect of TNFα neutralization on the course of MCMV infection in CD8 subset-depleted mice. CD8-depleted mice were treated with antibodies to TNFα (immune rabbit serum), IL-4 or CD4. The control group received NRS. Two weeks after MCMV infection mice were compared with respect to virus production in salivary glands, lungs and spleen. Virus titres in individual mice (●) and median values (—) are shown. DL, Detection limit. There is a significant difference in virus titres between mice treated with anti-TFNα serum and the control serum for lungs ($P < 0{\cdot}008$) and salivary glands ($P < 0{\cdot}005$).

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

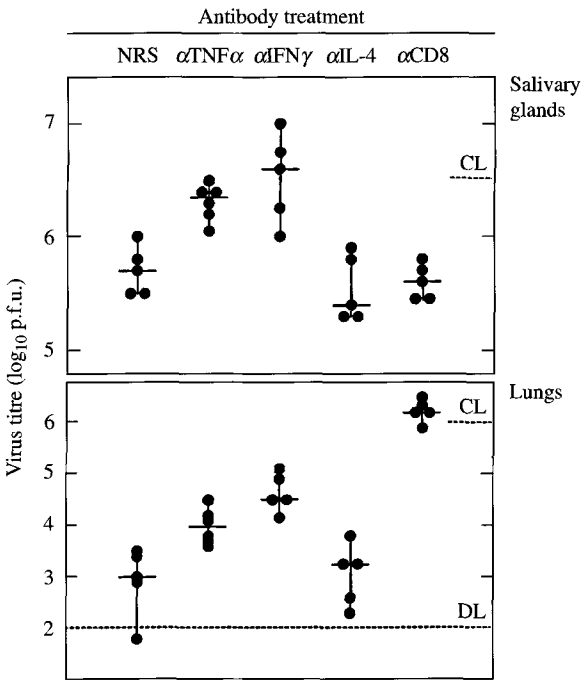

Fig. 5. Participation of endogenous TNFα in the antiviral activity of adoptively transferred lymphocytes. Splenocytes (2×10^5) obtained from immunocompetent MCMV-primed donors were transferred into γ-irradiated (6 Gy) and MCMV-infected syngeneic recipient mice. Recipient mice were divided into four subgroups receiving either NRS, rabbit anti-TNFα serum, MAb to IFNγ, IL-4 and CD8. Virus titres in individual recipient organs measured 2 weeks after adoptive transfer and infection (●) and median values (—) are shown. CL, (control level) represents the effect of cells obtained from non-primed control donors; DL, detection limit. There are significant differences between virus titres of mice treated with anti-TNFα serum and control serum in lungs ($P < 0{\cdot}01$) and salivary glands ($P < 0{\cdot}008$). Virus titres in lungs of anti-CD8-treated mice differed significantly ($P < 0{\cdot}001$) from virus titres in the control group.

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

Table 2. *Effect of TNFα and IFNγ neutralization on survival of mice after infection with salivary gland isolate of MCMV*

Treatment*		Survivors/ total number of mice
Anti-IFNγ	Anti-TNFα	
−	−	10/20 (50%)
+	−	1/8 (12%)
−	+	2/8 (25%)
+	+	0/8 (0%)

* 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 LD_{50} (1×10^5) of salivary gland isolate of MCMV. Antibody treatment was repeated 48 h after infection. The percentage of survivors is shown in parenthesis.

determined. As expected, depletion of IFNγ compromised the virus clearance from the salivary glands. A similar effect was seen after neutralization of TNFα but not after neutralization of IL-4 or depletion of the CD8 subset. The situation was different, however, when the lung virus titres were compared. As expected from previous studies (Lučin *et al.*, 1992), neutralization of endogenous IFNγ did affect but did not abolish the protective effect of transferred cells. Essentially the same effect was seen in the group of mice treated with anti-TNFα serum. However, depletion of CD8 T lymphocytes abolished the protection, yielding high virus titres in lungs. Since virus clearance from the lungs is predominantly the function of CD8 T lymphocytes this experiment confirmed that TNFα, as well as IFNγ, is less important for the control of MCMV infection in the lungs than CD8 subset depletion, but that TNFα represents an integral part of CD8 subset-mediated effector function. Remarkably, TNFα neutralization had no detectable effect in the spleen (data not shown) but this is not too surprising because the spleen is the organ that controls MCMV infection most thoroughly (Reddehase *et al.*, 1985). Thus, effector mechanisms in the spleen are obviously not affected upon neutralization of only a single cytokine.

Cytokine requirement for survival after infection with a lethal dose of MCMV

The effect of the neutralization of endogenous TNFα and IFNγ on the survival of mice infected with a high dose of MCMV was tested. Normal BALB/c mice were infected with 1×10^5 p.f.u. (1 LD_{50}) of a salivary gland isolate of MCMV (Jonjić *et al.*, 1988) and treated with neutralizing antibodies to TNFα, to IFNγ, or a combination of both. The results presented in Table 2 show that both cytokines are not only marginally involved in virus clearance but are essential for resistance to a lethal dose of MCMV. 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 (Jonjić *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 (Quinnan & 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 LD_{50} 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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