DEFECTIVE PRESENTATION TO CLASS I-RESTRICTED CYTOTOXIC T LYMPHOCYTES IN VACCINIA-INFECTED CELLS IS OVERCOME BY ENHANCED DEGRADATION OF ANTIGEN

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Cytotoxic T lymphocytes (CTL) recognize viral antigens in association with class I molecules of the MHC (1). Recent work has shown that the epitopes of proteins recognized by class I MHC-restricted CTL can be defined in the lysis assay with short synthetic peptides (2–8). In addition, target cells that express rapidly degraded fragments of viral proteins, which are not detectable serologically at the cell surface, are recognized efficiently by CTL (9–11). These results are consistent with the concept that class I-restricted CTL recognize protein antigens only after they have been degraded in the cytoplasm of the cell in which they are synthesized (10), or into which they have entered by fusion of virus with an endosome membrane (12–14). However, the pathways by which peptide epitopes are generated and transported to the surface of an infected or transfected cell are not known.

Additional evidence that expression of an intact viral protein at the surface of a target cell is not by itself sufficient for recognition by CTL has arisen from work with vaccinia virus as a vector for the expression of influenza hemagglutinin. While comparing early (E) (active before viral DNA replication) with late (L) (active after DNA replication) vaccinia promoters for expression of influenza hemagglutinin (HA), Coupar et al. (15) found that despite the presence of serologically detectable HA at the cell surface, presentation of HA epitopes to CTL was inhibited during the late phase of vaccinia infection.

In this paper, we extend these results by showing that the vaccinia-induced defect in presentation of influenza antigens exists in a less profound form during the early phase of infection, is selective for certain epitopes, and can be overcome by manipulations that induce enhanced degradation of the influenza protein in the vaccinia-infected cell. These results emphasize the association between degradation of

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[1] *Abbreviations used in this paper:* E, early; HA, influenza hemagglutinin; HAU, hemagglutinin units; L, late; NP, influenza nucleoprotein; PFU, plaque-forming units; Ub, ubiquitin.

endogenously synthesized antigens in the cytoplasm with recognition by class I-restricted CTL of epitopes at the cell surface. In addition, the specificity of the vaccinia inhibitory effect suggests diversity in the mechanisms by which different epitopes are presented at the cell surface in association with class I molecules.

Materials and Methods

Mice. 3-6-mo-old C57BL/10 or CBA mice used as responding cell donors were obtained from the National Institute for Medical Research, London, U.K., or the Sir William Dunn School of Pathology, Oxford, U.K.

In Vivo Priming of Mice. Mice were anesthetized with ether and primed by intranasal infection with five haemagglutinin units (HAU) of E61-13-H17 influenza virus as infectious allantoic fluid diluted in 50 μl PBS (16), or by intravenous injection of 10^7 PFU recombinant H1-VAC, 7.5K as described (17).

Peptides. Peptides were synthesized by solid phase techniques on a peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA) as described (2), and were kindly supplied by Jonathan Rothbard (Imperial Cancer Research Fund).

Recombinant Vaccinia Viruses: Hemagglutinin. The construction of a vaccinia-expressing influenza HA of the H1 subtype derived from A/PR/8/34 under the control of the 7.5K promoter (H1-VAC, 7.5K) has been described previously (18). Deletion of the NH_2-terminal signal sequence from the HA cDNA (amino acids 2-17, inclusive) has also been described (11). The signal-deleted H1 mutant and full length H1 cDNA were cloned into the vaccinia expression plasmid pRK19 (R. Kent and G. Smith, manuscript in preparation) downstream of the 4B late promoter. The derived recombinant vaccinia were termined Sig-H1-VAC 4B and H1-VAC 4B, respectively.

Recombinant Vaccinia Viruses: Nucleoprotein (NP). A full length cDNA copy of RNA segment 5 from influenza A/NT/60/68 (19) was cloned into the vaccinia expression plasmid pSC11 downstream of the 7.5K promoter (20), or the plasmids pRK19 and pKB14 (K. Breckling and B. Moss, manuscript in preparation) downstream of the 4B and 11K promoters, respectively. The derived recombinant vaccinia were termed NP-VAC 7.5K, NP-VAC 4B, and NP-VAC 11K, respectively.

The deletion mutant (IMP 1295) of NP coding for amino acids 1, 2, 327-498 was cloned as a partial Hind III 879-bp fragment of the plasmid pTK IMP1295 (21) into the Hinc II site of pUC9. The desired sequence was removed from this plasmid (pUC9 IMP1295) as a 595-bp (Bam HI) fragment and cloned into the vaccinia expression plasmids pSC11 (7.5K promoter), pRK19 (4B promoter), or pKB14 (11K promoter) as described above. The derived recombinant vaccinia were termed IMP1295-VAC 7.5K, IMP1295-VAC 4B, and IMP1295-VAC 11K, respectively.

The design of the ubiquitin(Ub)-NP fusions was based on the work of Bachmair et al. (22). The murine Ub gene was kindly provided by Dr. Tom St. John (Fred Hutchinson Cancer Research Center) as an Eco RI fragment (arf2, reference 23) subcloned into the plasmid pSJ118. The Ub cDNA was subcloned as a 319-bp Eco RI fragment into the Eco RI site of M13mp18AM4. A Sal I site was introduced into the sequence of the arf2 such that the central three bases of the Sal I site formed an Arg codon (CGA) immediately following the terminal Gly codon of Ub. The DNA sequence coding for the terminal 2 glycines of Ub in arf2 read GGT (Gly) GGC (Gly) ATC (Ile) ATT (Ile) before mutagenesis and GGT (Gly) GGT (Gly) CGA (Arg) CTT (Leu) after. The sequence of the mutagenic oligonucleotide was GCGTCTGCGCGGTGGTCGACTTGAGCCATCCCTTCG, and mutagenesis employed a standard procedure using amber selection in M13 as described (24). The complete mutated arf2 cDNA was sequenced by the dideoxy method. The full length NP cDNA (19) with an Nco I restriction site (CCATGG) at the start of translation was kindly provided by Ian Jones (NERC Institute for Virology, Oxford). The full length NP cDNA was cloned between the Nco I and Sal I sites of the plasmid pTAC-85 (25). The Ub-Arg-NP fusion was obtained as follows. pTAC-85-NP was digested with Nco I, and the ends blunted with mung bean nuclease using the buffers described (26) and 3 U of nuclease (Pharmacia Fine Chemicals, Piscataway, NJ) to digest 5 μg of DNA in 100 μl. The mutated Ub cDNA in M13 was digested with Sal I,

and the ends filled in with the Klenow fragment of DNA polymerase using standard procedures. The Ub cDNA was then isolated after Eco RI digestion. The Nco I cut, nuclease treated, ptac-85-NP was digested with Eco RI, and the fragment containing the NP sequence isolated and ligated to the prepared Ub cDNA fragment. The resulting plasmid contained the complete Ub sequence fused to nucleoprotein with an Arg codon replacing the Met codon of NP. The Ub-Met-NP fusion was obtained by following the same procedures but treating the Sal I cut Ub cDNA in M13 with Mung Bean nuclease, and the Nco I cut NP by filling in with the Klenow fragment of DNA polymerase. The resulting fusion retained the ATG codon of NP fused to the terminal Gly codon of Ub. The sequence of the fused regions were confirmed by dideoxy sequencing. The cDNA coding for the two Ub-NP fusions were subcloned as 2041-bp Eco RI-Sal I fragments into the vaccinia expression plasmids pSC11 (7.5K promoter), pRK19 (4B promoter), and pKB14 (11K promoter) as described above. The derived recombinant vaccinia were termed Ub-Arg-NP-VAC 7.5K, Ub-Arg-NP-VAC 4B, Ub-Arg-NP-VAC 11K, Ub-Met-NP-VAC 7.5K, Ub-Met-NP-VAC 4B, and Ub-Met-NP-VAC 11K.

Insertion into the vaccinia *tk* gene, isolation, and purification of recombinant viruses followed standard procedures (27).

Immunoprecipitation. The technique used has been described in detail elsewhere (10, 11). Briefly, for Fig. 1, 5×10^6 murine L929 cells were infected with vaccinia viruses at 20 plaque-forming units (PFU) per cell or with A/PR/8/34 influenza virus for 2 h at 37°C. Cells were washed once and resuspended at 10^6 cells per ml in complete medium. After incubation at 37°C for 7 h, cells were washed and incubated in methionine-free medium for 1 h. Cells were spun down, resuspended in 200 μl of methionine-free medium, and labeled with 120 μCi of [^{35}S]methionine for 30 min at 37°C. Cells were then diluted with 5 ml of complete medium containing 4 mM methionine, and the first sample (10^6 cells) was removed for lysis. The remaining cells were incubated, and further samples removed after 1 and 4 h. Samples were processed as described previously (10) using the mAb H36-18-2 for immunoprecipitation, and resolved on a 10% gel.

L/Db cells were infected with recombinant vaccinia expressing full length NP at 5 PFU per cell and those expressing the IMP fragment at 7 PFU per cell for 90 min (Fig. 3). They were then washed once with PBS and resuspended at $1-2 \times 10^6$ cells per ml in complete medium. Cells infected with vaccinia expressing inserts from the 7.5K promoter were incubated for 2 h, those infected with vaccinia expressing inserts from the 4B promoter were incubated for 5 h. The cells were then washed twice in PBS and resuspended in 0.5 ml of methionine-free RPMI 1640/5% FCS and incubated for a further hour before addition of 227 μCi of [^{35}S]methionine and further incubation for 30 min. Cells were then diluted with 3 ml of complete medium containing 4 mMol L-methionine. The first sample of cells (2×10^6) was removed for lysis. The remaining cells were incubated, and further samples were removed 30 and 120 min later. The samples were processed as described previously (10), with the minor modification that the lysates were pre-absorbed with 100 μl of 10% *Staphylococcus aureus* organism (Cambridge Bioscience, Cambridge, UK) overnight at 4°C. S146 antibody was added for 1 h, and the samples were treated as previously described. The immunoprecipitates were fractionated on a 12% polyacrylamide gel in the presence of 2-ME. The gel was fixed, treated with amplify (Amersham Corp., Bucks, UK), dried, and exposed to preflashed x-ray film.

L/Db cells were infected with vaccinia expressing wild type NP, Ub-arg-NP, Ub-met-NP, or the IMP1295 fragment (negative control) under the control of the 7.5K promoter at 7 PFU per cell for 1.5 h washed in PBS, resuspended at 10^6 cells per ml in complete medium for 3 h (Fig. 7). The procedure was then as described for Fig. 3, using the mAb to NP 5/1 (28), which does not bind the IMP1295 fragment.

Cytotoxic T Cell Cultures and Clones. Polyclonal CBA (H2-k) CTL specific for peptide 50-63 were used. 3-6-mo-old female CBA mice were infected intranasally with 5 HAU of virus E61-13-H17. At least 2 wk later, their spleen cells were restimulated in vitro with peptide. Cultures were prepared as described previously (29), but the stimulator cells were treated with peptide 50-63 at $1-5 \times 10^{-5}$ M/l for 1 h instead of being infected with influenza virus. Cultures initiated in this way (in the absence of exogenous IL-2) exhibited powerful peptide- and virus-specific cytotoxic activity after 5 d. CTL lines were maintained in exponential growth

by weekly stimulation with peptide-pulsed feeder cells and human rIL-2 (10 Cetus U/ml, Cetus Corp., Emeryville, CA).

Polyclonal CBA CTL Specific for Hemagglutinin. CBA mice were primed with 10^7 PFU i.v. of recombinant vaccinia expressing the HA of A/PR/8/34 under the control of the 7.5K promoter. 2 wk later spleen cells were restimulated in vitro with influenza virus A/PR/8/34 as described (11, 17), followed by a second restimulation in vitro 7 d later. After culture for 5 more days, the cells were harvested and used in the cytotoxicity assay described below.

Clone F5 is specific for peptide 365–379 derived from the NP A/NT/60/68 and has been described in detail previously (2).

Cytotoxic Assay. A standard 6-h Cr^{51} release assay was used as described previously using either L cells transfected with the class I gene H-2Db (10, 29) or untransfected L cells for experiments with hemagglutinin-specific CTL. In comparisons of vaccinia expressing proteins from early and late promoters, target cells were infected with 5–10 PFU per cell and labeled with Cr^{51} simultaneously for 90 min. They were then washed twice in PBS, resuspended at 10^6 per ml in complete medium for 4 h, and rewashed twice in PBS before use in the cytotoxicity assay. The timing of contact between targets and T cells was therefore comparable with the immunoprecipitation experiments. Percent-specific chromium release was calculated as: (release by CTL − medium release) × 100/(2.5% Triton release − medium release). All points were measured in duplicate against quadruplicate medium controls. Spontaneous Cr^{51} release in the absence of CTL ranged between 8% and 30% in all experiments.

Results

Influenza HA Expressed from the Vaccinia 4B (L) Promoter Is Inefficiently Recognized by CTL. Coupar et al. (15) used the vaccinia 11K promoter to express recombinant influenza A/PR/8/34 (H1) hemagglutinin. To confirm these results, we made a recombinant that expresses the same HA gene under the late 4B promoter. Fig. 1 (lanes *1–3*) shows the results of a pulse chase experiment confirming that serologically detectable HA was synthesized in the recombinant vaccinia-infected target cells. Immunofluorescence staining confirmed that >80% of infected cells stained brightly at the cell surface 6–8 h after infection, at levels comparable with a vaccinia expressing the HA gene from the 7.5K promoter (11), which functions both E and L (not shown).

Hemagglutinin-specific cytotoxic T cells, raised as described in Materials and Methods (11, 17), were tested for recognition of target cells infected with recombinant vaccinia that expressed hemagglutinin under the control of either the 7.5K (E and L) or 4B (L) promoters. Fig. 2 shows that whereas HA expressed under the control

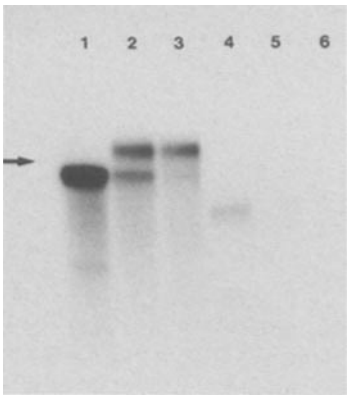

FIGURE 1. Immunoprecipitation and pulse chase of HA (lanes *1–3*) and SIG-HA (lanes *4–6*) expressed from the 4B promoter. Infected cells were pulse labeled for 30 min before removing the first aliquot for immunoprecipitation (lanes *1* and *4*), excess unlabeled Met was added and further aliquots removed after 1 h (lanes *2* and *5*) and 4 h (lanes *3* and *6*).

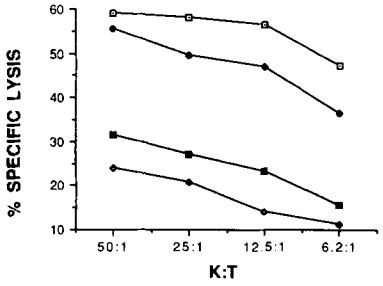

FIGURE 2. Deletion of the HA signal sequence overcomes the late defect in presentation of HA expressed from the 4B promoter. Recognition by hemagglutinin-specific polyclonal CBA CTL of L929 target cells infected with (◇) wild type WR-VAC; (◆) H1-VAC 7.5K; (■) H1-VAC 4B; (□) Sig-H1-VAC 4B.

of the vaccinia 7.5K promoter was efficiently recognized (11, 17), target cells expressing HA from the 4B promoter were lysed only marginally above the background on wild type vaccinia-infected cells. Presentation of epitopes to HA-specific CTL appeared to be inhibited in the target cells expressing HA under the control of the 4B (late promoter).

Deletion of the NH_2-terminal Signal Sequence of HA Overcomes the Vaccinia-induced Block to Presentation. In earlier work, we studied the presentation to CTL of a deletion mutant of influenza A/PR/8/34 hemagglutinin that lacked an NH_2-terminal signal sequence. Target cells that synthesized the signal deleted HA as a rapidly degraded cytoplasmic protein did not express any serologically detectable HA at the cell surface, but were recognized efficiently by class I-restricted CTL (11). These results were consistent with CTL recognizing a degradation product of the HA molecule, and were supported by experiments showing that the epitopes of both NP and HA could be defined with short synthetic peptides in vitro (2, 4, 8, 30).

It was therefore of interest to see what effect deleting the signal sequence would have on presentation of HA to CTL during the late phase of vaccinia infection. A new recombinant vaccinia was produced that expressed the signal-deleted HA (8) under the control of the 4B promoter. Fig. 1 (lanes *4–6*) demonstrates that the signal-deleted HA is detected as expected as a ~62-kD protein that shows no evidence of glycosylation, and is more rapidly degraded than full length HA with a half life of <1 h (11, 31).

HA-specific cytotoxic T cells recognize target cells expressing the signal-deleted HA under the control of the 4B promoter at least as efficiently as full length HA expressed from the 7.5K promoter (Fig. 2). In contrast, target cells expressing the full length HA from the 4B promoter were barely lysed above background. Deletion of the NH_2-terminal signal sequence therefore restored presentation to CTL of HA epitopes expressed during the late phase of vaccinia infection.

Presentation of Nucleoprotein Epitopes Is Selectively Inhibited in Vaccinia-infected Cells. To extend the results obtained with hemagglutinin, we examined recognition of influenza A/NT/60/68 nucleoprotein (NP) expressed under the control of the 7.5K (E and L) and the 4B (L) and 11K (L) promoters. NP was expressed efficiently from each promoter (Fig. 3 and data not shown). CTL specific for two different epitopes of NP, defined with peptides composed of amino acids 50–63 (4) and 365–379 (2), were compared for recognition of infected target cells. The targets were L cells (H-2k) transfected with the class I gene D^b (10), so that presentation of the two epitopes, restricted through K^k and D^b respectively, could be compared simultaneously.

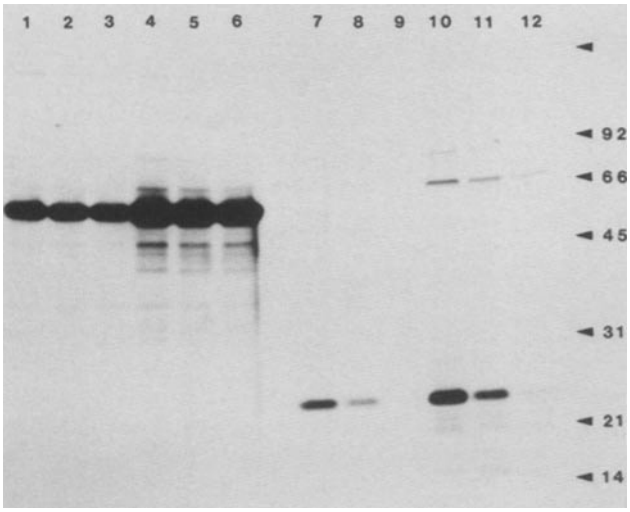

FIGURE 3. Immunoprecipitation and pulse chase of NP (lanes *1–6*) and the IMP1295 fragment (lanes *7–12*) expressed from the 7.5K promoter (lanes *1–3* and *7–9*) and 4B promoter (lanes *4–6* and *10–12*). Infected cells were pulse labeled for 30 min before removing the first aliquot for immunoprecipitation (lanes *1*, *4*, *7*, *10*); excess unlabeled Met was added and further aliquots removed after 30 min (lanes *2*, *5*, *8*, *11*) and 120 min (lanes *3*, *6*, *9*, *12*).

CTL cultures specific for the NH_2-terminal epitope (50–63) recognized target cells expressing NP from either the 7.5K or the 4B or 11K promoters as efficiently as influenza-infected cells. In contrast, clone F5 or polyclonal cultures from $H2^b$ mice specific for peptide 365–379 recognized the same target cells at a low or intermediate level when NP was expressed from the 7.5K promoter, or not at all when NP was expressed from the 4B or 11K promoters (Fig. 4 and data not shown).

These results showed that despite a high level of synthesis of the complete nucleoprotein in the target cells (Fig. 3), there was a marked disparity in the efficiency with which the two epitopes from different sites in the protein were presented to CTL in vaccinia-infected cells. Presentation of the D^b restricted epitope 365–379 was selectively inhibited to an intermediate extent when NP was expressed from the 7.5K promoter, and profoundly when expressed from the 4B or 11K L promoters. In contrast, the K^k-restricted epitope 50–63 was presented efficiently both early and late in vaccinia infection.

Enhanced Degradation of NP Partially Overcomes the Vaccinia-induced Block to Presentation. We produced two rapidly degraded derivatives of nucleoprotein that contained the COOH-terminal epitope 365–379. The first was a deletion mutant of NP (IMP1295, references 10, 21) that expressed amino acids 1, 2, 328–498 (lacking amino acids 3–327 inclusive). The second was based on the observations of Bachmair et al. (22) that a full length protein with certain amino acids replacing its NH_2-terminal methionine, when fused to Ub, was rapidly degraded in yeast cells (*Saccharomyces cerevisiae*). They termed this effect of the NH_2-terminal amino acid on protein stability the "N-end rule".

Expression of the Rapidly Degraded COOH-terminal Fragment of NP Overcomes the Defect in Antigen Presentation Associated with Vaccinia Infection. The expression of the deletion mutant (IMP1295) of influenza NP (amino acids 1, 2, 328–498) in Xenopus Oocyes and transfected L cells has previously been described (10, 21). L cells transfected with the fragment were recognized efficiently by CTL, despite the fact that the level of protein expressed was too low to detect by immunoprecipitation (10).

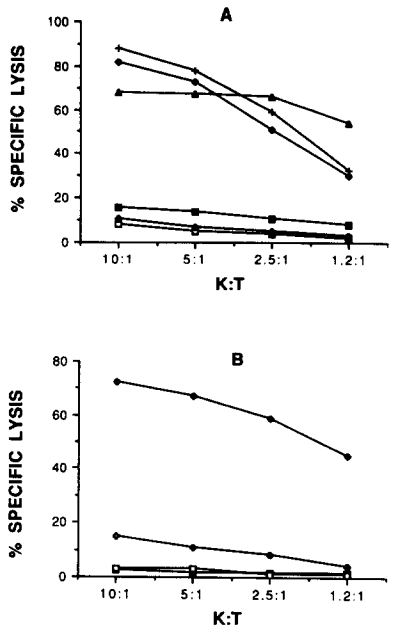

FIGURE 4. (*A*) Selective presentation (E) of NP epitopes expressed from the 7.5K vaccinia promoter. Recognition by clone F5 (specific for 365-379 + D^b) of L/D^b target cells: (□) uninfected; (▲) infected with E61-13-H17 influenza virus; (■) NP-VAC 7.5K. Recognition by CBA CTL (specific for 50-63 + K^k) of the same target cells: (◇) uninfected; (+) infected with E61-13-H17 influenza virus; (◆) NP-VAC 7.5K. (*B*) Selective presentation (L) of NP epitopes expressed from the 4B vaccinia promoter. Symbols as for (*A*).

Vaccinia expressing the fragment under the control of the 7.5K (E and L) and 4B (L) promoters were compared for expression. Fig. 3 shows that the fragment was synthesized in sufficient quantity from both promoters to be detected by immunoprecipitation with a polyclonal sheep serum. The molecular mass assessed by PAGE appeared slightly higher (22 kD) than the expected molecular mass of 19.2 kD (the significance of this finding is not clear). Lanes *7–9* and *10–12* of Fig. 3 demonstrate that the fragment was expressed in greater quantity from the 4B than the 7.5K promoter. In both cases, the fragment was short lived, with a half life of <30 min.

The effect on CTL recognition is shown in Figs. 5 and 6. The incomplete block to presentation of the epitope 365–379 detected during the early phase of vaccinia infection (7.5K promoter) is overcome by expressing it as part of the short lived fragment (Fig. 5). The profound block detected during the late phase (4B promoter) was also overcome, but the level of lysis of target cells expressing the fragment from the 4B promoter was always less than when it was expressed from the 7.5K promoter (Fig. 6).

Antigen Presentation and the N-end Rule. Two fusion proteins were produced by ligating the cDNA sequences coding for murine Ub (21) to the A/NT/60/68 influenza NP (19). In the first (control), the complete Ub was fused to the NH_2-terminal methionine of NP (Ub-met-NP). In the second, the NH_2-terminal methionine codon of NP was replaced by an arginine codon to produce Ub-arg-NP. The two fusion proteins therefore differed by a single amino acid (met/arg) at the NH_2 terminus of the NP sequence.

The two fusion proteins were expressed under the control of either the 7.5K (E plus L) or 4B (L) or 11K (L) vaccinia promoters. Fig. 7 compares the stability and level of expression of wild type NP (lanes *1–3*) with the Ub-arg-NP (lanes *4–6*) and the Ub-met-NP control (lanes *7–9*), expressed under the control of the 7.5K pro-

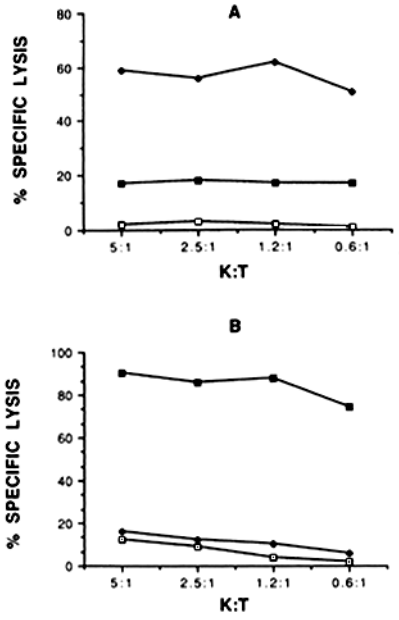

FIGURE 5. Expression of short-lived IMP1295 fragment from the 7.5K promoter overcomes the early defect in presentation of NP. (*A*) Recognition by clone F5 (specific for 365–379 + D^b) of L/D^b target cells: (□) uninfected; (■) infected with NP-VAC 7.5K; (◆) IMP1295-VAC 7.5K. (*B*) Recognition by CBA CTL (specific for 50–63 + K^k) of the same set of L/D^b target cells. Symbols as in (*A*).

moter. The apparent molecular masses of the two fusion proteins was the same as for wild type NP (~56 kD). This implied that they were cleaved at the Ub-NP junction rapidly after synthesis, and agrees with the results obtained by Bachmair et al. (22) using Ub-β-galactosidase fusions in yeast. The NP component of the Ub-

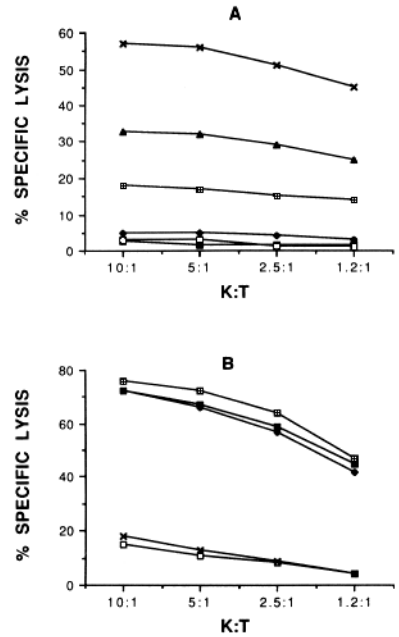

FIGURE 6. Expression of short-lived IMP1295 fragment or Ub-Arg-NP fusion protein partially overcomes the late defect in presentation of NP expressed from the 4B promoter. (*A*) Recognition by clone F5 (specific for 365–379 + D^b) of L/D^b target cells: (□) uninfected; (■) infected with NP-VAC 4B; (◆) Ub-Met-NP-VAC 4B; (⊞) Ub-Arg-NP-VAC 4B; (▲) IMP1295-VAC 4B; (X) IMP1295-VAC 7.5K (positive control for clone F5). (*B*) Recognition by CBA CTL (specific for 50–63 + K^k) of the same set of target cells. Symbols as in (*A*).

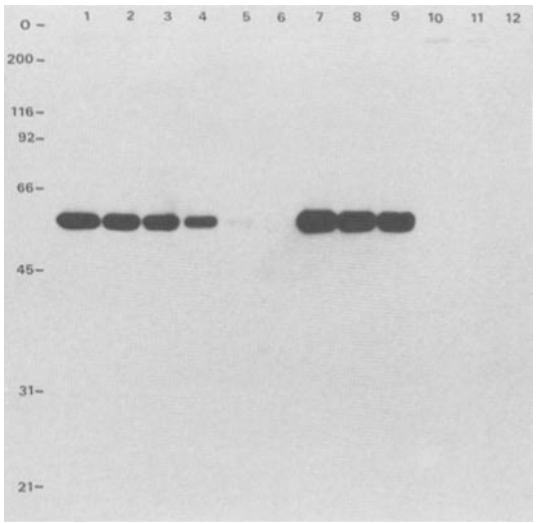

FIGURE 7. Immunoprecipitation and pulse chase of NP (lanes *1–3*), Ub-Arg-NP (lanes *3–6*), Ub-Met-NP (lanes *7–9*), and a negative control (lanes *10–12*), expressed from the 7.5K promoter. Infected L/D^b cells were pulsed labeled for 30 min before removing the first aliquot for immunoprecipitation (lanes *1*, *4*, *7*, *10*), excess unlabeled Met was added and further aliquots removed after 30 min (lanes *2*, *5*, *8*, *11*) and 120 min (lanes *3*, *6*, *9*, *12*).

arg-NP fusion was degraded rapidly, the half life being <30 min (Fig. 7, lanes *4–6*). In contrast, the half life of the NP component of the Ub-met-NP fusion (lanes *7–9*) was not detectably different from the wild type NP, which was stable during the 2-h duration of the pulse chase experiment. The same Ub-NP fusion proteins expressed under the control of the 4B or 11K late promoters were made in greater quantity, but decayed at the same rates as for the 7.5K promoter (data not shown). These results confirm those of Bachmair et al. and extend the applicability of the N-end rule to proteins expressed from vaccinia promoters in the higher eukaryotic cell.

Fig. 8 compares recognition of target cells expressing wild type NP, the Ub-met-NP, and the Ub-arg-NP fusions from the vaccinia 7.5K promoter. L/Db target cells expressing full length NP and the two Ub-NP fusion proteins were recognized equally efficiently by CBA T cells specific for peptide 50–63. This showed that rapid degradation did not impair presentation of the epitope defined by the sequence 50–63. In contrast, recognition by clone F5, specific for residues 365–379, of vaccinia infected cells expressing the full length NP was impaired (as shown above). However, target cells expressing the rapidly degraded Ub-arg-NP fusion were recognized to a level approaching that of influenza-infected target cells (Fig. 8 *A*). In four out of six repeats of this experiment, the control Ub-met-NP fusion was recognized by clone F5 indistinguishably from the wild type NP (as in Fig. 8 *A*). However, in two experiments recognition was at an intermediate level between wild type NP and the rapidly degraded Ub-arg-NP (data not shown).

The effect of these manipulations on presentation during the late phase (4B promoter) are shown in Fig. 6. Again the NP epitope 50–63 recognized by CBA T cells was readily detected on target cells expressing either wild type NP or either of the Ub-NP fusion proteins. In contrast, the epitope defined with clone F5, 365–379, appeared to be completely absent from the surface of the same target cells expressing either wild type NP or the Ub-met-NP fusion. Cells expressing the rapidly degraded ub-arg-NP fusion were recognized by clone F5, but at a level well below the positive control (Fig. 6).

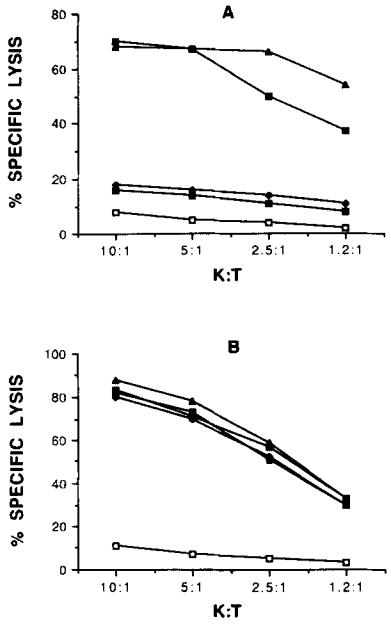

FIGURE 8. Expression of the short-lived Ub-Arg-NP fusion from the 7.5K promoter overcomes the early defect in presentation of NP. (*A*) Recognition by clone F5 (specific for 365-379 + D^b) of L/D^b target cells: (□) uninfected; (■) infected with NP-VAC 7.5K; (⊞) Ub-Arg-NP-VAC 7.5K; (◆) Ub-Met-NP-VAC 7.5K; (▲) influenza E61-13-H17. (*B*) Recognition by CBA CTL (specific for 50-63 + K^k) of the same set of L/D^b target cells. Symbols as in (*A*).

These results show that rapid degradation of NP induced by fusion to ubiquitin and mutation of the NH_2-terminal met to arg can partially overcome the profound block to presentation of the epitope 365-379 during the late phase of vaccinia infection. The incomplete block detected during the early phase (using the 7.5K promoter) was correspondingly more easily reversed.

Discussion

Coupar et al. (15) reported that late in vaccinia virus-infected cells there is a defect in presentation of epitopes to class I MHC-restricted CTL. We have extended these observations, showing that there is also a less profound defect in presentation early in vaccinia infected cells, and that neither defect is universal or absolute. Factors which may influence presentation of antigens to CTL include the subcellular localization of antigen, rate of proteolytic degradation, and efficiency of transport to the cell surface in association with a class I MHC molecule. The vaccinia-induced defect in antigen presentation may occur at any of these stages.

The enhanced recognition of HA lacking a signal sequence (Fig. 2) could be attributed to either altered location in the cell, or increased rate of degradation. The NH_2-terminal signal sequence of HA is required for transfer of the molecule through the membrane of the rough endoplasmic reticulum (ER), and its subsequent glycosylation, folding, trimerisation, and transport to the cell surface as an integral membrane protein (31). Preventing entry into the endoplasmic reticulum, by deleting the signal sequence, would be expected to increase the number of incorrectly folded molecules resident in the cytoplasm available as substrates for cytoplasmic proteases. The result emphasizes that signal-dependent transfer of viral proteins across the membrane of the ER does not play a role in presentation of HA epitopes to class I-restricted CTL.

Expression of nucleoprotein from a late vaccinia promoter is associated with an increase in the level of NP synthesis compared with the 7.5K promoter (Fig. 3). This establishes that the block to presentation of the 365–379 epitope is not related to the amount of complete NP available in the target cell. Either of the two strategies used to destabilize the molecule resulted in partial restoration of presentation without altering subcellular location as detected by immunofluorescence (21, and data not shown). This shows that degradation of NP and recognition by CTL of the sequence 365–379 are linked.

Evidence is accumulating for the general statement that CTL recognize the degradation products of viral proteins, rather than the folded proteins themselves (2–11). It is possible that vaccinia infection interferes with specific degradative enzymes involved in the generation of the immunodominant peptide epitopes of HA and the 365–379 epitope of NP, but has no effect on the generation of 50–63. Recently, sequences have been identified in cowpox and fowlpox viruses that are homologous to a family of eukaryotic serine protease inhibitors (32, 33). The cowpox protein had no identifiable hydrophobic signal sequence, and so would be expected to accumulate in the infected cytoplasm where it could inhibit specific proteases of the host cell. However, it is not yet known whether vaccinia, which is a closely related pox virus (34), can express an equivalent protein.

The enzymes involved in the degradation of the short lived proteins described here have not been identified. In yeast, the rapid degradation of a fusion between Ub and β-galactosidase was associated with polyubiquitination of the β-galactosidase, implying a role for the Ub-dependent degradative pathway (22). An alternative explanation for the N-end effect on NP is that the presence of an arginine at the free NH_2 terminus rather than acting as a specific signal for degradation, interferes with folding and results in the exposure of proteolytic cleavage sites that would normally be protected. From this point of view, the N-end effect on NP may be analogous to deleting the signal sequence of HA or deleting a large segment of NP, both of which are expected to interfere with folding. Work is in progress to assess the role of the Ub-activating enzyme in the degradation of our three short-lived proteins in the temperature-sensitive cell line ts85 (35).

The third stage of presentation suggested above is transport of the peptide epitope to the cell surface in association with a class I molecule. It is not known how peptides cross a bilipid membrane to reach the cell surface, nor where in the infected cell they associate with class I molecules. Vaccinia infection is associated with almost complete inhibition of host protein synthesis (34). It is therefore possible that lack of synthesis of class I molecules may contribute to the loss of presentation during the late phase of vaccinia infection, as suggested by Coupar et al. (15). We have observed that late during infection with vaccinia, target cells can be sensitized over the expected dose range with peptide 365–379 added to the medium surrounding the cells (2, 4, and data not shown). This shows that vaccinia infection does not interfere with the peptide-presenting function of D^b class I molecules at the cell surface. However, if peptides generated in the infected cell associate predominantly with newly synthesized class I molecules, en route to the surface from the endoplasmic reticulum, this explanation for our results could still be consistent. The positive effects of enhanced antigen degradation could then be explained by an increase in the concentration of peptide ligand available for binding to limiting concentrations of class

I molecules. The apparent specificity of the inhibitory effect may relate to differences in rates of transport of class I molecules (36) or rates of association of peptides. Coexpression of class I molecules and viral proteins from late vaccinia promoters will shed light on the issue.

Summary

Vaccinia infection interferes with the presentation of influenza Haemagglutinin (HA) and Nucleoprotein (NP) to class I-restricted CTL. The inhibitory effect is selective for certain epitopes, and is more profound during the late phase of infection. For influenza A/NT/60/68 NP, the block is present during both early and late phases of infection, and is selective for the COOH-terminal epitope defined by peptide 366–379, having no detectable effect on the presentation of the NH_2-terminal epitope 50–63. The presentation of HA is inhibited only during the late phase of vaccinia infection. For both proteins, presentation is partially (NP) or completely (HA) restored by expression of rapidly degraded protein fragments in the vaccinia infected target cell. For HA, deletion of the NH_2-terminal signal sequence completely overcomes the block. For NP, either a large NH_2-terminal deletion or the construction of a rapidly degraded ubiquitin-NP fusion protein partially restores presentation. These results illustrate the relationship between degradation of viral proteins in the cytoplasm of an infected cell and recognition of epitopes at the cell surface by class I-restricted T cells.

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