Recognition of Influenza A Virus Nucleoprotein by Human Cytotoxic T Lymphocytes

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SUMMARY

A recombinant vaccinia virus (NP-VAC) containing cDNA corresponding to segment 5, the nucleoprotein (NP) gene of influenza A/PR/8/34 virus was used to examine the specificity of human influenza virus immune cytotoxic T lymphocytes (CTL). Effector cell preparations from two donors recognized autologous lymphocytes that had been infected with NP-VAC. Lysis was specific because cells infected with vaccinia virus were not killed and recognition was HLA-restricted. In one donor, the influenza virus-specific CTL response changed with time so that his effector cells no longer recognized autologous lymphocytes infected with NP-VAC. However, a component that was NP-specific remained because these CTL lysed the more sensitive autologous B lymphoblastoid cells that had been infected with NP-VAC. In four other donors, no NP-specific CTL response could be detected using autologous lymphocyte targets. Thus NP, an internal virus protein, is one antigen that is recognized by human influenza A virus-specific CTL, but it is likely that other individual virus components contribute to the total CTL response.

INTRODUCTION

Cytotoxic T lymphocytes (CTL) specific for influenza A virus-infected target cells can be generated from peripheral blood lymphocytes of immune blood donors (McMichael & Askonas, 1978; Biddison et al., 1981). As in the mouse, these CTL are restricted by antigens of the major histocompatibility complex (MHC) and show exquisite specificity in their recognition of self HLA antigens (McMichael, 1978; Biddison et al., 1980). In contrast, the nature of the virus antigen recognized has been elusive. Polyclonal CTL show full cross-reactivity between all influenza A viruses that have been tested (McMichael & Askonas, 1978; Biddison et al., 1981). Their recognition of autologous infected cells is not inhibited by monoclonal antibodies specific for haemagglutinin, neuraminidase, matrix protein, nucleoprotein (NP) or combinations of these (Dongworth & McMichael, 1984); in contrast, recognition is readily inhibited by HLA-specific monoclonal antibodies (McMichael et al., 1981). The majority population of murine influenza virus-specific CTL also shows cross-reactivity in recognition of influenza A virus-infected cells (Zweerink et al., 1977; Effros et al., 1977; Braciale, 1977). Some cytotoxic T cell clones distinguish between different subtypes of influenza A virus and, by using reassorted viruses to infect cells, it was shown that these clones recognize haemagglutinin (Braciale et al., 1984), viral polymerase (Bennink et al., 1982) or NP (Townsend & Skehel, 1984). This approach could not be used to identify the target antigen of cross-reactive CTL. This question remained unresolved until Townsend et al. (1984) demonstrated that in C57 and C3H mice a major population of influenza A virus cross-reactive CTL recognized influenza A virus NP in L cells that had been transfected with cDNA for segment 5 of influenza A virus RNA. A similar result for BALB/c mice was found by Yewdell et al. (1985) who used...
recombinant vaccinia virus (Mackett et al., 1984; Smith et al., 1983) to insert NP into target cells. Neither set of data, however, excludes recognition of other virus antigens by CTL. Recent experiments by Yewdell et al. (1986) indicate that the non-structural protein 1, but not the matrix protein, can be detected by mouse CTL.

In this report we use a recombinant vaccinia virus expressing NP of influenza A/PR/8/34 virus (NP-VAC) to show that some human influenza A virus-specific CTL recognize NP. However, such recognition was not found in CTL from all donors tested and the pattern of recognition changed in one individual studied over a 9 month period.

**METHODS**

*Viruses.* Influenza A viruses, A/PR/8/34 (H1N1), A/X31 (H3N2) and A/NT/60/68 (H3N2) and influenza B/Hong Kong/68 virus were grown in chicken egg allantoic cavities, harvested and stored at −80 °C until use. Haemagglutination titres were determined and were between 1000 and 2000.

Recombinant vaccinia virus that expressed the nucleoprotein (NP-VAC) or haemagglutinin (H1-VAC) of influenza virus A/PR/8/34 were constructed using previously described methods (Mackett et al., 1984; Smith et al., 1983). Cloned copies of these genes were generously provided by Dr R. G. Webster (Mount Sinai School of Medicine, New York, N.Y., U.S.A.). 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 titres were determined on CV-1 cells.

*Induction of influenza A virus-specific CTL.* The methods were based on those described in detail previously (McMichael & Askonas, 1978; Biddison et al., 1981). Lymphocytes from venous blood were separated on Ficoll Hypaque and suspended at 3 × 10⁶/ml in RPMI 1640 medium (Gibco) without serum in aliquots of 4 ml in 50 ml tissue culture flasks (Sterilin). An equal volume of the same medium containing influenza A virus A/PR/8/34, A/X31 or A/NT/60/68 diluted 1 in 1000 was added to each flask. After 1 h, 0.5 ml foetal calf serum (FCS) (Gibco) was added and the flask incubated for 7 days. Cells were harvested and used for lytic assays. In experiments where sensitization was with vaccinia virus or recombinant vaccinia virus, 10% of the lymphocytes were infected with vaccinia virus as described below and then washed three times in RPMI 1640, added back to the uninfected lymphocytes and incubated in 10% FCS–RPMI 1640 for 7 days.

*Preparation of virus-infected target cells.* Target cells were peripheral blood lymphocytes (PBL), phytohaemagglutinin (PHA)-stimulated lymphoblasts or Epstein–Barr virus-transformed lymphoblastoid cells. Twenty µg PHA (Wellcome, Beckenham, U.K.) was added to 10⁶ PBL in 10 ml RPMI–10% FCS and cells were incubated for 3 days at 37 °C. Lymphoblastoid cell lines were generated by inoculating 10⁷ PBL with 1 ml of a gibbon cell line, B95.8, supernatant for 1 h, and resuspended at 1 × 10⁶/ml in RPMI–10% FCS in the presence of 2 µg/ml PHA. Lymphoblastoid B cells grew out of these cultures within 21 days.

For virus infection target cells were resuspended to 3 × 10⁶ in 100 µl RPMI 1640 medium. Virus (50 to 100 µl), was added to give a multiplicity of infection of 10 to 50, together with 150 µCi Na⁵¹CrO₄ (Amersham). After incubation for 1.5 h, cells were washed and resuspended in 3 ml RPMI 1640–10% FCS. They were incubated at 37 °C for 4 or 16 h (vaccinia virus) or 4 h (influenza A virus) washed three times in RPMI 1640 and resuspended for the lysis assay.

*Cell-mediated lysis.* Target cells were dispensed at 10⁴ cells/well in 96-well round-bottomed microtitre plates (Sterilin) in 50 µl RPMI–10% FCS and effectors added in 100 µl of the same medium. Killer: target (K:T) ratios between 5:1 and 100:1 were tested in each experiment. Experimental wells were dispensed in duplicate. Controls for background ⁵¹Cr release in the absence of effector cells (low controls) and maximal ⁵¹Cr release in the presence of 5% Triton X-100 (high controls) were prepared in quadruplicate. Plates were incubated for 5 h at 37 °C, centrifuged and 80 µl of supernatant was taken for counting. Lysis was calculated from the formula specific lysis = (experimental counts − low control) × 100/high control − low control. Low controls were normally less than 25% of total release, except for some cells infected with the wild-type vaccinia virus where backgrounds were up to 30% of high controls.

*Immunoprecipitation of influenza A virus nucleoprotein.* Lymphocytes or B lymphoblastoid cells were infected with influenza A virus, vaccinia virus or NP-VAC as described above. After the first 1.5 h infection they were resuspended at 10⁷ cells/ml in methionine-free RPMI 1640–10% FCS (Gibco). After incubation for 1 h, 50 to 100 µCi [³⁵S]methionine was added and incubation continued overnight at 37 °C. Cells were then lysed in 150 mm-NaCl, 5 mm-EDTA, 50 mm-Tris–HCl, 0.5% NP40, pH 7.4 for 30 min at 4 °C. After centrifugation in a microfuge, the supernatant was taken and cleared by incubation with 100 µl 10% formalin-fixed *Staphylococcus aureus* Cowan strain I cells (Pansorbin, Calbiochem). After 1 h the supernatant was incubated with anti-NP monoclonal antibody 5/1 (a gift from Dr R. G. Webster) at 5 µg/ml for 1 h. Then 100 µl 10% Pansorbin or 100 µl 5% Protein A-Sepharose (Sigma) was added. After 1 h the pellets were centrifuged, washed, the bound material was eluted with
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2% (w/v) SDS, 20% (w/v) sucrose, 5% (w/v) 2-mercaptoethanol, 0-02% bromophenol blue and run on a 10% polyacrylamide–SDS gel. This was treated with Amplify (Amersham), dried and autoradiographed.

Immunofluorescence and immunoperoxidase assays of NP-VAC-infected cells. Lymphocytes or lymphoblastoid cells were infected as described above. They were resuspended at 10⁶/ml in phosphate-buffered saline pH 7.4 containing 0.5% bovine serum albumin (PBS–0.5% BSA) and 50 μl aliquots dispensed in small plastic tubes. Anti-NP antibody or control antibody was added at a final concentration of 5 μg/ml. After 1 h the cells were washed twice in PBS–0.1% FCS and a second reagent was added. For fluorescence assays this was goat anti-mouse immunoglobulin, fluorescein-conjugated (Sigma). Cells were incubated for a further hour, washed and fixed in 1% formalin–1% BSA–PBS. They were then analysed by the Orthocytocytocytograph by Dr N. Carter, Nuffield Department of Surgery, John Radcliffe Hospital, Oxford.

For immunoperoxidase staining (Mason et al., 1982) a Cytospin preparation was made, fixed in acetone at −20°C and slides were incubated with the anti-NP antibody as described above. The second reagent was horseradish peroxidase-coupled rabbit anti-mouse immunoglobulin (Dako). After 1 h the cells were washed and the developing reagent, diaminobenzidine, was added with hydrogen peroxide. The preparations were then washed and counterstained with haematoxylin and eosin.

Antibodies. Anti-haemagglutinin monoclonal antibody H36-18-2 was kindly donated by Dr W. Gerhard (Wistar Institute, Philadelphia, Pa., U.S.A.) and the anti-NP antibody 5/1 by Dr R. G. Webster (St. Jude Children's Hospital, Memphis, Tenn., U.S.A.).

RESULTS

Expression of nucleoprotein by peripheral blood lymphocytes infected with NP-VAC

Preliminary experiments failed to detect NP on the surface of cells infected with NP-VAC. Consequently further preliminary experiments were carried out with recombinant vaccinia virus H1-VAC to monitor optimal conditions for expression of influenza virus antigen on these infected cells. H1 expression was measured on the surface of lymphocytes by immunofluorescence with an anti-H1 monoclonal antibody (H36-18-2). Over 80% of the cells expressed detectable H1 on the surface after infection at a multiplicity of 10 or greater, 5 h after adding virus. Viability of the recovered cells was greater than 80% at this time and was similar at 16 h although the total number of cells had decreased by about 50%. This cell loss was more marked after infection with the wild-type vaccinia virus used for controls.

After 6 to 16 h infection at a multiplicity of 10, lymphocytes infected with NP-VAC showed no detectable antigen on their surface by immunofluorescence or flow cytometry. However, NP could be detected in over 75% of acetone-fixed NP-VAC-infected lymphocytes by immunoperoxidase staining and it could be precipitated from the cells after labelling with [35S]methionine, using monoclonal or polyclonal antibodies (data not shown) and was visible after a short (4 h) exposure of the autoradiograph (Fig. 1). The NP-VAC virus was checked in this way four times during the course of the experiments and there was no evidence of any change in the activity of the virus. For cytotoxic T cell assays therefore the conditions described above were used.

Recognition of NP-VAC-infected lymphocytes by influenza A virus-specific CTL

Six donors whose lymphocytes gave an influenza A virus-specific CTL response after stimulation in vitro were tested for recognition of NP-VAC-infected autologous PBL. Although all six lysed influenza virus-infected autologous cells in an HLA-restricted fashion, only two lysed cells infected with NP-VAC at levels that were clearly greater than vaccinia virus controls. The response in one of these, CM, was studied in detail over the course of 9 months. Initially CM influenza A virus-specific CTL recognized autologous lymphocyte target cells that had been infected with NP-VAC. NP-VAC-infected lymphocytes that were mismatched for HLA were not lysed (Fig. 2); similar results were obtained on four occasions.

When the response of CM PBL was tested 3 months later there was a quantitative and qualitative change in the influenza A virus-specific CTL response. The level of lysis of autologous lymphocytes infected with influenza A virus had declined and these influenza A virus-specific CTL no longer recognized NP-VAC-infected target cells. However, it was found that a strong influenza A virus-specific CTL response could be obtained from CM lymphocytes when blood was taken after severe exercise (a 5 kilometre run in 20 min). This increased
lymphocyte recovery fivefold and the proportion of CD8- (T8)-positive lymphocytes by a factor of two (results not shown). From these cells a low level of recognition of NP-VAC-infected autologous lymphocytes was obtained (Fig. 3). The poor recognition of CM lymphocytes infected with NP-VAC at this time was not due to any defect in the recombinant vaccinia virus because at the same time as these experiments were carried out, lysis of NP-VAC targets was obtained from CTL from another donor, MG (Fig. 3). In addition when the same virus was used to infect the murine H-2\textsuperscript{b} cell line EL4, NP-specific CTL from C57 mice lysed targets effectively (data not shown). Also, NP could be precipitated from CM PBL infected with NP-VAC by specific antibody.

**Type of target cells used**

The poor CTL recognition of lymphocytes infected with NP-VAC but not influenza A virus in some of the experiments described above raised the possibility that PBL did not always present antigen appropriately. Therefore two other types of cells were tested as targets, autologous PHA lymphoblasts and lymphoblastoid cells. The results shown in Fig. 4 indicate that autologous lymphoblastoid cell lines infected with NP-VAC were more sensitive as target cells than similarly infected lymphocytes. Clear recognition of NP-VAC compared to vaccinia (Fig. 4) or other recombinant vaccinia viruses (data not shown) was seen on several occasions indicating that the small NP-specific response on PBL targets (Fig. 3) was real.
Fig. 3. Comparison of NP recognition by CTL from four donors (a, CM; b, MG; c, AM; d, JM). Each effector cell was tested on autologous PBL infected with influenza A/PR/8/34 virus (■), influenza B/Hong Kong virus (□), NP-VAC (●) or vaccinia virus (○). All of the responses shown were generated within 1 month of each other and over 4 months after the response shown in Fig. 2. The CM CTL were prepared from post-exercise PBL; the others were generated from PBL taken from resting donors.

Fig. 4. Comparison of NP-VAC-infected PBL (a), PHA lymphoblasts (b) and B lymphoblastoid cells (c) from CM as targets for influenza A virus-specific CTL. CM (post-exercise) influenza A virus-specific CTL were tested on NP-VAC (■) and vaccinia virus (□) infected autologous cells.
Fig. 5. Induction of influenza A virus-specific CTL with recombinant vaccinia virus. CM (post-exercise) PBL were stimulated for 7 days with autologous cells infected with influenza A/PR/8/34 (○, □), NP-VAC (●, ■) or vaccinia (▲, ▼) virus at a ratio of 10:1. They were tested (a) on CM PBL targets infected with influenza A/PR/8/34 virus, and (b) on CM PBL targets infected with influenza B/Hong Kong virus (○, ●, ▲) and SE (HLA mismatched) PBL infected with influenza A/PR/8/34 virus (□, ■, ▼).

Stimulation of influenza virus-specific CTL by NP-VAC-infected cells

CM PBL were incubated at a ratio of 10:1 with autologous lymphocytes that had been infected for 4 h with NP-VAC. After 7 days, the CTL were tested for their ability to lyse CM and SE (HLA mismatched) lymphocytes that had been infected with influenza A or B virus (Fig. 5). The results showed that an HLA-restricted influenza A virus-specific response was obtained but that it was weak compared to that found after stimulation with influenza A virus-infected cells. Also the numbers of cells recovered were much lower than in the experiments with influenza A virus sensitization. This is probably because the vaccinia virus replicates in lymphocytes and could thus infect the effector cells; in contrast, influenza A virus infection is abortive. For this reason it has not been possible to restimulate with recombinant vaccinia virus to obtain CTL lines specific for NP.

DISCUSSION

The results described here indicate that influenza A/PR/8/34 virus nucleoprotein, inserted into target cells by a recombinant vaccinia virus, can be recognized by human influenza A virus-specific CTL. These experiments thus demonstrate a virus antigen identified by human CTL.

When PBL were infected with recombinant vaccinia virus and used as target cells, CTL from only two out of six donors tested here showed detectable NP specificity. Furthermore, in one donor (CM) the CTL response changed quantitatively and qualitatively over 9 months, from a high lytic response with strong reactivity to NP (Fig. 2), to a low response unreactive with NP. Similar decreases in total responses have been described before and have been attributed to the limited lifespan of CTL memory cells (McMichael et al., 1983 a). At the later times, however, the CTL response measured in CM blood taken after exercise, which increased the yield of CD8-positive cells tenfold (unpublished results), showed good lysis of cells infected with whole influenza A virus and weak reactivity with NP-VAC-infected autologous lymphocytes. It is likely that the exercise mobilized normally non-circulating lymphocytes from sources such as the spleen. These cells contained a higher proportion of influenza A virus-specific CTL memory cells than were present in (resting) PBL at that time and a fraction of these were specific for NP.
The recognition of influenza A virus NP in these post-exercise CTL could be better revealed when autologous, or HLA-matched, B lymphoblastoid cells were infected with NP-VAC and used as target cells. These cells may make better targets for a variety of reasons. They may be more readily lysed by CTL (note the higher backgrounds in Fig. 4) or they may be infected more efficiently with vaccinia viruses. They could thus be more sensitive to lysis by a relatively low proportion of NP-specific CTL in the polyclonal killer cell population. The alternative possibility, that there had been a change in the quality of the NP-VAC was rejected because the result with MG CTL (Fig. 3) on NP-VAC-infected PBL was obtained at the same time as the poor response with the CM CTL, before exercise (not shown) or after exercise (Fig. 3) and NP could be easily immunoprecipitated from $[^{35}S]$methionine-labelled NP-VAC-infected lymphocytes at this time. Furthermore the virus is known to be stable and stores well at $-80^\circ$C. In recent, unpublished, experiments using NP-VAC and other recombinant vaccinia viruses, we have confirmed that influenza A virus-specific CTL from other donors contain an NP-specific component detectable only when B lymphoblastoid cell lines are used as target cells; in contrast both lymphocytes and B lymphoblastoid cells are killed equally effectively when infected with influenza A virus.

It has not been possible therefore to estimate the relative size of the NP-specific subpopulation in the influenza virus-specific CTL response, as estimates would differ according to the type of target cell used. It will be necessary to measure precursor frequency by limiting dilution. The approach used here however can be used to test which other virus components contribute to the CTL response, by using appropriate recombinant vaccinia viruses. As it is now clear that internal virus antigens can be recognized by CTL, we would expect other conserved internal antigens to be recognized, accounting for the influenza virus subtype cross-reactivity.

Similar findings to these have been made for murine influenza A virus-specific CTL (Townsend et al., 1984; Yewdell et al., 1985). In C57, C3H and BALB/c mice a substantial proportion of the CTL response to influenza A virus is directed against nucleoprotein (Townsend et al., 1984; Yewdell et al., 1985). However, in CBA mice it has been found that in occasional individuals, polyclonal CTL fail to recognize NP (A. R. M. Townsend & B. A. Askonas, personal communication). Furthermore, precursor frequency analysis has revealed a considerable variation in the percentage of CTL clones that respond to NP in different individuals within a given mouse strain (P. Pala, A. R. M. Townsend & B. A. Askonas, personal communication). In humans it is clear that there is also variability with time. These differences may reflect environmental influences such as the timing of the stimulating infection in vivo or, in the human, variability in circulating T cell populations. In addition they may reflect random events in the rearrangements of T cell receptor genes.

In mice, it is also clear that other internal proteins such as polymerase-2 (Bennink et al., 1982) and non-structural protein-1 (Yewdell et al., 1986) can be recognized by CTL. In contrast, recognition of surface haemagglutinin seems to be restricted to a minor population of CTL specific for a virus subtype (Braciale et al., 1984; Townsend et al., 1984). The mechanisms involved in the recognition of internal virus proteins are not clear. Yewdell et al. (1985) have found NP on the surface of NP-VAC-infected P815 cells but in our experiments NP was not detectable on similarly infected human lymphocytes or B lymphoblastoid cells using a monoclonal antibody (5/1) and a polyclonal sheep anti-NP serum. However, we cannot exclude the presence of low amounts of NP on these cells which could be recognized by CTL. Alternatively, as discussed by Townsend et al. (1984, 1985), CTL recognition may involve processing of virus antigen.

It is important to determine which virus antigens in addition to NP are recognized by human CTL because of the possibility of generating a vaccine that will stimulate cross-reactive and, it is hoped, cross-protective CTL (McMichael et al., 1983b). It will also be crucial to find out how best these internal components can be manipulated to stimulate cellular immune responses in vivo. Both partially purified NP (Wraith & Askonas, 1985) and NP-VAC (Yewdell et al., 1986) have been shown to stimulate primary influenza A virus-specific, but virus subtype cross-reactive, CTL immunity in mice in vivo.
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