IDENTIFICATION OF VIRAL MOLECULES RECOGNIZED BY INFLUENZA-SPECIFIC HUMAN CYTOTOXIC T LYMPHOCYTES

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Human cytotoxic T lymphocytes (CTL) specific for influenza A virus generated from peripheral blood lymphocytes of immune donors show exquisite specificity in their recognition of target cells that share class I molecules of the major histocompatibility complex (MHC) (1). Polyclonal human CTL show full cross-reactivity between all influenza A viruses (2, 3), but the nature of the viral antigens that are recognized together with self HLA antigens on the surface of infected cells is poorly defined. The influenza A virus genome codes for two antigenically variable transmembrane glycoproteins, haemagglutinin and neuraminidase, and eight more conserved internal proteins. Neutralizing antibody is directed toward the variable transmembrane glycoproteins and is subtype specific (4). In contrast, the virus determinants recognized by subtype crossreactive CTL now seem likely to be the more conserved internal antigens. Using a recombinant vaccinia virus expressing the nucleoprotein (NP-VAC)[1] it was shown that some human influenza A virus–specific CTL recognize the nucleoprotein molecule (5). Furthermore, a short synthetic peptide derived from the nucleoprotein sequence is recognized by CTL in a manner restricted to a single class I MHC antigen, HLA B37 (6, 7). Murine CTL have also been shown to recognize the individually expressed influenza virus haemagglutinin and nucleoprotein in transfected L cells (8, 9) or in cells infected with recombinant vaccinia viruses (10, 11). The nucleoprotein was found to be a major target antigen for murine crossreactive CTL, and one of the polymerases has also been recognized by a murine CTL clone (12 and J. Bastin, John Radcliffe Hospital, Oxford, personal communication).

If vaccines are to be produced that boost CTL memory, and which might offer

some degree of protection against new virus subtypes not provided by conventional antibody-producing vaccines, a detailed characterization of which internal antigens are recognized is necessary. To address this question, a series of vaccinia virus recombinants, each expressing an individual influenza virus protein, have been constructed. We report here the ability of CTL generated from donors of different HLA haplotypes to recognize autologous cells infected with these recombinant vaccinia viruses, and the influence that HLA type may have on this recognition. We show that at least three internal virus proteins may be seen by CTL with varying degrees of specific allelic HLA restriction. Some influenza proteins were not seen by any individual tested.

Materials and Methods

Viruses. Influenza viruses AX31 and A/NT/60/68 were grown in allantoic cavities of 11-d-old embryonated chicken eggs, harvested and stored at $-80°C$. Haemagglutinin titers were between 1,000 and 2,000. Thymidine kinase–negative recombinant vaccinia viruses containing influenza virus genes were constructed as described previously (13, 14). Recombinant vaccinia viruses PA-VAC, PB1-VAC, PB2-VAC, NP-VAC, NS1-VAC, and M1-VAC contained cDNAs from influenza A/PR/8/34 corresponding to acidic polymerase, basic polymerase 1, basic polymerase 2, nucleoprotein, nonstructural protein 1, and matrix protein 1, respectively. Recombinant vaccinia viruses NS2-VAC and M2-VAC contained cDNA copies of mRNAs coding for nonstructural protein 2 and matrix protein 2 of influenza A/Udorn/72; NA-VAC contains the neuraminidase gene of A/Cam/1/46 and H2-VAC contains the haemagglutinin gene of A/Jap/305/57 (14). All recombinant viruses have been shown to express the relevant influenza protein by immunoprecipitation of [^{35}S]methionine-labelled infected cell extracts using specific antiinfluenza antisera (5, 14, and G. Smith, unpublished data). NS2 and M2 were not expressed by NS1-VAC and M1-VAC, respectively.

Wild type vaccinia virus (strain WR) and recombinant viruses were grown in HeLa cells, purified from cytoplasmic extracts by sucrose density gradient centrifugation, and stored at $-80°C$. Plaque-forming unit titers were determined on CV-1 cells.

Blood Donors. All donors were healthy volunteers. HLA types were determined by the standard National Institutes of Health (NIH) technique. HLA types of all donors are given in Table I.

Induction of Influenza A Virus–specific CTL. The methods were based on those described previously (5). Briefly, peripheral blood lymphocytes (PBL) at 1.5×10^6 cells/ml were incubated in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY), in the absence of serum, with influenza virus at a final dilution of 1:2,000 for 1 h at $37°C$. FCS (Gibco Laboratories) was added to 5%, and the flask was incubated for six more days. Cells were harvested and resuspended in RPMI 1640, 10% FCS for use in lytic assays. Nonsensitized cells were incubated without virus for 6 d.

Preparation of Target Cells. Autologous Epstein-Barr virus–transformed lymphoblastoid cells, which were shown previously to form the most sensitive target cells, were resuspended to 10^6 in 100 μl RPMI 1640. Recombinant vaccinia virus, or wild type vaccinia, 50–100 μl was added to give a multiplicity of infection of 10–50 pfu/cell, together with 150 μCi Na^{51}Cr (Amersham Corp., Amersham, United Kingdom). After incubation for 1.5 h, cells were washed and resuspended in 1 ml RPMI 1640, 10% FCS. They were incubated for 4 h at $37°C$, washed three times in RPMI 1640 10% FCS, and resuspended for the lytic assay. It has been shown (5) that cells infected with recombinant vaccinia virus in this way have influenza virus antigen present, detectable by staining with appropriate monoclonal antibody or by immunoprecipitation.

Lytic Assay. A standard procedure was used (2, 15), and killer/target ratios used are shown in the figures. The percentage of specific ^{51}Cr released was calculated as [(release by CTL) − (medium only release)] $\times$ 100/[(detergent only release) − (medium only release)]. Each experimental point was measured in duplicate against quadruplicate

controls in medium alone. Spontaneous ^{51}Cr release by recombinant vaccinia–infected targets in medium alone varied between 15 and 25% of detergent-mediated lysis. Some cells infected with wild-type vaccinia had spontaneous release of up to 30% of detergent lysis.

Results

Recognition of Recombinant Vaccinia Virus–infected Target Cells.   CTL from six donors whose lymphocytes gave an influenza A virus–specific HLA-restricted response after stimulation in vitro were tested for recognition of recombinant vaccinia virus–infected autologous Epstein-Barr virus–transformed lymphoblastoid cells. All of these CTL lysed autologous cells infected with some, but not all, of the recombinant vaccinia viruses. In all cases, PBL that were cultured in the absence of influenza virus (nonsensitized controls) gave only background lysis of infected target cells (data not shown).

We found (Fig. 1) that target cells infected with PB2-VAC were clearly recognized and lysed by CTL from four out of six donors; those infected with NP-VAC by CTL from all donors; those infected with M1-VAC by all donors; and those infected with M2-VAC were lysed weakly by one out of five donors. Lysis of target cells infected with other recombinant vaccinia viruses, including H2-VAC and NA-VAC, were close to the background levels of lysis seen with uninfected cells.

HLA Restriction.   The HLA types of the donors are shown in Table I. IH, CM, and JM have been shown by immunoprecipitation and isoelectric focusing (IEF) of methionine-labeled cell lysates to have the commonest variant of HLA A2, A2.1, whereas AM has the basic variant HLA A2 molecule, A2.2 (16). MG and LG shared no HLA alleles with JM or IH, and yet CTL from all four of these donors recognized autologous targets infected with NP-VAC and M1-VAC. This implies that no one allele determined specificity for any one viral protein.

When influenza A virus–specific CTL were tested against HLA-A and -B mismatched target cells that were infected with the recombinant vaccinia viruses, they were not lysed (Fig. 2A and B). When tested on target cells that shared only one or two HLA-A or -B antigens, it appeared that different HLA antigens might influence which virus protein was recognized. Thus CM CTL lysed only M1-VAC-infected JM cells when only HLA-A2 was shared, but showed a preference for NP when A1 and B7 were shared. In the latter combination, M1-VAC-infected cells were only lysed slightly more than the background levels seen with NA-VAC (Fig. 2A). IH CTL also clearly recognized M1 through HLA-A2 (Fig. 2B).

Virus Specificity.   We have generated polyclonal CTL from the same donor by stimulating in vitro either with A virus X31, which is recombinant of ANT 60/68 and A/PR/8/34 with the 1968 surface glycoproteins (H3N2) but the 1934 internal proteins; or A/NT/60/68, with the 1968 surface glycoproteins (H3N2) and the 1968 internal proteins. Such CTL showed identical patterns of reactivity with the recombinant vaccinia–infected target cells (Fig. 3). The recombinant vaccinia viruses used to infect these target cells contained cDNA corresponding to genes from A/PR/8/34 influenza virus. Thus recognition of the internal virus proteins M1, NP, and PB2 was crossreactive with regard to virus subtype.
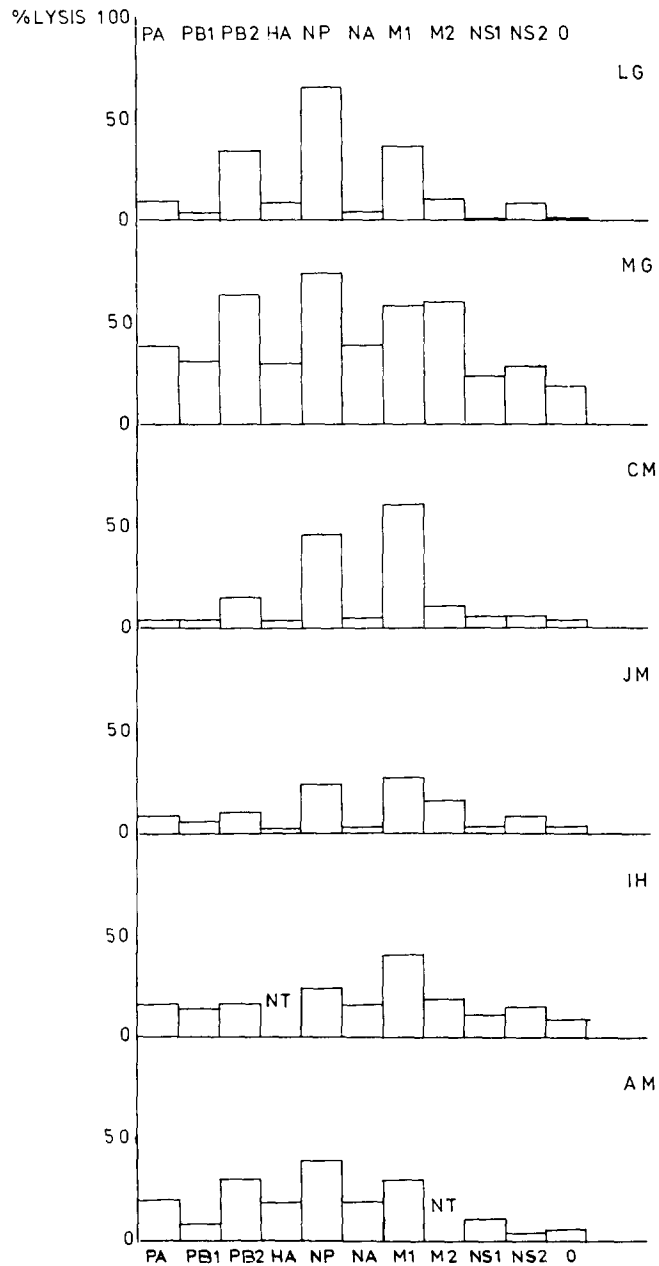
FIGURE 1. Recognition of autologous target cells infected with recombinant vaccinia virus by CTL from six donors. Lysis is shown on the y axis, measured at an E/T cell ratio of 40:1 for each donor. The recombinant vaccinia viruses used are indicated above and below the histogram. They were: *PA*, polymerase A; *PB1*, polymerase B1; *PB2*, polymerase B2; *HA*, haemagglutinin; *NP*, nucleoprotein; *NA*, neuraminidase; *M1*, matrix 1; *M2*, matrix 2; *NS1*, nonstructural protein 1; *NS2*, nonstructural protein; *0* uninfected cells.

TABLE I

HLA Types of Donors

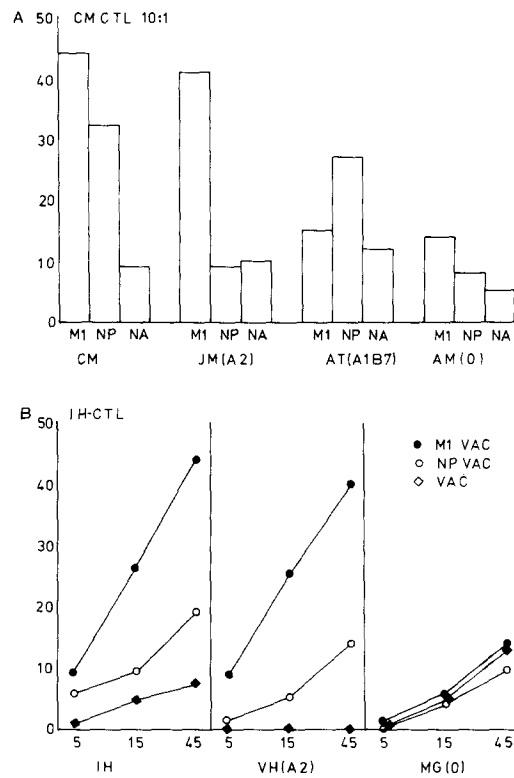| Donor	HLA							
	A		B		C		DR	
LG	1	29	44	37	6		ND	
MG	1	30	13	37	6		7	
CM	1	2	7	8	ND		2	3
JM		2	15	51	3		4	
IH	2	3	7	15	3	7	2	4
AM	2*	32	49	60	3	7		4

* Basic by IEF.

FIGURE 2.   (A) Recognition of recombinant vaccinia virus infected target cells by CM CTL. Target cells were CM (autologous), JM (shared A2), AT (shared A1 and B7), and AM (shared no HLA antigen), and were infected with M1-VAC (*M1*), NP-VAC (*NP*), or NA-VAC (*NA*). The last was representative of background nonspecific lysis. (B) Recognition of recombinant vaccinia virus infected target cells by IH CTL. E/T ratio on x axis, percent lysis, y axis. B lymphoblastoid target cells were IH (autologous), VH (shares HLA-A2), and MG (HLA missmatch), infected with M1 VAC, NP VAC or VAC.

Discussion

Conventional influenza vaccine, when administered, results in a humoral response with neutralizing antibodies directed towards surface viral glycoproteins, but poor stimulation of CTL memory (17). If an influenza vaccine that
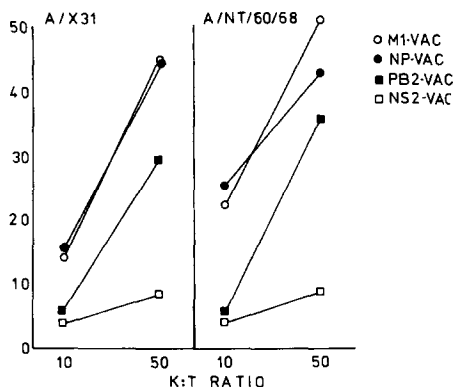
FIGURE 3. Virus specificity of CM polyclonal CTL produced with A/X31 (H3N2, 1934 internal proteins) or A/NT/60/68 (H3N2, 1968 internal proteins). E/T ratio on x axis, percent lysis on y axis. Target cells were autologous B lymphoblastoid cells infected with 10 pfu/cell vaccinia virus.

would stimulate crossreactive and crossprotective CTL is to be produced, it is important to determine which viral proteins are recognized by human CTL and whether HLA type influences this. It has been previously demonstrated, using reassorted viruses to infect cells, that relatively rare virus subtype–specific murine CTL clones recognized viral polymerase (12), haemagglutinin (15), or nucleoprotein (18). Crossreactive mouse influenza-specific CTL were shown to recognize nucleoprotein (10). Recognition of the matrix proteins by CTL has not been described before.

Here we used a complete set of recombinant vaccinia viruses containing cDNA that corresponded to each of the 10 influenza virus–encoded proteins. We were able to demonstrate that influenza A virus–specific but subtype crossreactive human CTL were able to recognize cells infected with NP-VAC (5), M1-VAC, and PB2-VAC. Target cells infected with H2-VAC and NA-VAC, where the influenza virus product was demonstrable on the surface with antibody (data not shown) were not recognized by A virus–polyclonal crossreactive CTL.

No subtype specificity has ever been seen in the human polyclonal CTL response to influenza virus (2). All donors used in this study were born before 1968 and may therefore be considered to have been exposed to H2 haemaglut-tinin, which was present on the influenza viruses that circulated between 1957 and 1968. However, as CTL memory is relatively short lived (19), CTL specific for H2 may no longer be present, explaining why H2 is not seen by crossreactive CTL. Viruses of the H1 subtype are currently prevalent, and one might therefore expect target cells infected with H1-VAC to be detected by H1-specific subtype CTL. However, when such target cells were tested with polyclonal CTL, HLA-unrestricted lysis was observed (data not shown), possibly due to a lectin-like effect. This phenomenon, which was not seen with H2-VAC or any of the other recombinant vaccinia viruses, has meant that we have been unable to determine whether subtype-specific CTL are present in these donors.

The lack of recognition of virus proteins other than NP, M1, and PB2 is not attributable to lack of expression, since all 10 influenza gene products have been detected by immunoprecipitation using specific antisera (G. Smith, unpublished

data). Moreover, NP, HA, PA, PB1, PB2, and NS1 targets have been recognized by autologous murine CTL (Bennink, J. R., J. W. Yewdell, G. L. Smith, and B. Moss; manuscript submitted for publication).

Recognition was HLA restricted. CTL from all the donors tested recognized targets infected with NP-VAC and M1-VAC; PB2-VAC and M2-VAC (weakly) were seen by some but not others. Two of the donors, MG and LG, shared no HLA alleles with three of the other donors, JM, IH, and AM, and yet all saw NP and M1. In MG and LG we know that nucleoprotein is seen in conjunction with HLA-B37 (6). IH saw matrix protein in conjunction with HLA-A2 and CM CTL appeared to see M1 with A2, and NP with A1 or B7. Such associations were not exclusive, however. We know that LG and MG CTL see a particular epitope of NP (amino acids 335–349) with B37 (7): obviously other epitopes exist and must associate with different HLA antigens.

It may be that the HLA type of an individual determines which virus antigen is seen predominantly, and indeed it is possible that some HLA antigens might offer favorable responses to influenza, with survival advantages. Conversely, there might be individuals totally unresponsive in terms of CTLs to influenza as a result of their HLA type, but as such types would have been selected against strongly they would be very rare. We have found that PBL from ~70% of donors in the United Kingdom fail to give a measurable influenza A virus–specific CTL response (17). This nonresponder population includes individuals with both HLA-A2 and -B37, and it seems likely that nonresponsiveness is normally a consequence of the limited duration of CTL memory (19).

CTL from each individual recognized at least two conserved viral antigens (normally thought of as internal) accounting for influenza virus subtype cross-reactivity, which is well documented. CTL prepared with both AX31 and A/NT/60/68, which contain the 1934 and 1968 internal proteins, respectively, reacted with M1, NP, and PB2 from the 1934 virus. The mechanism of recognition of internal virus proteins in association with MHC antigens is not clear, and nucleoprotein was not detectable on the surface of recombinant vaccinia–infected B lymphoblastoid cells using a monoclonal antibody 5/1 and a polyclonal sheep antinucleoprotein serum (5). It has been shown that recognition of autologous virus-infected target cells was not blocked by monoclonal antibodies specific for viral proteins (20), implying that the viral proteins are not recognized by CTL in their native form. The results are consistent with the view that CTL recognition may involve processing of the viral antigen by the target cell and that small peptide fragments of the antigen may be recognized in conjunction with MHC class I on the cell surface (6, 21).

Summary

Human cytotoxic T cells specific for influenza A virus were tested for recognition of each of the ten influenza A virus proteins expressed in target cells using recombinant vaccinia viruses. They recognized the matrix M1, polymerase PB2, and nucleoproteins of influenza virus in association with MHC class I antigens. These internal viral proteins were seen by CTL in conjunction with one or more of the available dependent HLA gene products. There was no detectable recognition of influenza virus surface glycoproteins in target cells.

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