

# THE INFLUENZA A VIRUS NUCLEOPROTEIN GENE CONTROLS THE INDUCTION OF BOTH SUBTYPE SPECIFIC AND CROSS-REACTIVE CYTOTOXIC T CELLS

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The specificity of the cytotoxic T lymphocyte (CTL)<sup>1</sup> response to influenza A virus infection in mouse and man has yet to be rigorously defined. The A virus genome codes for two antigenically variable transmembrane glycoproteins, hemagglutinin (HA), and neuraminidase (NA) in addition to up to eight more conserved non-glycoproteins that are expressed in both abortively and productively infected cells (1).

The majority of cytotoxic T cells detectable *in vivo*, and early in polyclonal cultures *in vitro*, cross-react on target cells infected with all strains of A viruses but not B viruses (2–6). These observations may account for the heterotypic protection observed *in vivo* after infection by serologically distinct A viruses (7–11) and they raise the question of the identity of the virus products recognized by cytotoxic T cells. Three lines of evidence have implicated the viral hemagglutinin as a target molecule for cross-reactive CTL: experiments using Sendai-mediated fusion of influenza glycoprotein containing liposomes to target cells (12), partial blockade of CTL recognition by monoclonal antibodies to the viral hemagglutinin (13), and recently, recognition by some cross-reactive CTL of L cells expressing a transfected HA gene (14). It has also been suggested that the structurally more conserved nonglycosylated virus proteins are involved in the recognition process (15–21).

In addition, there is a subpopulation of CTL detectable in the murine response, members of which specifically recognize only influenza A viruses closely related to the strain used for priming *in vivo* (3–5, 22). Cytotoxic cells with these properties have been selectively stimulated *in vitro* with purified hemagglutinin from A/JAP/305/57 (H2N2) virus (22, 23), but not, as yet, from other A strains. Similarly, subtype specific CTL clones isolated from A/JAP/305/57 primed animals display a recognition pattern on partially typed recombinant A viruses consistent with recognition of the A/JAP hemagglutinin on the infected target cell (24), and this has recently been confirmed using L cells expressing a transfected A/JAP HA gene (14).

These particular results conform with the idea that cytotoxic T cell recognition,

<sup>1</sup> *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; HA, influenza virus hemagglutinin; NA, influenza virus neuraminidase; NP, influenza virus nucleoprotein; PBS, phosphate-buffered saline; RIA, radioimmunoassay.

which is assumed to take place at the surface of the infected target cell, should be mainly directed at viral glycoproteins that are transported to and inserted in the target cell plasma membrane (25).

Recently, cytotoxic T cell clones have been isolated from mice primed with the recombinant A virus X31 (A/Aichi/68 × A/PR/8/34) (H3N2), or A/PR/8/34 (H1N1), which only lysed cells infected with specific A viruses (26, 27). However, when tested on appropriate genetically typed recombinant viruses it was found that the viral genes that controlled the expression of the antigens apparently recognized by these T cell clones did not code for glycoproteins. In one case recognition mapped to the A/PR/8/34 gene for P3(PB2) (26) and in our own example (28) to the X31 gene for nucleoprotein (which is derived from A/PR/8/34).

In this paper we extend these results by showing that CTL with specificity for the A/HK/8/68 (H3N2) nucleoprotein come to predominate in polyclonal cultures maintained *in vitro* by repeated stimulation with cells infected with the recombinant virus E61-13-H17 (A/HK/8/68 × A/PR/8/34) (H3N2), and that the influenza A nucleoprotein gene also plays a role in selecting CTL that are cross-reactive with influenza A viruses of different subtypes.

### Materials and Methods

*Mice.* 3–6-month old C57BL/6 female mice used as responding cell donors were obtained from OLAC (OLAC 1976 Ltd; Shaw's Farm, Bicester, Oxon, U.K.).

*In Vivo Priming of Mice.* Mice were anesthetized with ether and primed by intranasal infection with 5 hemagglutinin units E61-13-H17 or X31 virus as infectious allantoic fluid diluted in 50  $\mu$ l phosphate-buffered saline (PBS), exactly as described previously (22).

*Influenza Virus Strains.* The following virus strains were used: Natural isolates H1N1:A/PR/8/1934, A/Eng/1937, A/Bel/1942, A/Weiss/1943, A/Cam/1946, A/FM/1/1947, A/USSR/90/1977; H2N2:A/JAP/305/57; H3N2:A/Aichi/1/1968, A/Bangkok/1/1979; B virus: B/Hong Kong/8/1973; Recombinant A viruses: E61-13-H17, X31, X47, X61, X45, X57. The parental viruses used to make each of these recombinants and their resulting genotypes are given in Table I, and described in detail in references 29 and 30 (E61-13-H17 is referred to as recombinant number 33 in reference 30).

Virus was grown in the allantoic sacks of 11-d old embryonated chicken eggs, and stored as infectious allantoic fluid at  $-70^{\circ}\text{C}$ .

*Continuous Cytotoxic T Cell Cultures In Vitro.* The methods used to maintain influenza A specific cytotoxic T cell cultures in continuous growth *in vitro* have been described in detail elsewhere (31).

4 wk after priming by intranasal infection *in vivo*, two donor C57BL/6 spleens were pooled and a cell suspension made.  $1.2 \times 10^7$  responding cells were mixed with  $5 \times 10^6$  infected stimulator cells in 15 ml RPMI/10 (Gibco RPMI 1640, 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin,  $5 \times 10^{-5}$  M 2-mercaptoethanol) in 50-ml Nunc flasks (Gibco Ltd; Paisley, Scotland, U.K.).

Stimulator cells were syngeneic normal spleen cells irradiated with 2,000R, and infected in the absence of foetal calf serum with 0.5 ml infectious allantoic fluid/3 ml RPMI 1640/10<sup>8</sup> spleen cells. Stimulator cells were washed three times in PBS before addition to cultures.

After 1 wk incubation at  $37^{\circ}\text{C}$  the responding cells were harvested and restimulated with  $5 \times 10^6$  stimulator cells. After another week the responding cells were harvested and  $2 \times 10^6$  restimulated with  $5 \times 10^6$  stimulator cells in 12 ml RPMI/10 adjusted to 20% vol/vol with a crude preparation of rat T cell growth factors (R/S described below). Such cultures were maintained in exponential growth by repeated identical stimulations every

7 d. If the culture became obviously acidic before the seventh day, half the medium was replaced by fresh RPMI/10/10%R/S.

A lectin-free T cell growth factor preparation was made using a modification of the method described by Speiss and Rosenberg (32). A spleen cell suspension was prepared from PVG or Lewis rats (without lysing red cells) and adjusted to  $2 \times 10^7$  lymphocytes/ml in RPMI/10. Con A (Sigma Type III, Sigma London Chemical Co, Ltd; Poole, Dorset, U.K.) was added to a final concentration of 20  $\mu\text{g}/\text{ml}$  and the mixture incubated at 37°C for 2 h. The cells were then centrifuged at 400  $g$  for 10 min and washed twice in the same volume of PBS, before resuspension at  $5 \times 10^6$  cells per ml in RPMI/10 and incubation at 37°C for 48 h. The supernatant (R/S) was then harvested, millipore filtered, and stored at -40°C in aliquots.

*Cytotoxicity Assay.* A standard 6-h chromium<sup>51</sup> release assay was used as described previously (31). Briefly, EL4 (H-2b) target cells were labeled and infected for 90 min in suspension at 37°C with 150  $\mu\text{Ci}$  Cr<sup>51</sup> (Amersham International Ltd; Amersham, Bucks, U.K.) in 0.5 ml serum-free RPMI 1640 and 50–400  $\mu\text{l}$  of infectious allantoic fluid. After washing four times with 10 ml PBS,  $2 \times 10^4$  target cells/0.1 ml RPMI/10 were dispensed into 96-well flat-bottomed Costar microtiter plates (L. H. Engineering Co, Ltd; Bell's Hill, Stoke Poges, Bucks, U.K.), and effector CTL were then added in 0.1 ml RPMI/10. The plates were centrifuged for 1 min at 400  $g$  then incubated for 6 h, and recentrifuged for 5 min at 400  $g$  before harvesting 0.1 ml of supernatant for radioactivity determination. Percent specific chromium release was calculated as follows:

$$(\text{Release by CTL} - \text{Medium release}) \times 100 / (2.5\% \text{ Triton release} - \text{Medium release}).$$

All points were measured in duplicate against quadruplicate medium controls. Spontaneous chromium<sup>51</sup> release in the absence of CTL ranged between 9% and 34% in all experiments.

## Results

*Selection of an A Virus Subtype-Specific Population of CTL by Repeated Stimulation with E61-13-H17 Virus.* In a previous communication we described the effect of repeated stimulation in vitro with X31 virus-infected stimulator cells on spleen cells from X31-primed mice, in selecting CTL with specificity for a subgroup of A viruses that could then be cloned at high frequency (27). Target cell recognition by one of these clones, when tested on a discriminating set of recombinant A viruses mapped to the A/PR/8/34 gene for nucleoprotein (28). In order to examine this effect in more detail the E61-13-H17 virus was chosen in the present experiments to prime C57BL/6 mice. This virus differs from X31 only in its gene for NP which originates from its 1968 H3N2 parent (Table I and references 29, 30).

4 wk after priming, in vitro cultures were set up as described in Materials and Methods. Responding cells from two E61-13-H17-primed spleens were pooled and divided into two parts. One part was maintained in vitro by repeated stimulation with E61-13-H17-infected feeder cells, and the second part in identical conditions but stimulated by X31-infected feeder cells. The two parallel cultures were tested on week 2 (at which point a source of lectin free T cell growth factors was added) and week 7 of in vitro growth.

Fig. 1 shows the virus specificity of the two cultures at week 2 after two stimulations with antigen. In the left-hand panel the E61-13-H17-restimulated culture is shown; both the E61-13-H17- and X31-infected targets were lysed, the former more efficiently than the latter. In the right-hand panel the X31-stimulated half of the culture is shown; these effector cells clearly lysed the X31-

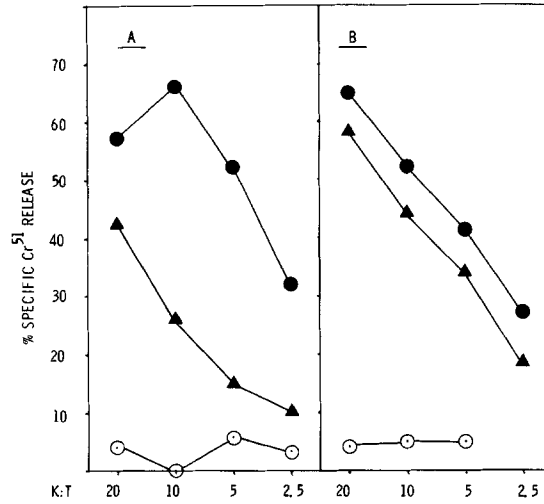


FIGURE 1. The specificity of the two cultures derived from C57BL/6 cells primed with E61-13-H17 virus in vivo after two stimulations at weekly intervals in vitro, *A*: with E61-13-H17-infected feeder cells; *B*: with X31 infected feeder cells. ●, E61-13-H17-infected; ▲, X31-infected; ○, uninfected EL4 target cells.

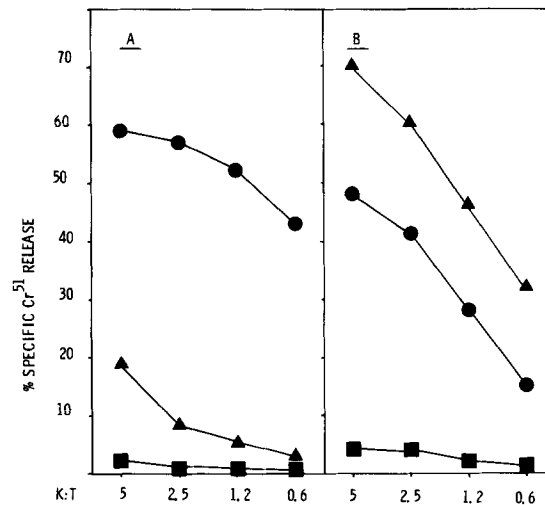


FIGURE 2. The specificity of the same two cultures after seven identical weekly stimulations in vitro, *A*: with E61-13-H17-infected feeder cells, *B*: with X31 infected feeder cells. ●, E61-13-H17-infected; ▲, X31-infected; ■, B/Hong Kong-infected EL4 target cells.

infected target as efficiently as the E61-13-H17 target. Thus, already at week 2 in vitro the E61-13-H17-restimulated culture was showing a detectable difference in the efficiency with which it could lyse target cells infected with two viruses that differ only in their NP genes.

This tendency becomes more marked with time. Fig. 2 depicts a repeat of the same test at week 7 in vitro after seven identical stimulations with virus-infected feeder cells. At this stage the E61-13-H17-restimulated portion of responding cells gives very weak lysis of X31-infected targets in comparison with the X31-

restimulated portion that lysed target cells infected with either X31 or E61-13-H17. The A virus specificity of both cultures is confirmed by the absence of lysis of B/Hong Kong/8/73 infected target cells.

*Mapping the Recognition of E61-13-H17 Specific CTL to the 1968 Nucleoprotein.* In order to confirm the specificity of the E61-13-H17-restimulated responder cells, a set of independent genetically typed recombinant viruses and natural isolates were used to infect target cells. Each of the six recombinant viruses resulted from a cross between an H3N2 virus and A/PR/8/34, and all have been described in detail in the literature (29, 30). The genotypes of each virus are shown in Table I.

Fig. 3 describes the recognition by the two CTL populations after 9 wk of growth in vitro, of target cells infected with these viruses. The first two panels confirm the E61-13-H17-restimulated culture recognized E61-13-H17-infected but not X31-infected cells. These two viruses differ only in their genes for NP. The H3N2 glycoprotein genes originated in these two viruses from A/Aichi/2/68 and A/HK/8/68, which are probably identical. This result, in conjunction with the results with wild type viruses described below, show that the H3N2 glycoproteins do not play a role in recognition by these selected effector cells. Minor differences in the H3N2 glycoproteins in the remaining four recombinant viruses (originating from H3N2 viruses isolated in different years) can therefore be ignored. These four recombinant viruses consist of two pairs (X61, X47, and X51, X45) each of which also differ only in the origin of their genes for NP (29). The remaining six panels of Fig. 3 show that the specificity of the E61-13-H17-restimulated CTL is determined by the gene for NP in viruses isolated after 1968, and are summarized in Table I.

In contrast, the half of the E61-13-H17-primed responder cells that was

TABLE I  
*Polyclonal CTL with Specificity for the Post-1968 Influenza A Nucleoprotein*

Test virus	Parental H3N2 virus	PB1	PA	PB2	HA	NA	NP	M	NS	Activity of CTL lines	
										A	B
E6113H17	A/HK/8/68				■	■	■			+	+
X31	A/Aichi/2/68				■	■				-	+
X47	A/Vic/3/75	■			■	■	■			+	+
X61	A/Texas/1/77	■			■	■				-	+
X45	A/Scot/840/74	■		■	■	■	■			+	+
X57	A/Vic/112/76	■		■	■	■				-	+
A/Aichi/68		■	■	■	■	■	■	■	■	+	+
A/PR/8/34										-	+

Summary of the tests done to map recognition by the two cultures described in Figs. 2 and 3 to the influenza A gene for nucleoprotein. All the recombinant viruses tested were derived from mixed infections with A/PR/8/34 (H1N1) and post-1968 H3N2 viruses (29, 30). In the table the H3N2 parental virus is listed for each recombinant virus and the genotype of each is shown by representing genes derived from the H3N2 parent by ■ and those derived from A/PR/8/34 as blanks. CTL line A, the polyclonal culture shown in Fig. 2A; CTL line B, the culture shown in Fig. 2B. PB1, PA, PB2, genes coding for polymerase proteins; HA, hemagglutinin; NA, neuraminidase; NP, nucleoprotein; M, matrix protein; NS, nonstructural proteins.

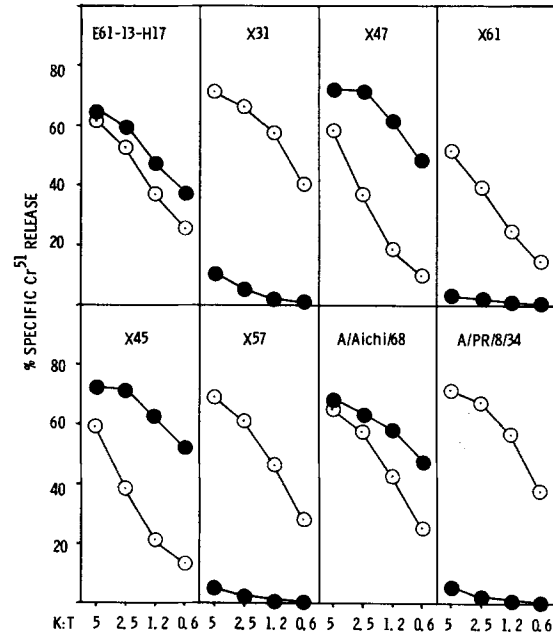


FIGURE 3. Recognition by the same two cultures of EL4 target cells infected with a discriminating set of recombinant A viruses. ●, E61-13-H17-primed and restimulated effectors; ○, E61-13-H17-primed, X31-restimulated effectors.

maintained in parallel by restimulation with X31-infected feeder cells *in vitro* remained cross-reactive on all the A virus-infected target cells tested, including the two widely separated natural isolates A/PR/8/1934 (H1N1) and A/Aichi/1968 (H3N2).

*Recognition of Natural Influenza A Virus Isolates by the E61-13-H17-Specific CTL Population.* The previously mentioned CTL clone with specificity that mapped to the A/PR/8/34 gene for NP, when tested on a range of natural A virus isolates, revealed a clear-cut division between those that could and those that could not sensitize target cells for lysis. Viruses isolated between 1934 (A/PR/8/34) and 1943 (A/Weiss/43) were recognized, those isolated in 1946 and later were not (27). The timing of this change coincides with an antigenic change in the nucleoprotein molecule defined using rabbit antisera to purified NP (33, 34) and certain monoclonal antibodies (35).

The activity of the E61-13-H17-restimulated effector cells on targets infected with a range of natural viruses is shown in Fig. 4 and summarized in Table II. In this case lysis of target cells infected with the 1934, 1937, 1942, and 1943 viruses was weak or absent (compared to background lysis of uninfected or B/Hong Kong-infected cells), whereas all the target cells infected with viruses isolated from 1946 onwards, including representatives of H1N1, H2N2, and H3N2 subtypes, were lysed efficiently by the E61-13-H17-restimulated CTL population. This result confirms that recognition of infected target cells by these effector cells is independent of the type of glycoproteins expressed, and gives the exact reciprocal pattern of our previously described CTL clone with specificity for the 1934 nucleoprotein (27).

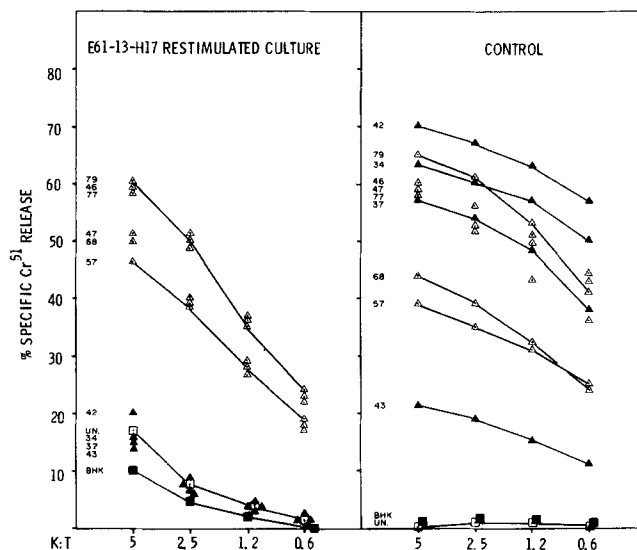


FIGURE 4. (A) Recognition by the E61-13-H17-restimulated culture of EL4 target cells infected with natural influenza A viruses isolated between 1934 (34) and 1943 (43), ▲; 1946 (46) and 1979 (79), △; B/Hong Kong infected, ■; and uninfected, □. (B) Recognition by a cross-reactive control culture of the same panel of target cells. The control culture was provided in this case by spleen cells primed in vivo with E61-13-H17 virus and restimulated twice at weekly intervals with X31 virus in vitro.

TABLE II  
*Recognition by the Nucleoprotein-specific Culture of Natural Influenza Viruses Isolated between 1934-1979*

Test virus	Subtype	Activity of CTL lines	
		E61-13-H17 Restimulated	Control
A/PR/8/1934	H1N1	-	+
A/Eng/1937	H1N1	-	+
A/Bel/1942	H1N1	-	+
A/Weiss/1943	H1N1	-	+
A/Cam/1946	H1N1	+	+
A/FM/1/1947	H1N1	+	+
A/USSR/90/1977	H1N1	+	+
A/Jap/305/1957	H2N2	+	+
A/Aichi/1/1968	H3N2	+	+
A/Bangkok/1/1979	H3N2	+	+

Summary of the recognition of target EL4 cells infected by natural influenza A viruses isolated between 1934 and 1979, by the E61-13-H17-restimulated culture and a cross-reactive control. The control was provided in this experiment by C57BL/6 spleen cells primed with E61-13-H17 in vivo and restimulated twice in vitro with X31 as described in Materials and Methods.

Cross-reactive control CTL lysed all the A virus-infected target cells, showing that the lack of lysis by the E61-13-H17-specific CTL of 1934-1943 virus-infected targets was not due to failure of these viruses to infect. The relatively inefficient infection of target cells by A/Weiss/43 is a consistent feature in our hands, and in four independent experiments the same results were obtained.

The cross-reactive control culture was provided by C57BL/6 spleen cells primed *in vivo* with E61-13-H17 virus, and restimulated *in vitro* twice with X31 virus as described in Materials and Methods.

### Discussion

In this paper we have extended our previous results to show that the genes for NP of both 1968 (H3N2) and 1934 (H1N1) influenza A viruses appear able to determine the recognition of infected target cells by cytotoxic T cells.

The antigenic determinant we have defined here with the E61-13-H17-restimulated culture appears to be immunodominant in C57BL/6 cultures *in vitro*. A polyclonal effector cell population that begins *in vitro* by showing lysis of both X31- and E61-13-H17-infected targets (Fig. 1), after 7 wk of stimulation with E61-13-H17-infected feeder cells loses the ability to recognize X31 while retaining efficient recognition of E61-13-H17-infected cells. The recognition of other recombinant A virus-infected targets (Fig. 3 and Table I) segregates with the gene for NP of viruses isolated after 1968. Furthermore, the ability to recognize target cells infected with natural A viruses isolated in the years between 1934 and 1979 (Fig. 4 and Table II) correlates with a change in the antigenic nature of the NP molecule defined with a rabbit antiserum (33, 34) and certain monoclonal antibodies (35).

In contrast, when the same spleen cells primed *in vivo* with E61-13-H17 are maintained *in vitro* for the same length of time by stimulation with X31-infected feeder cells, the selected effector cells remain cross-reactive on targets infected with A/PR/8/34 (H1N1) and A/Aichi/68 (H3N2) (Fig. 3). As X31 differs from E61-13-H17 only in its gene for NP (29, 30), this gene must play a role in the selection of both A virus cross-reactive and subgroup-specific cytotoxic T cells.

Influenza A nucleoprotein is detectable early in both abortive and productive infections, and becomes the predominant viral protein in the nuclei of infected cells (1, 36, 37). The primary structure of the NP molecule does not reveal a hydrophobic stretch of sequence analogous to that found in characterized integral membrane proteins (38, 39), although this does not rule out membrane association. There are reports that NP can be detected on the external surface of abortively infected cells (15, 16) and in the latter study a semiquantitative RIA method was used with several monoclonal antibodies to show that NP could be detected at a maximum of up to 10% of the amount of HA at the surface of influenza A virus-infected P815 cells. The significance of these observations to the recognition of infected cells by CTL is not known.

The possible roles of the influenza nucleoprotein in T cell recognition can be considered to involve either direct recognition of the nucleoprotein molecule by the cytotoxic T cell or indirect recognition of a determinant at the cell surface induced or controlled by the nucleoprotein. Direct recognition could be mediated either by the low level of cell surface expression previously described (16), or conceivably by a mechanism analogous to antigen processing involving fragmentation of the nucleoprotein and "presentation" by the infected cell. Indirect recognition could be mediated possibly by an effect of NP on one of the glycoproteins of the virus or by conformational changes induced in H-2 molecules by NP interacting with their cytoplasmic domains. However, the latter mecha-



nism appears less likely in the light of results by Murre et al. (40), who have shown that an L<sup>d</sup> molecule truncated by the loss of its cytoplasmic domain still functions efficiently as a restriction element for influenza A-specific CTL.

Considerations similar to these have been presented for the possible mechanisms involved in recognition by CTL of the SV40 large T antigen (41–43). Furthermore, in that system Tevethia et al. (42) have recently demonstrated that L cells transfected with a plasmid containing the large T gene became recognizable as targets for SV40-specific CTL. Recognition by subpopulations of T cells could be mapped using target cells expressing N or C terminal fragments of the protein (43). Similar experiments using cDNA copies of influenza genes to transfect target cells will be helpful in explaining the role of the NP in recognition by the cytotoxic T cells described in this report, and are in progress.

Sequence comparisons between the A/PR/8/34 (H1N1) (38) and A/NT/60/68 (H3N2) (39) nucleoprotein genes reveal only 30 amino acid changes out of 498 (94% conservation). Since the CTL selected in the E61-13-H17-specific culture can clearly discriminate between A/Weiss/43 and A/Cam/46 (Fig. 4), and the difference in sequence between the nucleoproteins of these two viruses is likely to be small, it appears that these cytotoxic T cells are sensitive to such small changes in nucleoprotein structure. At the same time, the fact that the NP molecule is 94% conserved in influenza A viruses also makes it a logical candidate as a target for CTL cross-reactive on all influenza A virus-infected cells.

By selecting immunodominant clones of cytotoxic T cells in the C57BL/6 mouse strain, the method used here of repeated stimulation with a genetically typed recombinant virus *in vitro* may oversimplify the range of CTL specificities produced. A recent analysis by Vitiello and Sherman (44) of cytotoxic T cell clones isolated by limiting dilution after one stimulation *in vitro*, implied a far greater diversity in the virus specificities defined in a C57BL/6J response. A similar experiment by Kees and Krammer (45), by using appropriately typed recombinant viruses to infect target cells, has shown that as many as 90% of such clones stimulated in limiting dilution cultures from C57BL/6 mice could differentiate between A viruses, and recognized determinants that were controlled by viral genes that segregated independently of the two glycoproteins. The proportion of these that map to the virus nucleoprotein awaits to be seen.

### Summary

Using genetically typed recombinant influenza A viruses that differ only in their genes for nucleoprotein, we have demonstrated that repeated stimulation *in vitro* of C57BL/6 spleen cells primed *in vivo* with E61-13-H17 (H3N2) virus results in the selection of a population of cytotoxic T lymphocytes (CTL) whose recognition of infected target cells maps to the gene for nucleoprotein of the 1968 virus. Influenza A viruses isolated between 1934 and 1979 fall into two groups defined by their ability to sensitize target cells for lysis by these CTL: 1934–1943 form one group (A/PR/8/34 related) and 1946–1979 form the second group (A/HK/8/68 related). These findings complement and extend our previous results with an isolated CTL clone with specificity for the 1934 nucleoprotein (27, 28).

It is also shown that the same spleen cells derived from mice primed with E61-

13-H17 virus in vivo, but maintained in identical conditions by stimulation with X31 virus (which differs from the former only in the origin of its gene for NP) in vitro, results in the selection of CTL that cross-react on target cells infected with A/PR/8/1934 (H1N1) or A/Aichi/1968 (H3N2).

These results show that the influenza A virus gene for NP can play a role in selecting CTL with different specificities and implicate the NP molecule as a candidate for a target structure recognized by both subtype-directed and cross-reactive influenza A-specific cytotoxic T cells.

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