Recognition of measles virus-infected cells by CD8+ T cells depends on the H-2 molecule

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Introduction

Infection with measles virus (MV) leads to acute measles which is characterized by fever and the typical rash. Usually, the disease is overcome within two weeks, but often complications such as encephalitis, pneumonia and diarrhoea, as well as secondary infections due to the marked immune suppressive effect of MV arise (for a review, see Katz, 1995). During infection, B and T cell responses arise. Observations in patients with agammaglobulinaemia indicate that B cell responses against MV are dispensable (Bruton, 1953; Good & Zak, 1956). In contrast, patients with defects in the cellular immune response are not able to clear the virus (Nahmias et al., 1967). Although CD4+ as well as CD8+ T cells are generated during acute infection, their contribution to overcoming infection remains to be elucidated. The frequency of CD4+ T cells and their highly lytic potential has been emphasized (Long & Jacobson, 1989). MV is lymphotropic and, at least in tissue culture, very cytopathic. Therefore, the generation of CD8+ T cells with MV-infected stimulator cells has been problematic, although with recent technical advances, a number of reports have described CD8+ T cell responses (Nanan et al., 1995; Uytdehaag et al., 1994). However, the contribution of CD4+ versus CD8+ T cells in overcoming disease is not yet known. Indirect evidence for the importance of CD8+ T cells is the fact that a high frequency of CD8+ T cells has been found in children recovering from acute measles (van Binnendijk et al., 1990). In the mouse model of MV-induced encephalitis (MVE), susceptibility and resistance of various inbred and H-2 congenic mouse strains depend on their MHC haplotype (Niewiesk et al., 1993). In resistant H-2d mice (like BALB/c), the CD8+ T cell response is restricted by the Ld molecule and in susceptible H-2k mice (like C3H) this response is restricted by the Kk molecule. BALB/c mice generate good CD8+ T cell responses which result in lysis of target cells infected with MV as well as cells infected with a vaccinia virus recombinant expressing the nucleocapsid (N) protein of MV (vvN). In contrast, infection of C3H mice with MV and stimulation of spleen cells with MV-infected stimulators give CD8+ T cells restricted by Kk which do not recognize MV-infected target cells, but readily lyse target cells infected with vvN (Niewiesk et al., 1993). CD8+ T cells from both mouse strains recognize target cells pulsed with the respective peptide epitopes [aa 281–289 on the N protein for Ld (Beauverger et al., 1993); aa 52–59 and aa 81–88 for Kk (Beauverger et al., 1994)]. In this

H-2d mice are resistant to measles virus-induced encephalitis (MVE) and develop Ld-restricted CD8+ T cells which lyse target cells infected with measles virus or with a vaccinia virus recombinant expressing the nucleocapsid protein of measles virus (vvN). In contrast, H-2k mice are susceptible to MVE and generate CD8+ T cells which lyse target cells infected with vvN, but not those infected with MV. We were able to demonstrate that this difference is not due to a defect in the antigen processing machinery, but that Kk molecules require 100-fold more peptide to sensitize target cells for lysis by CTL. vvN replicates well in target cells and therefore enhances the level of epitope peptide available for CTL recognition. In contrast, MV infection is abortive in mouse cells and low levels of epitope peptide are produced. As Ld requires 100-fold less peptide than Kk to sensitize target cells for lysis, the low level of epitope peptide is enough to induce lysis by CD8+ T cells, whereas for recognition via Kk, increased synthesis of protein is required. We propose that the differences in peptide binding between the two H-2 molecules will have consequences for the kinetics of the generation of CD8+ T cells as well as the absolute numbers of CD8+ T cells generated.

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paper, we demonstrate that recognition of MV via K\(^k\)-
restricted CTL can be achieved if target cells allow replication,
thus increasing the number of N protein molecules for
processing. In comparison to L\(^k\), K\(^k\) requires 100-fold more of
its epitope peptide to sensitize target cells for lysis by CD8\(^+\)
T cells.

Methods

**Mice.** Mice were bought from Harlan Winkelmann (Borchem) and
were specific pathogen-free (company specification). Every 6–8 months,
the animals were checked for pathogens by serological examination.
Animals were kept in a barrier system with light negative pressure
(150 Pa) and a 12 h day (artificial light), and were fed and watered ad
libitum. The room temperature (24 ± 2°C) and the humidity (70 ± 5%)
were regulated by air conditioning. Mice were used between the ages of
6–18 weeks.

**Infection of mice.** For the generation of CTL mice were infected
intraperitoneally (i.p.) with MV (Edmonston strain) or
\(\times 10^6\) p.f.u. of a vaccinia virus recombinant (vvR) expressing the
nucleoprotein (NP) of influenza A virus strain PR8.

**Cells, antibodies and peptides.** RMA-S cells that were stably
transfected with H2-K\(^k\) were obtained from Günther Hämmerling,
Heidelberg, Germany and cells that were stably transfected with L\(^k\)
were from Hans-Georg Rammmensee, Tübingen, Germany. P815 cells that
were stably transfected with H2-K\(^k\) were from Denis Gerlier and Chantale
Rabourdin-Combe, Lyon, France, and C1R-K\(^k\) cells were from Peter
Cresswell, Yale, USA.

All cell lines were cultured in RPMI/10% FCS. L929, L-L\(4\) and NS20Y
cells were cultured in MEM/10% FCS.

The MAb 11-4.1, which is specific for the K\(^k\) molecule, was purchased
from Pharmingen. Hybridoma F227, produced at the Institute of Virology
and Immunobiology, secretes MV N protein-specific antibody (Liebert
et al., 1990).

Peptides LDLVRLLI (from MV Edmonston strain N protein aa 52–59,
K\(^k\)-restricted), VESPQGLI (MV N protein aa 81–88, K\(^k\)-restricted),
YPALGLHEF (MV N protein aa 281–289) were synthesized and purified.

**Generation and culture of CTL.** For the generation of CD8\(^+\)
t cells, spleen cells from mice were irradiated (300 Gy) and either infected
with MV (Edmonston strain) at an m.o.i. of 1 or incubated with peptide
(50 \(\mu\)g/10\(^7\) cells) at 37°C for 2 h. Infected stimulators (3 \(\times 10^5\)) were
incubated in an upright 50 ml flask containing 15 ml RPMI/10% FCS
and 1.5 \(\times 10^6\) spleen cells from infected animals. After 7 days, living cells
were separated on a Percoll gradient (10–70% in PBS) for 3 min
and stained with the N protein-specific antibody F227 and a donkey ant-

**Cytotoxic assay.** Cells (10\(^6\)) were labelled with 3\(^7\) MBq Na\(^{51}\)CrO\(_4\)
(DuPont) for 80 min at 37°C and washed twice. If used, peptide was
added during the labelling step (50 \(\mu\)g/10\(^6\) cells). Labelled target cells
(10\(^5\)) in a volume of 50 ml were added to various numbers of T cells in
100 ml volumes in U-bottomed microtitre plates. After 5 h incubation at
37°C, 75 µl supernatant was harvested and counted. The percentage
lysis was calculated as follows: 100 \(\times (\text{experimental} – \text{spontaneous release})/\text{(total} – \text{spontaneous release})\). If not otherwise indicated, target

Results

**General influence on antigen processing?**

We have verified that MV-infected H-2k mice generate
CD8\(^+\) T cells which lyse vvN-infected target cells, but not
MV-infected cells (Fig. 2a). Some large DNA viruses interfere
by various mechanisms with the recognition of antigen irrespective of its origin. Adenovirus, for example, inhibits
CTL recognition of influenza A virus after co-infection of
target cells (Yewdell et al., 1988). We tested whether MV was
able to inhibit lysis by influenza A virus-specific CTL after co-
infecion of target cells with influenza A virus and MV. MV

infection did not influence the activity of influenza A virus-specific CTL (data not shown) and co-infection did not lead to enhanced recognition of MV by CTL (data not shown). Sometimes, faulty recognition of target cells due to an inhibitory effect of a virus on antigen processing can be overcome by the addition of IFN-γ. This cytokine changes the recruitment of different subunits of the proteasome and enhances the level of expression of the TAP transporters and the MHC class I and II molecules (Hengel et al., 1994; Sibille et al., 1992). Incubation of MV-infected cells with IFN-γ led to an enhanced level of Kk expression (Fig. 1a), but not to recognition of MV-infected cells by CD8+ T cells (data not shown). We investigated whether the Kk molecule might be retained in the cell as a result of MV infection. Staining with the Kk-specific MAb 11-4.1 and subsequent analysis by flow cytometry indicated that MV-infected cells expressed more Kk than non-infected cells (Fig. 1a). In addition, MV-infected cells could be rendered susceptible to lysis by addition of peptides 52–59 or 81–88 (Fig. 1b) indicating that there is no retention of the Kk molecule. These data confirm that the non-recognition of MV-infected target cells by Kk-restricted CD8+ T cells is specific for MV and not an overall effect on the cellular machinery.

**Impairment of antigen processing or presentation?**

The lack of recognition could in principle be due to a defect in antigen presentation (due to Ld or Kk) or processing (due to various genes). In BALB/c mice, which are resistant to MVE, Ld-restricted CD8+ T cells are generated which lyse MV-infected P815 cells well (Niewiesk et al., 1993). To test whether the generation of CD8+ T cells was dependent on the genes of the BALB/c mouse background, we assayed CTL activity in BaC3F1 and BALB/k mice. In BaC3F1 mice, every molecule is encoded by an allele of each parent. In BALB/k mice (congenic to BALB/c), H-2 is of the k haplotype whereas all other molecules are imprinted by BALB/c genes. Interestingly enough, the CTL response seemed to be governed by the H-2 molecule only. Kk-restricted CD8+ T cells (either derived from BALB/k or BaC3F1 mice) lysed target cells (L929) infected with vvN, but did not recognize MV-infected target cells (data not shown). In contrast, Ld-restricted CTL (also derived from BaC3F1 mice) lysed MV-infected cells (P815) (Fig. 2b).

To investigate the importance of cell type on CTL recognition, we tested Ld-restricted CTL (from BALB/c mice) on L929 cells (H-2k) transfected with Ld (L-Ld) (Fig. 2b). These cells were lysed after infection with MV, although never quite as efficiently as P815 cells. In the reverse experiment, CD8+ T cells from C3H mice specific for either Kk-restricted epitope were tested on P815 cells transfected with Kk. Infection with vvN but not MV led to recognition of target cells (Fig. 2c). These data indicate that the non-recognition of target cells is either due to differences in presentation by Ld and Kk or differences in processing of the respective epitopes.

**No defect in antigen processing**

As the level of protein content might differ between vvN- and MV-infected target cells, we estimated the level of protein...
Protein synthesis is important for recognition by CD8+ T cells

So far the only difference in antigen recognition was the difference in lysis between MV- and vvN-infected cells. A major difference between vaccinia virus and MV is that vaccinia virus replicates well in murine cells whereas MV replicates abortively. Although MV is taken up by L929 cells and its presence can be detected by Western blotting, immune precipitation showed that N was produced in large quantities only in vaccinia virus-infected cells (Fig. 3b). To investigate the effect of MV replication on lysis by CD8+ T cells, we used the semi-permissive mouse neuroblastoma cell line NS20Y and a fully permissive human B cell line (C1R) transfected with the Kk molecule. After infection of NS20Y cells, no more than 15% of cells expressed the N protein (as measured by flow cytometry) and in persistently infected NS20Y, no more than 45% were N protein-positive (data not shown). The extent of N protein expression correlated with lysis seen by CD8+ T cells (Fig. 5a). Similarly, the percentage of N protein-positive C1R-Kk cells after infection correlated with lysis (Fig. 5b). These data showed that a higher amount of N protein resulted in lysis.

Good binding of peptides to the Kk molecule

If more protein (and therefore more peptide epitope) is needed to sensitize target cells for lysis then this indicates a low binding affinity of the epitope peptides to the Kk molecule. We tested the ability of MV-derived epitope peptides (52–59 and 81–88) to sensitize RMA-S cells transfected with Kk for lysis by CD8+ T cells in a competition assay (Feltkamp et al., 1995). RMA-S cells are not able to transport peptide from the cytosol to the endoplasmic reticulum due to expression of a truncated TAP 2 molecule (Powis et al., 1992). Therefore, addition of peptide from the outside is the only possibility for stabilization and expression of MHC class I molecules on the cell surface. As a positive control, we chose a Kk-restricted peptide epitope from the NP of influenza A virus (aa 50–57) (Brown et al., 1994). In competition experiments, the two peptides 52–59 and 81–88, derived from MV N protein, bound at least as well as the peptide derived from influenza A virus NP (Fig. 6). In a different approach, we tested the off-rate (i.e. the rate of dissociation of the epitope peptide from the H-2 molecule) of MV-derived peptides. Twenty hours after peptide-pulsing, target cells were compared to freshly pulsed targets in a 51Cr-release assay in order to estimate the stability of peptide binding to Kk. Target cells pulsed with MV peptides 52–59 and 81–88 were killed more efficiently than cells loaded with influenza A virus peptide 50–57 (data not shown). This indicates a low off-rate and good binding of MV epitope peptides to the Kk molecule.

Fig. 2. Expression of the Kk or Ld molecule rather than the nature of the target cell determines recognition of MV-infected cells. (a) CD8+ T cells from C3H mice infected with MV were tested on L929 cells infected with vvN (■), MV (▲) and vvNP (○). (b) CD8+ T cells from BALB/c mice were tested on cells expressing the Ld molecule (P815, E:T ratio 30:1; L929-Ld, E:T ratio 6:1). ■, vvNP; □, MV; ▼ vvN. (c) CD8+ T cells from C3H mice were tested on cells expressing the Kk molecule (L929, E:T ratio 30:1; P815-Kk, E:T ratio 6:1). ■, vvNP; □, MV; ▼ vvN.

present in infected cells. After infection under standard conditions, protein was present in both cell samples (as seen by Western blotting; Fig. 3a). Therefore, both samples should be recognized by Kk- and Ld-restricted CD8+ T cells. As Kk-restricted epitopes are not recognized, antigen processing of these epitopes might be impaired. We tried to address specific steps in the antigen processing machinery by expressing the full-length N protein, the Kk-restricted epitope alone (to circumvent faulty protein degradation by the proteasome), and the epitope with the HA-1 signal peptide (to circumvent putatively inefficient transport by the TAP molecules) in vvR (Fig. 4). Kk-restricted CD8+ T cells recognized all constructs equally well so that no specific step in antigen processing could be defined as the cause of the non-recognition of MV-infected cells.
CTL in MV encephalitis

Fig. 3. MV N protein is present in MV-infected cells, but protein synthesis occurs after infection with vvN. (a) To test for the presence of MV N protein in L929 cells after infection by Western blotting, cell lysates from uninfected cells (lane 1), cells infected with vvN (lane 2) and MV (lane 3) were blotted onto nylon membranes and stained with a human serum containing MV-specific antibodies. (b) To test for synthesis of MV N protein by immune precipitation, labelled cell lysates of uninfected cells (lane 1), cells infected with vvNP (from influenza A virus, 56 kDa; lane 2), vvN (from MV, 60 kDa; lane 3) or MV (lane 4) were precipitated with a human serum containing antibodies specific for MV and influenza A virus and separated by PAGE.

Fig. 4. No difference in recognition of vvN expressing the full-length N protein, the peptide epitope or the epitope bound to the HA-1 leader peptide. L929 cells were infected with vvNP, MV, vvN (expressing the full-length N protein), vv52–59 (expressing the 52–59 peptide) and vvL+52–59 (expressing the 52–59 peptide with a leader peptide). Recognition of the different viruses was determined with CTL specific for the 52–59 peptide. The same set of experiments was performed with the 81–88 peptide and the same results were obtained. ■, E:T ratio 3:1; □, E:T ratio 30:1.

**Kk requires more peptide than Ld in a sensitization assay**

As the binding of Kk-restricted peptides seemed to be comparable to that of the influenza A virus epitope (which is well recognized after infection), we compared the ability of the Kk- and Ld-restricted peptides to sensitize their respective target cells for lysis. A 100-fold more Kk-restricted peptide was required to sensitize L929 cells for lysis than Ld-restricted peptide was required for P815 cells (Fig. 7a). In order to ensure that this effect was not due to differences in cell type, we compared sensitization efficiencies on RMA-S cells transfected with either Kk or Ld. The results were the same and could be reproduced with different T cell lines (Fig. 7b).

**Discussion**

In most virus infections, CD8+ T cells play a dominant role in clearing virus infection and protecting against disease (Koszinowski et al., 1991; Kagi & Hengartner, 1996). For MVE, it has been reported that, in the resistant BALB/c mouse, depletion of CD4+ T cells leads to breakdown of resistance (Finke & Liebert, 1994). In the light of recent findings, these data have to be re-evaluated. Firstly, it has been shown that without CD4+ T cell help, the clonal burst of CD8+ T cells specific for poorly replicating viruses (like MV in the mouse) is greatly diminished (Zimmermann et al., 1997) and secondly, even primed CD8+ T cells are not able to clear virus from the brain without CD4+ T cell help (Stohlman et al., 1998). In MVE, CTL activity correlates with resistance and susceptibility of different mouse strains (Niewiesk et al., 1993). Ld-restricted CD8+ T cells (from resistant BALB/c mice) recognize both target cells infected with MV and target cells infected with vvN. Kk-restricted CTL (from susceptible C3H mice) recognize the N protein after expression via a vvR but not after infection of target cells with MV. Infection with adenovirus leads to non-recognition of target cells co-infected with influenza A virus (Yewdell et al., 1988). After co-infection of target cells with influenza A virus and MV, IFN-γ, which helps to overcome inefficient antigen processing in cells infected with murine cytomegalovirus (Hengel et al., 1994), does not affect the recognition of MV-infected cells. These data indicate that there is no general effect of MV gene products on the antigen processing machinery of the cell. As CD8+ T cells restricted by Ld lyse MV-infected cells [L-Ld, P815 (Niewiesk et al., 1993) or CIR-Ld (C. Neumeister & S. Niewiesk unpublished)], this phenomenon seems to be due to either the Kk-molecule itself or to the processing of the Kk
peptide epitopes. In contrast to herpesvirus (Hill et al., 1995; Del Val et al., 1992; Wiertz et al., 1996; Ahn et al., 1996) and adenovirus (Jeffries & Burgert, 1990) infection, our investigations have shown that the transport of peptide and binding to the Kk molecule is not impaired and that the Kk molecule is not retained within the cell after MV infection. Some viruses, e.g. adenovirus 12, impair the proteasome activity and the TAP-dependent transport of peptides (Rotem-Yehudar et al., 1996). However, if the MV N protein is expressed by a vvR it is recognized by Kk-restricted CTL. The recognition is the same whether the full-length protein, an epitope or an epitope with a leader peptide enabling transport into the endoplasmic reticulum is expressed. This means that there is no specific defect in antigen processing. The important difference between MV and vvN infection of mouse cells is the poor replication of MV. It is known that MV usually does not replicate in murine cells (Horvat et al., 1996; Niewiesk et al., 1997). Also primary mouse B cells, which express the human MV receptor CD46 via a transgene, replicate MV in vitro after mitogen activation but not in vivo (Horvat et al., 1996). In agreement with these data, we found that infection of L929 cells with MV leads to uptake of virus (as detected by Western blot), but not to replication (as shown by immune precipitation). In contrast, vvN does replicate in L929 cells and is recognized by CD8+ T cells. In the MV semi-permissive NS20Y mouse neuroblastoma cell line, CTL recognition is partly restored. In human C1R cells
transfected with Kₖ, MV replicates well and these cells are readily lysed by CTL. It therefore seems that the amount of newly synthesized protein (and therefore epitope peptide produced), rather than the processing, limits CTL recognition. This does not completely rule out variation in the processing efficiency of the Kₖ and L₅₄ epitopes as has been described for other pathogens (Antón et al., 1997; Sijts et al., 1996). Our data demonstrate that the binding affinity of the Kₖ-restricted epitopes is at least as good as the affinity of the Kₖ-restricted influenza A virus epitope. In addition, the amount of peptide required to sensitize L₅₄-expressing cells correlates with binding studies of peptide to L₅₄ (Kageyama et al., 1995). However, in comparison the amount of epitope peptide required to sensitize cells expressing L₅₄ for CTL recognition is 100-fold less than the amount required to sensitize cells expressing Kₖ. It should be noted that, although the number of MHC–peptide complexes as well as T cell receptor affinity may influence the competition and sensitization assay, these systems seem to correlate well with peptide binding (Feltkamp et al., 1995).

In mice, MV replicates in brain tissue after intracerebral (i.c.) infection, whereas i.p. administration leads to abortive replication. Still, CD₈⁺ T cells develop after i.c. as well as i.p. infection and can be stimulated with MV-infected stimulator cells in vitro (Niewiesk et al., 1993). MV replicates in brain tissue of H-2k mice (Fennelly et al., 1995) and therefore CD₈⁺ T cells should be able to recognize it and protect against encephalitis. In a previous report, it has been demonstrated that the lytic ability of CD₈⁺ T cells in vitro correlates with their protective capacity in vivo (Del Val et al., 1991). Similarly, we find that Kₖ-restricted CD₈⁺ T cells have poor lytic ability and this might explain their lack of protection in vivo. Another important point might be the kinetics of infection and development of CD₈⁺ T cells. C3H mice die after i.c. infection with MV after 5–9 days. In the lymphocytic chorio-meningitis virus system, it has been shown that CD₈⁺ T cells against a major epitope develop after 6 days with peak activity on day 8, whereas CD₈⁺ T cells against a minor epitope were seen on day 8 with peak activity on day 10 (Weidt et al., 1998). Therefore, a delayed CD₈⁺ T cell response might occur too late to exert a protective effect.

In summary, we have shown that the difference in the CTL response in C3H mice susceptible to MVE and BALB/c mice resistant to MVE depends on the amount of newly synthesized viral protein and the binding abilities of the H-2 molecules.

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