Differential processing and presentation of the H-2D\textsuperscript{b}-restricted epitope from two different strains of influenza virus nucleoprotein

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The influenza virus strains A/NT/60/68 and A/PR/8/34 both have an immunodominant D\textsuperscript{b}-restricted epitope in their nucleoprotein (NP) at amino acid residues 366–374, with two amino acid differences between the epitopes. Cross-reactive cytotoxic T lymphocytes (CTLs) were generated by priming mice with the influenza virus A/NT/60/68 NP and restimulating \textit{in vitro} with influenza virus A/PR/8/34. CTLs that gave high levels of specific lysis recognized target cells infected with either strain of influenza virus with similar efficiency. Surprisingly, when target cells were infected with recombinant vaccinia viruses (VV) expressing the two different NPs, presentation of the D\textsuperscript{b}-restricted epitope from the A/NT/60/68 NP was extremely poor, whereas presentation of the equivalent epitope from the A/PR/8/34 NP was as efficient as in influenza virus-infected cells. This difference was observed in spite of the fact that the two NP sequences show 94\% identity at the amino acid sequence level. Experiments with additional cross-reactive CTL cell lines which recognized target cells less efficiently revealed a similar difference in presentation between the two NP epitopes in influenza virus-infected cells and showed a difference in the efficiency of presentation of the D\textsuperscript{b}-restricted epitope from the two NP molecules independent of VV infection. The results show that two equivalent epitopes in highly similar proteins are processed with very different efficiency, even though they are both immunodominant epitopes. They also suggest that the previously described inhibition of antigen presentation by VV is a general, non-specific effect, which is more apparent for epitopes that are processed and presented less efficiently.

Introduction

CD8\textsuperscript{+} cytotoxic T lymphocytes (CTLs) recognize short peptides, usually 8–10 amino acids long, presented at the cell surface by major histocompatibility complex (MHC) class I molecules. These peptides can be derived from intracellular pathogens such as viruses, tumour-specific proteins or endogenous self-proteins and are thought to be processed from intact polypeptides by proteasomes, which are abundant multi-subunit proteases. CTLs are an important part of the immune response for the defence against many virus infections and, consequently, during their co-evolution with the host’s immune system, certain animal viruses have acquired specific mechanisms with which they evade presentation by MHC class I molecules. For example, human cytomegalovirus encodes a gene, \textit{UL51}, whose product causes the rapid degradation of newly synthesized MHC class I molecules (Wiertz \textit{et al.}, 1996), whereas herpes simplex virus type I encodes a gene product, ICP47, which blocks the transport of peptides from the cytosol into the endoplasmic reticulum for association with MHC class I molecules (Hill \textit{et al.}, 1995). There are now many different examples of virus interference with the MHC class I presentation pathway (Miller & Sedmak, 1999).

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Vaccinia virus (VV), the prototypic orthopoxvirus, has a double-stranded DNA genome of 191 kb and replicates in the cell cytoplasm. The virus has been used extensively as an expression vector to study antigen presentation, but it interferes with the presentation of some CTL epitopes. This effect was first described with a recombinant VV expressing influenza virus haemagglutinin (HA) in which the presentation of HA epitopes to CTLs was inhibited during the late phase of infection (Coupar et al., 1986). Subsequently, we showed that the presentation of certain other influenza virus epitopes to CTLs is also inhibited by VV infection in murine cells and that the blockage is present during both the early and the late phases of infection, although the blockage is more extensive at late times (Townsend et al., 1988; Gould et al., 1991; Cossins et al., 1993). The inhibitory effect of VV is limited neither to a particular epitope nor to presentation by a particular MHC class I molecule.

Because the presentation of only certain epitopes is affected, the question arises as to whether or not this actually represents a mechanism of virus evasion of the immune response. It was proposed previously that the mechanism of interference with antigen presentation by VV is at the level of proteolytic processing of antigen (Townsend et al., 1988). This hypothesis is supported by the observation that the inhibitory effect can be overcome by expression of rapidly degraded forms of the antigen which are processed differently, such as a ubiquitin–nucleoprotein (NP) fusion protein (Townsend et al., 1988), or by redirecting HA into the cytosol by deletion of the amino-terminal signal sequence (Townsend et al., 1988; Gould et al., 1991). The subsequent identification of VV proteins with amino acid similarity to serine protease inhibitors (serpins) led to the suggestion that they may be involved in the inhibition of antigen proteolysis (Smith et al., 1989). However, no evidence for this hypothesis could be found when it was tested experimentally (Blake et al., 1995) and the mechanism of interference with antigen presentation by VV remains ill-defined.

In this study, we have investigated the presentation of the well-characterized D\(^b\)-restricted epitope in influenza virus NP using CTLs that are cross-reactive for two strains of influenza virus. Surprisingly, VV infection had very different effects on the presentation of this epitope from the two different strains of influenza virus NP. This was shown to be due to intrinsic differences in the efficiency of processing and presentation of the two D\(^b\)-restricted NP epitopes, even in the absence of VV infection. Therefore, the differential effects of VV infection are most likely to be due to a general decrease in antigen presentation, which has a more significant effect on epitopes that are presented less efficiently.

Methods

- **Influenza viruses.** Influenza viruses A/PR/8/34 (Mount Sinai strain, H1 subtype) and A/NT/60/68 (H3 subtype) were grown in the allantoic sacs of 11-day-old embryonated chicken eggs and used as infectious allantoic fluid. Dilutions of allantoic fluid were assayed for virus by agglutination of sheep red blood cells.

- **Synthetic peptides.** The synthetic peptides ASNENMETM and ASNENMDAM, corresponding to the D\(^b\)-restricted NP epitope of influenza A/PR/8/34 and A/NT/60/68, respectively, were purchased from GENOSYS Biotechnologies at a grade of greater than 95% purity. The peptides were dissolved in RPMI-1040 medium for use in assays.

- **Recombinant VV.** The Western Reserve (WR) strain recombinant viruses Ub-Arg-NP-VAC (Townsend et al., 1988), NT60NP-VAC (Townsend et al., 1988), PR8NP-VAC (Smith et al., 1987) and a recombinant of the modified virus Ankara (MVA) strain MVA.NP, which expresses the A/NT/60/68 NP (Hanke et al., 1998; Schneider et al., 1998), have all been described previously. All four recombinant viruses use the 7.5 KDa VV promoter for expression. MVA.pSC11, a negative control virus, was generated using the shuttle plasmid pSC11 (Chakrabarti et al., 1985) without a cloned insert by standard methods (Hanke et al., 1998).

- **Generation of cross-reactive NP-specific CTL cell lines and clones.** Female C57BL/6 (H-2\(^b\)) and CBA/Ca mice (H-2\(^k\)), 8–12 weeks old, were obtained from the specific-pathogen-free mouse-breeding unit (Sir William Dunn School of Pathology, University of Oxford, UK) or purchased from Harlan UK. Mice were immunized by intravenous injection of 10\(^7\) p.f.u. of recombinant VV Ub-Arg-NP-VAC (Townsend et al., 1988) for C57BL/6 mice and PR8NP-VAC (Smith et al., 1987) for CBA/Ca mice. Two weeks later, spleens were removed. Prepared spleen cells were then restimulated in vitro by using influenza A/PR/8/34 virus-infected syngeneic feeder spleen cells, as described previously (Gould et al., 1991). The effector cells were then restimulated with antigen at weekly intervals in the same way, and after 3 weeks of culture in vitro, human recombinant interleukin 2 (rIL-2) (Cetus) was added to a final concentration of 10 U/ml. CTL clones were established by limiting dilution to 0.5 cells per well in 96-well dishes. Actively growing clones were expanded into 24-well dishes and later into flasks, with weekly restimulation as described above. Effector cells were used in chromium-release assays on day 4 or 5 after restimulation with antigen.

- **Cytotoxic assay.** A standard 5 h \(^{51}\)Cr-release assay was used with modifications for the use of adherent target cell lines, as described previously (Townsend et al., 1984). The target cell lines used were L-D\(^b\) (K\(^+\)D\(^b\)D\(^b\)) (Townsend et al., 1985) and EL-4 (K\(^d\)D\(^b\)). Virus infections of target cells, the use of synthetic peptides and the calculation of the percentage of specific lysis were all described previously (Gould et al., 1991). Briefly, target cells (4 \times 10\(^5\)) were either left uninfected or infected with 10 p.f.u. per cell of either VV or influenza virus and labelled with \(^{51}\)Cr for 90 min. After two washes, target cells were left at 37 °C for 2 h to allow antigen expression, washed twice more and plated out in the assay. Each point was measured in duplicate against quadruplicate controls and average values are shown in all of the figures. All experiments were repeated at least twice, with similar results. Synchronous \(^{51}\)Cr-release was less than 23% in all experiments.

Results

**Difference in the recognition of A/NT/60/68 and A/PR/8/34 VV-expressed NPs (NP-VACs) by cross-reactive CTLs**

H-2D\(^b\)-restricted CTL cell lines recognizing the NP of both influenza virus strains, A/NT/60/68 and A/PR/8/34, were generated by priming H-2\(^b\) mice with a recombinant VV expressing a rapidly degraded form of the A/NT/60/68 NP, designated Ub-Arg-NP-VAC (Townsend et al., 1988), and
restimulation in vitro with influenza virus A/PR/8/34-infected feeder cells. Fig. 1 shows a chromium-release assay using such a line of effector cells which had been restimulated four times. CTLs efficiently recognized target cells that had been infected with influenza virus A/NT/60/68 or A/PR/8/34 or with PR8NP-VAC, but were unable to recognize cells infected with NT60NP-VAC (Fig. 1). Therefore, CTLs were cross-reactive for the two different NPs, but were unable to recognize the A/NT/60/68 NP when expressed by a recombinant VV, although they were able to recognize PR8NP-VAC-infected cells efficiently. As expected, these polyclonal CTLs recognized the D^b^-restricted epitope NP (amino acids 366–374) of both strains of virus, as shown by incubating the target cells with the respective synthetic peptides (data not shown and see Fig. 3).

Cross-reactive CTL clones discriminate between A/NT/60/68 and A/PR/8/34 NP-VACs

There were several possible explanations for the difference in recognition between the two NP-VACs, related either to the CTLs used in the cytotoxicity assay or to antigen processing and presentation in the target cells. Initial experiments were carried out using polyclonal cell lines and although they were clearly cross-reactive, there may have been some characteristic of these cell lines that favoured the recognition of the A/PR/8/34 NP epitope. To investigate this possibility, CTL clones were generated by limiting dilution of a polyclonal cell line and, after expansion, 12 individual clones were tested in a chromium-release assay with target cells that had been infected with either PR8NP-VAC or NT60NP-VAC (Fig. 2). The pattern of lysis was the same as that observed with the polyclonal CTL cell line, and for each clone recognition of the target cells infected with PR8NP-VAC was much more efficient than for NT60NP-VAC-infected cells (Fig. 2). Although it was possible that all 12 of the clones were derived from a common precursor, this was highly unlikely. The difference in efficiency of recognition was not simply due to a difference in the affinity of the T-cell receptor for the two different peptide epitopes as a titration of the two synthetic peptide epitopes with CTL clone 3 gave the same dose–response curves (Fig. 3).

The above results suggested that the differential recognition of the D^b^-restricted epitope in the two strains of NP in VV-infected cells was a result of differences in processing and presentation in the target cells. CTL clone 3 was used in additional chromium-release assays, and we confirmed that this clone was cross-reactive for cells infected with the two strains of influenza virus, but did not recognize target cells infected with NT60NP-VAC (Fig. 4A). There was no doubt that the target cells had been infected and were expressing the A/NT/60/68 NP because the same target cells were efficiently recognized by CTLs that were specific for the K^k^-restricted epitope in NP at amino acids 50–57 (Fig. 4B). The results showed clearly that the WR strain of VV inhibited the presentation of the D^b^-epitope in the A/NT/60/68 NP, but not in the A/PR/8/34 NP.

VV blocking effect is present using MVA

The MVA strain has become increasingly used for the generation of recombinant VV, largely because of its safety advantages and also because it is a good candidate for human vaccines. MVA has a significant number of changes, including deletions in its genome, compared with the WR strain (Antoine et al., 1998) and, therefore, we wished to test if the apparent blocking effect on antigen presentation was still present in MVA as a possible method of mapping any VV genes responsible for the effect. The presentation of the D^b^-restricted epitope from the A/NT/60/68 NP was assayed in MVA.NP-infected target cells using CTL clone 3 (Fig. 5A). The results were the same as those for the WR strain and, again, presentation of the D^b^-restricted epitope in the A/NT/60/68 NP was severely inhibited (Fig. 5A), although the same target cells were recognized efficiently by specific K^k^-restricted CTLs (Fig. 5B). Therefore, the apparent inhibitory effect on antigen presentation was present using both the WR and the MVA strains of VV.

Strain differences in the efficiency of presentation of the D^b^-NP epitope in influenza virus-infected cells

During the course of the experiments outlined above, a number of different, cross-reactive, polyclonal NP-specific CTL cell lines were generated and assayed using infected target cells. These different CTL cell lines varied in their killing efficiency, and experiments using CTLs with reduced killing efficiency gave unexpected results. L-D^b^-target cells infected with influenza virus A/PR/8/34 were recognized much more efficiently by NP-specific D^b^-restricted CTLs than by cells that...
**Fig. 2.** The differential recognition of A/NT/60/68 and A/PR/8/34 NP-VACs by CTL clones is shown. L-D^b target cells were infected with either PR8NP-VAC or NT60NP-VAC, as indicated, and incubated with 12 different CTL clones at an E:T ratio of 5:1 in a chromium-release assay.

**Fig. 3.** The effect of peptide epitope concentration on target cell recognition by CTLs is shown. L-D^b target cells were incubated with CTL clone 3 at an E:T ratio of 5:1 in the presence of synthetic peptides at the indicated concentrations in a chromium-release assay. The peptide sequences were ASNENMETM (A/PR/8/34 epitope) (▲) or ASNENMDAM (A/NT/60/68 epitope) (○).

**Fig. 4.** The differential recognition of A/NT/60/68 and A/PR/8/34 NP-VACs by CTL clone 3 is shown. L-D^b target cells were either uninfected (●) or infected with influenza virus A/NT/60/68 (▲), influenza virus A/PR/8/34 (○), NT60NP-VAC (▲) or PR8NP-VAC (■). Cells were used in a chromium-release assay at the indicated E:T ratios with either CTL clone 3 (A) or polyclonal K^k^-restricted NP-specific CTL effector cells (B).

**Fig. 5.** The inhibition of A/NT/60/68 NP Db epitope presentation by MVA is shown. L-D^b target cells were infected with influenza virus A/PR/8/34 (○), MVA.NP (▲) or MVA.pSC11 (●) and used in a chromium-release assay at the indicated E:T ratios with either CTL clone 3 (A) or polyclonal Kk-restricted NP-specific CTL effector cells (B).

**Fig. 6.** The differential recognition of A/NT/60/68 and A/PR/8/34 NPs in influenza virus-infected cells is shown. L-D^b target cells were either uninfected (●) or were infected with influenza virus A/PR/8/34 (○) or influenza virus A/NT/60/68 (▲). Cells were then used in a chromium-release assay at the indicated E:T ratios with either a polyclonal cross-reactive, D^b^-restricted NP-specific CTL cell line (A) or a polyclonal K^k^-restricted NP-specific CTL cell line (B).

There was no significant difference in the recognition of the same target cells by NP-specific K^k^-restricted CTLs (Fig. 6B). The differential presentation of the D^b NP epitope was also
found using influenza virus-infected EL-4 target cells (data not shown) and so was not an artefact of using L-D^b target cells. CTLs were cross-reactive for both strains of influenza virus, as shown by their recognition of both synthetic peptide epitopes (data not shown). Therefore, the differential presentation of the D^b NP epitope from two strains of influenza NP was independent of VV infection. These data suggest that the equivalent epitope in two different strains of influenza virus is processed and presented with different efficiency.

Discussion

In the present work, we have generated NP-specific, D^b-restricted CTLs which are cross-reactive for two different strains of influenza virus. These CTLs were used to investigate the presentation of different NP epitopes in both influenza virus- and VV-infected cells. We consistently found that when using efficient CTLs which gave high levels of specific lysis, there was no difference in recognition of target cells infected with influenza virus A/NT/60/68 or A/PR/8/34. However, using the same CTLs, there was a marked difference in presentation of the D^b epitope when the two NPs were expressed as recombinant VVs. We confirmed the previous finding that presentation of the D^b epitope in the A/NT/60/68 NP was very poor in VV-infected cells, even at early times in infection (Townsend et al., 1988). Surprisingly, however, presentation of the equivalent epitope in the A/PR/8/34 NP was very efficient in VV-infected cells. This difference occurred even though the two NPs share 94% amino acid identity and there are only two amino acid differences between the epitopes. The same result was obtained using both polyclonal CTLs and CTL clones and was not attributed to a difference in the affinity of the CTLs for the two peptide epitopes. Others have also demonstrated that cross-reactive CTLs have very similar affinities for these two NP epitopes (Haanen et al., 1999). The two equivalent NP peptide epitopes have been shown to have very similar affinities for the D^b molecule (Tourdot et al., 1997), and so a difference in epitope affinity for MHC binding is not the explanation for the difference. The results using the recombinant VVs suggest that the two NPs must somehow be processed for presentation differently, with the consequence that the A/PR/8/34 epitope is presented with greater efficiency during VV infection. Consistent with this hypothesis, a difference in the processing of these two NPs was also suggested by other studies using HLA-Aw68-restricted CTLs (Cerundolo et al., 1991). Further experiments with additional cross-reactive CTL cell lines that gave lower levels of specific lysis revealed a similar difference in efficiency of presentation of the two D^b-restricted NP epitopes in influenza virus-infected cells (Fig. 6), showing that the difference was independent of VV infection. Therefore, there is an intrinsic difference between the efficiency of presentation of the D^b-restricted NP epitope in influenza virus A/NT/60/68 and A/PR/8/34, but this only becomes apparent when the CTLs are recognizing either target cells inefficiently or VV-infected cells. It is well-established that different MHC class I-associated peptides may be produced from gene products with very different efficiency (Anton et al., 1997) and we propose that this is the case for the D^b epitope in the A/NT/60/68 NP as compared with the equivalent epitope in the A/PR/8/34 NP. Previous work using the proteasome inhibitor lactacystin has shown that generation of the D^b epitope from the A/NT/60/68 NP is extremely sensitive to the inhibitor, whereas generation of the A/PR/8/34 NP epitope is less so (Cerundolo et al., 1997). This is consistent with a difference in processing of the two equivalent epitopes. The reason why there should be such a difference in the processing of the two epitopes in the different strains of virus is unclear, but must be related to the 31 out of the 498 amino acid differences between the two NP molecules. An interesting possibility is that the A/PR/8/34 sequence leads to the formation of more NP-derived defective ribosomal products, which have been proposed to be a major source of peptide epitopes for presentation to CTLs (Schubert et al., 2000). VV has been extensively used as an expression vector for the investigation of CTL responses, but at the same time has been shown to interfere with the presentation of certain CTL epitopes (Bennink & Yewdell, 1990). Because VV is such a widely used vector and because of its potential use in vaccines, it is important to understand the mechanism of VV interference with antigen presentation and to establish whether or not a specific virus gene is involved. Our results suggest that rather than specifically interfering with the presentation of certain CTL epitopes, VV infection causes a general decrease in MHC class I-restricted antigen presentation, probably because of the induced shutdown of host cell protein synthesis (Moss, 1968). The differences in presentation that are observed with the recombinant NP-VACs may be explained by the intrinsic differences in efficiency of presentation of the two equivalent epitopes. This difference becomes significant because of the general reduction in antigen presentation that is caused by VV infection which caused the efficiency of presentation of the A/NT/60/68 NP epitope to be reduced below the threshold that is required for CTL recognition. VV infection would be expected to have a more significant effect on epitopes that are processed and presented less efficiently, and susceptibility to inhibition by VV infection may be a useful indicator of the efficiency of processing of a particular CTL epitope.

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