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Recognition of influenza virus proteins by cytotoxic T lymphocytes

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Recombinant DNA techniques have been used to express the proteins of influenza virus individually. Target cells expressing single viral proteins were then used to identify the molecules recognized by cytotoxic T lymphocytes (CTLs). Results have shown that, contrary to expectation, the majority of the proteins recognized by class I major histocompatibility complex-restricted CTLs are not transmembrane glycoproteins. Experiments with deletion mutants of the nucleoprotein (NP) gene showed that transport of epitopes to the membrane for recognition by CTLs was independent of a definable signal sequence. In addition, the epitopes recognized were contained within short linear sequences of amino acids, and rapid degradation of large NP fragments within the target cell did not prevent recognition by CTLs. These results led to the suggestion that the epitopes recognized by class-I-restricted CTLs resulted from degradation of viral proteins. If so, the epitopes should, like those for class-II-restricted T cells, be replaceable *in vitro* with short synthetic peptides. Five different epitopes of NP have now been demonstrated that can be defined with short peptides *in vitro*. Each peptide is recognized with a specific class I molecule (D^b, K^k, K^d and HLA B37). This has been extended to the influenza matrix protein, and a peptide epitope defined that is recognized by human CTLs in association with HLA-A2. The question arose as to whether a similar phenomenon would be found with viral proteins which are naturally inserted in the target cell membrane. A mutant haemagglutinin has been produced that lacks a hydrophobic signal sequence. This protein is expressed as a short-lived, unglycosylated, intracellular protein. However, target cells expressing this molecule were recognized efficiently by CTLs raised to the wild-type haemagglutinin and vice versa. These and more recent results with non-viral glycoproteins are consistent with the existence of a mechanism for degrading viral (and perhaps host) proteins and exposing them at the cell surface for recognition by cytotoxic T cells in association with class I molecules of the major histocompatibility complex.

Introduction

Mice and humans make a vigorous cytotoxic T lymphocyte (CTL) response to infection by influenza A virus (reviewed by Askonas *et al.* 1982). The majority of CTLs express the Lyt2 positive (CD8 in the human) surface phenotype (Swain & Dutton 1980) and recognize and kill infected target cells that share class I molecules of the major histocompatibility complex (MHC) with the host in which the CTLs developed (Blanden *et al.* 1975; Zinkernagel & Doherty 1979).

The work described here started with the chance observation that recognition by some murine CTLs was apparently controlled by genes of the influenza A virus that do not code for glycoproteins (Bennink *et al.* 1982; Townsend & Skehel 1982, 1984; Kees & Krammer 1984).

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The generally held view then was that cytotoxic T cells would recognize only viral proteins inserted in the plasma membrane of the infected target cell. However, the use of typed recombinant A viruses showed that a polymerase (PB2) gene and the nucleoprotein (NP) gene controlled the expression of antigens recognized by different populations of CTLs (Bennink *et al.* 1982; Townsend & Skehel 1984).

These observations led to the use of recombinant DNA techniques to express single viral genes in target cells. This was initially done by DNA-mediated gene transfer, and demonstrated that expression of nucleoprotein or haemagglutinin was sufficient to render target cells recognizable to different subpopulations of CTLs induced by infection (Townsend *et al.* 1984; Braciale *et al.* 1984). Later work with recombinant vaccinia viruses expressing individual viral proteins has shown that all the viral proteins can be recognized by subpopulations of class-I-restricted CTLs in either mouse or man (Gotch *et al.* 1987; Bennink *et al.* 1986).

Recognition of protein fragments

Nucleoprotein is not glycosylated, accumulates in the nuclei of infected and transfected cells, and has none of the sequence characteristics of an integral membrane protein (Winter *et al.* 1981; Huddlestone & Brownlee 1982). These results therefore raised the question of how such a protein is made available for recognition by CTLs at the cell surface. The following experiments were designed to identify possible signal sequences required for this phenomenon, and to localize epitopes.

Murine L cells were transfected with a series of overlapping deletion mutants of a nucleoprotein copy DNA (cDNA) that had been used to identify an internal sequence required for nuclear accumulation of the protein (Davey *et al.* 1985). The results showed that non-overlapping segments of protein were recognized by different subsets of T cells, implying that there was no unique signal within the amino acid sequence required for presentation at the cell surface. In addition, expression of short, unstable fragments (150 amino acids) resulted in efficient recognition by cytotoxic T cells. Recognition by individual T cells could be localized to regions of overlap less than 50 amino acids long (Townsend *et al.* 1985; Taylor *et al.* 1987). Similar results had been obtained previously with fragments of SV40 T antigen (Gooding & O'Connell 1983).

These experiments were extended to haemagglutinin (HA) by showing that target cells expressing a truncated form of HA lacking an *N*-terminal signal sequence were recognized by CTLs as efficiently as cells expressing the wild type. The truncated HA was detected with antibodies as a rapidly degraded cytoplasmic protein (Townsend *et al.* 1986*a*).

On the basis of these results we proposed that class-I-restricted T cells recognize the degradation products of proteins synthesized on free ribosomes in the cytoplasm (Townsend *et al.* 1985). This hypothesis required several components, including

(*a*) a degradation system in the cytoplasm distinct from that in endosomes and lysosomes;

(*b*) a transport system that provides a pathway for the peptide products of protein breakdown to cross a membrane and reach the topographical outside of the cell. A precedent for a peptide transporter exists in the prokaryotic oligopeptide permease (Hiles *et al.* 1987);

(*c*) association of peptides with class I molecules in a manner analogous to the demonstrated binding of peptides to class II (Babbit *et al.* 1985; Buus *et al.* 1986);

(*d*) a T-cell receptor repertoire capable of recognizing peptide epitopes in association with class I molecules of the MHC.

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Recognition of peptides

Peptides derived from an appropriate NP sequence were tested for their ability to induce specific proliferation by class-I-restricted T-cell clones and for sensitizing target cells for lysis (Townsend *et al.* 1986*b*). Four epitopes of NP have now been defined with short peptides. In each case the peptide was recognized with a unique class I molecule (366–379 + D^b, 50–63 + K^k, 147–158 + K^d and 335–349 + HLA B37), and responder status in man and congenic strains of mice was controlled predominantly by the corresponding class I gene (Taylor *et al.* 1987; McMichael *et al.* 1986). These findings have been extended by identifying peptide epitopes in influenza matrix and haemagglutinin proteins, and in HLA CW23 (Gotch *et al.* 1987; Braciale *et al.* 1987; Maryanski *et al.* 1986), and several more examples are in the press.

Peptides were recognized either when target cell and T cell were bathed in medium with peptide in solution, or when the target cell was pre-incubated with peptide and then washed before exposure to the T cell (Townsend *et al.* 1986*b*). Peptides were therefore able to associate with the surface of the target cell and implied binding to class I molecules before recognition by the T-cell receptor. These results were also consistent with the crystal structure of the HLA A2 molecule described recently by Bjorkman *et al.* (1987). The groove lying between two α-helices formed by the α_1 and α_2 domains of the molecule has various properties expected of a peptide-binding site. The polymorphic residues of class I molecules cluster in the walls of the groove, and the size of the groove is appropriate for binding peptides of the size defined in the cytotoxic T-cell recognition experiments.

Little is known about the binding of peptides to class I molecules. An estimate of the level of saturation of class I molecules required for efficient T-cell recognition can be made by applying the binding-affinity measurements for peptides to class II molecules (Babbit *et al.* 1985; Buus *et al.* 1986). In most experiments plateau levels of CTL lysis in a 6 h period are achieved with peptides in solution in contact with the target-cell surface at between 10^{-9} and 10^{-6} mol l^{-1} (Townsend *et al.* 1986; Bastin *et al.* 1987; Taylor *et al.* 1987; Bodmer *et al.* 1988; Braciale *et al.* 1987). Using the approximation

$$\text{percentage saturation} = \frac{100\,[\text{peptide}]\,K_a}{(1+[\text{peptide}]\,K_a)}$$

and substituting gives a figure of 0.5% saturation at equilibrium for a peptide concentration of 10^{-8} mol l^{-1} with a K_a of 5×10^5 l mol^{-1}. Assuming an average of 10^5 class I molecules per cell, this represents about 500 molecules of bound peptide per cell surface. True estimates await direct-binding analysis of peptides for class I molecules.

Information about the specificity of the association of peptide with class I molecules can be obtained indirectly by screening peptides for their ability to inhibit at molar excess the recognition of a test peptide. We have used the D^b-restricted peptide NP 366–379 as the test ligand and screened related and unrelated peptides for competition (Bodmer *et al.* 1989). We find that selected peptides of unrelated sequence to 366–379 can compete for presentation with D^b. For instance, NP 50–63 is an efficient competitor, whereas NP 147–158 is not. Neither is discernibly related in sequence to NP 366–379, and neither is an epitope recognized in H-2b mice immunized with influenza. The result implies that the D^b binding site may accommodate a broader range of structures than an antibody-combining site, as might be expected.

If degradation of viral proteins occurs in the cytoplasm before recognition by CTLs, the

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mechanism is expected to be distinct to presentation of exogenous proteins with class II molecules (Unanue 1984; Townsend *et al.* 1985). Morrison *et al.* (1986) have elegantly demonstrated this distinctness by comparing the recognition of class-I- to class-II-restricted T cells of influenza haemagglutinin. They found that newly synthesized haemagglutinin was presented selectively to class-I-restricted T cells, whereas exogenous haemagglutinin was presented selectively to class-II-restricted T cells.

These results could initially be interpreted by Morrison *et al.* as favouring recognition by class-I-restricted CTLs of complete HA inserted in the membrane of the infected cell. However, the subsequent evidence of the HA signal-depletion experiment (described above), combined with the definition of epitopes with short peptides, has strengthened the idea that separate pathways exist for the presentation of epitopes with class I and class II molecules of the MHC (Townsend *et al.* 1986*a*,*b*; Morrison *et al.* 1986).

Recent work by Yewdell *et al.* (1988) and Moore *et al.* (1988) has shown that entry into the cytoplasm either by fusion of inactivated virus with an endosomal membrane or micro-injection through pinosome lysis results in presentation to class-I-restricted T cells. These results again highlight the cytoplasm as the most likely site of proteolysis of proteins presented with class I molecules.

Proteolysis and CTL recognition

The proteolytic system involved in presentation of epitopes with class I is likely to be constitutive as healthy transfected L cells present NP epitopes to peptide specific CTL clones (Townsend *et al.* 1984, 1986*b*). On this evidence we suggested that presentation of the proteolytic products of their endogenous proteins may be a continuous activity of all cells (Townsend *et al.* 1985). A candidate pathway for proteolysis is the ubiquitin-dependent system, as it is cytoplasmic and may be constitutively active (Ciechanover *et al.* 1984).

This concept may shed light on the nature of alloreactivity as conceived by Matzinger & Bevan (1977), and the identity of minor histocompatibility genes. The high frequency of T cells apparently able to recognize foreign MHC may be explained by the presence on the cell surface of an array of complexes of self-derived peptides with each class I molecule, each one stimulating a different set of T cells. This avoids limiting the interactions of class I to other integral membrane proteins as in the original hypothesis (Matzinger 1981). The identity of minor H gene products has remained elusive, mainly because they have not been definable with antibodies raised to integral membrane proteins. This would be expected if they resulted from the presentation of polymorphic non-membrane proteins, analogous to influenza NP in a transfected cell.

A useful tool would be a selective inhibitor of presentation of antigens with class I. This may exist in the form of vaccinia virus. Coupar *et al.* (1986) noticed that influenza HA was presented to CTLs by recombinant vaccinia-infected cells efficiently if expressed from an early promoter, but weakly if expressed late.

We have compared CTL recognition of haemagglutinin and nucleoprotein expressed during the early and late phases of infection by using the 4B late promoter (Townsend *et al.* 1988). We find that for nucleoprotein the inhibitory effect is selective for certain epitopes. The presentation of the D^b-restricted sequence 366–379 is profoundly inhibited compared with the K^k-restricted sequence 50–63, which appears to be unaffected. For both NP and

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haemagglutinin, presentation is either partly or completely restored by expression of unstable versions of these proteins in the vaccinia-infected target cell. For HA, deletion of the *N*-terminal signal sequence overcomes the block to presentation. For NP, either a large *N*-terminal deletion or the construction of a rapidly degraded ubiquitin–NP fusion protein (as described by Bachmair *et al.* 1986) partly restores presentation. It would seem that offering the vaccinia-infected cell a more efficient substrate for proteolysis restores presentation to CTLs, and suggests that vaccinia may be interfering with protein degradation.

In this next context it may be relevant that cow-pox virus expresses a protein with sequence homology to a family of serine protease inhibitors related to antithrombin III (Pickup *et al.* 1986). In addition, the sequence described by Pickup *et al.* did not contain an *N*-terminal signal sequence, and so might be expected to accumulate in the cytoplasm of the host cell. It is therefore possible that vaccinia synthesizes one or more protease inhibitors that selectively interfere with enzymes involved in cleavage of some epitopes and not others, which would account for our observations.

Another possibility is that the inhibitory effect is mediated through the shutdown of host protein synthesis during vaccinia infection (Moss 1985). Coupar *et al.* (1986) suggested that if co-synthesis of class I molecules with the viral antigen was required for efficient presentation, then loss of host protein synthesis might result in loss of presentation. The fact that the vaccinia effect is selective makes this explanation less likely, although as different allelic class I molecules move to the cell surface at different rates, the effect on intracellular pools may vary for different allelic products (Williams *et al.* 1985). The two epitopes of NP were restricted through different class I molecules.

These two explanations are not mutually exclusive and are testable by appropriate manipulations of vaccinia virus.

Conclusions

The evidence reviewed here suggests that class-I-restricted T cells can recognize the degraded fragments of proteins that have passed through the cytoplasm of the target cell, either as a result of new synthesis or entry from outside through the membrane of an endosome. This mechanism may be quite distinct from the typically pH-dependent presentation of exogenous protein antigens, which are thought to remain effectively topographically outside the cell and which are usually recognized by class-II-restricted T cells. The unifying concept that all T cells recognize degraded forms of protein antigens bound to class I or II MHC molecules is consistent with evidence that both T-'killer' and T-'helper' cells share a common pool of T-cell V-receptor genes.

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