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Late phase inhibition of murine cytomegalovirus replication by synergistic action of interferon-gamma and tumour necrosis factor

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We have shown previously that the antiviral function of $CD4^+$ T lymphocytes against murine cytomegalovirus (MCMV) is associated with the release of interferon-γ (IFN-γ). We now demonstrate that IFN-γ and tumour necrosis factor alpha (TNF-α) display synergism in their antiviral activity. As little as 2 ng/ml of IFN-γ and TNF-α reduced the virus yield by about three orders of magnitude. There was no effect on immediate early (IE) and early (E) gene expression as far as the candidate genes IE1, E1 and those encoding the major DNA-binding protein and the DNA polymerase were concerned. Late gene transcription, assayed by the candidate genes encoding glycoprotein B and the MCMV homologue of ICP 18·5, was blocked and MCMV DNA replication was found to be reduced but not halted. The most prominent finding of the cytokine effect, seen by electron microscopy, was an alteration of nucleocapsid formation. Altogether, the synergism is multifaceted and acts at more than one stage during viral morphogenesis. Because the cytokines clearly do not act at an early stage of infection we conclude that the mode of cytokine activity differs between alpha- and betaherpesviruses.

Introduction

Cytomegalovirus (CMV), a member of the herpesviridae family, establishes both acute and chronic infections. Infection threatens the immunocompromised rather than the immunocompetent host. Studies on infection of mice with murine CMV (MCMV), a model for human CMV disease, have shown that natural killer cells provide resistance to infection with high doses of virus (Welsh *et al.*, 1991), whereas T lymphocytes are required for virus clearance and survival (reviewed by Koszinowski *et al.*, 1990). $CD8^+$ T lymphocytes represent the major protective principle, but cannot prevent horizontal infection in the absence of $CD4^+$ T lymphocyte functions (Jonjić *et al.*, 1989). In the absence of $CD8^+$ T lymphocytes, $CD4^+$ lymphocytes fully replace their function and provide virus clearance (Jonjić *et al.*, 1990). The function of the $CD4^+$ T cells is known to be associated with the release of interferon-γ (IFN-γ) because neutralization of endogenous IFN-γ prevents $CD4^+$ T lymphocyte function *in vivo* (Lučin *et al.*, 1992). Therefore, IFN-γ must play a central role in the control of MCMV replication *in vivo*.

IFN-γ on its own has only a moderate antiviral effect against MCMV *in vitro*, and therapy with recombinant IFN-γ cannot replace antiviral functions of T cells *in vivo* (Lučin *et al.*, 1992). We therefore reasoned that the activity of IFN-γ against CMV may be solely mediated by a supportive function for the antiviral activity of T lymphocytes. This supportive function could be associated with the increase in the expression of IFN-γ sensitive genes, for instance those encoding the MHC class I and II molecules. Increased expression of these proteins might result in enhanced antigen presentation. Another possibility, not mutually exclusive of the former, is that IFN-γ must associate with other cytokines to exhibit a direct antiviral effect. It has been reported that tumour necrosis factor (TNF) and IFN-γ in combination have synergistic antiviral activity against DNA viruses such as adenovirus type 2 (Mayer *et al.*, 1992; Wong & Goeddel, 1986), herpes simplex virus type 1 (HSV-1) (Feduchi *et al.*, 1989) and HSV-2 (Wong & Goeddel, 1986) and pseudorabies virus (Schijns *et al.*, 1991). The TNFs α and β (TNF-α/β) are synthesized by monocytes and lymphocytes respectively, bind to the same receptor and have indistinguishable biological activities (Aggarwal *et al.*, 1985; Beutler & Cerami, 1989; Paul & Ruddle, 1988). We have therefore studied the effect of IFN-γ and TNF-α on MCMV replication *in vitro*.

Here we demonstrate that these cytokines display synergy in their antiviral function. We report that IFN-γ

interferes with MCMV replication whereas TNF-α has very little effect on MCMV replication but strongly augments the antiviral function of IFN-γ. The antiviral state is achieved with non-toxic concentrations between 1 and 5 ng/ml of both cytokines and results in a reduction of the yield of infectious virus by more than three orders of magnitude. The cytokines interfere with MCMV maturation mainly during the late phase of the virus replication cycle.

Methods

Cells and viruses. Sucrose gradient-purified mouse embryo fibroblast culture-propagated MCMV (strain Smith; code VR-194; ATCC) was used. BALB/c secondary embryonal fibroblasts were prepared as described previously (Keil *et al.*, 1985) and propagated in MEM (Gibco) supplemented with 5% (v/v) fetal calf serum (Gibco), 2 mM-L-glutamine, 100 µg/ml of streptomycin and 100 units/ml of penicillin. Cells were infected with MCMV using the technique of centrifugal enhancement of infectivity at 800 *g* for 30 min (multiplication factor of 20).

Reagents and antibodies. Recombinant (r) murine IFN-γ and rTNF-α were generously provided by G. R. Adolf, Bender, Vienna, Austria. Specific activity of rIFN-γ stock (lot no. H3.RD48) was 10^7 units/mg, as determined by the L cell encephalomyocarditis virus bioassay. Specific activity of rTNF-α stock was 5×10^7 units/mg, as determined by a standard cytotoxicity assay on L929 cells in the presence of actinomycin D (ActD) (Aggarwal *et al.*, 1985). To inhibit transcription and translation, ActD (5 µg/ml) and cycloheximide (CH) (50 µg/ml) were used, respectively. Antiserum to pp89 (IE1) non-structural MCMV protein was produced in rabbits after immunization with the synthetic peptide P(34–53) (del Val *et al.*, 1988); the monoclonal antibody (MAb) 20/234/28 (mouse IgG1) recognizes nuclear early protein E1 of 36K and 38K (Bühler *et al.*, 1990); antiserum against glycoprotein B (gB) was produced by rabbit immunization with a vaccinia virus–gB recombinant (Rapp *et al.*, 1992); MAb against vertebrate actin (clone C4, mouse IgG1) was obtained from Boehringer Mannheim.

Immunoprecipitation. Cells were labelled with 150 µCi/ml of [^{35}S]methionine (Amersham) and lysed in buffer containing 10 mM-Tris–HCl pH 7·5, 150 mM-NaCl, 1% NP40, 0·1% SDS, 1% sodium deoxycholate and 1 mM-PMSF. Immunoprecipitation, SDS–PAGE and fluorography were carried out as described previously (Keil *et al.*, 1985). In brief, samples of cell lysates equivalent to 2×10^5 cells were incubated with 10 µl of antiserum or with 3 µl of ascitic fluid containing monoclonal antibodies. Antigen–antibody complexes were precipitated with Protein–A Sepharose (Pharmacia).

RNA analysis. Murine embryonal fibroblasts (MEF) were infected with MCMV at an m.o.i. of 10. Total cellular RNA was isolated according to the method of Chirgwin *et al.* (1979). Denatured RNA samples were size-fractionated by agarose gel electrophoresis and transferred to nylon membranes (GeneScreen, NEN) according to the instructions of the manufacturer. Membranes were prehybridized for 3 h in 5 × SSC, 5 × Denhardt's solution (1 × solution contains 0·02% of each of BSA, polyvinylpyrrolidone and Ficoll 400), 1% SDS, 10% dextran sulphate and 250 µg/ml salmon sperm DNA at 68 °C. Hybridizations were carried out overnight, under the same conditions, with a radioactively labelled probe. DNA probes used for hybridization were labelled with [α-^{32}P]dCTP (Amersham) by using a randomly primed DNA labelling system (Boehringer Mannheim).

The following genomic fragments were used as probes. (i) An 821 bp *Eco*RI fragment (map units 0·396 to 0·393) from the MCMV *Hin*dIII D region served as a probe for the gene encoding the major DNA-binding protein (MDBP) (Messerle *et al.*, 1992). (ii) A 1734 bp *Pst*I fragment (map units 0·383 to 0·376) from the MCMV *Hin*dIII D region served as a probe for the gene encoding protein ICP 18·5 (Messerle *et al.*, 1992). (iii) An 850 bp *Xba*I/*Pvu*II fragment (map units 0·3663 to 0·370) from the MCMV *Hin*dIII D region served as a probe for the gB gene (Rapp *et al.*, 1992). (iv) A 1190 bp *Hin*dIII/*Sma*I fragment (map units 0·351 to 0·356) from the MCMV *Hin*dIII D region served as a probe specific for the MCMV DNA polymerase gene (Elliot *et al.*, 1991). (v) Full-length cDNA from the human fibroblast cytoplasmic β actin gene was used as probe for a cellular gene (Gunnig *et al.*, 1983).

DNA analysis. Cells were lysed in a buffer containing 0·5% SDS, 0·1 M-NaCl, 0·01 M-Tris–HCl, 0·025 M-EDTA and 0·2 µg/ml proteinase K pH 8·0 for 16 h at 56 °C. Total cellular DNA was extracted by phenol–chloroform–isoamyl alcohol treatment and digested overnight with *Hin*dIII. Southern blotting to a nylon membrane (GeneScreen, NEN) was performed after electrophoresis of DNA fragments on a 0·6% agarose gel. The membranes were prehybridized at 65 °C for 3 h in a buffer containing 5 × SSPE, 5 × Denhardt's solution, 0·1% SDS and 100 µg/ml denatured salmon sperm DNA. After hybridization with the radioactively labelled probes under the same conditions for 20 h filters were washed and subjected to autoradiography.

To analyse the DNA content of released virions, cell-free culture supernatants were centrifuged at 30000 *g* for 1 h and pelleted virions were then blotted onto a nylon filter (GeneScreen) by using a vacuum blotter (Bio-Rad). After denaturation with 0·5 M-NaOH in 1 M-NaCl and neutralization with 3 M-NaCl, 0·5 M-Tris–HCl pH 7·4, the filters were subjected to hybridization as described above.

For hybridization, a fragment from plasmid IE 111 (Keil *et al.*, 1987), comprising the IE1 and IE3 genes, was labelled with [α-^{32}P]dCTP (Amersham), using a randomly primed DNA labelling system (Boehringer Mannheim).

Electron microscopy. Twenty-four and 48 h post-infection (p.i.) cells were trypsinized and fixed with 2·5% glutaraldehyde in 0·1 M-sodium cacodylate buffer pH 7·2 for 2 h, washed and soaked in 1% osmium tetroxide. Specimens were dehydrated in propanol, propylene oxide and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Philips 301 electron microscope at 80 kV.

Results

Synergistic effect of IFN-γ and TNF-α on MCMV replication

The combined activity of IFN-γ and TFN-α on MCMV replication was assessed by virus plaque number reduction and by the quantification of virus production. Because simultaneous infection and cytokine treatment had no effect on virus replication (data not shown) MEF were first incubated with cytokines for 48 h and then infected with MCMV. As demonstrated in Fig. 1 cytokine treatment resulted in significant inhibition of MCMV replication. Pretreatment of cells with IFN-γ alone clearly reduced virus plaque formation in a dose-dependent manner, whereas TNF-α on its own exerted a weak inhibitory activity (Fig. 1*a*). In accordance with our previous observations (Lučin *et al.*, 1992) a concentration of about 5 ng/ml of IFN-γ was required to

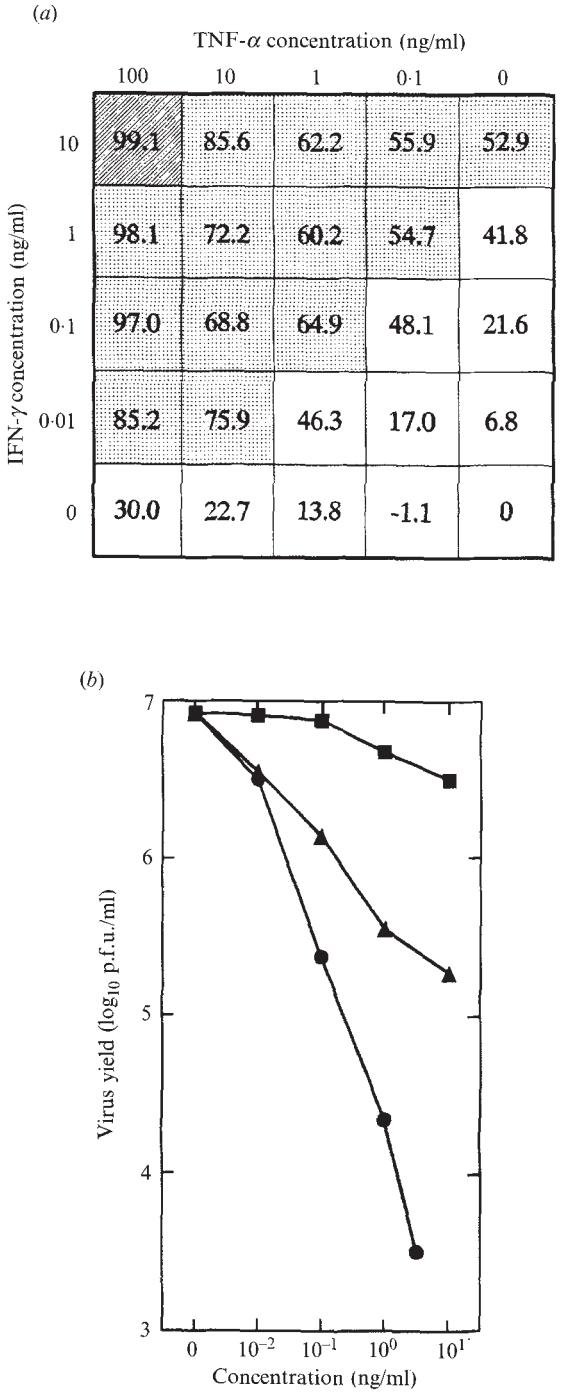

Fig. 1. Synergistic effect of rIFN-γ and rTNF-α on MCMV replication. Virus plaque formation (*a*) and virus yield (*b*) were determined in MEF after pretreatment with cytokines. The cells were treated for 48 h with recombinant cytokines, the cytokines were removed, and the cells were infected with MCMV (100 p.f.u./well). Number of plaques (*a*) and total virus yield (*b*) were determined after 4 days and 3 days, respectively. The results represent the mean percent reduction from triplicate culture (*a*) and the mean virus yield from four replicate cultures (*b*). Hatched areas indicate toxic concentrations of cytokines and dotted areas indicate concentrations of cytokines resulting in plaques of significantly smaller size. (■) TNF-treated; (▲) IFN-treated; (●) TNF/IFN-treated.

reduce the number of plaques by 50%. Although high concentrations of a single mediator were required for plaque reduction, after addition of both cytokines an effect was seen even at physiological mediator concentrations. In combination with 1 ng/ml of TNF-α a 100-fold lower concentration of IFN-γ (0·05 ng/ml) was sufficient to achieve 50% plaque reduction. Thus, the combination of both cytokines has a synergistic effect which results in a significant reduction of plaque numbers.

The individual cytokines were not toxic, as assessed by crystal violet staining, even at the highest concentrations used (50 ng/ml of IFN-γ and 500 ng/ml of TNF-α; data not shown). The combination of IFN-γ and TNF-α had some inhibitory effects on cell growth. The hatched areas in Fig. 1 (*a*) indicate concentrations at which such effects were detectable. The combination of 5 ng/ml of IFN-γ and 5 ng/ml of TNF-α was not toxic for MEF after 48 h of treatment and resulted in an 85% reduction of plaque numbers.

The plaques formed in monolayers pretreated with cytokines were of a significantly smaller size (showed by dotted area on Fig. 1 *a*). This finding was suggestive of an effect upon virus production. To test this, virus yield was determined 72 h after infection (Fig. 1 *b*). Pretreatment with IFN-γ resulted in a nearly 1·5 $\log_{10}$ reduction in virus yield, whereas pretreatment with TNF-α did not have a significant effect. As expected, the combination of both cytokines acted synergistically and reduced virus yield by almost three orders of magnitude. This is exemplified by the fact that 0·1 ng/ml of IFN-γ in combination with 0·1 ng/ml TNF-α sufficed to achieve the effect of 10 ng/ml of the IFN-γ in absence of TNF-α. In conclusion, the combination of IFN-γ with TNF-α results in a moderate reduction of plaque numbers and a strong reduction of virus yield.

These results suggest a physiological function of these cytokines in the reduction of virus production at the level of the individual cell and of the subsequent transmission of infection to neighbouring cells. Notably, infection of cultures with a low m.o.i. (0·001), i.e. the infection of only few cells at the start of the experiment, resulted in a more than 2 $\log_{10}$ lower virus yield in cells treated with IFN-γ alone and an almost 4 $\log_{10}$ lower virus yield when both cytokines were applied . In contrast, infection with a higher m.o.i. (1), i.e. the simultaneous infection of the majority of cells, reflects the antiviral activity of cytokines at the individual cell level. In this case virus yield was reduced only 10-fold after IFN-γ treatment and about 100-fold after combined IFN-γ and TNF-α treatment (data not shown). Therefore, the individual virus dose and the dynamics of tissue infection need to be taken into account for full evaluation of the antiviral activity of these cytokines.

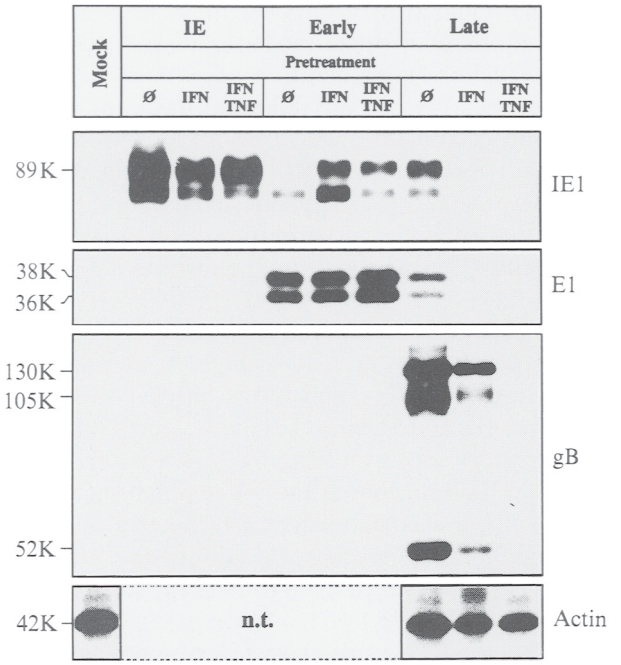

Fig. 2. Selective effect of cytokines on the synthesis of MCMV proteins. After incubation with rIFN-γ (5 ng/ml) or rIFN-γ and TNF-α (both at concentrations of 5 ng/ml) for 30 h cells were infected with MCMV (m.o.i. 10). To study the synthesis of IE proteins, the infection was carried out in the presence of CH for 3 h which was replaced by ActD and [^{35}S]methionine for an additional 4 h. Early proteins were labelled with [^{35}S]methionine from 3 to 7 h. Late proteins were labelled from 18 to 24 h p.i. Cell lysates were immunoprecipitated with rabbit antiserum to peptide P(34–53) of pp89 (IE1), MAb 20/234/28 to E1 protein, and rabbit antiserum to gB. The MAb C4 to actin served as a control for a cellular gene.

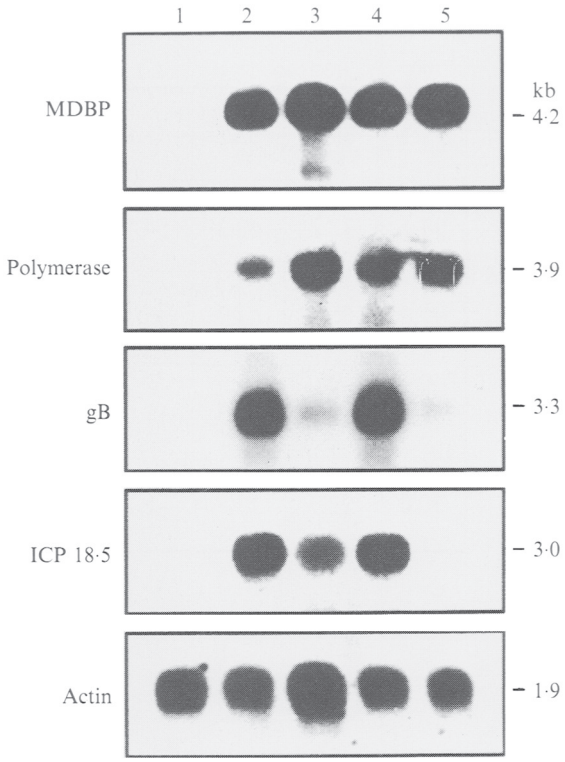

Fig. 3. Cytokine effect on MCMV transcripts. RNA was isolated from mock-infected cells (lane 1), MCMV-infected cells (m.o.i. 10) without cytokine preincubation (lane 2), or cells incubated for 30 h with 5 ng/ml of TNF (lane 3), IFN (lane 4) or TNF and IFN (lane 5) prior to MCMV infection. Total cellular RNA was isolated 24 h p.i., separated by agarose gel electrophoresis and hybridized with the specific probes.

Inhibition of late phase protein synthesis

The synergistic effect of IFN-γ and TNF affects a step before or at the transcription of immediate early (IE) genes of HSV-1 (Feduchi *et al.*, 1989). To elucidate at which stage during MCMV replication the cytokines exert their inhibitory activity, the synthesis of viral proteins representative of the IE, early (E) and late phases of the MCMV replication cycle was studied. Cells were labelled with [^{35}S]methionine and lysates were immunoprecipitated with antibodies specific for the candidate proteins. Immunoprecipitation with pp89 (IE1)-specific antiserum showed some inhibitory cytokine effect on the synthesis rate of this protein during the IE phase, whereas during the E phase cytokine treatment enhanced the synthesis of pp89 and during the late phase synthesis was prevented (Fig. 2). The differential abundance of pp76, a degradation product of pp89 (Keil *et al.*, 1985), was not related to a cytokine effect as it is also observed in samples not treated with cytokines. The synthesis of the E phase products of the E1 gene was, if affected at all, enhanced during the early phase. Similar results were observed for both the 53K and the 69K early proteins which belong to a second set of early genes (Bühler *et al.*, 1990) (data not shown). The finding that the synthesis of the candidate early proteins was not reduced by cytokine treatment indicated that the observed minor inhibitory effect on IE1 synthesis during the IE phase was functionally irrelevant. At late times of MCMV infection the combination of cytokines reduced IE1 protein synthesis. The synthesis of gB, a late phase protein, was significantly inhibited following pretreatment with IFN-γ and almost completely prevented after application of both cytokines. These results suggest that the mode of antiviral activity of these cytokines differs between herpesviruses and that one important manifestation of the cytokine-induced block in MCMV replication is a selective reduction of the synthesis of viral proteins during the late phase. Note that the effect of cytokines on viral protein synthesis was not paralleled by a similar effect on cellular protein synthesis since no difference in the synthesis of actin was observed. In addition, as was expected, the synthesis of IFN-γ-responsive proteins (eg. MHC class I gene-encoded

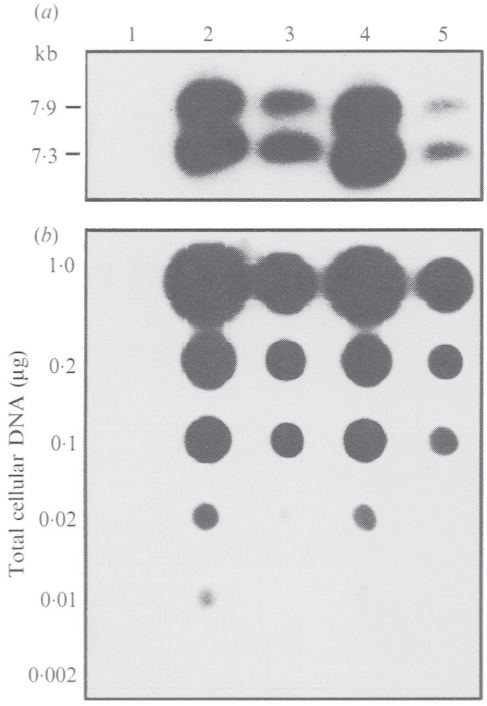

Fig. 4. Analysis of DNA from cytokine-treated cells. Total cellular DNA was isolated 24 h p.i. from cells mock-infected (lane 1), MCMV-infected (m.o.i. 10) without cytokine preincubation (lane 2), or cells incubated for 30 h with 5 ng/ml of TNF (lane 3), IFN (lane 4) or TNF and IFN (lane 5) prior to MCMV infection. After digestion with *Hin*dIII Southern blot (*a*) and dot-blot (*b*) analysis were performed using a *Hin*dIII K/L-specific probe.

proteins) was increased by 10- to 100-fold (data not shown).

Inhibition of late phase transcription

Reduced late phase protein synthesis could be due to the inhibition of transcription or to an effect on transcript stability. Therefore, Northern blot analysis was performed at different times after MCMV infection. Transcription of MCMV genes during the IE (IE1) and E (E1) phases was not affected (data not shown). In the late phase (24 h p.i.), a selective effect was seen in that the accumulation of certain transcripts was inhibited. The accumulation of MDBP gene and polymerase gene transcripts appeared to be normal or even stimulated in cells pretreated with cytokines (Fig. 3). Both the MDBP and polymerase genes represent E genes with maximal transcription at the end of the E phase and at the beginning of the late phase (Messerle *et al.*, 1992; Elliot *et al.*, 1991). In contrast, transcripts of certain late genes (gB and ICP 18·5) were found to be significantly reduced following IFN-γ treatment and particularly following combined IFN-γ/TNF-α treatment. Therefore, preincubation of cells with these cytokines induces an antiviral state which affects late phase transcription, late transcript stability, or both.

Effect of IFN-γ and/or TNF-α on viral DNA synthesis

Inhibition of viral DNA synthesis by phosphonoacetic acid or Gancyclovir prevents the expression of late phase genes (Keil *et al.*, 1984). Thus, reduced expression of late genes could be a consequence of inhibited viral DNA synthesis caused by cytokine treatment. To quantify the cytokine effect on MCMV DNA synthesis, Southern blot analysis was performed. Total DNA was extracted from infected cells and digested with *Hin*dIII. Ethidium bromide staining after separation on an agarose gel showed the same fragment pattern in infected normal cells and in cytokine-treated cells, suggesting that elongation of newly synthesized DNA occurred normally (data not shown). Southern blot analysis with a probe specific for the *Hin*dIII K and L fragments supported this observation and demonstrated quantitative effects of cytokine pretreatment on viral DNA synthesis (Fig. 4*a*). Titration of digested DNA and subsequent dot-blot analysis using the same probe revealed that viral DNA synthesis was reduced but not completely prevented. The reduction was about fivefold in IFN-γ-treated cells and about 10-fold in IFN-γ/TNF-α-treated cells (Fig. 4*b*). Clearly, a reduction in infectious progeny number by three orders of magnitude can not be explained by this limited effect upon viral DNA synthesis.

Assembly of nucleocapsids and maturation of virions in cytokine-treated cells

Reduced transcription and translation of MCMV late genes should have direct consequences on the maturation of MCMV virions. Therefore, MCMV morphogenesis was analysed by electron microscopy 24 and 48 h after infection. In the absence of cytokine treatment nucleocapsids, predominantly those with an electron-lucent core, arise in the nuclei of MCMV-infected cells together with morphological changes in nuclear structures (Weiland *et al.*, 1986) (Fig. 5*a* and *c*). Formation of CMV inclusion bodies and nuclear dense bodies precedes the appearance of nucleocapsids (Fig. 5*a*). The cytoplasm contains typical cytoplasmid multicapsids as well as monocapsids enveloped by cellular vacuoles (Fig. 5*a*). At 24 h p.i. the cells were heterogeneous with respect to the phase of virus replication since 24·4% of cells did not contain capsids, 29·5% of cells contained only nuclear capsids and 46·1% of cells contained both nuclear and cytoplasmic nucleocapsids. At 48 h p.i., however, nucleocapsids were found in both the nuclei and cytoplasm of almost all cells (Table 1).

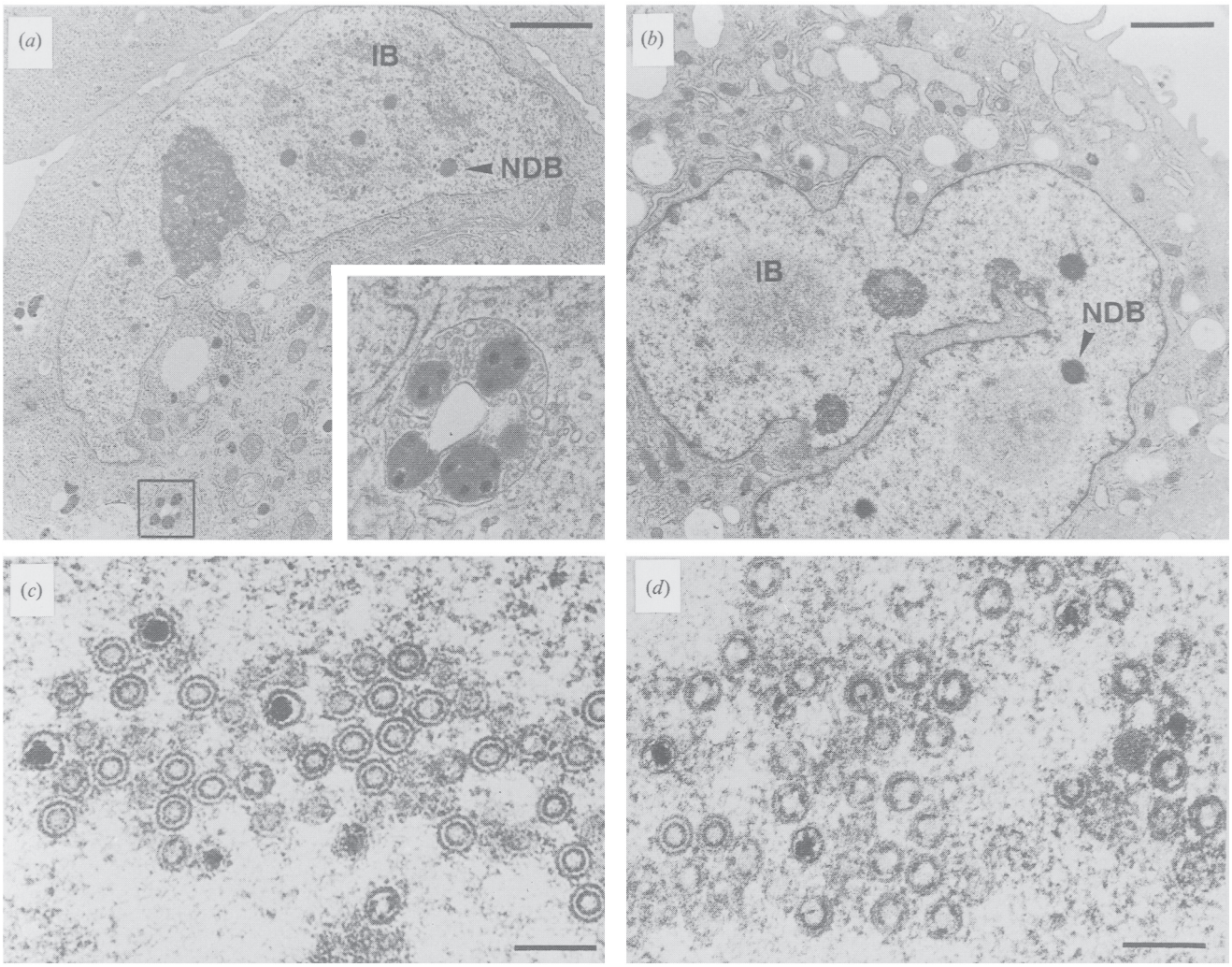

Fig. 5. Cytokine effect on MCMV morphogenesis. Cells were treated as outlined in Table 1. Electron microscopy was carried out 24 h p.i. (*a*, *b*) and 48 h p.i. (*c*, *d*). (*a*) Distribution of nucleocapsids and MCMV-induced morphological changes. (*b*) Incompletely formed nuclear inclusion body and the absence of nucleocapsids in IFN-γ/TNF-α-treated cells 24 h p.i. (*c*) Predominance of electron-lucent-core capsids in the nucleus 48 h p.i. (*d*) Appearance of nucleocapsids in IFN-γ/TNF-α-treated cells 48 h p.i. IB, Inclusion body. NDB, Nuclear dense body. Bar markers represent 2 μm (*a* and *b*) and 200 nm (*c* and *d*).

Both IFN-γ and IFN-γ/TNF-α pretreatment of cells altered the morphogenesis of MCMV. At 24 h p.i. only a few cells with nucleocapsids could be detected (Table 1). Many cells contained incompletely formed inclusion bodies which is suggestive of an inhibition of morphogenesis prior to the appearance of nucleocapsids (Fig. 5*b*). At 48 h p.i. the majority of IFN-γ-treated cells contained nucleocapsids both in the nucleus and cytoplasm, while in IFN-γ/TNF-α-treated cells 58·1% of cells lacked cytoplasmic nucleocapsids (Table 1). Inspection of the cells at a higher magnification revealed not only a delayed but also an altered morphogenesis. In the nucleus of IFN-γ/TNF-α-treated cells capsids with electron-lucent cores predominated. The inner shell in most of these capsids was interrupted and contained discrete accumulations of electron-dense material (Fig. 5*d*). This type of nucleocapsid appears to represent a transient stage during MCMV morphogenesis since these forms represent only a minority in normal cells (Fig. 5*c*). The numeric increase of these incompletely formed electron-lucent-core nucleocapsids was the prominent characteristic of IFN-γ/TNF-α-treated cells. With respect to cytoplasmic nucleocapsids only quantitative differences and no alterations in morphology were seen. Accumulation of diffusely arranged amorphous material representing nuclear inclusion bodies was typical for cytokine-treated cells that did not, either at 24 or 48 h p.i., contain nucleocapsids (Fig. 5*b*). Therefore, it seems that morphogenesis of MCMV is altered at multiple stages after IFN-γ and IFN-γ/TNF-α treatment.

Table 1. *Cytokine effect on formation and distribution of nucleocapsids*

Time p.i. (h)	Treatment*	Formation and distribution of nucleocapsids† (%) No capsids	Only nuclear	Nuclear and cytoplasmic
24	0	24·4	29·5	46·1
	IFN-γ	97·6	1·6	0·8
	TNF-α	37·5	9·2	53·3
	IFN-γ/TNF-α	99·0	0·5	0·5
48	0	1·1	6·9	92·0
	IFN-γ	27·9	2·7	69·4
	TNF-α	5·8	3·6	89·6
	IFN-γ/TNF-α	14·2	43·9	41·9

* To MEF rIFN-γ (5 ng/ml) or rTNF-α (5 ng/ml) or both cytokines was added. Infection with MCMV at an m.o.i. of 10 was carried out 30 h later. Cells were subjected to electron microscopy 24 and 48 h p.i.

† Two-hundred individual cells were analysed by electron microscopy.

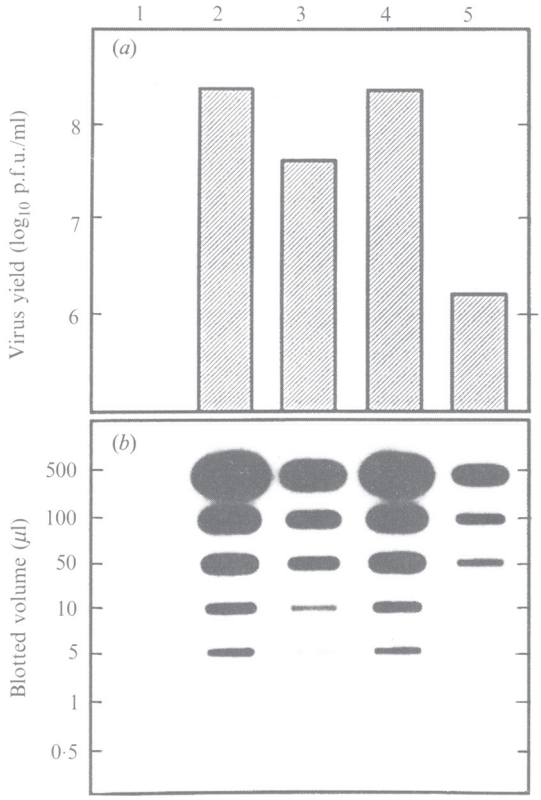

Fig. 6. Effect of cytokine treatment on virion infectivity. Cells were mock-infected (lane 1), MCMV-infected (m.o.i. 1) without cytokine preincubation (lane 2), or incubated for 30 h with 5 ng/ml of TNF (lane 3), IFN (lane 4) or TNF and IFN (lane 5) prior to MCMV infection (m.o.i. 1). Forty-eight hours after infection the number of infectious virus units was determined by a plaque assay (*a*) and the total viral DNA in supernatants was estimated by blotting of an aliquot of pelleted virions and subsequent hybridization with a *Hin*dIII K/L-specific probe (*b*).

The maturation of nuclear inclusion bodies, the assembly of nucleocapsids and the egress of mature nucleocapsids from the nucleus appear to be major points of cytokine action during MCMV morphogenesis. Treatment of cells with TNF-α alone did not detectably affect the assembly of MCMV virions; this was expected from the minor effect on virus productivity and the biochemical analysis.

Infectivity of virions released from cytokine-treated cells

A reduced synthesis of viral DNA and the decrease in late gene expression should result in the release of a smaller number of MCMV particles. In addition, many of these particles should have a reduced infectivity or lack this property altogether owing to their defects in morphogenesis and lack of gB. To test this, the cells were infected at an m.o.i. of 1 and virus yields in supernatants were determined. At this m.o.i., pretreatment of cells with IFN-γ reduced the release of infectious particles eight- to 10-fold, but the combined cytokine treatment reduced infectious virus yield by two orders of magnitude (Fig. 6*a*). Blotting of virions from supernatant onto the nylon filter and subsequent hybridization with an MCMV DNA-specific probe showed a fourfold reduction in DNA content after IFN-γ treatment and after combined cytokine treatment the DNA content was reduced by a factor of eight (Fig. 6*b*). If the DNA content is taken as an estimate for the genome number we must conclude that 90% of these genomes are contained in non-infectious particles. Therefore, both a reduced release of virions and a reduced infectivity of these virions are the consequences of the synergistic action of IFN-γ and TNF.

Discussion

The cytokines IFN-γ and TNF exhibit synergism in the induction of resistance against certain RNA and DNA viruses. Among DNA viruses, adenovirus (Wong *et al.*, 1986; Mayer *et al.*, 1992), African swine fever virus (Esparza *et al.*, 1988), and the alphaherpesviruses HSV (Wong *et al.*, 1986; Feduchi *et al.*, 1989) and pseudorabies virus (Schijns *et al.*, 1991) have been found to be susceptible to this combination of cytokines. Here we report that the replication of MCMV, a betaherpesvirus, is also susceptible to their synergistic action. Thus, in order to explain the antiviral effect of $CD8^+$ and $CD4^+$ T lymphocytes against CMV *in vivo*, not only the direct cytolytic activity of these effector cells, but also, and probably much more importantly, the effect of cytokines released by these cells have to be considered.

Strong effects are seen even at low cytokine concentrations which may be reached locally during the

initiation of an immune response *in vivo*. The general notion that the induction of an antiviral state requires exposure of cells to the cytokine prior to infection also applies in this case. Because of its slow replication cycle CMV is probably particularly vulnerable to this type of immune control. Recognition of viral proteins expressed during the IE or E phase of the replication cycle by activated T lymphocytes could perhaps trigger a cytokine release sufficient for the induction of an antiviral state in cells neighbouring the infected cell even before infectious progeny is released. Evidence in support of this hypothesis is our finding that neutralization of IFN-γ completely cancels the antiviral effect of $CD4^+$ T lymphocytes in the salivary gland and prevents virus clearance (Lučin *et al.*, 1992).

Although the combined cytokines TNF and IFN-γ act on both alpha- and betaherpesviruses, surprisingly the specific mode of action is different. In their study on HSV-1 replication Feduchi *et al.* (1989) placed the synergistic action at a very early step, after virus entry but before or at the transcription of IE genes, because the expression not only of early thymidine kinase and late (γ0) genes but also of IE genes (α22) was inhibited. Earlier data on the inhibitory function of interferons in the absence of TNF on HSV-1 and -2 also indicated a suppression of IE and E gene expression exemplified by the genes for ICP4 and DNA polymerase (Domke-Opitz *et al.*, 1986).

In our study on the targets of the cytokine action we tested a selection of individual genes from the three temporal phases of the MCMV replication cycle that have been defined. The analysis of these candidate genes allowed us to reach the firm conclusion that the mode of action in MCMV must differ from that described for HSV. We would place the effect mainly in the late phase and in the transitional step between the E and late phases. We reach this conclusion for the following reasons: a significant effect at IE and E times is unlikely because the expression of the IE1 gene product pp89 was not affected; moreover there was no inhibition of the E1 gene product and there was no effect on transcription of the E genes encoding the DNA polymerase and MDBP, two of the seven E genes involved in the control of herpesviral DNA synthesis (Wu *et al.*, 1988). Yet, MCMV DNA replication was found to be reduced by a factor of five- to 10-fold. Host cell mechanisms are required for the transport of the HSV-1 MDBP to the nucleus and to replication compartments, and components of the cellular DNA replication apparatus contribute to the efficiency of HSV DNA synthesis (De Bruyn Kops & Knipe, 1988). Additional targets for the cytokine action, therefore, may be the host cell components that are involved in viral DNA replication. An observation that the cytokines inhibit cellular DNA synthesis (P. Lučin & U. H. Koszinowski, unpublished data) and the notion that MCMV requires events associated with the host S phase for initiation of DNA replication (Muller & Hudson, 1977) support this hypothesis. Finally, by concentrating solely on a few candidate viral genes we may have missed a step at which the cytokine action could have interfered with E gene function. Altogether it is clear, however, that a reduction of DNA replication by one order of magnitude at maximum cannot account for a 1000-fold reduction in the yield of infectious virus.

Of true late genes only one candidate, the MCMV gB gene (Rapp *et al.*, 1992), was amenable to analysis. This gene encodes a glycoprotein which is conserved in all herpesviruses that have been characterized at the molecular level. The expression of this glycoprotein is essential for herpesvirus infectivity (Cai *et al.*, 1988). Even if we make the conservative assumption that this effect is the only alteration in the expression of late proteins, the drastic reduction in gB expression under combined IFN-γ/TNF-α treatment would by itself explain the biological findings. We assume that the combined cytokine effect acts essentially at the level of transcription of gB because the reduction was not accompanied by a detectable alteration of the post-translational processing of the protein. Although the significant reduction of gB synthesis could explain the experimental findings the situation is probably more complex. The difference in viral morphogenesis we observed is suggestive of an effect on additional genes. Two findings are worth recollecting. First, although there was no difference observed in the sequential steps of the replication cycle, nucleocapsid formation in the nucleus and particle export into the cytoplasm for further maturation were reduced and very slow in cytokine-treated cells. This maturation defect was not merely a delay because the analysis after 72 and 96 h still showed the same reduction in virus titres (data not shown). Secondly, the alteration of nucleocapsid formation has to be considered. In normal cells the majority of electron-lucent nucleocapsids take the form of double shells and nucleocapsids with an incomplete inner shell represent a minority. Remarkably, the latter form predominates in cytokine-treated cells. The enzymatic degradation of the inner shell of the capsid probably represents a step prior to the take-up of the electron-dense core material (Weiland *et al.*, 1986). If this order of events is correct, the function responsible for this inner shell degradation might represent a target for the cytokine effect, and this results in the accumulation of these otherwise transient forms. Insufficient synthesis of late proteins that are involved in nucleocapsid assembly, as exemplified by the product of the ICP 18·5 gene homologue (Addison *et al.*, 1990), also presumably

contributes to the different morphogenesis of MCMV in cytokine-treated cells. In addition, reduced DNA synthesis and inhibited expression of late glycoproteins results in release of reduced numbers of virions, the majority of which are non-infectious. Taken together, these multiple targets or multiple consequences of the antiviral cytokine action on the MCMV replication cycle explain the synergistic effect.

In conclusion, our studies in a model system for human CMV disease have pointed to an important and synergistic role of cytokines in the control of virus clearance. In theory these two cytokines in combination could represent an antiviral preparation suitable for medical use. Unfortunately, for various reasons systemic application is probably not the method of choice. These cytokines act locally and their potential activity can be effectively demonstrated when they are produced at the site of virus replication, for instance by recombining the respective cytokine gene into the virus under study (Kohonen-Corish *et al.*, 1990; Sambhi *et al.*, 1991).

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