

HLA B37 DETERMINES AN INFLUENZA A VIRUS NUCLEOPROTEIN EPITOPE RECOGNIZED BY CYTOTOXIC T LYMPHOCYTES

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In both humans and mice the majority of influenza A virus-specific, cytotoxic T lymphocytes (CTL) recognize determinants that are shared between different subtypes of the virus (1–3). The nature of the antigen that was recognized remained elusive for several years. Attempts to define it by blocking CTL target cell recognition with monoclonal antibodies specific for individual influenza virus proteins failed (4). The problem was solved only when it became possible to insert single influenza A virus proteins into target cells. This was first achieved by transfecting cDNA copies of single-virus RNA segments (genes) into mouse lymphoblastoid cells. It was then found that, whereas hemagglutinin was recognized by rare subtype-specific CTL (5, 6), nucleoprotein was the major target antigen for crossreacting CTL, even though nucleoprotein was present in only very low amounts on the cell surface. Yewdell et al. (7), in an alternative approach, used recombinant vaccinia to insert a single influenza A virus gene, as cDNA, into target cells by infection. They also found that nucleoprotein was a major epitope. Similar results have been obtained with human CTL (8), and it has been shown in addition that two other proteins, matrix and polymerase PB2, are recognized.¹ The virus glycoprotein, hemagglutinin was seen by a minor subpopulation of subtype-specific CTL in mice (5, 6) but such a population has not been found in humans, possibly because humans have been naturally immunized with several strains and subtypes of influenza A viruses. Recognition of the other glycoprotein, neuraminidase, has not been seen.

Further characterization of the influenza A virus nucleoprotein (NP)² epitope was made by Townsend et al. (9) who transfected truncated NP cDNA into target cells. By using overlapping cDNA preparations, they localized the epitope recognized by CTL of C57 and CBA mice. These studies also showed that short fragments of the protein could be recognized by CTL that were specific for the virus, and that a common signal sequence was not needed to transmit protein fragments to the membrane. These results implied that CTL, like helper T cells, might recognize protein antigens (9) with their native conformation disrupted.

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² *Abbreviations used in this paper:* BCL, Epstein-Barr virus-transformed B lymphoblastoid cell line; NP, influenza A virus nucleoprotein.

TABLE I
Amino Acid Sequences of Peptides Used

Peptide	Virus*	Sequence [‡]
335-349	1968	SAAFEDLRVLSFIRG
336-349	1968	AAFEDLRVLSFIRG
337-349	1968	AFEDLRVLSFIRG
338-349	1968	FEDLRVLSFIRG
339-349	1968	EDLRVLSFIRG
335-349	1934	SAAFEDLRVLSFIKG
365-380	1968	IASNENMDAMESSTLE

* The 1968 sequences were derived from the nucleoprotein of influenza A/NT/60/68 and the 1934 sequence was from influenza A/PR/8/34 virus (11).

[‡] The single-letter amino acid code is used.

This was tested directly using synthetic peptides covering the C-terminal third of the nucleoprotein from amino acids 325 to 487. This experimental approach demonstrated that CTL clones from C57 mice recognized the synthetic peptide that corresponded to residues 365-379 of nucleoprotein (10). In addition, CTL from one human donor, MG, which were known to recognize NP, lysed target cells treated with another synthetic peptide 335-349 (10).

In the experiments described here, we have screened CTL prepared from donors of other HLA types for recognition of the peptide 335-349. CTL from three other donors sharing HLA B37 recognized this peptide, but CTL from 10 other donors did not. Continuously growing CTL lines were prepared from lymphocytes of two of the B37⁺ donors, and the determinant was further localized to stretch of eight amino acids.

Materials and Methods

Donors. Blood donors known from previous experiments (e.g., reference 8) to give an influenza-specific CTL response in vitro were used in these experiments. We are grateful to Dr. Frank Ennis (Worcester, MA) for the gift of the lymphocytes of donor XY and to Dr. A. Rickinson (Birmingham, United Kingdom) for the lymphocytes of donors CD and JD. HLA types had been determined by Dr. A. Ting (Nuffield Department of Surgery, Oxford), using the standard National Institutes of Health technique. Lymphoblastoid cell lines were prepared by transformation with Epstein-Barr virus as previously described (8).

Viruses. Influenza A/X31 and A/NT/60/68 viruses were grown as previously described. The recombinant vaccinia virus, NP-VAC, in which cDNA was coding for influenza A/PR/8/34 nucleoprotein was a gift from Dr. G. Smith and Dr. B. Moss (National Institutes of Health, Bethesda, MD) and has been described before (7).

Induction of Influenza A Virus-specific CTL. This was carried out exactly as described previously (8) by adding influenza A virus to peripheral blood lymphocytes in culture at a multiplicity of infection of 1:10. CTL were tested 1 wk after initiation of culture.

Peptides. Synthetic peptides were prepared on a synthesizer (Applied Biosystems, Inc., Foster City, CA) and purified by HPLC. Each peptide was at least 90% pure. Peptide sequences were based on published information (11). The peptides used are listed in Table I.

Treatment of Target Cells with Peptides. Two methods were used as described previously (10). In the first, CTL and ⁵¹Cr (Radiochemical Center, Amersham, United Kingdom) labeled target cells were mixed in 150 μ l of RPMI 1640 with 10% FCS (Gibco-Biocult, Paisley, United Kingdom), in microtiter wells. Peptide, diluted in RPMI 1640 was then

TABLE II
Recognition of NP Peptide 335–349 by Human Influenza A Virus-specific CTL

Donor	HLA type	E/T ratio	Percent lysis of autologous target cells		
			A/X31	335–349	Nil
MG	A1,31;B13,37	30:1	48	32	5
LG	A1,29;B44,37	40:1	55	51	2
CD	A1,2;B62,37	20:1	45	23	5
JD	A1,29;B44,37	20:1	19	17	1
EG	A25,30;B44,13	60:1	52	0	3
FM	A2,2;B62,49	60:1	44	0	5
IH	A2,3;B7,15	40:1	23	8	7
VH	A1,2;B8	40:1	14	0	0
CM	A1,2;B7,8	20:1	15	3	0
AB	A2,3;B51,39	30:1	26	6	1
JJ	A2,11;B35,50	60:1	35	10	14
XY	A2,28;B22,35	25:1	37	14	18
JM	A2,2;B15,51	40:1	25	4	9
CB	A1,30;B7,44	60:1	20	1	0

added, in a volume of 15 μ l, to the required dilution. In the second method, B lymphoblastoid cells were centrifuged and resuspended in 0.1 ml RPMI/10% FCS. An equal volume of ^{51}Cr (3 $\mu\text{Ci/ml}$) was added followed by an equal volume of peptide to the required concentration. After 1 h cells were washed three times in RPMI 1640, counted, and dispensed in wells for the CTL assay.

Cytotoxicity Assay. This was carried out exactly as described previously (1, 6, 8, 10).

Cold Target Inhibition Experiments. Autologous B lymphoblastoid cells lines were infected with influenza A virus or treated with peptide exactly as for preparation of target cells but without ^{51}Cr labeling. These were added to the standard chromium-release assay wells immediately after the effector cells to give ratios of cold inhibitor/target up to 40:1.

Results

Recognition of Nucleoprotein Peptide 335–349 Is Strongly Associated with HLA B37. Our initial observation was that influenza-specific CTL prepared from one donor, MG, recognized the peptide 335–349, restricted through HLA B37 (10). Influenza A virus-specific CTL were prepared from donors known to give a measurable response and tested on autologous or fully HLA-matched B lymphoblastoid target cells that had been pretreated with peptide 335–349. Donors selected for the presence of HLA B37, normally present in 3% of the population, were also tested. The results, shown in Table II, indicate that all 4 HLA B37-positive CTL recognized the peptide, whereas the other 10 did not. Three other HLA B37-positive donors were tested but gave no measurable influenza A virus-specific response and no lysis of peptide-treated cells. Although two of the B37⁺ donors were related (LG is the daughter of MG), the others were not related. The results suggest that the 335–349 epitope is selected directly or indirectly by HLA B37 for CTL recognition.

Establishment of IL-2-dependent, Peptide-specific CTL Lines. Primary cultures from MG and LG lymphocytes, stimulated for 7 d with influenza A/X31 virus, were restimulated with equal numbers of an irradiated (3,000 rad) autologous

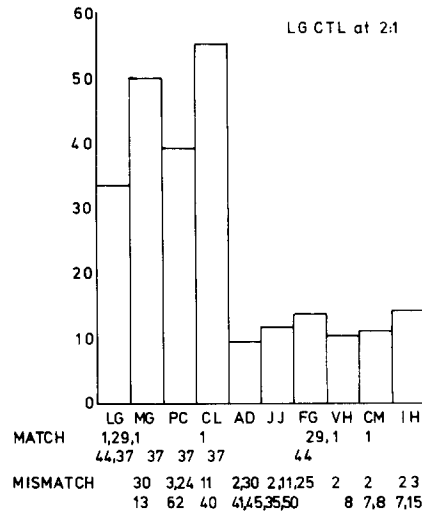


FIGURE 1. HLA restriction of LG CTL line grown by restimulation with the peptide 335–349 treated LG BCL and IL-2 for 8 wk. Effectors were tested on the target BCL shown at a 2:1 ratio, each pulsed with the peptide 335–349 for 1 h at 0.4 μ M before washing and adding to killer cells. Shared and mismatched HLA A and B antigens are shown. Results are shown on the y axis as percent specific lysis.

EBV-transformed lymphoblastoid B cell line (LBCL) that had been pulsed with peptide 335–349 at 60 μ M for 60 min. These cells were washed and then added to CTL at a ratio of 1:1. Supernatants from the gibbon IL-2-producing cell line MLA144 were added to 25–30%. In both cases the cells responded by growing and were maintained by thrice-weekly feeding with IL-2 and once-weekly addition of fresh peptide-pulsed cells. Lines were generated from each donor on several occasions and have been maintained for >12 wk in culture without loss of specificity.

Specificity of CTL Lines. MG and LG CTL lines maintained specificity for peptide 335–349, lysing autologous or HLA B37-matched target cells efficiently (>50% specific lysis) at killer/target ratios as low as 0.5:1. They were regularly tested against other synthetic peptides derived from the nucleoprotein amino acid sequence, including 365–380, and showed no crossreactivity. After growing for 8 wk, the CTL were tested for virus specificity and HLA restriction (Fig. 1). They lysed autologous target cells infected with influenza A/X31 virus and NP-vaccinia virus, but not targets infected with vaccinia virus. HLA restriction was present in both lines and was tested extensively for the line LG. Each lymphoblastoid target cell was pulsed with peptide 335–349. Only those that shared B37 were lysed (Fig. 2).

Whether or not the corresponding region of the nucleoprotein was seen on the surface of virus-infected cells was tested by cold target inhibition of CTL-mediated lysis. Influenza A virus-infected autologous lymphoblastoid cells inhibited lysis of cells that had been pulsed with peptide 335–349 (Fig. 3), indicating that the epitope of peptide 335–349 is present on the surface of cells infected with whole virus.

Fine Specificity of Peptide-specific CTL. MG and LG CTL lines were added to

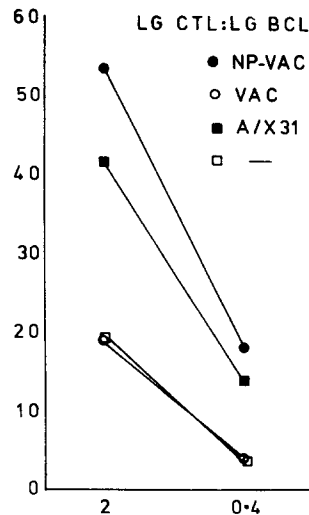


FIGURE 2. Virus specificity of LG CTL line grown by restimulation with peptide 335-349 (1968)-treated LG BCL and IL-2 for 8 wk. Target cells were LG BCL, (■) infected with influenza A/X31 virus, (□) uninfected, (●) infected with NP-VAC, or (○) infected with vaccinia virus. Percent specific lysis is shown on the ordinate; killer/target ratios on the abscissa.

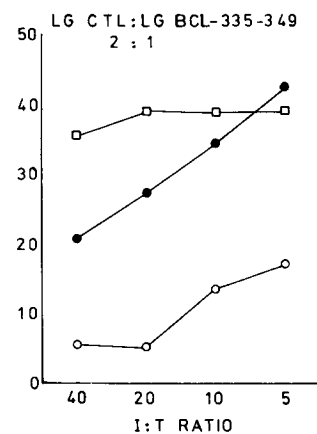


FIGURE 3. Influenza A virus-infected cells display the epitope of peptide 335-349. LG CTL were added at a ratio of 2:1 to ^{51}Cr -labeled LG BCL that had been treated with peptide 335-349. Unlabeled LG BCL inhibitor cells which were added at ratios of 5:1 to 40:1 were either (□) uninfected, (●) infected with influenza A/X31 virus, or (○) pulsed with peptide 335-349. Percent specific lysis is shown on the y axis.

autologous lymphoblastoid cell lines in the presence of peptide at a range of dilutions from 0.025 to 30 μM . The titration curves shown in Fig. 4 reveal that LG CTL recognized target cells at dilutions of peptide 20 times greater than did MG CTL. This result was reproducible with CTL lines prepared on different occasions. It suggests that the LG CTL recognized peptide on target cells with greater efficiency than the MG CTL.

Both CTL lines were tested in a similar manner with a nested set of peptides 335-349 to 339-349. Both lines lysed cells pulsed with lower concentrations of

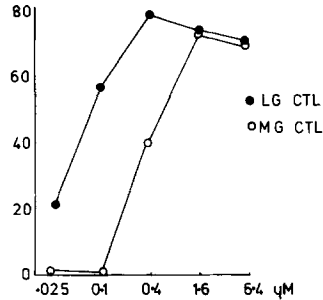


FIGURE 4. (●) LG and (○) MG CTL lines were tested on MG BCL at killer to target ratio of 2:1 in the presence of peptide 335–349 at the final concentrations shown. Percent specific lysis is shown on the y axis.

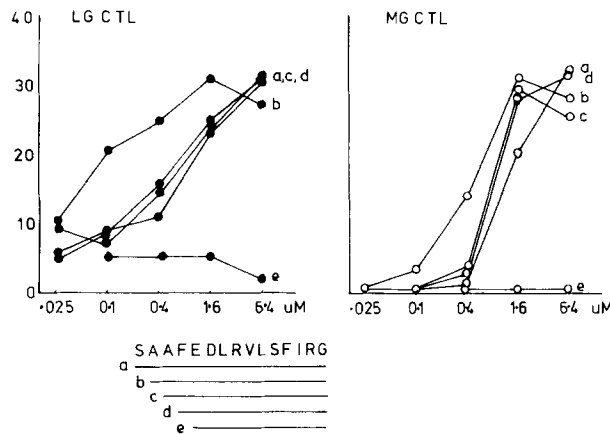


FIGURE 5. (●) LG and (○) MG CTL lines were tested on MG BCL at a killer to target ratio of 2:1 in the presence nested set of peptides shown. Percent specific lysis is shown on the y axis.

336–349 than 335–349 while the peptides 337–349 and 338–349 were not recognized as well. The 11-mer peptide 339–349 could not act as a target cell for either type of CTL line (Fig. 5).

Both CTL lines reacted equally well with the peptide derived from the 1968 sequence (as used above) and the peptide derived from the 1934 sequence; these differ only by an arginine (1968) substituted for a lysine (1934) at position 348.

Phenotype of CTL Lines. The CTL lines were stained with monoclonal antibodies specific for CD4, CD8, and CD3. Both lines were >90% positive for CD8, <10% for CD4, and 95% for CD3.

Discussion

In the experiments described here, influenza A virus-specific CTL from 4 of 14 human donors were found to lyse autologous target cells that had been treated with the influenza nucleoprotein peptide 335–349. One of these responders, LG, was the daughter of MG, but the others were unrelated. All were positive for the HLA antigen, HLA B37, which suggests that this molecule plays a dominant role in determining the fine specificity of the CTL. Three other

B37⁺ donors were tested, but it was not possible to generate influenza A virus-specific responses from their cells; currently, we can do so from ~30% of donors (12). Of the 10 influenza-specific CTL preparations that failed to recognize autologous cells that had been treated with peptide, none was positive for HLA B37.

It is known that CTL from at least five of the B37⁻ donors specifically lyse target cells infected with the recombinant vaccinia virus, NP-VAC, and thus recognize NP in association with other HLA antigens.¹ These CTL must therefore recognize other regions of NP, or other peptide epitopes, in association with other HLA class I antigens.

These data give strong support to the determinant selection hypothesis, which was originally formulated to explain immune response gene function (13) and has been extended to explain MHC class I function and polymorphism (14). The simplest interpretation is that the HLA antigen B37 and peptide 335–349 associate in a unique way which is immunogenic to CTL. Specific but low-affinity interactions between peptide antigens and Ia molecules have been described (15, 16). The finding of Townsend et al. (9) that CTL from CBA (H-2k) and C57 (H-2b) mice recognize different regions of the NP molecule also supports the notion. However, the involvement of other polymorphic genes in epitope selection cannot be excluded.

Experiments on the specificity of CTL were facilitated by the finding that specific CTL lines grew after restimulation with the 335–349 peptide, as shown by irradiated autologous B lymphoblastoid cells in the presence of MLA144 supernatant (IL-2). The rapid growth of the CTL in this manner contrasts with the difficulty that we have encountered in the past in growing CD8⁺ CTL on influenza virus-infected cells. The CTL lines were >90% positive for the CD8 antigen, and were therefore distinct from the CD4 cytolytic T cell clones that recognize NP, as described by Fleischer et al. (17).

The CTL lines were specific for the 335–349 peptide. They were regularly tested against the 365–380 peptide, which is seen by the murine CTL, and also other peptides, with negative results. The CTL lines maintained their ability to kill virus-infected cells that show specificity for nucleoprotein when recombinant vaccinia virus was used to infect targets. The relationship between the epitope defined by peptide 335–349 and that presented on the surface of influenza A virus-infected cells was further explored by cold target-cell inhibition. It was found that influenza virus-infected cells could inhibit recognition of peptide-treated cells by the CTL line, though less efficiently than unlabeled peptide-treated cells. This indicates that this epitope is represented on the surface of influenza virus-infected cells.

The CTL lines also maintained their HLA specificity. Several HLA-mismatched target cells were pulsed with peptide and tested for lysis. None was seen confirming the unique association with B37. The results also failed to reveal any alloreactivity by these T cells, which, although not monoclonal, shared recognition of precisely defined antigens (18).

The fine specificity of recognition was further analyzed by testing CTL with related peptides. Both CTL lines crossreacted fully with the 1934 peptide that differs in the amino acid at position 348 (11). When the amino-terminal residues

were removed, the recognition was improved for peptide 336–347 for both CTL lines. Removal of amino acids 336 and 337 gave results very similar to the full-length peptide, but removal of the phenylalanine at position 338 abolished activity. This indicates that the most critical residues for T cell recognition probably lie between residues 339 and 347.

The efficiency of recognition of peptide differed between the two lines. The LG CTL line recognized 20-fold less peptide than did the MG CTL. Thus, although both lines recognized peptide in association with the same HLA antigen, there may be at least two ways of doing this. This could reflect a molecular difference in the T cell receptor combinations used by clones within the two lines. However, we cannot assume an affinity difference without direct binding measurements; other explanations for the finding, such as differences in T cell mobility or activation state, are possible.

These results have a practical implication. Attempts are currently being made to synthesize peptides based on virus protein sequences for possible use as vaccines. For stimulation of CTL activity, at least, we could not expect any single peptide to be very efficient; the 335–349 peptide should only stimulate CTL in the 3% of the population that are positive for HLA B37. Although CTL are probably less important than antibody in conferring prophylactic immunity to influenza, they do have a well-defined role to play in clearance of virus (reviewed in 19) and, unlike antibody, could offer subtype-crossreactive protection.

In conclusion, these results define an epitope on the influenza A virus nucleoprotein that is recognized by CTL of individuals who are positive for HLA B37. The close association between the epitope and HLA B37, and the fact that we have not been able to identify other haplotypes that recognize this region imply that each HLA antigen will present a set of peptide fragments of foreign proteins to CTL, and that each set will differ. Thus HLA type must have a qualitative effect on the nature of an individual's CTL response. It will be important to define the variety of peptides with which a given HLA antigen can interact, because this may determine the extent of the HLA polymorphism needed for survival in a potentially hostile environment.

Summary

Human influenza A virus-specific, cytotoxic T cells have been shown previously to recognize the virus nucleoprotein on infected cells. CTL preparations from four HLA B37-positive donors were shown to recognize a synthetic peptide that corresponded to amino acids 335–349 of the nucleoprotein sequence. Influenza-specific CTL from 10 donors of other HLA types failed to recognize this epitope. CD8⁺ CTL lines were derived from lymphocytes of two HLA B37-positive donors and used to show that the peptide was represented on virus-infected cells and to determine the probable boundaries of the epitope.

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Note added in proof: Since this manuscript was submitted we have tested influenza A virus-specific CTL from four more donors for recognition of NP peptide 335–349. Two that were positive for HLA B37 lysed autologous peptide-treated cells to levels comparable with MG CTL; two that were negative for HLA B37 failed to kill peptide-treated target cells.

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