# CYTOTOXIC T LYMPHOCYTE RECOGNITION OF THE INFLUENZA HEMAGGLUTININ GENE PRODUCT EXPRESSED BY DNA-MEDIATED GENE TRANSFER

By T. J. BRACIALE, V. L. BRACIALE, T. J. HENKEL, J. SAMBROOK, and M.-J. GETHING

*From the Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110; and the Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724*

Cytotoxic T lymphocytes (CTL)[1] are a readily detectable component of the cellular immune response of most mammalian species to infection by viruses (1). Typically, CTL recognize with great specificity the immunizing antigen when it is displayed on the surface of a histocompatible target cell. However, CTL that occur in both mice and humans in response to type A influenza virus show an unusually high degree of cross-reactivity for target cells infected with serologically distinct type A influenza strains (2–5). This broader spectrum of reactivity reflects the existence of distinct subpopulation(s) of type-specific CTL (2, 4, 6). Indeed, antiinfluenza CTL clones have been isolated that display either this cross-reactive phenotype (7, 8) or more restricted specificities (7, 9, 10).

Although there is no clinical evidence that cross-reactive cellular immunity confers protection against infection with pandemic strains of influenza virus, there are convincing data that CTL promote recovery of experimental animals from influenza virus infection (11–15). It was therefore of interest to define further the antigens recognized on the target cell surface by cross-reactive and other subpopulations of antiinfluenza virus CTL. To date, several different viral proteins have been detected serologically on the surface of cells infected with influenza virus (16–21). To assess the relative contribution of one of these putative target antigens to the heterogeneity of the CTL response, we have used the techniques of DNA-mediated gene transfer to obtain stable transformed lines of murine cells expressing the gene product of a single influenza gene—the hemagglutinin (HA). In this paper, we report the recognition of the cloned HA gene product by both heterogeneous and homogeneous (cloned) populations of type A influenza-specific CTL.

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[1] *Abbreviations used in this paper*: CTL, cytotoxic T lymphocytes; FBS, fetal bovine serum; HAT, hypoxanthine, aminopterin, thymidine; HA, influenza hemagglutinin; M, matrix protein; NA, influenza neuraminidase; NP, nucleocapsid protein; NS, nonstructural protein; RIA, radioimmunoassay; VSV, vesicular stomatitis virus.

## Materials and Methods

*Animals.* Male CBA/J (H-$2^k$) and BALB/c ByJ (H-$2^d$) mice were purchased from The Jackson Laboratory, Bar Harbor, ME, and were used at 7–14 wk of age.

*Viruses.* Influenza virus strains A/PR/8 (A/Puerto Rico/8/34 [$H_1N_1$]), A/WSN/33 ($H_1N_1$), A/JAP/57 (A/Japan/305/57[$H_2N_2$]), A/PC/75 (A/Port Chalmer/75 [$H_3N_2$]), A/AA/67 (A/Ann Arbor/7/67 [$H_2N_2$]), and B/Lee were grown in the allantoic cavity of 10-d-old embryonated eggs of chickens and stored as infectious allantoic fluid, as previously described (4).

*Immunizations.* Mice were inoculated intraperitoneally with 100–300 hemagglutinating units of infectious influenza virus of the A/JAP/57, A/WSN/33, or B/Lee strains as described (4). Mice were used as responder spleen cell donors 3 or more wk after immunization.

*In Vitro Secondary and Tertiary Responses.* Splenocytes from virus-immune mice provided the source of CTL precursors for in vitro stimulations. The induction of in vitro secondary and tertiary CTL responses was carried out as described in detail previously (4, 7). Activated CTL were used 5–7 d after in vitro stimulation or restimulation.

*Establishment and Maintenance of CTL Clones.* CTL clones were generated from A/JAP/57 immune CBA/J splenocyte precursors according to established procedures (7, 22). Clones were maintained in continuous culture for at least 6 wk before cytotoxicity assays on L929 transformants. Cloned CTL were usually used in the assays 4–6 d after routine subculture in the presence of syngeneic, irradiated, A/JAP/57-infected splenocytes and conditioned media from concanavalin A-activated rat splenocytes.

*Establishment and Maintenance of Cell Lines Expressing A/JAPAN/305/57 HA.* Cell lines 1E2 and 2A1 were derived from LMtk$^-$ cells transfected with a recombinant plasmid pTKSVHA-1 containing the *Escherichia coli* β-lactamase gene, the chicken thymidine kinase (tk) gene, and the A/JAP/57 HA gene inserted between the SV40 virus early promoter and SV40 early sequences containing RNA-processing signals (23) (Fig. 1*A*). The recombinant plasmid was introduced into the LMtk$^-$ cells by the calcium phosphate coprecipitation technique (24) and the tk$^+$ clones were selected and grown in hypoxanthine, aminopterin, thymidine (HAT) medium (24). Cell line L10 was derived from LMtk$^-$ cells, cotransfected with a recombinant viral vector SVLHA-8 (31), in which the A/JAP/57 HA gene has replaced the DNA sequences coding the T antigen in the SV40 early region, and a recombinant plasmid, pOD3, containing the chicken thymidine kinase gene (26), inserted into a derivative of pBR322 (D. Hanahan, unpublished results) (Fig. 1*B*). These two plasmids were simultaneously introduced by the calcium phosphate coprecipitation method and tk$^+$ clones selected in HAT medium. Cell line pOD3 was derived by transfection of LMtk$^-$ cells with plasmid pOD3 alone. The various cell lines were examined for HA, DNA, and RNA content by Southern and Northern blot hybridization (25) and for HA antigen expression by radioimmunoassay (RIA) (see Fig. 2). The full characterization will be presented in detail elsewhere (Gething and Sambrook, manuscript in preparation). In brief, the 1E2, 2A1, and L10 cell lines contain ~2–10 copies of the HA gene per cell and express poly(A)$^+$ HA mRNA and HA protein at ~1/100th the level produced by influenza virus-infected LMtk$^-$ cells. Transformed cell lines were passaged and maintained in HAT medium consisting of Dulbecco's medium supplemented with 10% fetal bovine serum (FBS) and hypoxanthine (15 μg/ml), aminopterin (0.44 μg/ml), and thymidine (9.7 μg/ml). LMtk$^-$ cells were passaged and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Cells were maintained as monolayers in 75-cm$^2$ tissue culture flasks. Cell monolayers were disrupted by treatment with trypsin (0.25% vol/vol) / EDTA (0.02% wt/vol) before reseeding and use in CTL assays.

*Assay of Cell-mediated Cytotoxicity.* Cytotoxicity was assayed by release of $^{51}$Cr as described elsewhere (4), except that the cells were reseeded on 9-cm diam polymethylpentene petri dishes (Nalge Co., Rochester, NY) 24 h before $^{51}$Cr labeling or virus infection. The procedure facilitated resuspension of the cells without trypsinization. Labeling and infection of the cells were carried out as described (4). Cytotoxicity assays were carried out for 7–8 h or for 16 h as indicated in tables. Spontaneous release of $^{51}$Cr from targets ranged from 5 to 12% in 7–8-h assays and from 15 to 20% in 16-h assays. Release values represent

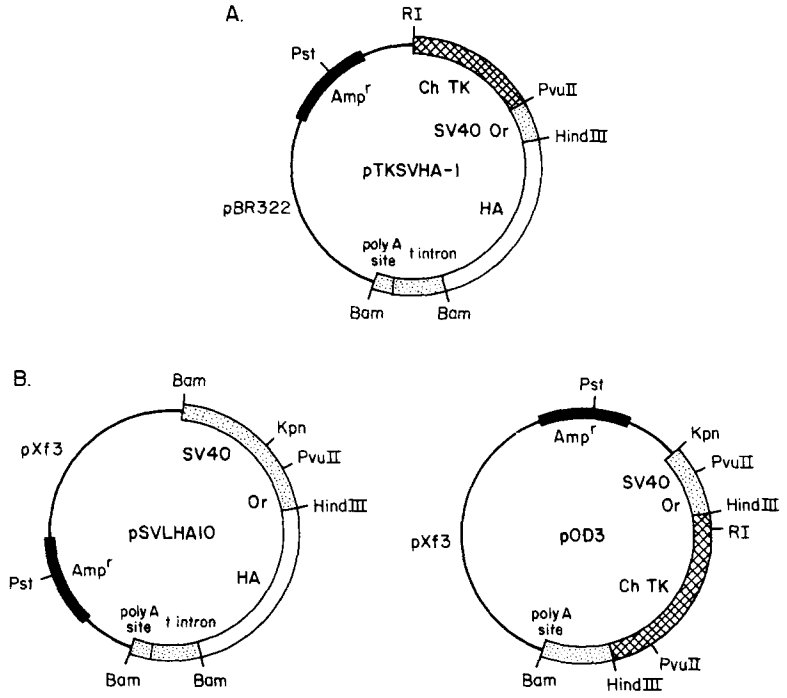


FIGURE 1. Recombinant plasmids designed for the constitutive expression of HA in continuous lines of murine cells.

the means of percent specific $^{51}Cr$ release from three or four replicate wells. Standard errors, calculated by the method of least squares, were always <5% of mean value and have been omitted from the tables.

*Radioimmunoassay.* The HA antigen in transformants expressing the HA gene was quantitated in cell extracts by RIA using high titer rabbit anti-HA antibody, as described in detail elsewhere (27).

*Flow Cytofluorometry.* Cells were examined for expression of HA on the surface by quantitative cytofluorometry using a FACS IV flow cytometer (Becton, Dickinson & Co., Mountain View, CA) with a log fluorescence intensity scale. Cell lines were reseeded on polymethylpentene plates and incubated for 15–17 h at 37°C in HAT medium. In some experiments, 1E2 or pOD3 cells were infected with A/JAP/57 virus at the time of reseeding and examined 12 h later concomitantly with uninfected 1E2 and pOD3. Cells were incubated with a 1:10 dilution of a high titer rabbit antibody specific for the A/JAP/57 HA (16) for 30 min at 0°C. After repeated washings, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (Cappel Laboratories, Cochranville, PA) and incubated again at 0°C for 30 min. After further washing, the cell suspensions were examined by quantitative cytofluorometry.

## Results

*Recognition of HA$^+$ Transformants by Antiviral CTL.* We examined the capacity of murine CTL obtained after inoculation of A/JAPAN/305/57 virus into mice of the CBA/J strain (H-2$^k$ haplotype) to lyse a panel of L cell (H-2$^k$) transformants. Three of the lines, 1E2, 2A1, and L10-1, all of which expressed A/JAP/57 HA, were derived by transfection of the parental LMtk$^-$ cells with eukaryotic vectors that carried the gene for the A/JAP/57 HA, as well as the chicken thymidine

kinase gene (Materials and Methods). Two additional cell lines served as controls: the parental LMtk$^-$ cells and the tk$^+$ pOD3 cells, which are derivatives obtained by transfection of LMtk$^-$ cells with a recombinant plasmid that carried the chicken thymidine kinase gene but no HA sequences. As demonstrated in Table I, Exp. 1, the three HA$^+$ cell lines were susceptible to lysis by a heterogeneous population of A/JAP/57-specific CTL over several effector to target ratios. Neither the parent LMtk$^-$ line nor the pOD3 cell line were susceptible to lysis by virus-specific CTL. After infection with A/JAP/57 virus, both the HA$^+$-transformed cell lines (1E2, 2A1, and L10) and the control cells (LMtk$^-$ and pOD3) were equally susceptible to lysis by antiviral CTL (Table I, Exp. 2).

The three HA$^+$ transformants were also observed to differ in their relative susceptibility to lysis by A/JAP/57-specific CTL, with line 1E2 being most susceptible and line 2A1 the least, at all effector/target ratios tested (Table I, Exp. 1). The difference in reactivity of the three cell lines with CTL may be a reflection of the amounts of HA protein that they express since the number of molecules of HA produced per cell by each transformant, measured by indirect RIA (Fig. 2), roughly correlates with the susceptibility of the cell lines to lysis. Evidence from experiments in which cells of the three lines were superinfected with A/JAP/57 virus (e.g., Table I, Exp. 2) indicates that their different reactivities cannot be explained by variability in the intrinsic susceptibility of the lines to CTL-mediated lysis. Because the 1E2 transformant was consistently lysed to the greatest extent, all subsequent experiments have been carried out with this line and either the LMtk$^-$ or the pOD3 cell lines as controls.

It is also evident from the results in Table I that HA$^+$ transformants became more susceptible to CTL-mediated lysis after superinfection with A/JAP/57 virus. This enhanced lysis may be a consequence of the ~100-fold greater

TABLE I

*Recognition by A/JAP/57-specific CTL of Transformed HA$^+$ Cell Lines**

| Exp. | Effector to target ratios‡ | Percent specific $^{51}$Cr release from target cells§ | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | LMtk$^-$ | | pOD3 | | 2A1 | 1E2 | | L10 |
| 1 | 1:1 | 0 | | 0.2 | | 0.7 | 2 | | 1 |
| | 5:1 | 0.2 | | 0.2 | | 4 | 10 | | 9 |
| | 50:1 | 0.3 | | 0.5 | | 20 | 45 | | 35 |
| | | LMtk$^-$ | | pOD3 | | 1E2 | | L10 | |
| | | Uninfected | A/JAP/57 infected | Uninfected | A/JAP/57 infected | Uninfected | A/JAP/57 infected | Uninfected | A/JAP/57 infected |
| 2 | 6:1 | 3 | 67 | 0 | 39 | 19 | 64 | 13 | 51 |
| | 30:1 | 7 | 97 | 3 | 79 | 46 | 85 | 40 | 83 |

* Spleen cells from pools of two to three immune CBA/J donors 3 or more wk after inoculation with infectious A/JAP/57 virus were cultured with A/JAP-infected, irradiated, syngeneic stimulator splenocytes. After 5–7 d of culture, CTL effectors were used in this analysis.

‡ $1 \times 10^4$ $^{51}$Cr-labeled uninfected (Exp. 1) or uninfected and A/JAP/57-infected (Exp. 2) L cell targets were added per well.

§ Values are the mean from four replicate wells with spontaneous release subtracted. Assay times are 7–8 h.

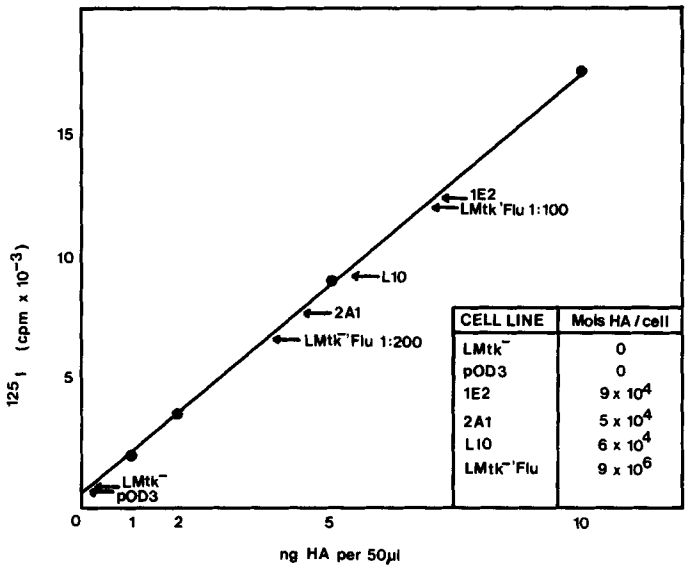


FIGURE 2. Analysis of HA content in cell extracts by solid-phase radioimmunoassay (27). Abscissa indicates nanograms of HA present in 50 µl of sample. Ordinate indicates amount of $^{125}$I-labeled rabbit anti-HA IgG bound to HA. Standard curves were generated by binding purified HA to solid-phase matrix. HA content of various cell extracts is indicated by arrows. Molecules (mols) of HA per cell as measured in assay are given in box.

amounts of HA produced in influenza virus-infected cells (Fig. 2). Because the RIA measures total cell-associated HA, it was necessary to examine quantitatively the expression of HA on the surface of the cells. Fig. 3 shows the result of a flow cytofluorometric analysis of the 1E2 line before and after viral infection, using high titer rabbit antibody raised against the isolated A/JAP/57 HA (16). 1E2 cells infected with A/JAP/57 virus showed a 50–100-fold higher surface expression of HA than the uninfected 1E2 cell line which itself demonstrated less than a twofold higher peak fluorescence than the background on uninfected pOD3 cells.

An alternative explanation of the enhanced susceptibility is that HA was expressed at a level necessary for efficient recognition by CTL in only a proportion of the transformed cell population. To test whether this latter possibility was due to genetic heterogeneity that had occurred since the cell line was originally isolated, we subcloned the 1E2 cell line and examined a panel of 10 independent clones for their susceptibility to lysis by A/JAP/57 virus-specific CTL. All were lysed with approximately the same efficiency as the original 1E2 line (not shown). We therefore conclude that the relative inefficiency with which 1E2 cells are lysed by virus-specific CTL is not a consequence of heritable differences in the levels of expression of either HA or the major histocompatibility antigen. However, we cannot exclude the possibility that the expression of either or both of these antigens (or the efficiency with which they associate on the cell surface) is modulated at different stages in the cell cycle or is affected by cell density or contact.

*Recognition of Cells Expressing the HA Gene Product by Heterogeneous Populations of*

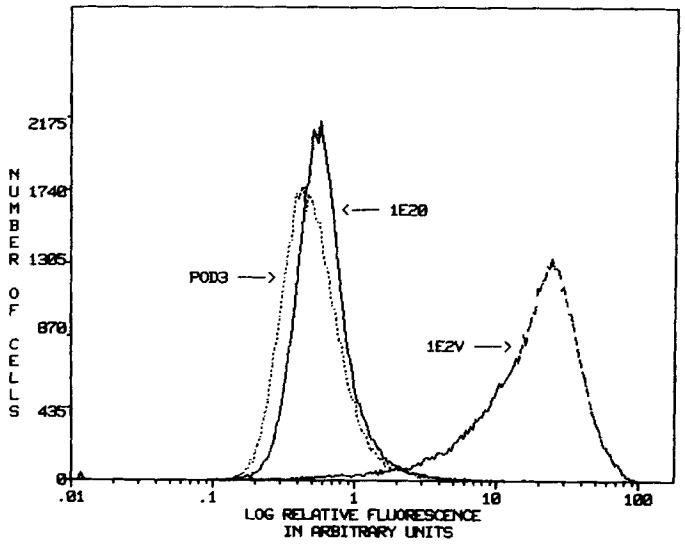


FIGURE 3. Level of A/JAPAN/305/57 HA expression on transformed and virus-infected cells. Three cell lines, the $tk^+$ pOD3 line (POD3), the $tk^+$ $HA^+$ 1E2 line (1E2$\phi$), and the 1E2 line superinfected with A/JAPAN/305/57 virus (1E2V), were examined for the binding of hyperimmune rabbit anti-HA antiserum by a two-step procedure using a fluoresceinated IgG fraction of goat anti-rabbit IgG antiserum.

*Cross-reactive CTL.* The results given above show that the L cells expressing the A/JAP/57 HA after DNA-mediated gene transfer are lysed by A/JAP/57-specific CTL. It was important to assess whether the HA is recognized in an H-2-restricted fashion. Representative results of such an analysis are shown in Table II. As discussed above, heterogeneous populations of A/JAP/57-specific CTL derived from histocompatible CBA/J ($H\text{-}2^k$) donors readily lyse uninfected 1E2 cells expressing HA antigen but do not lyse the uninfected parental $LMtk^-$ cells. Both types of cell are equally susceptible to lysis after infection with A/JAP/57 virus. By contrast, neither uninfected nor infected 1E2 cells were more susceptible than uninfected $LMtk^-$ cells to lysis by a population of A/JAP/57-specific CTL effectors derived from histoincompatible BALB/c ($H\text{-}2^d$) mice (Table II, Exp. 1). The elevated background of cytolytic activity for target cells of the $H\text{-}2^k$ haplotype exhibited by antiinfluenza CTL of $H\text{-}2^d$ origin has been previously reported (4, 28) and presumably reflects the presence of "dual specificity" CTL that simultaneously express $H\text{-}2^d$-restricted recognition of influenza virus and alloreactivity for the $H\text{-}2^k$ haplotype (29).

CTL directed against a related but serologically distinct virus (influenza virus type B) would not be expected to react with cells expressing A/JAP/57 HA (2, 4, 28). As demonstrated in Table II, Exp. 3, the A/JAP/57 $HA^+$-transformed line 1E2 was susceptible to lysis by antiviral CTL directed to influenza B/Lee only after infection of the line with B/Lee virus. Furthermore, recognition of the expressed HA product on the 1E2 line by A/JAP/57-specific CTL was not significantly affected by superinfection with B/Lee virus (Table II, Exp. 3).

Thus, by the criteria of (*a*) H-2 restriction in recognition and (*b*) specificity for the target antigen, the A/JAP/57 HA product expressed after DNA-mediated

TABLE II

*Specificity and H-2 Restriction of HA Gene Product Recognition by Heterogeneous CTL Populations**

| Exp. | Effectors | Effector to target ratios‡ | Percent specific $^{51}Cr$ release from target cells§ | | | |
|---|---|---|---|---|---|---|
| | | | LMtk⁻ | | 1E2 | |
| | | | Uninfected | A/JAP/57 infected | Uninfected | A/JAP/57 infected |
| 1 | CBA/J anti-A/JAP/57 | 2:1 | 0.4 | 40 | 12 | 40 |
| | | 10:1 | 1 | 70 | 40 | 73 |
| | CBA/J anti-A/WSN | 2:1 | 3 | 25 | 11 | 31 |
| | | 10:1 | 12 | 56 | 38 | 62 |
| | BALB/c anti-A/JAP/57 | 2:1 | 4 | 5 | 3 | 3 |
| | | 10:1 | 17 | 18 | 16 | 15 |
| 2 | CBA/J anti-A/JAP/57 | 2:1 | 4 | 20 | 10 | 34 |
| | | 10:1 | 9 | 54 | 38 | 74 |
| | CBA/J anti-A/WSN | 2:1 | 5 | 23 | 4 | 16 |
| | | 10:1 | 18 | 46 | 18 | 52 |

| Exp. | Effectors | Effector to target ratios‡ | pOD3 | | | 1E2 | | |
|---|---|---|---|---|---|---|---|---|
| | | | Uninfected | A/JAP/57 infected | B/Lee infected | Uninfected | A/JAP/57 infected | B/Lee infected |
| 3 | CBA anti-A/JAP | 0.2:1 | 1 | 9 | 0 | 4 | 19 | 0 |
| | | 2.1 | 1 | 46 | 4 | 36 | 78 | 25 |
| | CBA anti-A/WSN | 0.2:1 | 0 | 7 | 0 | 5 | 20 | 0 |
| | | 2.1 | 1 | 36 | 3 | 31 | 70 | 24 |
| | CBA anti-B/Lee | 0.2:1 | 0 | 0 | 6 | 0 | 1 | 11 |
| | | 2.1 | 0 | 0 | 41 | 3 | 7 | 50 |

* As in Table I except that effector populations in Exp. 3 were derived from tertiary CTL population generated by restimulation of in vitro secondary CTL at day 10 of in vitro culture.

‡ As in Table I.

§ As in Table I except that assay times are 16 h.

gene transfer was recognized in a manner similar to that of HA expressed as a consequence of influenza virus infection. It was therefore of interest to determine if HA expressed on the surface of L cells could serve as a target antigen for the subpopulation of cross-reactive CTL generated in the murine response to inoculation with type A influenza viruses (2–4). For this purpose, CTL were generated in response to A/WSN/33 virus ($H_1N_1$ subtype) whose HA is serologically distinct from that of A/JAP/57 virus ($H_2N_2$ subtype). It has been shown previously that a cross-reactive subpopulation of anti-A/WSN CTL is able to mediate lysis of A/JAP/57-infected target cells (4). Table II shows the results of three representative experiments. In most cases (e.g., experiments 1 and 3), A/WSN/33-specific effectors lyse uninfected 1E2 cells much more efficiently than unin-

fected control LMtk$^-$ or pOD3 cells. Thus, the influenza virus HA can be directly implicated as a target for cross-reactive CTL.

On the other hand, in several experiments (e.g., experiment 2), the lysis of uninfected 1E2 cells by CTL directed against A/WSN/33 virus was no greater than that of uninfected control cells. Both the HA$^+$ cells and the parental cells were, however, recognized by A/WSN/33 effector CTL after superinfection with A/JAP/57 virus. These observations raised the possibility that the influenza virus HA may be a target antigen for only a subset of cross-reactive CTL. This hypothesis was further supported by the repeated observation that superinfection of the HA$^+$ cells with the appropriate virus (e.g., type A viruses but not B/Lee) increased the level of lytic activity exhibited by both A/JAP/57-specific and A/WSN/33-specific CTL.

*Recognition of Endogenously Expressed HA by Cloned CTL.* To test the possibility that only a subset of virus-specific CTL recognized the endogenously expressed HA and to obtain more definitive information about the range of CTL specificities directed to the HA, we examined the ability of cloned CTL populations to recognize A/JAP/57 HA. Table III shows the results for six representative CTL clones (of more than 20 examined) selected for A/JAP/57 virus recognition from

TABLE III

*Recognition of the A/JAP/57 HA Gene Product by Cloned Populations of A/JAP-specific CTL**

| Clone‡ | Effector to target ratios§ | Percent specific $^{51}$Cr release from target cells‖ | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1E2 | | pOD3 | | | | |
| | | Uninfected | A/JAP infected | Uninfected | A/PR8 ($H_1N_1$) | A/JAP ($H_2N_2$) | A/AA ($H_2N_2$) | A/PC ($H_3N_2$) |
| 36-7 | 0.2:1 | 14 | 47 | 6 | 3 | 20 | 3 | 3 |
| | 2:1 | 65 | 91 | 8 | 6 | 68 | 9 | 4 |
| | 5:1 | 80 | 99 | 9 | 8 | 81 | 15 | 7 |
| 36-9 | 0.2:1 | 17 | 39 | 5 | 3 | 12 | 3 | 0 |
| | 2:1 | 54 | 80 | 3 | 1 | 57 | 14 | 0 |
| | 5:1 | 75 | 91 | 5 | 2 | 70 | 21 | 1 |
| 35-6 | 0.2:1 | 1 | 46 | 4 | 18 | 28 | 26 | 23 |
| | 2:1 | 14 | 88 | 5 | 90 | 85 | 78 | 84 |
| | 5:1 | 31 | 96 | 7 | 100 | 92 | 90 | 89 |
| 36-1 | 0.2:1 | 0 | 39 | 3 | 27 | 30 | 28 | 28 |
| | 2:1 | 2 | 90 | 5 | 86 | 84 | 82 | 84 |
| | 5:1 | 7 | 95 | 9 | 91 | 94 | 83 | 91 |
| 36-2 | 0.2:1 | 0 | 30 | 3 | 11 | 15 | 15 | 15 |
| | 2:1 | 2 | 79 | 7 | 66 | 68 | 66 | 65 |
| | 5:1 | 6 | 91 | 9 | 86 | 89 | 80 | 86 |

* CTL clones were derived from three separate A/JAP/57 virus-immune CBA/J donors as described (7). Clones were maintained in continuous culture for 4–6 wk before use.

‡ As determined by H-2 restriction in proliferation (22), clones 35-6, 36-1, and 36-2 were restricted by H-2D$^k$, and clones 36-7 and 36-9 by H-2K$^k$ (unpublished results).

§ As in Table I.

‖ As in Table II.

three separate cloning experiments.

A virus-unique/subtype-specific pattern of viral antigen recognition was exemplified by clones 36-7 and 36-9 (Table III), which exhibited a high degree of lysis of both uninfected and A/JAP/57-infected 1E2 cells. These A/JAP/57 virus-specific clones failed to lyse A/PR/8 ($H_1N_1$)- or A/PC/75 ($H_3N_2$)-infected pOD3 targets but showed a high degree of lysis on A/JAP/57-infected targets and lysed to a lower but significant degree target cells infected with the serologically related A/AA/67 ($H_2N_2$) virus. This pattern of viral recognition by CTL has previously been described by us (7) at the clonal level. Our studies on the CTL response to isolated HA (30) and on the recognition of recombinant viral strains by CTL clones (7) suggested that HA was the primary target antigen for these unique and subtype-specific CTL. The finding of specific recognition of the $HA^+$ 1E2 cells by clones with this specificity confirms this point.

A less frequent pattern of CTL recognition is represented by clone 35-6, which is cross-reactive for the major type A influenza subtypes (as demonstrated on the various virus-infected pOD3 targets) and also exhibits a lower but still significant level of lysis of uninfected $HA^+$ 1E2 cells. Thus, we could directly demonstrate at the clonal level HA-specific CTL with cross-reactive specificity for type A influenza viruses and thereby confirm the observations (Table II) made with heterogeneous CTL populations.

The predonimant pattern observed (12 of 20 clones) was that represented by clones 36-1 and 36-2, which failed to lyse uninfected 1E2 cells but exhibited a high degree of specific lysis on A/JAP/57-infected 1E2 cells. When tested on control pOD3 cells infected with a panel of type A influenza viruses of different subtypes, all targets were lysed with equal efficiency. This finding directly supports the concept that there are cross-reactive CTL clones which fail to recognize the HA gene product at least at the level expressed in 1E2 cells.

The demonstration, at the clonal level, of CTL that failed to recognize the endogeneous HA provided an obvious explanation for the enhanced lysis of $HA^+$ transformants after viral infection (Tables I and II) and supported the notion that other viral antigens might serve as the targets for these CTL. On the other hand, three CTL clones that were defined as HA specific also showed to varying degrees enhanced lysis of 1E2 after viral infection. This latter observation might be explained by increased CTL-target interaction, resulting in enhanced lysis after viral superinfection, since the expression of cell surface A/JAP/57 HA is substantially increased after viral infection (Figs. 2 and 3).

## Discussion

The findings reported here demonstrate that the protein product of the cloned A/JAP/57 HA gene, constitutively expressed in continuous cell lines after DNA-mediated gene transfer, can act as a suitable target antigen for H-2-restricted CTL. Our results, along with other recent findings using the cloned HA gene in several eukaryotic expression vectors (23, 27, 31), strongly suggest that the product of the cloned HA gene is indistinguishable in its antigenic and biological properties from the HA synthesized in influenza virus-infected cells and further substantiates the usefulness of DNA-mediated gene transfer to study the structure and function of the HA molecule. More importantly, our results suggest that it

should now be possible to use recombinant DNA technology to probe HA for antigenic structures involved in CTL recognition.

In this report, we were also able to demonstrate unambiguously, using cells expressing HA and no other influenza virus gene product, that the type A influenza HA can serve as a target antigen for cross-reactive antiinfluenza CTL. Cross-reactive, HA-specific killing was demonstrable both with heterogeneous and cloned populations of CTL. These results substantiate previous observations of one of us (33) that one or both viral glycoproteins (HA or neuraminidase) was a target antigen for cross-reactive CTL. Future work can now be directed towards defining the structural basis for this cross-reactive recognition by CTL of serologically distinct HA. In this context, it is of interest that in the vesicular stomatitis virus (VSV) system where the VSV G protein has been implicated as the target for cross-reactive CTL (33), monoclonal G-specific antibodies that simultaneously recognize serologically distinct VSV serotypes have been reported (35). Furthermore, among the type A influenza viruses, a type-specific (cross-reactive) determinant in the HA-2 portion of the HA molecule has recently been demonstrated after low pH treatment of the cleaved HA (36). Thus, it is possible that HA of different subtypes of type A influenza carry a conserved epitope(s) which is immunodominant for CTL recognition/activation but very poorly recognized by the humoral response. Alternatively, cross-reactive epitopes might be generated by "processing" of the HA and/or interaction of the HA molecule with other cell surface constituents (e.g., class I major histocompatible complex products). The availability of the cloned HA gene and expression system for CTL recognition will make it possible for us to determine the structural basis of this cross-reactive CTL recognition.

We have also demonstrated here that the influenza HA can serve as the target antigen for strain-specific, subtype-specific CTL. This finding directly confirms previous conclusions from an analysis of the CTL response to isolated HA (6, 30). The fact that the HA can serve as the target antigen for both highly strain-specific and cross-reactive CTL suggests that the clonal repertoire of HA-specific CTL may, like the antibody repertoire, be large. It should be emphasized that in this report we have not attempted to determine the full range of CTL specificities attributed to the HA, but rather to evaluate the feasibility of using DNA-mediated gene transfer to permit analysis of the CTL response to a single virus gene product. Repertoire analysis can, however, now be undertaken in a systematic fashion. The extent of repertoire diversity, i.e., the range of CTL reactivity patterns to the HA, will certainly depend on a variety of factors including virus and mouse strains, immunization protocols, and CTL cloning methodology.

The detection of cross-reactive CTL clones that failed to recognize the HA-expressing 1E2 line supports the possibility that virus-specified polypeptides, other than HA, can serve as target antigens for these cross-reactive CTL. A similar conclusion has been reached by Lamb et al. (37) using cloned populations of noncytolytic human T cells. A likely target for these cross-reactive CTL is the virus-coded neuraminidase (NA), and this hypothesis can now be tested directly since CTL clones with putative NA specificity have recently been obtained, albeit at a very low frequency (T. J. Braciale, unpublished results). In addition to the

NA, other possible target antigens for these cross-reactive CTL include the nucleocapsid protein (NP), matrix protein (M), the nonstructural protein (NS), and the transcriptase-associated P3 (PA) protein. These molecules are highly conserved among type A influenza strains and evidence which indicates the cell surface expression of the NP, M, and $NS_1$ has been reported (9, 16–21). Again, it may now be possible to evaluate the role of each of the viral polypeptides in CTL recognition by gene transfer techniques.

An alternative explanation for the failure of some cross-reactive CTL clones to recognize the uninfected 1E2 line in the face of efficient lysis of virus-infected 1E2 rests on our observation that A/JAP/57 HA-specific CTL clones also showed some enhanced lytic activity after superinfection of the 1E2 cells with A/JAP/57 virus. This result is consistent with the existence of CTL clones with low avidity for HA antigen, since the expression of HA is 50–100-fold lower in the 1E2 line than in virus-infected cells. Several lines of evidence (e.g., reference 38) suggest that CTL clones may indeed differ in their avidities for target antigen. In the current instance, the clones identified as HA-specific by lysis of uninfected 1E2 cells may represent a subclass of clones with high avidity for HA. At least some CTL clones that failed to lyse uninfected 1E2 cells could be HA specific but have an avidity too low to be demonstrable towards the uninfected $HA^+$ transformant. The recent development of murine cell lines transformed with an HA-bovine papilloma virus recombinant and expressing ~50-fold higher levels of HA (Sambrook, unpublished results) will allow us to investigate this issue in the near future.

Although expressed at a rather low level compared with the infected cell, the HA on the surface of L cell transformants was readily detected by antiinfluenza CTL. The fact that target antigen recognition by the CTL can be so efficient may in part explain why it has been difficult to demonstrate blocking of target cell lysis by antiviral CTL using antiviral antibody (1). Furthermore, our results suggest that selected CTL populations might be used as sensitive probes for the cell surface expression of products derived from genes introduced by various eukaryotic expression vectors.

In conclusion, we have demonstrated in this report that the product of an exogeneously introduced DNA copy of the A/JAP/57 HA gene can be recognized by CTL in an H-2-restricted manner and in particular that the HA is indeed a target antigen for at least a subset of cross-reactive (type A specific) CTL. These findings now open the way for mapping specific CTL epitopes on the HA by site-directed mutagenesis. Furthermore, they provide an opportunity to assess the importance of clonal avidity in CTL recognition as well as a basis for analyzing interactions between the HA and class I gene products on the cell surface.

## Summary

We have used the technique of DNA-mediated gene transfer to examine cytotoxic T lymphocyte (CTL) recognition of the product of the cloned A/JAPAN/305/57 hemagglutinin (HA) gene in murine (L929) cells. Using both heterogeneous and homogeneous (clonal) populations of type A influenza-specific CTL, we have demonstrated that the HA molecule can serve as a target antigen

for both the subtype-specific and the cross-reactive subpopulations of influenza-specific CTL. Our results also raise the possibility that other virus-specified polypeptides may serve as target molecules for cross-reactive CTL.

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