Cytokines restore MHC class I complex formation and control antigen presentation in human cytomegalovirus-infected cells

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CD8$^+$ cytotoxic T cell (CTL) clones with specificity for defined minor and major histocompatibility (H) antigens were used to monitor antigen presentation in human cytomegalovirus (HCMV)-infected skin fibroblasts At the immediate early stage of virus replication antigen presentation was intact, but was abolished during the early and late phase Lack of CTL recognition was not selective for certain antigens but was associated with decreased steady state levels of nascent MHC class I complexes and unassembled MHC class I heavy chains, whereas free β_2-microglobulin remained abundant HCMV also affected the stability of both immature endoglycosidase H (Endo H)-sensitive and mature Endo H-resistant MHC class I molecules, suggesting that the virus interferes with antigen presentation at more than one step during maturation of the MHC class I complex The action of interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) lifted the block of MHC class I complex formation by stimulating synthesis, assembly and stability of MHC class I molecules This resulted in restored antigen presentation provided that cells were exposed to the factors before HCMV infection Because few MHC molecules suffice for CTL recognition these cytokines compensated for the negative viral effect on the antigen presentation function Nevertheless, the viral interference with MHC class I complex formation was still active The data imply that specific cytokines limit the immune evasion potential of HCMV from CD8$^+$ T lymphocyte control

Introduction

CD8$^+$ T lymphocytes recognize major histocompatibility complex (MHC) class I molecules that are formed as heterotrimeric complexes consisting of a highly polymorphic glycoprotein heavy chain, a noncovalently linked nonpolymorphic light chain protein, β_2-microglobulin (β_2m), and a short peptide derived from antigenic proteins (Townsend & Bodmer, 1989, Rotzschke *et al*, 1990) After infection with virus, processing of viral antigens requires protein degradation by cytoplasmic proteinase into peptides (Kelly *et al*, 1991, Martinez & Monaco, 1991, Ortiz-Navarrete *et al*, 1991) which are subsequently translocated into the endoplasmic reticulum (ER) by ATP-dependent peptide transporters of the ABC family (Powis *et al*, 1991, Spies *et al*, 1990) or by mechanisms independent of ATP (Levy *et al*, 1991) In this compartment, the assembly of trimolecular MHC class I complexes involves folding the MHC class I heavy chain (Townsend *et al*, 1990), transient interaction with chaperonins (Rajagopalan & Brenner, 1994) and peptide transporters (Ortmann *et al*, 1994, Suh *et al*, 1994), stable association with β_2m (Kvist & Hamann, 1990), and binding of the antigenic peptide that is held in the polymorphic peptide binding groove MHC class I molecules lacking peptide display a different conformation and are deficient with respect to surface transport and stability (Hsu *et al*, 1991, Lie *et al*, 1990) Like viral antigens recognized by virus-specific cytotoxic T lymphocytes (CTL), minor H (mH) antigens are peptides and elicit MHC class I-restricted T cell responses (Goulmy *et al*, 1977, De Bueger *et al*, 1992) Thus, like major histocompatibility antigens, mH antigens also represent serious barriers for successful organ and bone marrow transplants between individuals

Human cytomegalovirus (HCMV) is a member of the herpesvirus family and establishes both acute and chronic infections Primary infection is followed by lifelong persistence in a latent state from which reactivation can occur While infection of the immunocompetent host is usually asymptomatic, HCMV causes severe and fatal disease in immunocompromised individuals such as organ transplant recipients or AIDS patients (Drew & Mintz, 1984, Ho, 1982) In organ and bone marrow recipients CMV infection is also a major problem in graft

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rejection and graft-versus-host disease (Meyers *et al*, 1986, Miller *et al*, 1986) Cell transfer experiments as well as studies with recombinant vaccines expressing a class I restricted epitope (Reddehase *et al*, 1987) demonstrated that $CD8^+$ T lymphocytes play an essential role in the host defence against murine cytomegalovirus (MCMV) and mediate protective immunity Adoptive immunotherapy of CMV infections in humans with antiviral MHC class I-restricted $CD8^+$ T lymphocytes supports this notion (Riddell *et al*, 1992)

Previous studies demonstrated that murine cytomegalovirus can interfere with the MHC class I antigen presentation pathway by an early (E) gene function (Del Val *et al*, 1989) MCMV prevents presentation of peptides derived from immediate early (IE)-expressed proteins by blocking the transport of the peptide loaded MHC class I complexes through the Golgi compartment (Del Val *et al*, 1992) This ability of MCMV to inhibit antigen presentation and to evade $CD8^+$ T cell-mediated immune control is overcome by the action of interferon-γ (IFN-γ) *in vitro* as well as *in vivo* (Hengel *et al*, 1994) Like MCMV, HCMV is able to decrease MHC class I expression (Barnes & Grundy, 1992) Beersma *et al* (1993) reported that nascent MHC class I molecules are unstable in infected cells. Although HCMV-infected cells were deficient in presentation of an externally loaded synthetic peptide to CTL (Warren *et al*, 1994), the functional consequences of this interference with antigen presentation of endogenously processed peptide antigens are not clear The interference of HCMV with $CD8^+$ T cell recognition was considered to be specific for IE antigen (Gilbert *et al*, 1993) despite the fact that HCMV-IE antigen-specific $CD8^+$ T cell responses can be elicited *in vitro* (Borysiewicz *et al*, 1988)

Here we report that infection of skin fibroblasts with HCMV, which leads to a loss of MHC class I complex formation and surface expression due to an E gene function of the virus, is paralleled by the absence of presentation of constitutively expressed minor or major H antigens The viral interference showed no antigen selectivity The block of antigen presentation was overcome by pretreatment of cells with IFN-γ or tumour necrosis factor (TNF-α) Although these cytokines can also have direct antiviral activities (Lucin *et al*, 1994) the effect upon antigen presentation is due to a strong increase of the steady state levels of MHC class I complexes, thereby restoring peptide presentation while the viral E gene mechanism was still active

Methods

Cells The human foreskin fibroblast cell lines VH3 and VH7 were established in primary culture after standard procedures by mincing the skin and subsequent stirring in prewarmed trypsin (Wiedbrauk & Johnston, 1992) Recovered fibroblasts were passaged in DMEM supplemented with 10% FCS, penicillin, streptomycin and 2 mM-glutamine and used between culture passages 6 to 12

Virus and infection conditions Virus stocks were prepared by propagating HCMV strain AD169 (a gift of M Mach, Erlangen, FRG) in fetal human lung fibroblasts (passages 6 to 20) grown in DMEM supplemented with 10% FCS Infectious supernatants were harvested when 100% of the cells showed a cytopathogenic effect The titres of virus stocks were determined by standard plaque assays after centrifugation at 800 *g* for 30 min and had an infectivity of 5×10^6 to 3×10^7 p f u /ml For infections, subconfluent monolayers of VH cells were incubated with HCMV at the m o i indicated and cultures were centrifuged at 800 *g* for 30 min to enhance the efficiency by a factor of 10 to 20 In all experiments, control settings showed more than 97% of cells positive for HCMV E gene expression in immunofluorescence microscopy (Gleaves *et al*, 1984) Selective expression of HCMV IE gene products was achieved by infection of cells with HCMV in the presence of cycloheximide (50 μg/ml), which was replaced 4 h later by actinomycin D (5 μg/ml) followed by incubation for 7 h before the cells were further analysed Late phase gene expression was prevented by the use of phosphonoacetic acid (PAA, 250 μg/ml), which arrests CMV-infected cells in the E phase

Reagents and antibodies Recombinant human IFN-γ and TNF-α were obtained from Boehringer Mannheim, and human IFN-α was purchased from Paesel and Lorei (Frankfurt/Main, Germany) The following monoclonal antibodies (MAbs) were used W6/32 (anti-HLA class I complexes) (ATCC HB 95), BB7 2 (anti HLA-A2) (ATCC HB 82), BBM 1 (anti-human β_2m) (ATCC HB 28), anti-HLA A,B,C heavy chain (Olympus) (Bushkin *et al*, 1986), anti-actin (Boehringer Mannheim), anti-HCMV early nuclear antigen (Du Pont NEN), anti-HCMV late nuclear antigen (Du Pont NEN), anti-ICAM-1 (Immunotech), anti-transferrin receptor (Oncogene Science), and anti-CD44 (Immunotech) Polyclonal rabbit anti-mouse IgG was from Dianova

Flow cytometry Isolated VH cells were pre-incubated in 5% goat serum and then stained with MAbs Bound antibodies were visualized by addition of fluorescein-conjugated goat anti mouse isotype-specific (Medac) antibodies As a negative control cells were incubated with the second antibody alone A total of 10^4 cells was analysed for each fluorescence profile on a FACScan IV (Becton Dickinson)

Metabolic labelling, immunoprecipitation and endoglycosidase H treatment Cells were labelled with [^{35}S]methionine (1200 Ci/mmol, Amersham) at a concentration of 500 μCi/ml as described previously (Del Val *et al*, 1992) In brief, anti-actin and rabbit anti-mouse IgG were used to remove actin molecules Subsequently, quantitative precipitations were performed sequentially with protein G purified preparations of MAb W6/32, followed by BBM1 and anti-HLA A,B,C To ensure quantitative retrieval of immune complexes, the lysates were incubated twice more with Protein A–Sepharose (Pharmacia) before addition of the next MAb Immune complexes were mock treated or digested with 2 mU of endoglycosidase H (Endo H) (Boehringer Mannheim) overnight at 37 °C, eluted with sample buffer, and analysed by 10–15% polyacrylamide gradient gel electrophoresis The gels were treated with En^{3}Hance (Du Pont NEN), dried, and exposed to X-Omat AR films at −70 °C for 1–7 days

Cytolytic assays The generation and maintenance of the $CD8^+$ CTL clones used in this study was described in detail earlier (De Bueger *et al*, 1992, Van Els *et al*, 1992) The CTL clone 3E7 is allo-A2 specific, the clone 1R35 specific for the male H-Y antigen is HLA-A2 restricted, and the clone 5HO11 recognizing the HA 3 mH antigen is HLA-A1 restricted In brief, the CTL clones were cultured in RPMI 1640 supplemented with antibiotics, 15% pooled human serum and 20 U/ml

rIL-2, and they were expanded by weekly restimulation with irradiated EBV-immortalized BLCL plus freshly isolated PMNC from random donors. Cryopreserved CTL were thawed for immediate usage as effector cells in ^{51}Cr-release assays or kept for 2–4 days with 20 U/ml rIL-2 before use. After washing and treatment with trypsin, VH cells were labelled with ^{51}Cr for 1 h. The viability of cells was determined by trypan blue exclusion and suspended targets were used at 1000 cells per well in a standard 4 h ^{51}Cr-release assay. In all experiments, effector to target (E/T) ratios ranged from 20:1 to 0·16.1. Spontaneous ^{51}Cr release in the experiments given did not exceed 30% of the maximal release values measured in the presence of 1% Triton X-100.

Results

HCMV affects CD8$^+$ T cell recognition of major and minor histocompatibility antigens

The recognition of different viral epitopes by CD8$^+$ CTL is inhibited by HCMV functions. Since the recognition of individual antigens is affected to a different extent, the virus function appeared to be selective for IE antigen, but independent of MHC expression (Gilbert *et al.*, 1993). Such features complicate the analysis of inhibitory functions when HCMV-specific CTL are used to assess the antigen presentation function during infection. We took advantage of the detection of constitutively expressed cellular peptides by MHC class I-restricted CTL. The HLA-A2-restricted human mH antigen H-Y and the HLA-A1-restricted antigen HA-3 are expressed by human foreskin fibroblast cells (HFF) (Goulmy, 1988; De Bueger *et al.*, 1992). HFF cells from randomly selected donors were prescreened for HLA A-2 expression and antigen presentation to peptide-specific CTL clones. The HFF cell lines VH3 and VH7 were identified as suitable targets for H-Y and HA-3 as well as for HLA-A2 specific CTL clones. To follow presentation of mH peptides and of HLA-A2 during permissive HCMV infection, VH cells were infected at an m.o.i. of 15 and tested throughout the HCMV replication cycle. As shown in Fig. 1, after viral gene expression for 12 h, HLA-A2 and mH peptide antigens could be recognized by the respective CTLs. However, later stages of HCMV infection were associated with inhibited presentation of both H-Y and HA-3 peptide, and with delayed kinetics the recognition of HLA-A2 molecules was also abolished at 96 h post-infection (p.i.) (Fig. 1). Impaired CTL recognition of the HLA class I-dependent antigens after HCMV infection was observed for both VH3 and VH7

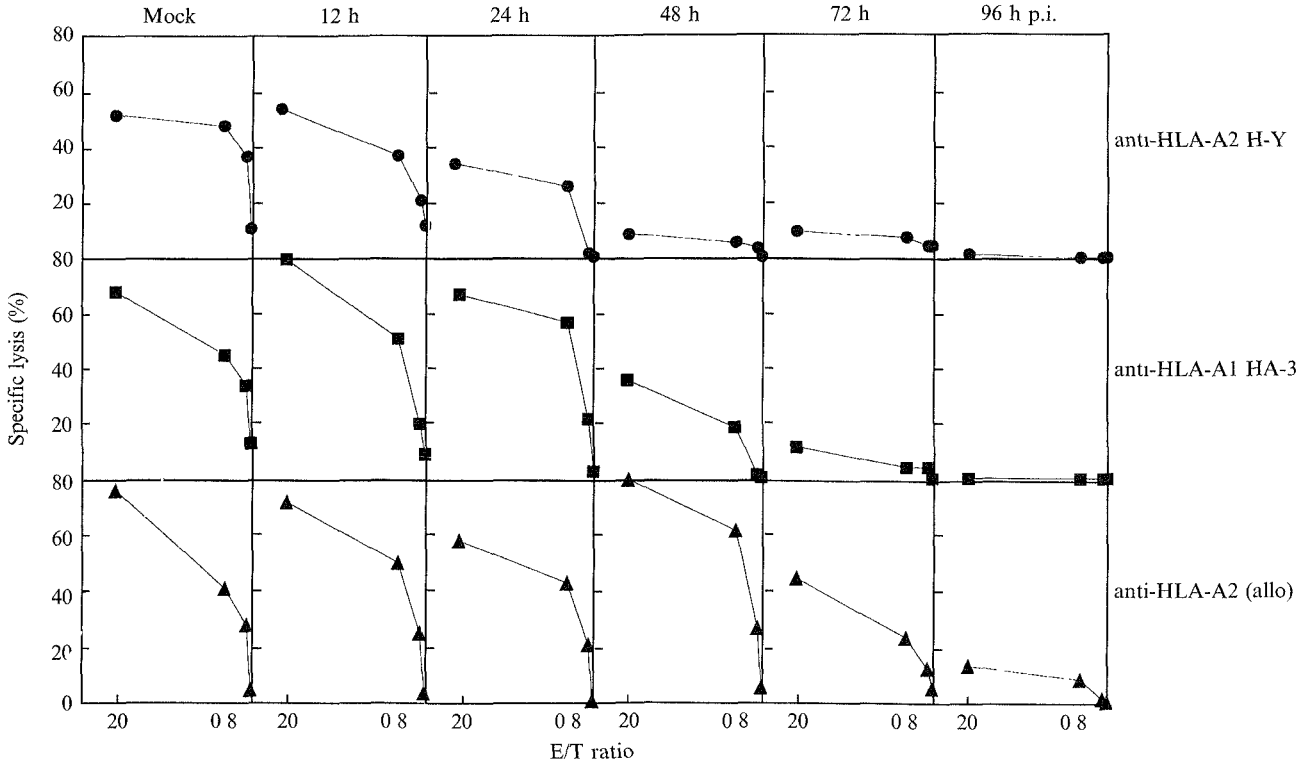

Fig. 1. Effect of HCMV infection on CD8$^+$ CTL recognition of minor and major histocompatibility antigens. mH antigen H-Y- and HA-3-expressing HLA-A2$^+$ fibroblasts (VH7) were infected with CMV (m.o.i. 15) at the time points indicated before cells were labelled for 60 min with ^{51}Cr and tested in a 4 h standard Cr-release assay with graded numbers of effector cells. The effectors were CTL clone 1R35 specific for H-Y antigen (upper panels, ●), CTL clone 5HO11 specific for HA-3 (middle, ■), and CTL clone 3E7 that recognizes HLA-A2 as an allo-antigen (lower panels, ▲).

cells, and occurred with identical kinetics at all virus doses tested (m.o.i. 3, 15 and 75) without affecting cell viability and spontaneous ^{51}Cr release (data not shown). Thus HCMV interferes with the recognition of endogenous MHC class I antigens in an antigen-independent manner.

In permissively infected cells, CMV gene expression is regulated in a cascade fashion characteristic for herpesviruses. Metabolic inhibitors of viral gene expression can be used to separate the different phases of infection, i.e. the immediate early (IE), early (E) and late (L) phase. In order to assign interference of the virus with HLA class I antigen recognition to a distinct phase of HCMV gene expression, cells were infected (as described in Methods) in the presence of cycloheximide, an inhibitor of protein synthesis; this was replaced after 4 h by the inhibitor of transcription actinomycin D, resulting in a restriction of HCMV gene expression to the IE phase. Under these conditions of selective IE expression recognition of VH fibroblasts by the CTL clones 12 h p.i. was actually augmented compared to mock controls (data not shown). When an arrest of the E phase was achieved by infection of cells in the presence of PAA, which blocks viral DNA replication and the subsequent expression of late phase genes, lysis by H-Y- and HA-3-specific CTL was completely abolished at 72 h p.i. In contrast to permissively infected cells showing late gene expression, HLA-A2-specific CTL still recognized these targets to some extent (data not shown). These experiments confirmed a previous observation that the viral gene(s) inhibiting CTL recognition are already being expressed in the E phase of productive HCMV infection (Warren *et al.*, 1994).

An early gene function of HCMV selectively reduces MHC class I expression

Cytomegaloviruses, both mouse and human, are known to interfere with MHC class I molecule expression. In the case of MCMV, retention of nascent correctly assembled MHC class I complexes in the ER/*cis*-Golgi compartment during the viral E phase results initially in inhibited presentation of newly synthesized antigens, and later on in a general reduction of MHC class I molecules at the cell surface (Del Val *et al.*, 1992). HCMV was reported to destabilize MHC class I complexes within 24–40 h without affecting class I heavy chain mRNA levels (Beersma *et al.*, 1993; Yamashita *et al.*, 1993; Browne *et al.*, 1990). To follow the fate of resident MHC class I molecules during the course of HCMV infection in VH cells, expression of MHC class I molecules was tested in parallel with the adhesion molecules ICAM-1 (CD54), pgp-1 (CD44), and the transferrin receptor (CD71). As assessed by cytofluorometric analysis, the surface densities of these marker molecules remained essentially unaffected during infection. As reported by Downes & Grundy (1993), levels of ICAM-1 molecules were even slightly increased during the course of permissive infection (data not shown). In contrast, the density of HLA class I complexes continuously decreased during HCMV infection resulting in a loss of about 90% of the molecules at 96 h p.i. Next, sequential and quantitative immunoprecipitation experiments after [^{35}S]methionine labelling of HCMV-infected VH cells were performed to study the fate of nascent class I molecules in HCMV-infected VH cells. The MAb W6/32 recognizes only β_2m-assembled HLA molecules. Using this antibody, under IE conditions (data not shown) or 12 h after HCMV infection, a slight increase of steady state levels of complexed class I molecules was seen that was followed by a dramatic reduction in recovered class I complexes at 72 h p.i. and later stages of infection (Fig. 2*a*). Subsequent precipitation of free β_2m revealed an increased expression of this molecule (Fig. 2*b*). The abundance of isolated HLA class I heavy chains was reduced, although not to the extent of β_2m-assembled class I heavy chains (Fig. 2*c*). Transferrin receptor molecules were expressed in constant amounts (data not shown). Restriction of HCMV gene expression to the E phase by PAA treatment was also associated with significantly reduced amounts of HLA class I complexes, although under these conditions the viral effect was less pronounced than in the absence of PAA, when cells were allowed to express late phase genes (*vide infra*, Fig. 4*a*, lane 3). Although occurring in VH cells with delayed kinetics, these findings revealed the HCMV–MHC class I interaction that specifically affects the stability of the association of HLA heavy chains with β_2m (Beersma *et al.*, 1993) and differs from that described for MCMV (Del Val *et al.*, 1992). As found for MCMV, the viral mechanism is expressed as an E gene(s) function and results in deficient antigen presentation to $CD8^+$ T lymphocytes.

Cytokines restore antigen presentation to $CD8^+$ CTL during HCMV infection

HCMV infection *in vitro* impairs the ability of infected cells to present antigen via MHC class I molecules. However, *in vivo* $CD8^+$ T lymphocytes are effective in the control of HCMV (Riddell *et al.*, 1992). Lack of antigen presentation in the MHC class I pathway is not compatible with this finding. Therefore, we suspected that the block of antigen presentation by HCMV-infected cells *in vitro* does not reflect the situation *in vivo*. Distinct soluble factors can modulate several steps in the intracellular pathway of antigen processing and presentation (Wong *et al.*, 1983; Klar & Hämmerling, 1989;

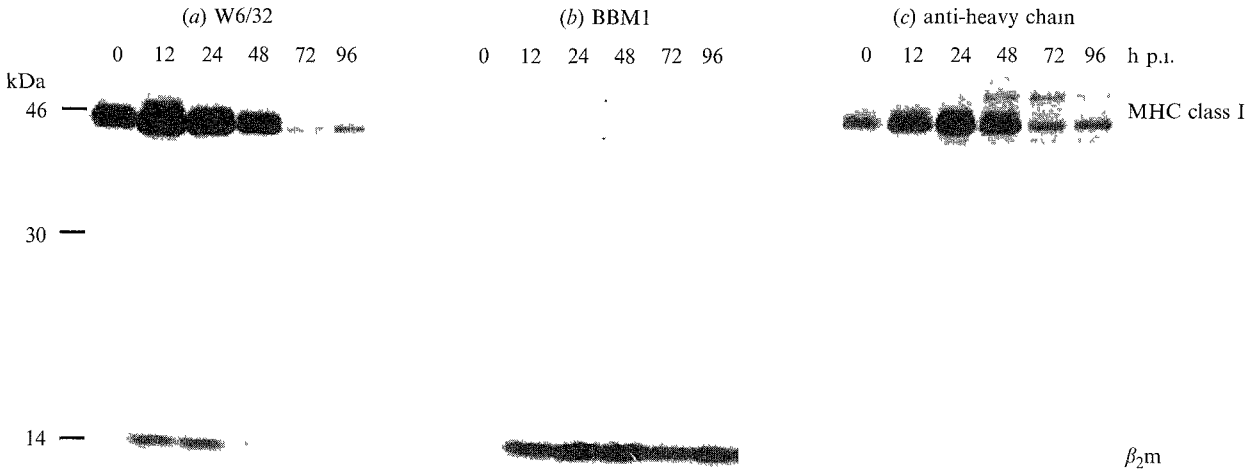

Fig. 2. Steady state levels of MHC class I complexes, of free β_2m and of free MHC heavy chains during HCMV infection After HCMV infection (m o.i 15) of VH cells for the time indicated, metabolic labelling with [^{35}S]methionine was performed for 90 min. The cell lysates were immunoprecipitated sequentially, first with MAb W6/32 (*a*) specific for assembled MHC class I complexes, then with MAb BBM1 (*b*) reacting with β_2m and finally with an anti-heavy chain MAb (*c*). Precipitates were separated on 12–15% gradient SDS–PAGE gels.

Ortiz-Navarrete *et al.*, 1991). To test whether the HCMV E gene effect can be lifted by such cytokines, IFN-γ was investigated as a candidate. VH cells infected for 72 h were exposed to 500 U IFN-γ/ml for different periods of time before infection and targets were analysed for antigen presentation. As shown in Fig. 3(*a*), IFN-γ rescued presentation of both HY and HLA-A2 antigen but with different kinetics. While the addition of IFN-γ at the same time as infection had no effect, pre-incubation for 24 h sufficed to restore HLA-A2 recognition while the HLA-A2-restricted presentation function to H-Y-specific CTL was not improved. Pretreatment for 48 h, however, fully restored presentation of both antigens compared to uninfected controls. This action of IFN-γ followed a dose-dependent relationship (Fig. 3*b*). Presentation of HLA-A2 responded at low concentrations of the cytokine while optimal presentation of mH peptides required 500 U/ml of the cytokine.

The ability of IFN-γ to restore presentation of HCMV-infected cells prompted us to test TNF-α and type I interferons (α, β), which have been reported to increase MHC class I expression (Collins *et al.*, 1986; Pestka *et al.*, 1987; Pfitzenmaier *et al.*, 1987). Fig. 3(*c*) shows that these factors, like IFN-γ, also restore MHC class I-dependent antigen presentation of HCMV-infected cells when pre-incubated for 48 h. In addition, TNF-α can cooperate in mH antigen presentation with IFN-γ (Fig. 3*d*). Finally, conditioned supernatants from HCMV-infected fibroblast cultures stimulate the presentation of mH antigens after pre-incubation of target cells and this effect was sensitive to anti-IFN-β antibodies (data not shown). From these experiments two conclusions were drawn: first, IFN-γ, TNF-α and type I interferons are able to compensate for the negative effect of HCMV on MHC class I presentation, and, second, that these cytokines cannot restore defective antigen presentation in cells that are already infected.

Overexpression of MHC class I molecules by IFN-γ provides ternary complexes for antigen presentation

IFN-γ displays multiple effects on both viral as well as cellular genes (Pestka *et al.*, 1987). It is able to enhance the expression of MHC class I and β_2m genes (Wong *et al.*, 1983). In MHC class I-deficient tumour cells that constitutively express unassembled heavy chains (Klar & Hämmerling, 1989; Sibille *et al.*, 1992) IFN-γ stimulates direct association of MHC class I heavy chains with β_2m. To decide by which mechanism IFN-γ lifts the negative viral effect on MHC class I complex formation, VH cells were pretreated with cytokines and infected for 72 h. Cells were metabolically labelled for 90 min and cellular lysates were sequentially subjected to quantitative immunoprecipitations by the MAb W6/32 which selectively precipitates assembled class I complexes. Fig. 4(*a*) shows a strong increase in steady state levels of β_2m-associated heavy chains in HCMV-infected cells pre-incubated with either IFN-γ, TNF-α or type I IFN, compared to untreated cells. The amount of recovered MHC class I complexes, however, did not reach the level for cytokine-conditioned mock-infected VH cells as indicated by the fainter β_2m bands of MHC complexes in HCMV-infected samples, showing that the inhibitory E gene function was still active. The pattern of viral proteins induced after IFN-γ pretreatment showed no

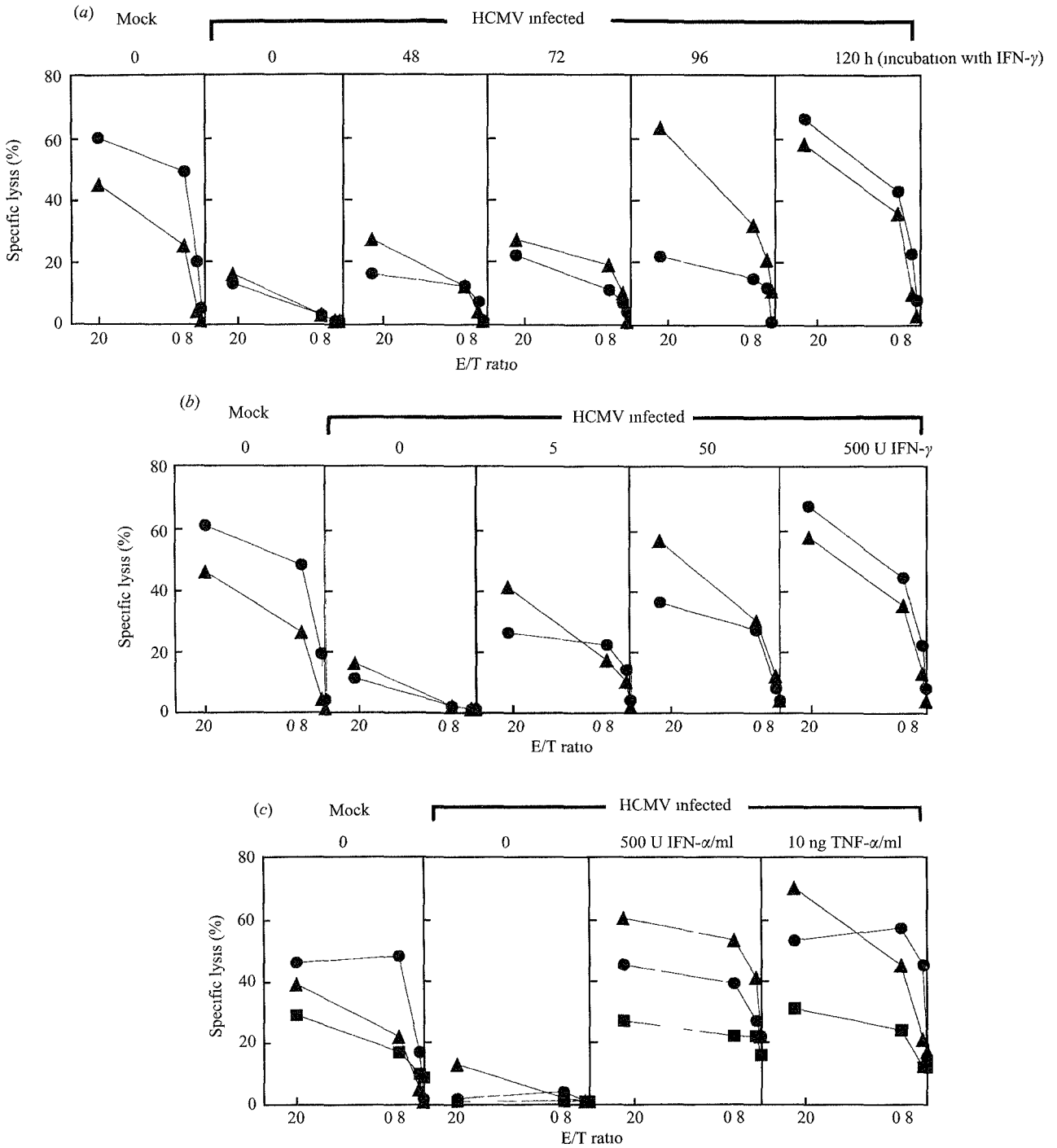

Fig 3 For legend see opposite

detectable differences when analysed with polyclonal human sera (data not shown) Immunoprecipitation of the viral 72 kDa early protein with a MAb (Fig 4*b*) revealed a moderate stimulation by interferons of HCMV early gene expression In addition, the 68 kDa late protein was present in HCMV-infected cells exposed to INF-γ or TNF-α (Fig 4*c*), suggesting that the activity on MHC class I gene expression and not an effect on viral genes is responsible for the restoration of antigen presentation

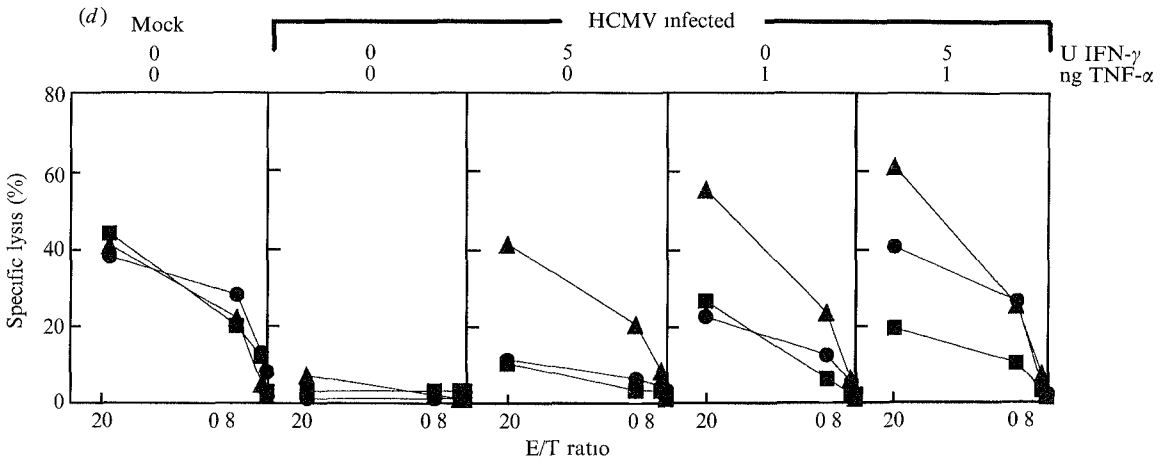

Fig 3 (*a*) Kinetics of restoration by cytokines of mH antigen and HLA-A2 presentation in HCMV-infected cells After various periods of pretreatment with IFN-γ (500 U/ml), VH3 cells were infected with HCMV (m o i 15) for 72 h before ^{51}Cr-release assay at the E/T ratios indicated IFN-γ was present until cells were harvested for the CTL assay ▲, CTL clone 3E7 recognizing HLA-A2 allo-antigen, ●, CTL clone 1R35 specific for HLA-A2 H-Y (*b*) For 48 h before HCMV infection (m o i 15), VH3 cells were exposed to titrated concentrations of IFN-γ, 72 h after infection, cells were tested with anti-HLA-A2 (▲) and anti-HLA-A2 H-Y (●) CTL (*c*) VH7 fibroblasts were pre-incubated with 500 U IFN-α/ml or 10 ng TNF-α/ml for 48 h before infection with HCMV (m o i 15) in the presence of cytokines for 72 h ▲, CTL clone 3E7 recognizing HLA-A2 allo-antigen, ●, CTL clone 1R35 specific for HLA-A2 H-Y, ■, CTL clone 5HO11 specific for HLA-A1 HA3 Targets were assayed with CTL at the E/T ratios indicated (*d*) IFN-γ and/or TNF-α were added to VH7 cells 48 h before HCMV infection (m o i 15) ▲, CTL clone 3E7 recognizing HLA-A2 allo-antigen, ●, CTL clone 1R35 specific for HLA-A2 H-Y, ■, CTL clone 5HO11 specific for HLA-A1 HA3

Stabilization of MHC class I molecules by IFN-γ

The failure to present endogenous peptide antigens during HCMV infection is associated with a down-regulation of assembled trimolecular class I complexes. IFN-γ increases steady state levels of MHC class I complexes in HCMV-infected cells. In order to analyse whether IFN-γ affected the half-life of nascent class I complexes during HCMV infection, pulse-chase experiments were performed. Class I complexes precipitated by MAb W6/32 were subjected to Endo H digestion. This enzyme preferentially cleaves immature *N*-linked oligosaccharides characteristic of glycoproteins that have not reached the *medial*-Golgi complex (Kobata, 1979). Further processing of the oligosaccharide chain by enzymes located in the *medial*-Golgi compartment renders the glycan structure resistant to Endo H cleavage and thus characterizes mature class I molecules (Kornfeld & Kornfeld, 1985). After a pulse with [^{35}S]methionine for 25 min, a significant proportion of the class I molecules synthesized in mock-infected cells and a smaller number in HCMV-infected cells already exhibited Endo H resistance. These molecules were represented by several slower migrating heavy chain bands while Endo H-susceptible class I glycoproteins showed a faster mobility resulting from Endo H cleavage (Fig. 5, left). After a 30 min chase nearly all class I complexes lost sensitivity to Endo H digestion in mock-treated VH cells, while class I molecules in HCMV-infected cells were processed to Endo H-resistant forms with slightly delayed kinetics. While in mock-infected VH cells a significant proportion of class I complexes was still stable after 270 min chase, the bulk of class I complexes in HCMV-infected cells disappeared within 90 min, including even those molecules that had matured to an Endo H-resistant phenotype. Compared to untreated cells, a strong induction of nascent class I complexes was noted in IFN-γ-treated cells (Fig. 5, right) resulting in an overexposure of this part of the autoradiograph. Notably, the majority of labelled class I molecules in HCMV-infected VH cells was still present after 270 min chase as in mock-infected cells. A shorter exposure of the film showed an almost comparable strong band for MHC class I heavy chains (data not shown). This observation indicates that IFN-γ can protect MHC class I complexes from destabilization by HCMV gene functions. The restoration of antigen presentation therefore consists of two events. The first is the enhanced expression of class I molecules. Secondly, MHC molecules loaded with peptides escape the viral interference, maintain stability as ternary complexes, and are transported to the cell surface.

Discussion

Previous studies have demonstrated that MHC class I molecules are downregulated by HCMV without reducing the mRNA levels of MHC class I gene products (Barnes & Grundy, 1992; Browne *et al.*, 1990; Warren *et al.*, 1994). This viral effect is associated with both a

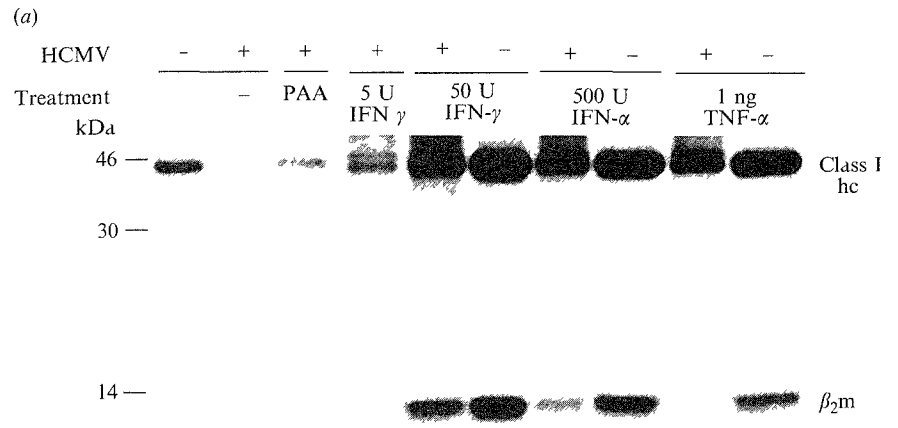

Fig 4 MHC class I complex formation in the presence of PAA, in cytokine pretreated HCMV-infected VH3 cells and in cytokine pretreated mock infected VH3 cells Cells were exposed to IFN γ, IFN-α or TNF-α at the concentrations indicated for 48 h before HCMV infection (m o i 15) 72 h after infection cells were metabolically labelled with [^{3}S]methionine for 90 min lysed and immunoprecipitated with an anti-actin MAb followed by MAb W6/32 (*a*) W6/32 precipitates were separated on 12–15% SDS PAGE gradient gels Transition of infection from E to late gene expression was prevented by the continuous presence of PAA (250 μg/ml) (*b c*) Cytokine pretreated HCMV infected cells express unchanged levels of HCMV 72 kDa E protein (*b*) and 68 kDa late protein (*c*) Cytokine treatment HCMV infection and metabolic labelling were performed as described in Methods HCMV proteins were immunoprecipitated sequentially by specific MAbs and precipitates subjected to gel electrophoresis on 15% SDS–PAGE gels

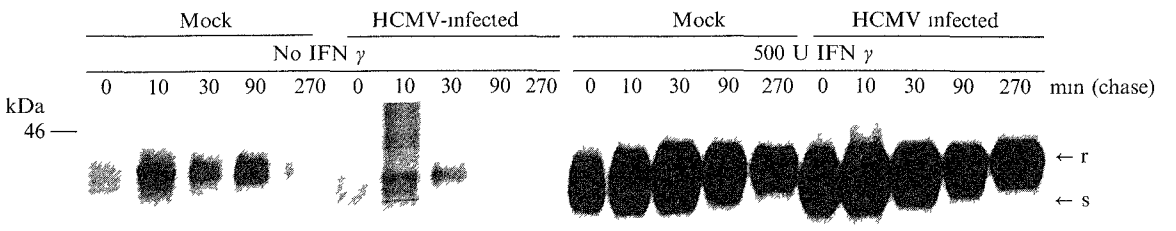

Fig 5 Stabilization of MHC class I complexes in IFN-γ pretreated HCMV infected cells Mock-infected or HCMV-infected VH cells were pre incubated with 500 U IFN γ/ml for 48 h as indicated Cells were pulse-labelled with [^{35}S]methionine for 25 min and then chased for the indicated times Immunoprecipitation was performed with MAb W6/32 Before gel electrophoresis all samples were digested with Endo H The bands resulting from enzymatic digestion are indicated s (for susceptible) or r (for resistant) Only the heavy chain region of the autoradiogram is shown

reduced stability of MHC class I heavy chains and decreased steady state levels of assembled ternary MHC class I complexes. This mechanism is thought to affect presentation and CTL recognition of HCMV peptide antigens. In this study, two aspects were addressed concerning the evasion of HCMV from $CD8^+$ T cell surveillance. First, we determined the extent and kinetics by which HCMV infection alters the endogenous peptide presentation function of infected cells. Second, the antagonistic influence of selected cytokines on the viral effect was studied.

In terms of MHC class I-restricted T cell recognition, different HCMV epitopes derived from nonstructural IE and structural late antigens are differentially sensitive to the viral effect (Gilbert *et al.*, 1993). The apparently selective effect on different viral antigens is thought not to be MHC linked. However, when only HCMV-specific CTL are used as a probe, such properties may render the functional analysis of the effect on MHC class I expression difficult. Therefore, we decided to analyse HCMV-infected cells with MHC class I-restricted T lymphocytes that detect not viral but cellular peptides that are constitutively presented. We find a stringent and general HCMV control of MHC class I antigen presentation.

As observed by others (Beersma *et al.*, 1993; Yamashita *et al.*, 1993) the viral inhibition of MHC class I complex formation is initiated during the E phase of infection and is most prominent at late times of the HCMV replication cycle. This event is paralleled by a decrease in mH peptide recognition by $CD8^+$ CTL. The presentation kinetics of individual peptide antigens was different; also the recognition of allo-HLA-A2 was susceptible to the viral effect. A reason for the sustained allo-reactivity could be that the $CD8^+$ T cell clone 3E7 not only recognizes trimolecular HLA-A2 complexes but also unassembled HLA-A2 heavy chains. Conversely, restoration of antigen presentation by IFN-γ and TNF-α occurred at lower cytokine concentrations for HLA-A2 allo-recognition compared to minor peptide recognition.

Although the occurrence of a defect in MHC class I complex formation caused by HCMV strain AD 169 correlated with the decrease of $CD8^+$ T cell recognition by VH skin fibroblasts, the kinetics of this event is much slower than reported recently (Beersma *et al.*, 1993; Warren *et al.*, 1994; Yamashita *et al.*, 1993). This discrepancy can be explained by the properties of different cell types. In human fetal lung fibroblasts Endo H cleavage patterns indicate that the breakdown of MHC class I complexes occurs primarily in a pre-Golgi compartment. As a consequence, the exit of class I complexes from the ER is drastically reduced, and a more rapid loss of $CD8^+$ T cell recognition is found compared with VH cells, which are human dermal fibroblasts (data not shown). The latter express higher amounts of MHC class I molecules and the viral interference not only affects Endo H-sensitive MHC molecules but also destabilizes more mature, Endo H-resistant class I complexes, suggesting that the viral effect can operate in or beyond the *cis*-Golgi compartment. These differences suggest that some cell types are more susceptible to the viral effect than others. Particular cell types may even be constitutively resistant, which would explain why HCMV-specific CTL are generated after infection (Borysiewicz *et al.*, 1988). On the other hand, our data imply that the $CD8^+$ T cell-mediated clearance of infection in fibroblast-like cells requires the regulating activities of cytokines like interferons and TNF-α. *In vivo* experiments with MCMV support this idea (Hengel *et al.*, 1994). The mode of interference with the MHC class I presentation pathway differs between mouse and human CMV. While the latter reduces the stability of class I molecules, the former has no influence on MHC class I complex formation and stability, but prevents the exit of peptide-charged molecules from the ER/*cis*-Golgi (Del Val *et al.*, 1992). In addition to this inhibition of nascent MHC class I molecules, an independently expressed MCMV gene function affects resident MHC class I complexes at the cell surface (Thäle *et al.*, 1995). Therefore, our analysis in HCMV-infected fibroblasts required us to determine the decrease of both nascent and resident class I molecules. The kinetics we observed by using $CD8^+$ CTL clones that detect cellular peptides does not indicate that HCMV interferes with resident MHC class molecules before effects on nascent MHC class I molecules are initiated.

In both HCMV and MCMV, IFN-γ functions as an antagonist that restores antigen presentation. Recently, the gene product of the herpes simplex virus (HSV) IE gene ICP 47 was identified as a viral factor that inhibits antigen presentation to $CD8^+$ T lymphocytes (York *et al.*, 1994) by blocking peptide transporters (Hill *et al.*, 1995; Früh *et al.*, 1995). Its expression as an IE gene product may contribute to the finding that minor peptide-specific CTL lysis is already affected at 5 h p.i. (Kuzushima *et al.*, 1990). It is not known whether this effect can also be overcome by IFN-γ. More than one cytokine can rescue antigen presentation. Their mode of action is complex, but it is a common feature that their effect results in an increased abundance of MHC class I complexes. Our experiments focused on MHC class I molecule biosynthesis and complex stability. Any positive effect of the cytokines on earlier steps like antigen processing, ER translocation of peptides or chaperonin expression should also increase the yield of final processed peptide and thus contribute to higher levels of ternary class I complexes (Klar & Hämmerling, 1989; Sibille *et al.*, 1992; Spies *et al.*, 1990).

The data presented here demonstrate a block on presentation of endogenous, non-viral antigens due to HCMV E gene functions and imply that there is also viral interference with the presentation of HCMV antigens Experimental evidence for this was presented by Gilbert *et al* (1993) who demonstrated weak $CD8^+$ CTL recognition of HCMV IE-specific T cell lines during permissive HCMV infection Antigen-specific mechanisms, like competition between viral peptide ligands and weaker cellular peptides (Kuzushima *et al*, 1990), and selective properties of individual HCMV antigens (Gilbert *et al*, 1993) have been discussed in order to explain the viral effect If the effect is indeed dominated by peptide competition, continuous presentation of certain self peptides, retained allo-A2 CTL recognition and stable expression of MHC class I complexes should be expected In addition, external loading of HCMV-infected fetal lung fibroblasts with synthetic antigenic peptide of the influenza virus matrix protein also failed to sensitize target cells for HLA-A2-restricted CTL lysis, indicating a critical loss of class I molecules (Warren *et al*, 1994)

In organ and bone marrow transplants, HCMV infection is associated with graft rejection and graft-versus-host disease (Miller *et al*, 1986, Reddehase *et al*, 1987, Rubin, 1990) Here we document that presentation is regulated by cytokines either produced by the HCMV-infected cell itself (i e type I interferon) or by antiviral T lymphocytes (IFN-γ, TNF-α) Inflammation in response to viral infection may represent a direct link that also increases the extent of mH peptide presentation in uninfected cells (De Bueger *et al*, 1992), leading to a break in T cell tolerance, allograft rejection and graft-versus-host disease (Krammer *et al*, 1984)

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