

## Lack of MHC class I complex expression has no effect on spread and control of cytomegalovirus infection *in vivo*

Bojan Polić,<sup>1</sup> Stipan Jonjić,<sup>1</sup> Ivica Pavić,<sup>1</sup> Irena Crnković,<sup>1</sup> Irena Zorica,<sup>1</sup> Hartmut Hengel,<sup>2</sup> Pero Lučin<sup>1</sup> and Ulrich H. Koszinowski<sup>2\*</sup>

<sup>1</sup> Department of Physiology and Immunology, Medical Faculty, University of Rijeka, B. Branchetta 20, 51000 Rijeka, Croatia and <sup>2</sup> Department of Virology, University of Heidelberg, Im Neuenheimer Feld 324, D-69120 Heidelberg, Germany

It has been claimed that MHC class I proteins serve as receptors for murine cytomegalovirus (MCMV) and that this interaction is the most important mechanism for virus entry in most cells. This claim is based on the observation that the MHC haplotype contributes to the susceptibility to cytomegalovirus (CMV) infection *in vivo*. Results from *in vitro* studies support the concept that stable expression of correctly folded MHC class I molecules contributes to infection, since the individual properties of MHC class I alleles, the availability of  $\beta_2$ -microglobulin ( $\beta_2m$ ) and also the degree of peptide charging of the MHC class I heavy chain  $\beta_2m$  heterodimers determined the infection phenotype of cell lines. To assess the biological relevance of proper MHC class I expression we investigated CMV infection in  $\beta_2m$ -deficient mice which fail to express ternary MHC

class I complexes and lack peripheral CD8<sup>+</sup> T lymphocytes. We found that organ virus titres and virus clearance kinetics were not altered in  $\beta_2m$  mutant mice. In addition, there was no indication of diminished virus propagation in  $\beta_2m^{-/-}$  embryonic fibroblasts.  $\beta_2m^{-/-}$  mice suffered from the lack of CD8<sup>+</sup> T lymphocytes that was partially compensated for by the function of CD4<sup>+</sup> T lymphocytes. An organ-specific anti-virus function of natural killer (NK) cells was observed, independent from the  $\beta_2m$  deletion. The immune control unique for salivary gland infection was maintained. From the data presented here, we confirm the role of MHC class I molecules in the immune surveillance of CMV infection but question the biological impact of correct MHC class I complexes for productive infection.

### Introduction

Published reports have suggested that the MHC proteins serve as receptors for murine cytomegalovirus (MCMV) (Price *et al.*, 1990; Wykes *et al.*, 1992, 1993; Price, 1994). The sensitivity of macrophages and certain cell lines to MCMV infection is associated with the MHC class I haplotype and correlates with the sensitivity of mice to MCMV infection *in vivo* (Chalmer *et al.*, 1977; Price *et al.*, 1990). Studies using mutant and transfected cell lines expressing various MHC class I genes indicated that all class I proteins confer sensitivity to MCMV infection, but D<sup>d</sup> and K<sup>b</sup> were the most efficient (Wykes *et al.*, 1993). MAbs directed against MHC class I molecules can inhibit the infection, which is indicative of the role of these molecules in virus entry (Price *et al.*, 1990; Wykes *et al.*, 1993).

The finding that human CMV (HCMV) is coated with  $\beta_2$ -microglobulin ( $\beta_2m$ ) led to the suggestion that HLA class I molecules may serve as receptors for HCMV by forming a bridge between the virion-surface-bound- $\beta_2m$  and MHC class I heavy chains on the cell surface (Grundy *et al.*, 1987*a,b*; McKeating *et al.*, 1987). This proposal was also supported by the observation that the infection with HCMV of permissive fibroblasts that were propagated in  $\beta_2m$ -free medium was impaired unless the cultures were supplemented with  $\beta_2m$  (Grundy *et al.*, 1987*b*). Subsequent reports on the interaction between HCMV and class I molecules, however, have argued against this hypothesis (Beersma *et al.*, 1991).

The absence of  $\beta_2m$  from the virion envelope of MCMV and the moderate enhancement of infection after the addition of  $\beta_2m$  to the culture medium (Wykes *et al.*, 1992), suggested that the function of  $\beta_2m$  could not be to provide a bridge between the virus and MHC class I molecules. A cell line in which reduced sensitivity to MCMV infection was associated with the lack of  $\beta_2m$  gene expression indicated that stable expression of

\* Author for correspondence. Fax +49 6221 563953.  
e-mail Koszino@novsrvi.piol.uni-heidelberg.de

correctly folded class I molecules was required for infection (Wykes *et al.*, 1993). Similarly, a requirement to expose ternary MHC class complexes at the cell surface for MCMV entry was demonstrated by using cells which lack a peptide transporter (TAP) gene and consequently fail to express peptide filled MHC class I complexes at the cell surface. Stabilization of empty class I molecules by cognate peptides improved MHC class I surface expression and infectivity with MCMV (Wykes *et al.*, 1993).

This report investigates the biological role of MHC class I molecules in MCMV infection by using  $\beta_2m$ -deficient mice which fail to express ternary MHC class I complexes, and which are consequently devoid of CD8<sup>+</sup> T lymphocytes (Zijlstra *et al.*, 1990). These mice thus represent a model that can be used to assess the virological importance of MHC class I expression for virus spread and tropism to different tissues, and allow the general resistance of MHC class I-deficient mice to CMV infection to be determined. Altogether, the results do not support a major virological role for correctly folded MHC class I molecules during MCMV infection.

## Methods

**Animals.**  $\beta_2m^{-/-}$  mice (129/Sv  $\times$  C57BL/6, H2<sup>b</sup>) were kindly provided by Rudolf Jaenisch (Whitehead Institute of Biomedical Research, Cambridge, USA) (Zijlstra *et al.*, 1989). The  $\beta_2m^{-/-}$ -genome-transmitting chimeras were mated with the C57BL/6 strain mice. In the F<sub>2</sub> generation, 25% of mice were homozygous for the  $\beta_2m^{-/-}$  genotype. The homozygous mice were then mated with their heterozygous littermates to obtain 50% of each genotype. The genotype of individual mice was determined by cytofluorometric screening of CD8<sup>+</sup> cells in the peripheral blood. Briefly, 100  $\mu$ l of blood from a tail vein was preserved from coagulation with 5  $\mu$ l of 0.5 M-EDTA (pH 8.0). FITC-conjugated MAbs to CD8 (rat anti-mouse Lyt-2 FITC; Becton Dickinson) were added to this suspension. After a 30 min incubation period at 4 °C, red blood cells were lysed by lysis solution (Becton Dickinson) and the remaining cells were analysed by a FACScan cytofluorometer (Becton Dickinson). Individual animals were considered homozygous if CD8<sup>+</sup> cells were completely absent from the sample.

**Viruses.** The Smith strain of MCMV (VR-194, ATCC) was propagated in mouse embryo fibroblasts and purified by ultracentrifugation on a 15% sucrose gradient, as described previously (Reddehase *et al.*, 1984). The mice were infected with  $1 \times 10^5$  p.f.u. of MCMV in the footpad.

Salivary gland isolates of MCMV (SGV) were obtained from  $\gamma$ -irradiated (6 Gy) MCMV-infected weaning mice of strain C57BL/6. Two weeks after infection, salivary glands were collected, homogenized and stored at -70 °C (Osborn & Walker, 1970).

**Depletion of lymphocyte subsets.** Depletion of lymphocyte subsets was performed as described previously (Cobbold *et al.*, 1984; Jonjić *et al.*, 1990). Briefly, purified MAbs were used for the elimination of CD4<sup>+</sup> (YTS 191.1.2.) (Cobbold *et al.*, 1984), CD8<sup>+</sup> (YTS 169.4.2.) (Cobbold *et al.*, 1984) or natural killer (NK) NK 1.1<sup>+</sup> (PK-136) (Koo *et al.*, 1986) cell subsets.

Groups of mice were injected with antibody for three successive days before infection, and then every fifth day until the end of the

experiment (Jonjić *et al.*, 1990). The efficacy of cell depletion was monitored by a two-colour cytofluorometric analysis of spleen and lymph node cells. Reagents used for FACS analysis were: anti-Lyt-2 FITC (Becton Dickinson), anti-L3T4 PE (Becton Dickinson), anti-NK 1.1 biotin (Pharmingen), anti-CD2 PE (Pharmingen) and streptavidin FITC (Becton Dickinson).

**Detection of MCMV in tissues.** Virus titres in tissues were determined by plaque assay (Reddehase *et al.*, 1985). The detection limit of the assay was extended to 1 p.f.u. per organ homogenate as described previously (Jonjić *et al.*, 1994). The statistical significance of the differences between experimental groups was determined by the Mann-Whitney exact rank sum test. Virus titres (X and Y) were considered significantly different for  $P(X \text{ versus } Y) < a = 0.05$  (one sided), where  $P$  is the observed probability value and  $a$  is a selected significance level.

**Detection of serum antibodies by ELISA.** Virus-specific antibodies from murine sera were detected by an ELISA as described previously (Jonjić *et al.*, 1988). Infected murine embryonic fibroblasts were used as a source of virus antigens. Isotype specificities of the antibodies were determined by using isotype-specific peroxidase-conjugated antibodies (Serotec). Concentrations of anti-virus antibodies were determined using the standard curves of isotype-specific immunoglobulins (Klein-Schneegans *et al.*, 1989).

**Virus neutralization assay.** A virus neutralization assay was used to determine the capacity of sera from infected  $\beta_2m^{+/-}$  and  $\beta_2m^{-/-}$  mice to neutralize MCMV (Reddehase *et al.*, 1994). Sera obtained at 22 weeks after infection from each group of mice were pooled and diluted in 96-well microtitre plates in minimal essential medium (MEM) (Gibco) containing 3% FCS in a volume of 0.1 ml. For controls, pooled sera from non-infected  $\beta_2m^{+/-}$  and  $\beta_2m^{-/-}$  mice were used. Tissue-culture-grown MCMV was diluted to obtain 200 p.f.u. per well and was added to the diluted sera. After an incubation period of 1 h (37 °C, 5% CO<sub>2</sub>) virus titres were determined by plaque assay.

**Infection of  $\beta_2m^{-/-}$  cells in vitro.** The productivity of MCMV infection was tested by an *in vitro* assay. Mouse embryonic fibroblasts (MEF) obtained from  $\beta_2m^{-/-}$  or  $\beta_2m^{+/-}$  mice were grown in 24-well plates (Greiner) in MEM (Gibco) supplemented with 3% FCS. Alternatively, sera derived from  $\beta_2m^{-/-}$  or  $\beta_2m^{+/-}$  mice were used instead of FCS. The expression of cell surface MHC class I molecules was monitored by flow cytometry (FACScan, Becton Dickinson). The cells were stained with MAbs either to K<sup>b</sup> (B8-24-3) (Kohler *et al.*, 1981) or D<sup>b</sup> molecules  $\beta 28-14-8S$  (Ozato *et al.*, 1980). Cells were infected with 100 p.f.u. per well. After incubation periods of 3, 4 and 5 days the plates were frozen and thawed to release intracellular virus, and samples from three wells for each incubation period were pooled and tested in the plaque assay.

## Results

### $\beta_2m^{-/-}$ mice control MCMV infection

With the exception of virus control in the salivary glands, the clearance of MCMV from tissues is a function of CD8<sup>+</sup> T lymphocytes (Jonjić *et al.*, 1989). After elimination of CD8<sup>+</sup> T lymphocytes in adult mice, CD4<sup>+</sup> T lymphocytes compensate for the deficit (Jonjić *et al.*, 1990) and clear the virus with an efficiency similar to that of fully immunocompetent mice. This compensatory function of the CD4<sup>+</sup> subset is not mediated by antibodies since CD8<sup>+</sup>-depleted and B-cell-deficient mice

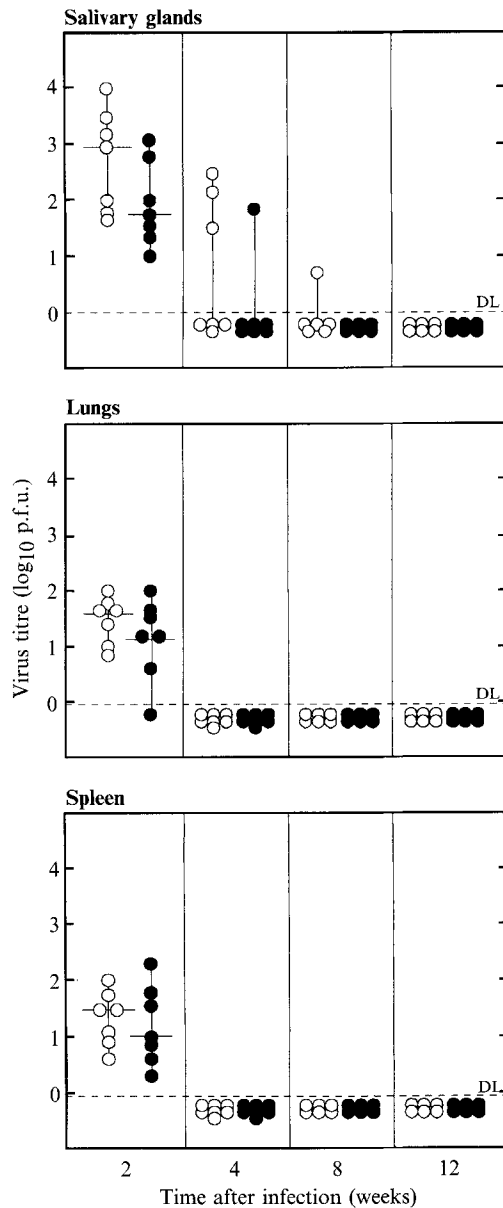


Fig. 1. Clearance kinetics in MCMV-infected  $\beta_2m^{-/-}$  and  $\beta_2m^{+/-}$  mice. Virus titres in salivary glands, lungs and spleen were compared at 2, 4, 8 and 12 weeks after infection with  $10^5$  p.f.u. of MCMV. Open circles ( $\circ$ ) and closed circles ( $\bullet$ ) represent individual virus titres in  $\beta_2m^{+/-}$  and  $\beta_2m^{-/-}$  mice, respectively. Median values ( $-$ ) and detection limits (DL) are shown.

are still able to control virus infection with clearance kinetics similar to that of their seropositive littermates (Jonjić *et al.*, 1994).

The result of primary infection with tissue-culture-grown MCMV in  $\beta_2m^{-/-}$  mice and their heterozygous littermates was investigated by measuring infectious virus titres in organs at different time points after infection with tissue-culture-grown MCMV. The absence of class I molecules and  $CD8^+$  T cells did not alter virus

titres, i.e. spread and clearance of MCMV, since no significant differences could be detected between the  $\beta_2m^{-/-}$  and heterozygous mice (Fig. 1). Note that small differences in organ titres observed at 2 weeks after infection, in this particular experiment, could not be reproduced (not shown). Thus, similar virus titres are reached in different organs in the presence or absence of correctly folded MHC class I molecules. Secondly, the delayed clearance of MCMV from the salivary gland can not be associated with an unusual MHC class I expression in this organ. Thirdly, the lack of  $CD8^+$  T cells can also be compensated for by other effector functions of the immune system when mice are born with a  $CD8^+$  T cell deficit.

#### Permissive infection of $\beta_2m^{-/-}$ cells with MCMV *in vitro*

Fibroblasts derived from  $\beta_2m^{-/-}$  mice and their heterozygous littermates ( $\beta_2m^{+/-}$ ) were tested for cell surface expression of class I molecules by flow cytometry (Fig. 2a). Almost no expression of class I molecules could be detected on  $\beta_2m^{-/-}$  cells whereas cells derived from mice heterozygous for the  $\beta_2m$  mutation were all class I positive. The same type of results was observed for K<sup>b</sup> molecules (data not shown).

The  $\beta_2m^{-/-}$  and  $\beta_2m^{+/-}$  fibroblasts were infected with MCMV and virus production *in vitro* was determined. No differences in the kinetics of virus production and virus yield were observed with either low (Fig. 2b) or high m.o.i. (not shown). Thus, the lack of ternary class I complexes had no influence on the infectivity and productive infection in permissive cells *in vitro*. MHC class I complexes are required neither for virus entry into the cell nor for the morphogenesis of progeny virus. To exclude the putative role of serum-derived  $\beta_2m$  on virus infection and productivity, fibroblasts were cultured in medium supplemented with 2% normal mouse serum derived from  $\beta_2m^{-/-}$  mice instead of FCS. As a control, serum derived from  $\beta_2m^{+/-}$  mice was also included (Fig. 2c). No differences in virus productivity were observed between the cultures containing either  $\beta_2m^{-/-}$  or  $\beta_2m^{+/-}$  sera.

#### Analysis of MCMV control in $\beta_2m^{-/-}$ mice

To study whether essential components of the immune response that control MCMV infection differ between  $\beta_2m^{-/-}$  and  $\beta_2m^{+/-}$  mice, animals were treated prior to infection with anti-CD4, anti-CD8 or anti-NK 1.1 MAb in order to deplete  $CD4^+$ ,  $CD8^+$  and NK lymphocytes, respectively. Virus titres in tissues were determined 2 weeks later. Depletion of the  $CD4^+$  subset compromised the capacity of  $\beta_2m^{-/-}$  mice as well as of  $\beta_2m^{+/-}$  mice to

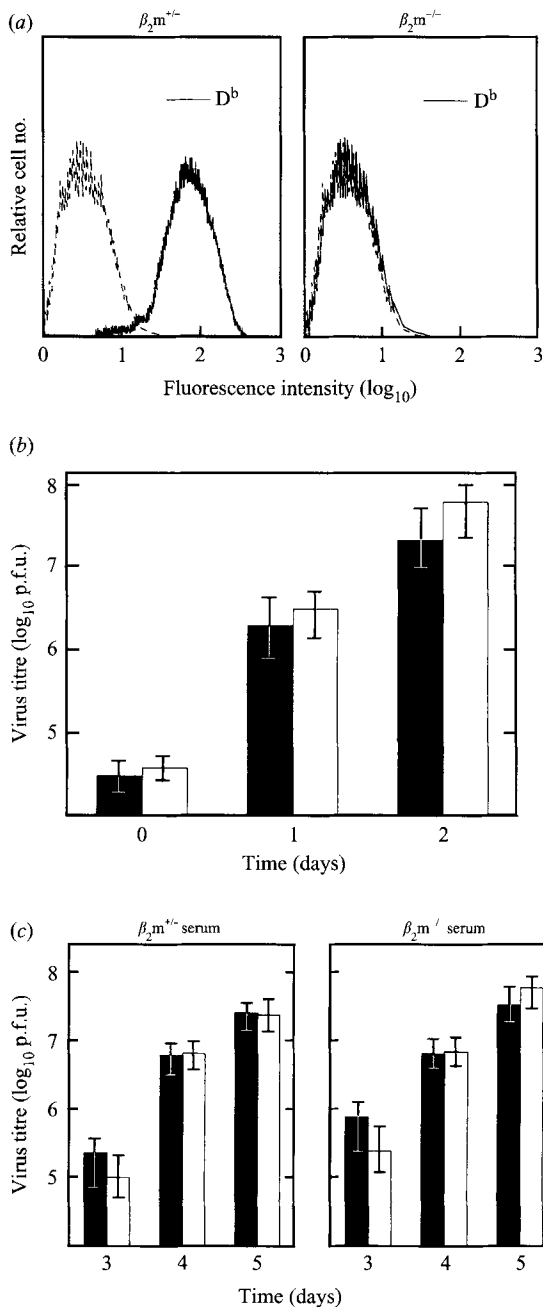


Fig. 2. MCMV propagation in cells deficient in MHC class I complexes. (a) Absence of cell surface expressed MHC class I molecules on MEF derived from  $\beta_2m^{-/-}$  mice. MEF were labelled with MAb 28-14-8S that recognizes D<sup>b</sup> molecules. Surface expression of class I molecules was measured by FACS using goat anti-mouse FITC-conjugated antibodies as a second reagent. (b) *In vitro* productivity of MCMV-infected MEFs derived from  $\beta_2m^{-/-}$  and  $\beta_2m^{+/-}$  mice. MEF were infected with 0.001 p.f.u. per cell without centrifugal enhancement. At 3, 4 and 5 days after infection the yield of virus was determined. Virus productivity in MEF derived from  $\beta_2m^{+/-}$  (unshaded bars) and  $\beta_2m^{-/-}$  (shaded bars) mice, respectively, cultured in medium supplemented with FCS are shown. The data represent the mean value and range of virus titres of triplicate cultures. (c) As for (b) except that the culture medium was supplemented with sera from  $\beta_2m^{-/-}$  or  $\beta_2m^{+/-}$  mice.

control the virus in salivary glands. A similar effect on virus titres in this tissue was observed after the depletion of NK cells, whereas the depletion of the CD8<sup>+</sup> subset had no effect on the salivary gland clearance, irrespective of the mouse strain used (Fig. 3).

Different results were obtained when the virus titres in other organs were tested. Depletion of the CD4<sup>+</sup> subset did not alter the virus content in the lungs and spleen of  $\beta_2m^{+/-}$  mice but resulted in significantly higher virus titres in  $\beta_2m^{-/-}$  mice. Apparently, the residual cells could not fully compensate for the lack of the CD4<sup>+</sup> subset in  $\beta_2m^{-/-}$  mice, whereas in  $\beta_2m^{+/-}$  mice this function was provided by CD8<sup>+</sup> T cells in the absence of CD4<sup>+</sup> T cells (Jonjić *et al.*, 1989). Altogether, the organ-specific differences in virus control are probably only due to the lack of CD8<sup>+</sup> T lymphocytes and not to the lack of correctly folded MHC molecules. This conclusion is based on similar findings after selective depletion of T cell subsets (Jonjić *et al.*, 1989, 1990).

Remarkably, anti-CD8 treatment showed a small, but reproducible effect on virus titres in the lungs of  $\beta_2m^{-/-}$  mice, indicating that some CD8<sup>+</sup> T cells are also present and can be functional in  $\beta_2m^{-/-}$  mice, as reported by other authors (Apasov & Sitkovsky, 1993; Correa *et al.*, 1992; Lehmann-Grube *et al.*, 1994). The depletion of NK cells had no detectable effect on the control of MCMV in lungs and spleen, suggesting that these cells are not essential for virus control in tissues other than those of the salivary glands under the experimental conditions used. In addition, the data show that the function of NK cells was not affected by MHC class I expression. These results indicate that NK cells do not represent the major compensatory immune mechanism in a CD8<sup>+</sup>-depleted host.

The virus-specific antibody response was diminished in  $\beta_2m^{-/-}$  mice compared to their heterozygous littermates (Fig. 4a). Levels of IgG isotypes were significantly reduced in  $\beta_2m$ -deficient animals, which was also found after vaccinia virus infection (Spriggs *et al.*, 1992). To assess whether this quantitative difference in IgG levels has functional consequences, an *in vitro* virus neutralization assay was performed (Fig. 4b). The neutralizing capacity of the serum derived from MCMV-primed  $\beta_2m^{-/-}$  mice was indeed lower by 2–3 log<sub>2</sub> steps when compared to the control serum derived from  $\beta_2m^{+/-}$  mice.

#### Control of virulent MCMV in $\beta_2m^{-/-}$ mice

Mice deficient in the MHC class I-restricted effector mechanism were able to control the infection with low virulence tissue-culture-grown virus. Their resistance to the infection with the virulent SGV isolate of MCMV was questionable. Therefore, groups of  $\beta_2m^{-/-}$  and  $\beta_2m^{+/-}$  mice were infected with various doses of SGV and the

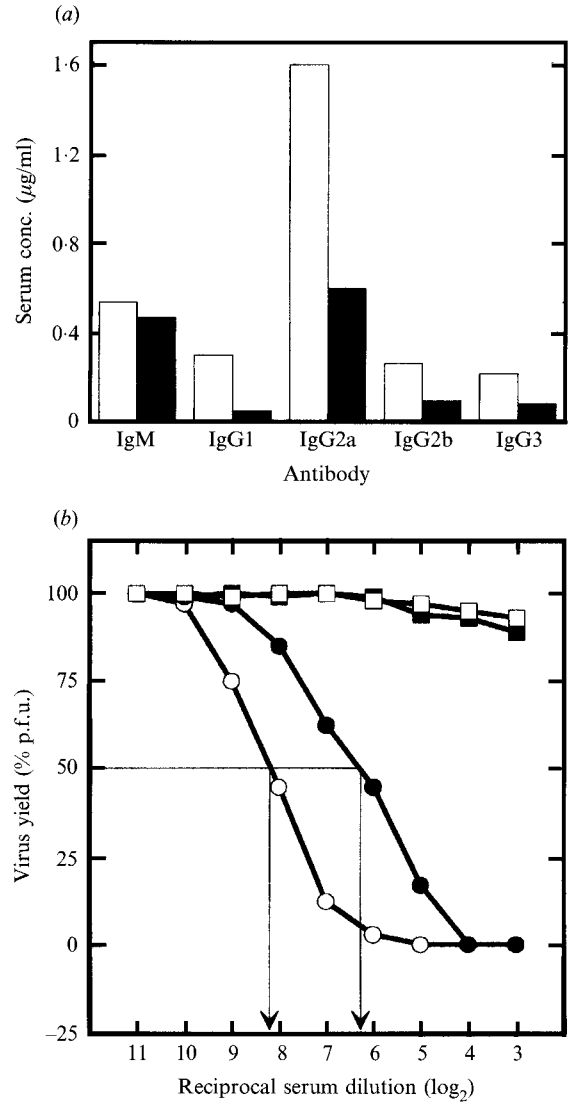
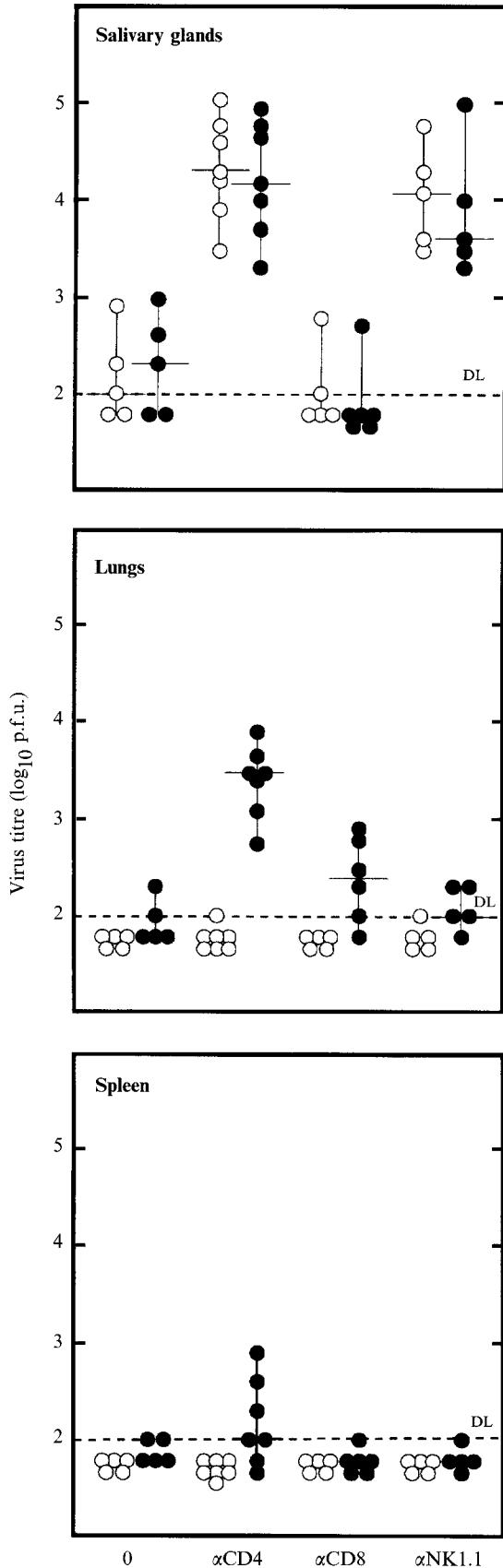


Fig. 4. Antibody responses in  $\beta_2m^{-/-}$  mice. (a) Comparison of specific IgG anti-virus antibody responses between  $\beta_2m^{-/-}$  mice and their heterozygous littermates. Specific anti-virus antibody concentrations in pooled sera of  $\beta_2m^{+/+}$  (unshaded bars) and  $\beta_2m^{-/-}$  (shaded bars) mice, respectively, are shown. Virus-specific antibodies were determined by ELISA. (b) Neutralization capacity of serum from  $\beta_2m^{-/-}$  mice. A comparison of the neutralization capacity of pooled sera from six immune  $\beta_2m^{+/+}$  (○) and six immune  $\beta_2m^{-/-}$  (●) mice is shown. A virus plaque reduction of 50% was used to determine the differences in neutralization titres. Non-immune sera from  $\beta_2m^{+/+}$  (□) and  $\beta_2m^{-/-}$  (■) mice were used as controls.

Fig. 3. Effect of lymphocyte subset depletion on virus clearance *in vivo*. Individual virus titres in salivary glands, lungs and spleen of  $\beta_2m^{+/+}$  (○) and  $\beta_2m^{-/-}$  (●) mice were compared after treatment with anti-CD4 (MAb YTS 191.1.2.), anti-CD8 (MAb YTS 169.4.2.) and anti-NK 1.1. (MAb PK 136) antibodies. The virus titres were determined 2 weeks after footpad infection with  $10^5$  p.f.u. of MCMV. Median values (—) and detection limit (DL) are shown. There is a significant difference in the virus titres in salivary glands between non-treated (both  $\beta_2m^{+/+}$  and  $\beta_2m^{-/-}$ ) and anti-CD4 and anti-NK treated animals ( $P < 0.001$ ).

Table 1. Susceptibilities of  $\beta_2m^{+/-}$  and  $\beta_2m^{-/-}$  mice to SGV infection\*

SGV (p.f.u.)	Survivors/total no. of mice	
	$\beta_2m^{+/-}$	$\beta_2m^{-/-}$
$5 \times 10^4$	6/6	5/11
$1 \times 10^5$	5/6	0/6
$2 \times 10^5$	3/6	0/6
$4 \times 10^5$	1/6	0/6

\* The mice were infected intraperitoneally.

survival of animals was monitored daily (Table 1).  $\beta_2m^{-/-}$  mice were more susceptible to SGV in comparison to age-matched controls. The LD<sub>50</sub> for the control group of mice was  $2 \times 10^5$  p.f.u. of SGV, in contrast to  $5 \times 10^4$  p.f.u. for  $\beta_2m^{-/-}$  mice. This compromised virus resistance in  $\beta_2m^{-/-}$  mice could be due to the lack of CD8<sup>+</sup> cells (Zijlstra *et al.*, 1990), a defective NK cell response (Liao *et al.*, 1991), the impaired antibody response, or a combination of these deficiencies. To assess the putative role of the CD8<sup>+</sup> subset in the control of acute infection with a virulent virus,  $\beta_2m^{+/-}$  mice were depleted of CD8<sup>+</sup> T lymphocytes and infected with half the LD<sub>50</sub> dose of SGV (Fig. 5). Most of the  $\beta_2m^{+/-}$  CD8-depleted animals succumbed to the infection with kinetics similar to those of  $\beta_2m^{-/-}$  mice, whereas all non-treated  $\beta_2m^{+/-}$  mice survived the infection. Thus, the presence or absence of class I-restricted CD8<sup>+</sup> T cells is considered to be responsible for the differences in the control of MCMV infection with a high dose of virulent virus.

## Discussion

The requirement of stable expression of correctly folded MHC class I proteins for MCMV infection was evaluated by using  $\beta_2m^{-/-}$ -deficient mice lacking ternary MHC class I complexes (Zijlstra *et al.*, 1989). We showed that for  $\beta_2m^{-/-}$  mice the virus titres in organs and the clearance kinetics were indistinguishable from those found in the heterozygous littermates. Furthermore, embryonic fibroblasts derived from the MHC class I negative mice showed similar infectibility and virus productivity to control cells with a high expression of trimolecular class I complexes. Collectively, these results do not support published *in vitro* data, which suggest that MHC class I molecules serve as receptors for MCMV (Wykes *et al.*, 1992, 1993) and conclude that MHC class I molecules represent the most important mechanism for infection in most murine cells (Price, 1994).

Based on published *in vitro* data we expected  $\beta_2m$ -deficient animals, and also cells derived from them, to exhibit a certain degree of resistance to MCMV infection.

Fibroblasts derived from  $\beta_2m$  negative animals, however, were equally permissive for infection as class I positive control cells. These results are not easily reconciled with the reported findings of a reduced sensitivity to MCMV infection by the RIE/TL8X.1 cell line when compared to its parental R1.1 cell line (Wykes *et al.*, 1993). The RIE/TL8X.1 cells synthesize class I heavy chains, but they do not produce  $\beta_2m$  (Williams *et al.*, 1989). Accordingly, class I heterodimers cannot be efficiently assembled and transported to the cell surface (Williams *et al.*, 1989; Rock *et al.*, 1991). One explanation for these opposing reports is that another molecule may serve as the dominant receptor for MCMV and that MHC class I molecules at best may modulate the function of this unknown receptor. The fact that certain cell lines lacking functional class I molecules can still be infected with MCMV suggests the existence of other modes of virus entry besides MHC class I molecules (Wykes *et al.*, 1993). Thus, the embryonic fibroblasts used in our study are heterogenous concerning the mode of MCMV entry, whereas R1.1 cells which may be deficient in the expression of the dominant virus receptor MHC class I molecules may represent a supportive mode for virus entry.

An alternative explanation is that the virus can use the free class I heavy chains as a receptor. Indeed, it was observed by Wykes *et al.* (1993) that D<sup>b</sup> transfected to  $\beta_2m$ -deficient RIE.TL8.X1 cells improves their infectibility. The possibility that free heavy chains can be expressed on the cell surface of  $\beta_2m^{-/-}$  embryonic fibroblasts cannot be excluded (Allen *et al.*, 1986; Hansen *et al.*, 1988). However, free heavy chains are usually detected only on cells with normal class I expression but not on the  $\beta_2m$ -deficient cell line (Williams *et al.*, 1989; Rock *et al.*, 1991). Therefore, it appears that these molecules arise from previously assembled class I molecules (Rock *et al.*, 1991). RMA-S cells have a mutation at the TAP site and form unstable complexes between heavy chains and  $\beta_2m$ , which reach the cell surface but dissociate rapidly at 37 °C (Ljunggren *et al.*, 1990). Thus, these cells do exhibit probably much more free class I heavy chains than cells from  $\beta_2m^{-/-}$  mice. Nevertheless, these cells are also quite resistant to MCMV infection, unless the MHC class I complex is stabilized (Wykes *et al.*, 1993). Collectively, the results do not support suggestions that the isolated heavy chains function as virus receptors (Price *et al.*, 1990; Wykes *et al.*, 1993).

A putative role for extracellular  $\beta_2m$  in MCMV infection was also addressed in this work and no effect was seen. Previously published data show that exogenously added  $\beta_2m$  facilitates MCMV infection *in vitro* (Wykes *et al.*, 1992). However, the rather modest effect on infectivity and the finding that  $\beta_2m$  was not associated

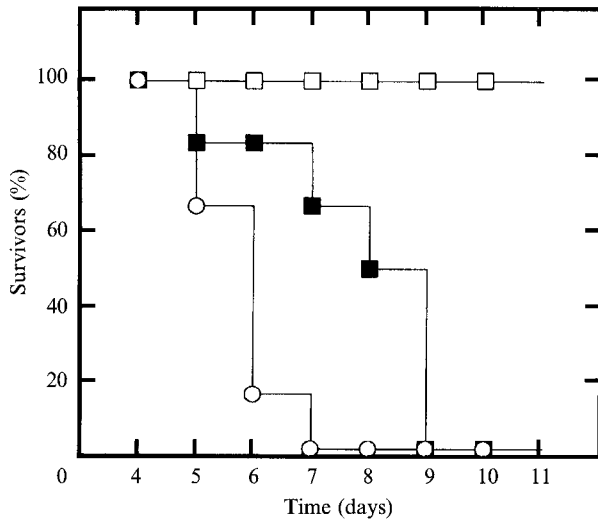


Fig. 5. Reduced resistance of  $\beta_2m^{-/-}$  mice to acute infection with virulent MCMV.  $\beta_2m^{-/-}$  mice ( $n = 9$ ) were infected with half the  $LD_{50}$  of SGV and depleted of  $CD8^+$  T cells ( $\circ$ ), or treated with PBS ( $\square$ ). The group of  $\beta_2m^{-/-}$  mice ( $n = 9$ ) was infected with SGV and treated with PBS ( $\blacksquare$ ). The survival of mice was monitored daily.

with the virion envelope led these authors to conclude that extracellular  $\beta_2m$  is of limited importance for MCMV infection. Using sera derived from  $\beta_2m^{-/-}$  and  $\beta_2m^{+/-}$  mice we also found no difference in productive MCMV infection *in vitro*. Altogether, the results of our study question the biological significance of MHC class I molecules in the process of MCMV entry and MCMV productivity.

The mouse lacking  $\beta_2m$  gene expression has been shown to be a suitable model for studying compensatory immune effector mechanisms which operate in the absence of MHC class I-restricted effector functions [reviewed by Raulat (1994)]. We previously reported that in the absence of  $CD8^+$  T cells the remaining functions of the immune system can compensate (Jonjić *et al.*, 1990; Lučin *et al.*, 1992; Pavić *et al.*, 1993). This mechanism of virus control in the  $CD8$ -deficient host is supported by  $CD4^+$  T lymphocytes (Jonjić *et al.*, 1990). However, this study indicates that the plasticity of immune effector functions is limited and that MHC class I-restricted  $CD8^+$  T cells represent the main mechanism for the survival of an acute infection with a high dose of virulent virus. This is in accordance with the data reported for the infection of  $\beta_2m^{-/-}$  mice with a virulent strain of influenza virus (Bender *et al.*, 1992; Eichelberger *et al.*, 1991). Apart from the lack of  $CD8^+$  cells, a defective antibody response could contribute to the enhanced sensitivity to SGV. However, this is unlikely since mice that are genetically deficient in the production of B cells are not more susceptible to acute MCMV infection (Jonjić *et al.*, 1994). In addition, the depletion of  $CD8^+$  T cells in

normal animals reduced their resistance to SGV to a level similar to that observed in  $\beta_2m^{-/-}$  animals.

The data confirm the organ-specific function of effector mechanisms. The virus infection in salivary glands, which also shows a distinct immune control in the immunocompetent host (Jonjić *et al.*, 1989, 1990), is controlled by mechanisms dependent on the  $CD4^+$  subset, and is irrespective of the presence of  $CD8^+$  T cells. It appears that NK cells contribute to virus clearance in this particular tissue and require the supportive function of  $CD4^+$  helper T cells. This NK cell function could be detected so far only in the salivary glands. There is no need to assume that the function of NK cells is defective in  $\beta_2m^{-/-}$  mice. It is known that the expression of MHC class I molecules on target cells correlates inversely with the resistance to lysis by NK cells (Ljunggren & Karre, 1990; Hoglund *et al.*, 1991a; Liao *et al.*, 1991). Published data indicate that  $\beta_2m^{-/-}$  mice exhibit an impaired NK cell response, in spite of the normal development of this subset (Hoglund *et al.*, 1991a,b; Denkers *et al.*, 1993). These mice failed to reject an allogeneic bone marrow transplant (Liao *et al.*, 1991), and also, the cytotoxicity against the NK tumour target YAC-1 and other target cells was reduced (Denkers *et al.*, 1993). Yet, NK cells in  $\beta_2m^{-/-}$  mice are still a considerable source of cytokines, especially interferon- $\gamma$  (IFN- $\gamma$ ), which plays a substantial role in the resolution of parasite infections (Denkers *et al.*, 1993). IFN- $\gamma$ , however, also plays a role in the control of MCMV infection (Lučin *et al.*, 1992; Hengel *et al.*, 1994).

To summarize, this study demonstrates that the absence of correct MHC class I complexes does not affect the capacity of MCMV to spread and replicate *in vivo* and *in vitro*.

This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 352) and project Ko 571/11 and by a BMFT grant (project KI 9308) and by the Ministry of Science of Croatia (Grant 3-01-169).

## References

- ALLEN, H., FRASER, J., FLYER, D., CALVIN, S. & FLAVELL, R. (1986). Beta-2 microglobulin is not required for cell surface expression of the murine class I histocompatibility antigen H-2D<sup>b</sup> or of a truncated D<sup>b</sup>. *Proceedings of the National Academy of Sciences, USA* **83**, 7447-7451.
- APASOV, S. & SITKOVSKY, M. (1993). Highly lytic  $CD8^+$ ,  $\alpha\beta$  T-cell receptor cytotoxic T cells with major histocompatibility complex (MHC) class I antigen-directed cytotoxicity in  $\beta_2$ -microglobulin, MHC class I-deficient mice. *Proceedings of the National Academy of Sciences, USA* **90**, 2837-2841.
- BEERSMA, M. F. C., WERTHEIM VAN DILLEN, P. M. E., GEELLEN, J. L. M. C. & FELTKAMP, T. E. W. (1991). Expression of HLA class I heavy chain and  $\beta_2$ -microglobulin does not affect human cytomegalovirus infectivity. *Journal of General Virology* **72**, 2757-2764.
- BENDER, B. S., CROGHAN, T., ZHANG, L. & SMALL, P. A., JR (1992). Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality

- after influenza virus challenge. *Journal of Experimental Medicine* **175**, 1143–1145.
- CHALMER, J. E., MACKENZIE, J. S. & STANLEY, N. F. (1977). Resistance to murine cytomegalovirus linked to the major histocompatibility complex of the mouse. *Journal of General Virology* **37**, 107–114.
- COBBOLD, S. P., JAYASURIYA, A., NASH, A., PROSPERO, T. D. & WALDMAN, H. (1984). Therapy with monoclonal antibodies by elimination of T-cell subsets *in vivo*. *Nature* **312**, 548–550.
- CORREA, I., BIX, M., LIAO, N.-S., ZIJLSTRA, M., JAENISCH, R. & RAULET, D. (1992). Most  $\gamma\delta$ T cells develop normally in  $\beta_2$ -microglobulin-deficient mice. *Proceedings of the National Academy of Sciences, USA* **89**, 653–657.
- DENKERS, E. Y., GAZZINELLI, R. T., MARTIN, D. & SHER, A. (1993). Emergence of NK1.1<sup>+</sup> cells as effectors of IFN- $\gamma$  dependent immunity to *Toxoplasma gondii* in MHC class I-deficient mice. *Journal of Experimental Medicine* **178**, 1465–1472.
- EICHELBERGER, M., ALLAN, W., ZIJLSTRA, M., JAENISCH, R. & DOHERTY, P. C. (1991). Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8<sup>+</sup> T cells. *Journal of Experimental Medicine* **174**, 875–880.
- GRUNDY, J. E., MCKEATING, J. A. & GRIFFITHS, P. D. (1987a). Cytomegalovirus strain AD169 binds  $\beta_2$ -microglobulin *in vitro* after release from cells. *Journal of General Virology* **68**, 777–784.
- GRUNDY, J. E., MCKEATING, J. A., WARD, P. J., SANDERSON, A. R. & GRIFFITHS, P. D. (1987b).  $\beta_2$ -microglobulin enhances the infectivity of cytomegalovirus and when bound to the virus enables class I HLA molecules to be used as a virus receptor. *Journal of General Virology* **68**, 793–803.
- HANSEN, T. H., MYERS, N. B. & LEE, D. R. (1988). Studies of two antigenic forms of Ld with disparate  $\beta_2$ -microglobulin associations suggests that  $\beta_2$ m facilitates the folding of the alpha 1 and alpha 2 domains during de novo synthesis. *Journal of Immunology* **140**, 3522–3527.
- HENGEL, H., LUČIN, P., JONJIĆ, S., RUPPERT, S. & KOSZINOWSKI, U. H. (1994). Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. *Journal of Virology* **68**, 289–297.
- HOGLUND, P., GLAS, R., OHLEN, C., LJUNGGREN, H.-G. & KARRE, K. (1991a). Alteration of the natural killer repertoire in H-2 transgenic mice, specificity of rapid lymphoma cell clearance determined by the H-2 phenotype of the target. *Journal of Experimental Medicine* **174**, 327–334.
- HOGLUND, P., OHLEN, C., CARBONE, E., FRANKSSON, L., LJUNGGREN, H.-G., LATOUR, A., KOLLER, B. & KARRE, K. (1991b). Recognition of  $\beta_2$ -microglobulin-negative ( $\beta_2$ m<sup>-</sup>) T-cell blasts by natural killer cells from normal but not from  $\beta_2$ m<sup>-</sup> mice, nonresponsiveness controlled by  $\beta_2$ m<sup>-</sup> bone marrow in chimeric mice. *Proceedings of the National Academy of Sciences, USA* **88**, 332–336.
- JONJIĆ, S., DEL VAL, M., KEIL, G. M., REDDEHASE, M. J. & KOSZINOWSKI, U. H. (1988). A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection. *Journal of Virology* **62**, 1653–1658.
- JONJIĆ, S., MUTTER, W., WEILAND, G., REDDEHASE, M. J. & KOSZINOWSKI, U. H. (1989). Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4-positive T-lymphocytes. *Journal of Experimental Medicine* **169**, 1199–1212.
- JONJIĆ, S., PAVIĆ, I., LUČIN, P., RUKAVINA, D. & KOSZINOWSKI, U. H. (1990). Efficacious control of cytomegalovirus infection after long-term depletion of CD8<sup>+</sup> T lymphocytes. *Journal of Virology* **64**, 5457–5465.
- JONJIĆ, S., PAVIĆ, I., POLIĆ, B., CRNKOVIĆ, I., LUČIN, P. & KOSZINOWSKI, U. H. (1994). Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. *Journal of Experimental Medicine* **179**, 1713–1717.
- KLEIN-SCHNEEGANS, A. S., GAVERIAUX, C., FONTENEAU, P. & LOOR, F. (1989). Indirect double sandwich ELISA for the specific and quantitative measurement of mouse IgM, IgA and IgG subclasses. *Journal of Immunological Methods* **119**, 117–125.
- KOHLER, G., LINDHAL, K. F. & NEUSSER, C. (1981). Characterization of a monoclonal anti-H-2Kb antibody. *Immune System* **2**, 202–208.
- KOO, G. C., DUMONT, F. J., TUTT, M., HACKETT, J. & KUMAR, V. (1986). The NK1.1-mouse, a model to study differentiation of murine NK cells. *Journal of Immunology* **137**, 3742–3747.
- LEHMANN-GRUBE, F., DRALLE, H., UNTERMÖHLEN, O. & LÖHLER, J. (1994). MHC class I molecule-restricted presentation of viral antigen in  $\beta_2$ m-microglobulin-deficient mice. *Journal of Immunology* **153**, 595–603.
- LIAO, N.-S., BIX, M., ZIJLSTRA, M., JAENISCH, R. & RAULET, D. (1991). MHC class I deficiency, susceptibility to natural killer (NK) cells and impaired NK activity. *Science* **253**, 199–202.
- LJUNGGREN, H.-G. & KARRE, K. (1990). In search of the 'missing self', MHC molecules and NK recognition. *Immunology Today* **11**, 237–243.
- LJUNGGREN, H.-G., STAM, N. J., OHLEN, C., NEEFFJES, J. J., HOGLUND, P., HEEMELS, M.-T., BASTIN, J., SCHUMACHER, T. N. M., TOWNSEND, A., KARRE, K. & PLOEGH, H. L. (1990). Empty MHC class I molecules come out in the cold. *Nature* **346**, 476–480.
- LUČIN, P., PAVIĆ, I., POLIĆ, B., JONJIĆ, S. & KOSZINOWSKI, U. H. (1992). Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. *Journal of Virology* **66**, 1977–1984.
- MCKEATING, J. A., GRIFFITHS, P. D. & GRUNDY, J. E. (1987). Cytomegalovirus in urine specimens has host  $\beta_2$ -microglobulin bound to the viral envelope: a mechanism of evading the host immune response? *Journal of General Virology* **68**, 785–792.
- OSBORN, J. E. & WALKER, D. L. (1970). Virulence and attenuation of murine cytomegalovirus. *Infection and Immunity* **3**, 228–236.
- OZATO, K., HANSEN, T. H. & SACHS, D. H. (1980). Monoclonal antibodies to mouse MHC antigens, antibodies to H-2Ld antigen, the products of a 3rd polymorphic locus of the mouse major histocompatibility complex. *Journal of Immunology* **125**, 2473–2477.
- PAVIĆ, I., POLIĆ, B., CRNKOVIĆ, I., LUČIN, P., JONJIĆ, S. & KOSZINOWSKI, U. H. (1993). Participation of endogenous tumour necrosis factor alpha in host resistance to cytomegalovirus infection. *Journal of General Virology* **74**, 2215–2223.
- PRICE, P. (1994). Are MHC proteins cellular receptors for CMV? *Immunology Today* **15**, 295–296.
- PRICE, P., GIBBONS, A. E. & SHELLAM, G. R. (1990). H-2 class I loci determine sensitivity to MCMV in macrophages and fibroblasts. *Immunogenetics* **32**, 20–26.
- RAULET, D. H. (1994). MHC class-I-deficient mice. *Advances in Immunology* **55**, 381–421.
- REDDEHASE, M. J., KEIL, G. M. & KOSZINOWSKI, U. H. (1984). The cytolytic T lymphocyte response to the murine cytomegalovirus. I. Distinct maturation stages of cytolytic T lymphocytes constitute the cellular immune response during acute infection of mice with the murine cytomegalovirus. *Journal of Immunology* **132**, 482–489.
- REDDEHASE, M. J., WEILAND, F., MUNCH, K., JONJIĆ, S., LUSKE, A. & KOSZINOWSKI, U. H. (1985). Interstitial murine cytomegalovirus pneumonia after irradiation, characterization of cells that limit viral replication during established infection of the lungs. *Journal of Virology* **55**, 264–273.
- REDDEHASE, M. J., BALTHESSEN, M., RAPP, M., JONJIĆ, S., PAVIĆ, I. & KOSZINOWSKI, U. H. (1994). The conditions of primary infection define the load of latent viral genome in organs and the risk of recurrent cytomegalovirus disease. *Journal of Experimental Medicine* **179**, 185–193.
- ROCK, K. L., GAMBLE, S., ROTHSTEIN, L., GRAMM, C. & BENACERRAF, B. (1991). Dissociation of  $\beta$ -2 microglobulin leads to the accumulation of a substantial pool of inactive class I MHC heavy chains on the cell surface. *Cell* **65**, 611–620.
- SPRIGGS, M. K., KOLLER, B. H., SATO, T., MORRISSEY, P. J., FANSLAW, W. C., SMITHIES, O., VOICE, R. F., WIDMER, M. B. & MALISZEWSKI, C. R. (1992).  $\beta_2$ -microglobulin-, CD8<sup>+</sup> T-cell-deficient mice survive inoculation with high doses of vaccinia virus and exhibit altered IgG responses. *Proceedings of the National Academy of Sciences, USA* **89**, 6070–6074.
- WILLIAMS, D. B., BARBER, B. H., FLAVELL, R. A. & ALLEN, H. (1989). Role of  $\beta_2$ -microglobulin in the intracellular transport and surface expression of murine class I molecules. *Journal of Immunology* **142**, 2796–2806.
- WYKES, M. N., PRICE, P. & SHELLAM, G. R. (1992). The effects of  $\beta_2$ -

- microglobulin on the infectivity of murine cytomegalovirus. *Archives of Virology* **123**, 59–72.
- WYKES, M. N., SHELLAM, G. F., MCCLUSKEY, J., KAST, W. M., DALLAS, P. B. & PRICE, P. (1993). Murine cytomegalovirus interacts with major histocompatibility complex class I molecules to establish cellular infection. *Journal of Virology* **67**, 4182–4189.
- ZIJLSTRA, M., LI, E., SAJJADI, F., SUBRAMANI, S. & JAENISCH, R. (1989). Germ-line transmission of a disrupted  $\beta_2$ -microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature* **342**, 435–438.
- ZIJLSTRA, M., BIX, M., SIMISTER, N. E., LORING, J. M., RAULET, D. H. & JAENISCH, R. (1990).  $\beta_2$ -microglobulin deficient mice lack CD4<sup>-</sup> 8<sup>+</sup> cytolytic T cells. *Nature* **344**, 742–746.

---

(Received 11 July 1995; Accepted 21 September 1995)