Brief Definitive Report

A 15 AMINO ACID FRAGMENT OF INFLUENZA NUCLEOPROTEIN SYNTHESIZED IN THE CYTOPLASM IS PRESENTED TO CLASS I-RESTRICTED CYTOTOXIC T LYMPHOCYTES

By KEITH GOULD,* JUDY COSSINS,* JUDY BASTIN,‡ GEORGE G. BROWNLEE,* and ALAIN TOWNSEND‡

*From the *Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE; and the ‡Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom*

Recent evidence suggests that CTL recognize the peptide degradation products of viral proteins presented at the surface of infected cells in association with class I molecules of the MHC (1-12). However, the mechanisms by which peptide epitopes are generated and transported to the cell surface in vivo are not known.

There are two models that could explain the mechanism of presentation of a viral peptide epitope at the cell surface. Viral proteins may be degraded by proteases in the cytoplasm to form short peptide epitopes. These peptides would then have to contain all the necessary information to cross a bilipid membrane in order to reach the cell surface (1, 2). Alternatively, larger protein fragments may be transported across a bilipid membrane before degradation, in which case signals for transport could be encoded separately to those forming the minimal epitope.

In previous work (1, 13) we have shown that presentation of epitopes does not depend on a single identifiable signal sequence in either the influenza nucleoprotein (NP) or haemagglutinin (HA). However, these experiments involved expression of protein fragments >130 amino acids long, and could not rule out a role for amino acid sequences outside the minimal epitopes defined with synthetic peptides. A recent report (14) describes recognition by CTL of cells that synthesize a hydrophobic fragment of 41 amino acids derived from the transmembrane region of influenza HA. However, this fragment is also longer than the minimal epitope contained within it (6).

To try and resolve this issue, we have constructed a recombinant vaccinia virus designed to express a minimal peptide epitope consisting of 15 amino acids of the influenza NP in the cytoplasm of infected cells. We report here that target cells infected with this recombinant vaccinia virus are recognized efficiently by class I-restricted CTL, suggesting that transport of the epitope to the cell surface requires no additional sequence information. This observation is consistent with the first suggested model and implies that a peptide transport system exists in eukaryotic cells.

This work was supported by the Medical Research Council of Great Britain.

Materials and Methods

Construction of 366-379-NP-VAC 7.5K. Two oligonucleotides 66 bases long were synthesized on a DNA synthesizer (381A; Applied Biosystems, Inc., Foster City, CA). They were designed so that when annealed they would consist of a sequence that would ligate to a Hind III site, the initiator methionine codon together with the preceding nine nucleotides of untranslated sequence of the influenza A/NT/60/68 NP gene (15), and the codons for amino acids 366–379 of NP, followed by two stop codons and a sequence that would ligate to Bam HI site. The sequences of the oligonucleotides were: (5')-AGCTTATCAAAATCATGGCTTCAAATGAAAACATGGATGCTATGGAATCAAGTACTCTTTGATAAG-(3') and (5')-GATCCTTATCAAAGAGTACTTGATTCCATAGCATCCATGTTTTCATTTGAAGCCATGATTTTGATA-(3'). They encode the amino acid sequence met-ala-ser-asn-glu-asn-met-asp-ala-met-glu-ser-ser-thr-leu. The gel-purified oligonucleotides were annealed and ligated into Hind III and Bam HI cut pKG18 downstream of the 7.5-kD vaccinia promoter, which is active during both early and late phases of infection (16). pKG18 was derived from pGS62 (16) and contains extra cloning sites for expression (K. Gould; unpublished results). After the ligation, the construct was checked by DNA sequencing.

Production of recombinant vaccinia virus by insertion into the *tk* gene followed standard procedures (16). Recombinants were identified by hybridization with one of the ^{32}P-labeled oligonucleotides described above, and plaque purified twice. The vaccinia expressing residues 366–379 from influenza A/NT/60/68 NP will be referred to as 366-379-NP-VAC 7.5K.

A control vaccinia expressing a rapidly degraded form of the full-length influenza A/NT/60/68 NP fused to murine ubiquitin (Ub-Arg-NP-VAC 7.5K) has been described (17). We have previously shown that L/D^b cells infected with this virus are recognized by class I-restricted CTL from CBA (H2-k) and C57BL (H2-b) mice as efficiently as influenza-infected cells (17).

CTL Cultures, Clones, and Peptides. Clone F5 is specific for the sequence 366–379 derived from the NP of influenza A/NT/60/68 in association with H-2D^b and has been described previously (2). Polyclonal CTL derived from CBA mice and specific for the amino acid sequence 50–63 derived from the NP of A/NT/60/68 in association with H-2K^k were induced in influenza-infected mice as described previously (5). Peptides were synthesized by solid phase techniques on a synthesizer (430A; Applied Biosystems, Inc.) as described (2) and were kindly supplied by Jonathan Rothbard (Imperial Cancer Research Fund, London, UK).

Cytotoxic Assay. A standard 6-h ^{51}Cr release assay was used as previously described using either L cells transfected with the class I gene H-2D^b (18), or untransfected L cells. 2–5 × 10^6 target cells were infected with 10 plaque-forming units (PFU) per cell of recombinant vaccinia and labeled with ^{51}Cr for 90 min. After being washed twice, the cells were resuspended at 10^6 cell/ml in RPMI/10% FCS for 4 h at 37°C, washed twice, and used in the assay. In experiments with cycloheximide, target cells were resuspended in RPMI/10% FCS containing 2 × 10^{-4} M cycloheximide for 30 min before infection with vaccinia virus. All subsequent manipulation of the cells (including the washes) were as above but included cycloheximide at the same concentration. In experiments where peptides were included in the assay, these were present throughout the period of contact between CTL and target cells as described (2), at a final concentration of 2 × 10^{-5} M. Percent specific chromium release was calculated as: 100 × (release by CTL − medium release)/(2.5% Triton release − medium release). All points were measured in duplicate against quadruplicate controls. Spontaneous ^{51}Cr release in the absence of CTL ranged between 12 and 19% in all experiments.

Results

The CTL clone F5 recognizes the peptide 366–379 of NP in association with the class I molecule D^b (2). Fig. 1 *a* shows that F5 recognized L/D^b cells (L cells transfected with the gene coding for D^b) infected with 366-379-NP-VAC 7.5K as efficiently as L/D^b cells infected with a recombinant vaccinia virus expressing a rapidly degraded form of the complete NP molecule (17) or influenza virus (data not shown). Recognition of 366-379-NP-VAC 7.5K–infected cells by F5 was class I re-

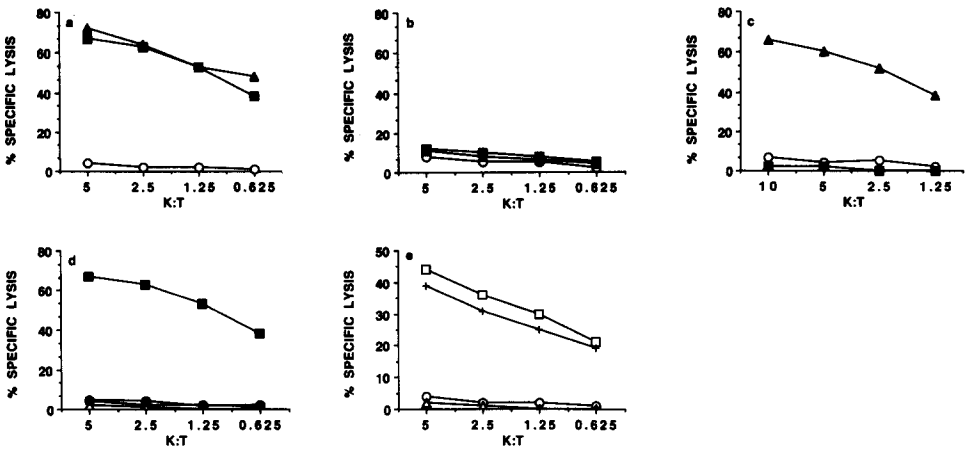

FIGURE 1. Recognition by the H-2 D^b-restricted CTL clone F5 (*a*, *b*, *d*, and *e*), or polyclonal H-2 K^k-restricted CBA CTL (*c*) of target cells treated in different ways: (*a*) F5 CTL and L/D^b targets, uninfected, O; infected with 366-379-NP-VAC 7.5K, ■; infected with Ub-R-NP-VAC 7.5K (17), ▲; (*b*) F5 CTL and untransfected L targets, uninfected, O; infected with 366-379-NP-VAC 7.5K, ■; infected with Ub-R-NP-VAC 7.5K, ▲; (*c*) polyclonal CBA CTL and untransfected L targets, uninfected, O; infected with 366-379-NP-VAC 7.5K, ■; infected with Ub-R-NP-VAC 7.5K, ▲; (*d*) F5 CTL and L/D^b targets, uninfected, O; uninfected and cycloheximide treated, Δ; infected with 366-379-NP-VAC 7.5K, ■; cycloheximide treated and infected with 366-379-NP-VAC 7.5K, ●; (*e*) F5 CTL and L/D^b targets, uninfected, O; uninfected and treated with cycloheximide, Δ; exposed to peptide 365–379 of NP, □; treated with cycloheximide and exposed to peptide 365–379 of NP, +.

stricted, as F5 failed to recognize infected L cells that did not express D^b (Fig. 1 *b*). Fig. 1 *c* demonstrates that recognition of 366-379-NP-VAC 7.5K–infected cells was antigen specific, because a polyclonal CTL line derived from CBA (H-2k) mice specific for the NP sequence 50–63 (5) did not lyse 366-379-NP-VAC 7.5K–infected L cells. The polyclonal CTL line did lyse L cells infected with Ub-arg-NP-VAC 7.5K that expressed the complete molecule containing the epitope defined with peptide 50–63 (Fig. 1 *c*).

To show that clone F5 recognized the peptide sythesized in infected target cells, and not free peptide contaminating the recombinant vaccinia preparation, cycloheximide was used to inhibit protein synthesis in the 366-379-NP-VAC 7.5K–infected L/D^b cells. Fig. 1 *d* shows that presentation of the epitope 366–379 in recombinant vaccinia-infected cells to clone F5 was completely inhibited by 2×10^{-4} M cycloheximide. In contrast, Fig. 1 *e* shows that an identical treatment of target cells with cycloheximide had no significant effect on presentation by L/D^b of externally added peptide. These results are consistent with newly synthesized peptide being recognized by clone F5 at the surface of 366-379-NP-VAC 7.5K–infected target cells.

To show that recognition by clone F5 was not due to release of peptide from cells damaged by vaccinia infection, we did a mixing experiment. Equal numbers of uninfected ^{51}Cr-labeled L/D^b cells and unlabeled 366-379-NP-VAC 7.5K–infected L/D^b cells were mixed and incubated with clone F5. We could find no evidence for transfer of peptide between target cells (Fig. 2).

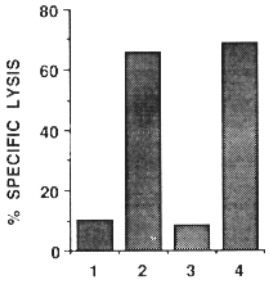

FIGURE 2. Mixing experiment to test for transfer of peptide between target cells. Clone F5 was incubated in U-bottomed wells at a ratio of 10:1 with L/D^b target cells treated as follows: (*1*) ^{51}Cr-labeled, uninfected; (*2*) ^{51}Cr-labeled, 366-379-NP-VAC 7.5K infected; (*3*) ^{51}Cr-labeled uninfected mixed with an equal number (10^4) of unlabeled cells infected with 366-379-NP-VAC 7.5K; (*4*) unlabeled, uninfected cells mixed with an equal number of ^{51}Cr-labeled 366-379-NP-VAC 7.5K-infected cells. The ^{51}Cr release assay was done as described in Materials and Methods. All points were measured in quadruplicate.

Discussion

We have previously shown that target cells synthesizing deletion mutants of both influenza NP and HA that lacked hydrophobic signal sequences were recognized efficiently by CTL, implying that signal-dependent transport across the membrane of the rough endoplasmic reticulum did not play a role in presentation of epitopes to class I-restricted CTL (1, 13). This was particularly clear for HA, as deletion of the NH_2-terminal signal sequence actually enhanced presentation of epitopes to CTL in recombinant vaccinia-infected target cells, while preventing transport of the molecule into the endoplasmic reticulum, as determined by the lack of detectable glycosylation of nascent signal-deleted HA chains (17).

We extend these results by showing that a minimal peptide epitope, synthesized by recombinant vaccinia virus in infected cells, is presented to class I-restricted CTL. Although there is no direct evidence that only a 15-amino acid peptide of NP is synthesized in the infected cells, the expression construct contains two sequential termination codons in frame with the epitope sequence. If alternative translational initiation sites were used, either even shorter NP peptides or sequences unrelated to NP would be made. Neither of these would be recognized by clone F5 (2).

Vaccinia virus carries out its entire life cycle in the cytoplasm of infected cells, so that the NP peptide should be synthesized in the cytosol. This implies that the NP epitope, consisting of residues 366–379, contains sufficient information to be transported from the cytoplasm to the cell surface. It is conceivable that vaccinia-encoded proteins may have some effect in assisting transport, although vaccinia infection tends to inhibit rather than assist presentation of epitopes to class I-restricted CTL (19).

An additional point of interest is that the inhibitory effect of vaccinia infection on presentation of the NP epitope 366–379 in cells expressing the full-length molecule (17) is overcome by expressing the epitope as a peptide. This is consistent with the vaccinia inhibitory effect acting at a point before transport and association of the epitope with class I molecules, and would be explained by specific inhibition of proteolysis.

Our results are consistent with a previously proposed hypothesis that large protein antigens are degraded in the cytoplasm to short peptides, which at least in the case of the epitope studied here, contain sufficient information to allow passage across the bilipid membrane (1, 2). We can only speculate on the nature of the peptide transport mechanism. It is likely to be constitutive as it must be active in uninfected cells expressing transfected viral genes (18, 20, 21). It must also have a broad specificity

in order to transport peptides of widely differing sequence (1-12). It is noteworthy that the sequence 366-379 of NP contains nine charged or polar residues and is hydrophilic.

Summary

A recombinant vaccinia has been designed to express amino acids 366-379 of influenza nucleoprotein, previously shown to be the minimal epitope recognized by a class I-restricted cytotoxic T cell clone. Target cells infected with the recombinant vaccinia virus expressing this peptide are recognized by CTL as efficiently as target cells expressing the complete nucleoprotein. The results imply the existence of a peptide transport system that constitutively passes the products of degraded proteins from the cytoplasm into a membrane-bound compartment of the cell.

We thank Jonathan Rothbard for supplying peptides, Cetus Corp. (Emeryville, CA) for supplying rIL-2, and Hilary Scotney for technical support.

Received for publication 23 February 1989 and in revised form 5 June 1989.

References

1. Townsend, A. R. M., F. M. Gotch, and J. Davey. 1985. Cytotoxic T cells recognize fragments of influenza nucleoprotein. *Cell.* 42:457.
2. Townsend, A. R. M., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell.* 44:959.
3. Maryanski, J. L., P. Pala, G. Corradin, B. R. Jordan, and J. C. Cerottini. 1986. H-2 restricted cytotoxic T cells specific for HLA can recognize a synthetic HLA peptide. *Nature (Lond.).* 324:578.
4. Taylor, P. M., J. Davey, K. Howland, J. Rothbard, and B. A. Askonas. 1987. Class I MHC molecules, rather than other mouse genes dictate influenza epitope recognition by cytotoxic T cells. *Immunogenetics.* 26:267.
5. Bastin, J., J. Rothbard, J. Davey, I. Jones, and A. Townsend. 1987. Use of synthetic peptides of influenza nucleoprotein to define epitopes recognized by class I-restricted cytotoxic T lymphocytes. *J. Exp. Med.* 165:1508.
6. Braciale, T. J., V. L. Braciale, M. Winkler, I. Stroynowski, L. Hood, J. Sambrook, and M. J. Gething. 1987. On the role of the transmembrane anchor sequence of influenza hemagglutinin in target cell recognition by class I MHC-restricted, hemagglutinin-specific cytolytic T lymphocytes. *J. Exp. Med.* 166:678.
7. Gotch, F., J. Rothbard, K. Howland, A. Townsend, and A. McMichael. 1987. Cyotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. *Nature (Lond.).* 326:881.
8. Clayberger, C., P. Parham, J. Rothbard, D. S. Ludwig, G. K. Schoolnik, and A. M. Krensky. 1987. HLA-A2 peptides can regulate cytolysis by human allogeneic T lymphocytes. *Nature (Lond.).* 330:763.
9. Moore, M. W., F. R. Carbone, and M. J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen presentation. *Cell.* 54:777.
10. Whitton, J. L., J. R. Gebhard, H. Lewicki, A. Tishon, and B. A. Oldstone. 1988. Molecular definition of a major cytotoxic T-lymphocyte epitope in the glycoprotein of lymphocytic choriomeningitis virus. *J. Virol.* 62:687.
11. Kumar, S., L. H. Miller, I. A. Quackyi, D. B. Keister, R. A. Houghten, W. L. Maloy,

B. Moss, J. A. Berzofsky, and M. F. Good. 1988. Cytotoxic T cells specific for the circumsporozoite protein of Plasmodium falciparum. *Nature (Lond.).* 334:258.

12. Del Val, M., H. Volkmer, J. B. Rothbard, S. Jonjic, M. Messerle, J. Schickedanz, M. J. Reddehase, and U. H. Koszinowski. 1988. Molecular basis for cytolytic T-lymphocyte recognition of the murine cytomegalovirus immediate early protein pp 89. *J. Virol.* 62:3965.
13. Townsend, A. R. M., J. Bastin, K. Gould, and G. G. Brownlee. 1986. Cytotoxic T lymphocytes recognize influenza haemagglutinin that lacks a signal sequence. *Nature (Lond.).* 234:575.
14. Sweetser, M. T., V. L. Braciale, and T. J. Braciale. 1988. Class I MHC-restricted recognition of cells expressing a gene encoding a 41 amino acid product of the influenza hemagglutinin. *J. Immunol.* 141:3324.
15. Huddleston, J. A., and G. G. Brownlee. 1982. The sequence of the nucleoprotein gene of human influenza A virus, strain A/NT/60/68. *Nucleic Acids Res.* 10:1029.
16. Smith, G. L., J. Z. Levin, P. Palese, and B. Moss. 1987. Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. *J. Virol.* 160:336.
17. Townsend, A., J. Bastin, K. Gould, G. Brownlee, M. Andrew, B. Coupar, D. Boyle, S. Chan, and G. Smith. 1988. Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. *J. Exp. Med.* 168:1211.
18. Townsend, A. R. M., A. J. McMichael, N. P. Carter, J. A. Huddleston, and G. G. Brownlee. 1984. Cytotoxic T cell recognition of the influenza nucleoprotein and haemagglutinin expressed in transfected mouse L cells. *Cell.* 39:13.
19. Coupar, B. E., M. E. Andrew, G. W. Both, and D. B. Boyle. 1986. Temporal regulation of influenza hemagglutinin expression in vaccinia virus recombinants and effects on the immune response. *Eur. J. Immunol.* 16:1479.
20. Gooding, L. R., and K. A. O'Connell. 1983. Recognition by cytotoxic T lymphocytes of cells expressing fragments of the SV40 tumour antigen. *J. Immunol.* 131:2580.
21. Braciale, T. J., V. L. Braciale, T. J. Henkel, J. Sambrook, and M. J. Gething. 1984. Cytotoxic T lymphocyte recognition of the influenza hemagglutinin gene product expressed by DNA-mediated gene transfer. *J. Exp. Med.* 159:341.