

# IMMUNOLOGIC RECOGNITION OF INFLUENZA VIRUS-INFECTED CELLS

## II. Expression of Influenza A Matrix Protein on the Infected Cell Surface and its Role in Recognition by Cross-Reactive Cytotoxic T Cells

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Cytotoxic T cells generated in the course of viral infection of mice show two types of specificity. Cytotoxic T cells specific to one virus group will not lyse target cells infected with unrelated viruses (1). Furthermore, for lytic interactions to occur, the virus-infected targets must either share the *H-2K*<sup>1</sup> and/or *H-2D* region of the major histocompatibility complex with the cytotoxic T cells (1) or, in the case of radiation chimeras (2), must share *H-2K* or *H-2D* products with the chimeric environment in which the T cells were sensitized. In a previous report<sup>2</sup> we demonstrated a further level of specificity of cytotoxic T cells, that is, the presence of two distinct subpopulations of cytotoxic T cells in the primary and secondary response to type A influenza viruses of the A (H0/N1) and A (H2/N2) subtype. One subpopulation is specific for the immunizing virus. The other subpopulation exhibits a high degree of cross-reactivity between virus subtypes. This report investigates the possibility that distinct viral antigens, expressed on the surface of the infected cell, are recognized by the two different subpopulations of influenza-specific cytotoxic T cells. Since influenza viruses of the A (H0/N1) and A (H2/N2) subtypes have serologically distinct hemagglutinin and neuraminidase antigens, one or both of these virion surface glycoprotein antigens could serve as the target of the virus strain-specific cytotoxic T cells, but these antigens are less likely candidates for recognition by cross-reactive T cells. All type A influenza viruses do, however, possess two serologically crossreacting nonglycosylated antigens—the matrix (M) protein and ribonucleoprotein complex (RNP), both of which are internally located in the virion (3). Evidence to date also suggests that these antigens are not present on the surface of the influenza-infected cells (3).

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<sup>1</sup> Abbreviations used in this paper: 2-DG, 2-Deoxy-D-Glucose; EID, egg infectivity dose; FCS, fetal calf serum; H, influenza virus hemagglutinin antigen; H-2, mouse major histocompatibility complex; M, influenza virus matrix protein antigen; N, influenza virus neuraminidase antigen; RNP, influenza virus ribonucleoprotein antigen complex; T cell, thymus-derived lymphocyte.

<sup>2</sup> Braciale, T. J. 1977. Immunologic recognition of influenza virus-infected cells. I. Generation of a virus strain-specific and a cross-reactive subpopulation of cytotoxic T cells in the response to type A influenza viruses of different subtypes. *Cell Immunol.* In press.

In this paper data are presented which demonstrate that influenza A matrix protein, but not RNP, is present on the surface of target cells infected with a variety of influenza A viruses of different subtypes. Furthermore, inhibition of glycoprotein synthesis in infected target cells with 2-Deoxy-D-Glucose (2-DG) abrogates reactivity of infected target cells to lysis by virus strain-specific, but not cross-reactive cytotoxic T cells. These findings support the possibility that the influenza glycoproteins (hemagglutinin and/or neuraminidase) and matrix protein are the targets for the virus strain-specific and cross-reactive cytotoxic T cells, respectively.

### Materials and Methods

**General.** Male BALB/c mice (7-12 wk of age) were used throughout. P815 mastocytoma cells, maintained in tissue culture, were used as target cells in all experiments. Dulbecco's Modified Eagle's Minimal Essential Medium (GIBCO, Grand Island, N. Y. Catalogue no. H-16) was used as medium in all cytotoxicity assays. Grade III 2-DG was obtained from Sigma Chemical Co., St. Louis, Mo. Details on the immunization of mice with influenza virus, generation of secondary influenza-specific cytotoxic cells in vitro, and  $^{51}\text{Cr}$  release assay have been presented in a previous report.<sup>2</sup>

**Viruses.** Influenza virus strains A/WSN (H0/N1), A/PR/8 (H0/N1), A/JAP (H2/N2), A/Ann Arbor (H2/N2), A/Port Chalmers (H3/N2), and a high growing recombinant between A/Port Chalmers and A/PR/8 - A/Port Chalmers-PR/8 (H3/N2) - were grown in embryonated eggs and stored as described previously.<sup>2</sup>

**Antisera.** Rabbit antisera to influenza type A matrix protein, type A ribonucleoprotein complex, and subtype H2 hemagglutinin were kindly provided by Dr. W. G. Laver. The purified viral antigens were obtained from egg-grown influenza viruses and prepared as previously described (4). Isolated matrix protein, ribonucleoprotein, and hemagglutinin were obtained from virus strains A/Port Chalmers-PR/8 (H3/N2) (a high growing recombinant strain), A/Berk (H2/N2), and A/JAP (H2/N2), respectively. Rabbits were immunized in both foot pads and intramuscularly in the thighs with isolated antigen in a 50% suspension of Freund's complete adjuvant. On the 40th day the animals received a second intramuscular dose of antigen in Freund's complete adjuvant. The animals were bled 7-9 days later. Sera were separated and stored at  $-20^{\circ}\text{C}$ . The sera were judged to be specific by standard serologic criteria. Antisera to the matrix protein and RNP reacted exclusively with their respective antigens in immunodiffusion and showed no activity in the hemagglutination inhibition test; similarly, the anti-hemagglutinin antiserum demonstrated a high titer ( $>1/1,000$ ) in the hemagglutination inhibition test with homologous H2/N2 virus (A/JAP) but showed no specific activity against type A virus of different subtypes or type B virus. This antiserum was also unreactive in immunodiffusion with purified internal virion antigens. Day 0 bleeds from the respective immune serum donors served as preimmune sera in the cytotoxicity assay. All sera were heat-inactivated at  $56^{\circ}\text{C}$  for 30 min before use.

**Assay for Antibody Plus Complement-Mediated Cytotoxicity.** P815 cells were labeled with  $^{51}\text{Cr}$   $\text{O}_4$  at a concentration of  $50-150 \mu\text{Ci}/10^6$  viable cells for 1 h at  $37^{\circ}\text{C}$  in serum-free medium. After washing, the cells were resuspended in serum-free medium and samples of  $1-4 \times 10^6$  cells in 0.5 ml vol were infected in suspension with 10 egg infectivity doses (EID)<sub>50</sub> of virus (as infectious allantoic fluid) per cell. The cells were incubated at  $37^{\circ}\text{C}$  for 30 min to allow virus adsorption then washed and resuspended in medium supplemented with 10% heat-inactivated fetal calf serum (FCS). The cells were maintained in suspension for 6.5 h at  $37^{\circ}\text{C}$  in a humid atmosphere of 10%  $\text{CO}_2$ , 7%  $\text{O}_2$ , and 83%  $\text{N}_2$  to allow viral antigen expression. The cells were then pelleted and resuspended in fresh medium plus 10% FCS at a concentration of  $4 \times 10^5$  cells/ml. 0.05-ml samples of cells (containing  $2 \times 10^4$  cells) were distributed into individual wells of 96 well (6 mm diameter) plastic tissue culture trays (Linbro Chemical Co., New Haven, Conn.). Serial twofold dilutions of immune or preimmune serum in medium plus 10% heat-inactivated FCS were added to the wells in 0.05 ml vol. The trays were incubated at room temperature ( $18-22^{\circ}\text{C}$ ) for 45-60 min before the addition of complement (0.1 ml of a 1:5 dilution of guinea pig serum per well). The trays were then incubated at  $37^{\circ}\text{C}$  for 3 h in a humid atmosphere of 10%  $\text{CO}_2$ , 7%  $\text{O}_2$ , and 83%  $\text{N}_2$ . After incubation, 0.1 ml of supernate was removed from each well and the  $\gamma$ -emissions in the supernate were counted in a Packard Auto-Gamma Spectrometer, Packard Instrument Co., Inc., Downers Grove, Ill. Total releasable radioactivity from the targets (water lysis) was obtained by adding 0.05 ml of labeled

target cells ( $2 \times 10^4$  cells) to 20 vol of distilled water, incubating the cells at  $37^\circ\text{C}$  in parallel with the assay and determining the number of counts in one-half of the lysate supernate. Target cells were also incubated either with medium alone, with medium plus immune serum, or with medium plus complement. The highest  $^{51}\text{Cr}$  release from these three controls was taken as spontaneous  $^{51}\text{Cr}$  release. This value usually ranged from 5 to 12% and was always less than 15%. Percent specific  $^{51}\text{Cr}$  release was obtained from the formula:

$$\frac{\text{Test counts} - \text{spontaneous release}}{\text{Water lysis counts} - \text{spontaneous release}} \times 100$$

All values are the mean percent specific  $^{51}\text{Cr}$  release of four replicate wells. The standard errors of the means were always less than  $\pm 3\%$  and are omitted from the figures for clarity.

Experiments with the inhibitor of glycoprotein synthesis 2-DG were carried out essentially as described above. After the 30-min period of virus absorption the suspensions of infected P815 cells were divided into two equal portions. One portion of cells was transferred into medium plus 10% FCS containing 2-DG at a concentration of 5 mM. The other portion of cells was maintained in medium plus 10% FCS alone. Both samples were incubated for 6.5 h at  $37^\circ\text{C}$  and assayed in parallel as described above. Cells infected in the presence of 2-DG were assayed for antibody plus complement-mediated lysis in the presence of 5 mM 2-DG, i.e. both the dilutions of sera and complement contained 2-DG at a concentration of 5 mM. 2-DG appeared to have no effect on the rate of spontaneous  $^{51}\text{Cr}$  release from infected P815 cells as these values were comparable both with and without 2-DG.

*Complement.* Fresh frozen guinea pig serum served as the complement source. The serum was adsorbed with agarose (80 mg/ml of serum) just before use. A one to five dilution of serum in medium plus 10% heat-inactivated FCS was used in the assay. The complement source was not cytotoxic for P815 cells as judged by  $^{51}\text{Cr}$  release from target cells in the presence of complement alone.

*Assay for Cell-Mediated Cytotoxicity.* The cytotoxicity assay with unlabeled infected competitor cells was carried out as described in a previous report.<sup>2</sup> Cytotoxicity assays were carried out in parallel in the presence and absence of 5 mM 2-DG.  $^{51}\text{Cr}$ -labeled P815 cells were infected with 10 EID<sub>50</sub> doses of influenza virus per cell. After a 30-min incubation at  $37^\circ\text{C}$ , the cells were washed and divided into two equal portions. One portion of cells was transferred into cold ( $4^\circ\text{C}$ ) medium plus 10% FCS containing 2-DG at a concentration of 10 mM. The other portion of cells was transferred into medium plus 10% FCS without 2-DG. The final cell concentration in each case was  $2 \times 10^5$  cells/ml. The cells were immediately dispersed into wells of the assay trays ( $2 \times 10^4$  cells/well in 0.1 ml vol) as described.<sup>2</sup> Mixtures of secondary influenza-immune cytotoxic effector cells generated *in vitro*<sup>2</sup> and unlabeled infected competitor cells were then added to the wells in a 0.1-ml vol of medium plus 10% FCS without 2-DG. The final concentration of 2-DG in wells containing 2-DG was 5 mM. The assay trays were incubated at  $37^\circ\text{C}$  for 6.5 h and percent specific  $^{51}\text{Cr}$  release from the assay wells was determined as described.<sup>2</sup> Since 2-DG was expected to inhibit glycoprotein synthesis in both the  $^{51}\text{Cr}$ -labeled targets and unlabeled competitor cells, the competitor cells were infected 6.5 h before the labeled targets and maintained in suspension at  $37^\circ\text{C}$  for this time period. This procedure allowed expression of viral antigens by the competitor cells before they were added to assay wells containing 2-DG.

## Results

*Lysis of Influenza Virus-Infected P815 Cells by Anti-Matrix Antibody and Complement.* To determine if either or both internal virion antigens (matrix protein and RNP) are expressed on the surface of infected cells,  $^{51}\text{Cr}$ -labeled P815 cells were infected with A/WSN (H0/N1), A/JAP (H2/N2), or B/Lee virus and tested along with uninfected cells in the  $^{51}\text{Cr}$  release assay for their susceptibility to complement-dependent lysis by antisera specific for influenza A-matrix protein, RNP, or hemagglutinin of the H2 subtype. The latter antiserum was included as a specificity control since the hemagglutinin antigen has been demonstrated on the surface of influenza-infected cells by several different

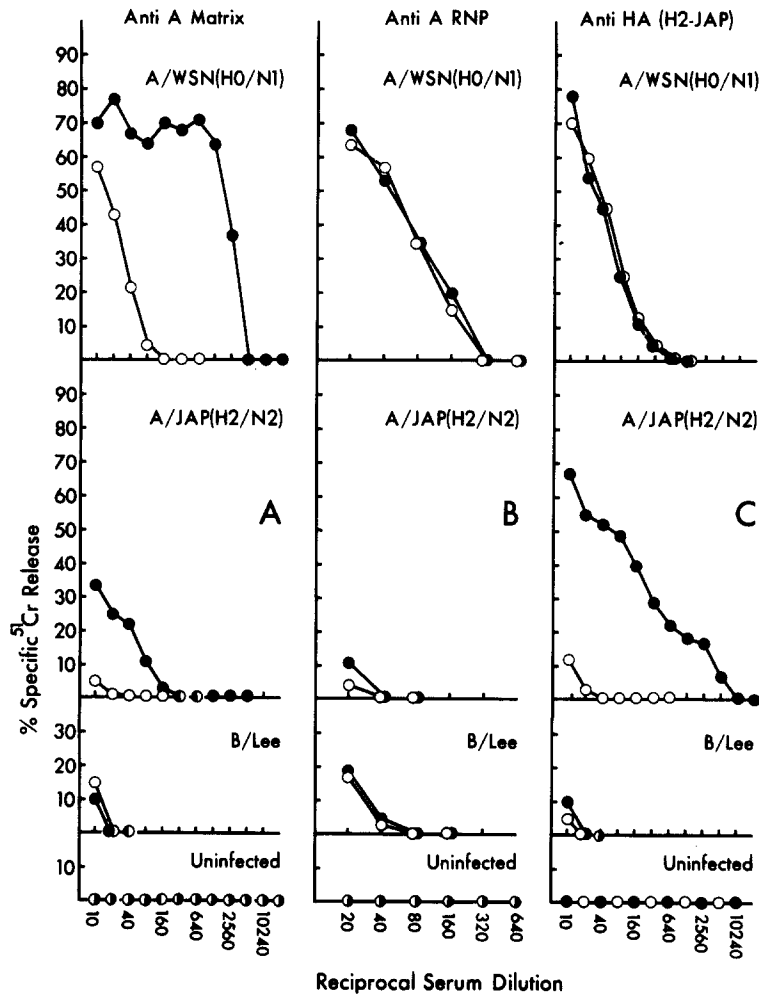


FIG. 1. Cytotoxicity of specific anti-influenza antisera for influenza-infected P815 cells. P815 cells were infected with the indicated influenza strains (A/WSN, A/JAP, B/Lee) and tested along with uninfected P815 cells for complement-dependent lysis in the presence of rabbit antiserum to influenza A matrix protein (Panel A), to influenza A ribonucleoprotein (Panel B) or to influenza subtype H2 hemagglutinin (Panel C). Closed circles (●—●) represent the lytic activity of the various immune sera. Open circles (○—○) represent the lytic activity of preimmune serum from the same donor. Details in Materials and Methods.

techniques (5-7). Hyperimmune rabbit antiserum to purified influenza A-matrix protein antigen in the presence of complement specifically lyses cells infected with A/WSN (H0/N1) and A/JAP (H2/N2) but not uninfected cells or influenza B/Lee-infected cells (Fig. 1 A). On the other hand, antiserum to the RNP antigen shows no significant lysis on any cell above the background seen with pre-immune serum (Fig. 1 B). Furthermore, antiserum to the H2 (A/JAP) hemagglutinin specifically lyses only with A/JAP (H2/N2) infected cells. These findings indicate that both influenza A-matrix protein and viral hemagglutinin are expressed on the surface of infected P815 cells during the course of infection

at a time (6.5 h) when these targets are susceptible to lysis by cytotoxic T lymphocytes.<sup>2</sup>

Although matrix protein could be detected on surfaces of both A/WSN and A/JAP-infected cells, the cytotoxic potency of the anti-matrix antiserum was greater for the former strain (dilution end point = 1/5,120; maximum <sup>51</sup>Cr release = 70%) than for the latter (dilution end point = 1/320; maximum <sup>51</sup>Cr release = 30%). To assess whether this difference in susceptibility to lysis was unique to these two viruses or perhaps reflected differences between virus subtypes, target cells infected with several other type A viruses of different subtypes were examined in the <sup>51</sup>Cr release assay with anti-matrix antibody. The virus strains tested were: A/PR/8 (H0/N1), A/Ann Arbor (H2/N2), and A/Port Chalmers (H3N2). A fourth strain, A/Port Chalmers-PR/8 (H3/N2) – a high growing recombinant virus between Port Chalmers and PR/8 which contains the virion surface antigens of the Port Chalmers strain – was also included since the anti-matrix antiserum was produced against the matrix protein of this strain. As Fig. 2 shows, infected targets of all three subtypes are specifically lysed by anti-matrix antibody and complement but to different degrees. Target cells infected with the H0/N1 virus, PR/8, were most susceptible to lysis, while Ann Arbor (H2/N2)-infected targets were least susceptible. These results taken together with the data in Fig. 1 A suggest that target cells infected with type A influenza virus of different subtypes differ in their susceptibility to lysis by anti-matrix antibody and complement. In this connection, it is worthwhile noting that target cells infected with the A/Port Chalmers-PR/8 recombinant which probably contains the A/PR/8 matrix protein (W. G. Laver, personal communication) appear to be as susceptible to lysis as targets infected with A/PR/8 or A/WSN (Figs. 1 A and 2). Two explanations for this phenomenon seem most likely: (a) type A influenza viruses of different subtypes differ in the kinetics and/or degree of expression of M protein on the cell surface; (b) matrix protein antigens of different subtypes cross-react to different degrees with a given antiserum. Experiments now in progress may distinguish between these two possibilities.

Grant et al. (8) have reported that oncornavirus antigen passively adsorbed to cell surfaces can render these cells susceptible to lysis by specific antibody and complement. This finding raises the possibility that matrix protein may also be passively adsorbed to the surface of infected cells. To test this possibility, cell mixing experiments were performed in which unlabeled A/WSN infected cells were added immediately after infection to <sup>51</sup>Cr-labeled uninfected or B/Lee-infected P815 cells. The cell mixtures were then incubated for 6.5 h to allow viral antigen expression and tested for their susceptibility to lysis by anti-A matrix protein antibody and complement in the <sup>51</sup>Cr release assay. No lysis of uninfected or B/Lee-infected cells could be detected above that seen in Fig. 1 A with the addition of up to a 10-fold excess unlabeled A/WSN-infected cells. Furthermore, at 6.5 h into infection, medium release (release of <sup>51</sup>Cr from cells incubated in medium without antibody and complement) from both infected and uninfected cells was equivalent (5–12%). Also, both infected and uninfected cells showed greater than 95% viability as measured by trypan blue exclusion. Hence there is no evidence for death or damage of infected cells at the time of assay as judged by these criteria. These results, while not formally ruling out the

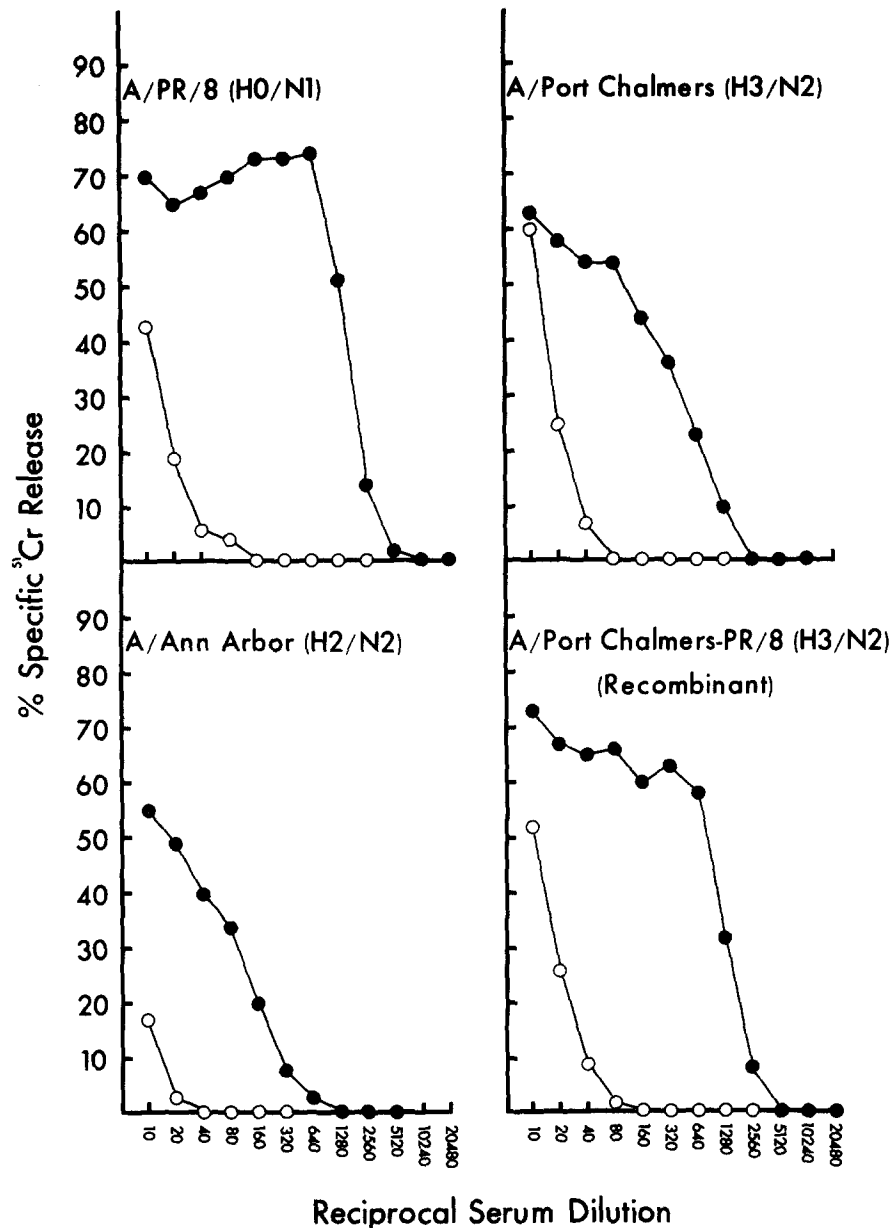


FIG. 2. Cytotoxicity of anti-A matrix protein antisera for P815 cells infected with type A influenza viruses of different subtypes. Symbols as in Fig. 1. Details in text.

possibility of adsorption of matrix protein to the cell surface, do suggest that under the conditions described here, passively adsorbed matrix protein, released from damaged cells, is not a major contributor to the matrix protein detectable on the infected cell surface.

In the above experiments, varying degrees of nonspecific lysis were observed with preimmune sera (Figs. 1 and 2). This nonspecific activity was not observed

on uninfected target cells and could not be adsorbed out with uninfected cells. The degree of nonspecific lysis obtained with any immune serum-preimmune serum pair appeared to depend on the infecting virus strain. A/WSN and A/PR/8-infected cells showed the highest degree of nonspecific lysis and A/JAP and B/Lee the lowest. This phenomenon either could be due to some alteration in the cytoplasmic membrane as a result of virus infection which renders the cell more susceptible to nonspecific lysis or, as has been shown in herpes virus-infected cells (9), could be due to the expression of immunoglobulin receptors on the cell surface after virus infection which could facilitate nonspecific adsorption of immunoglobulin to the cell surface.

*Effect of 2-DG on the Susceptibility of Influenza-Infected P815 Cells to Lysis by Cytotoxic T Lymphocytes and Antibody.* The above experiments demonstrate that influenza matrix protein which is an internal antigen in the virion is expressed on the surface of P815 cells infected with type A influenza virus of different subtypes. Since the matrix protein antigens of all type A influenza viruses are serologically related, this antigen could be recognized by the subpopulation of cytotoxic T cells which cross-reacts between subtypes. On the other hand, virus strain-specific cytotoxic T cells may recognize the viral antigens specific for an influenza A subtype (hemagglutinin and/or neuraminidase). One test of this possibility is to inhibit the expression of particular viral antigens on the target cell surface and to test the susceptibility of the target cells to lysis by either strain-specific or cross-reactive cytotoxic T cells.

Influenza hemagglutinin and neuraminidase are glycoproteins (10, 11) and their formation is therefore susceptible to inhibitors of glycoprotein synthesis. Influenza matrix protein is nonglycosylated (3) and its formation should not be susceptible to these inhibitors. The glucose analogue 2-DG has been shown to inhibit the synthesis (12, 13) and glycosylation (14, 15) of the influenza glycoproteins in influenza-infected cells. Furthermore, Jackson et al. (16) have recently shown that 2-DG significantly inhibits (80–90%) glycoprotein synthesis in P815 cells while inhibiting total protein synthesis to a lesser extent (30–40%).

To directly determine the effect of 2-DG on the expression of viral antigens at the cell surface, virus-infected cells, treated with 2-DG, were tested for their susceptibility to lysis by anti-hemagglutinin or anti-matrix antibody and complement. <sup>51</sup>Cr-labeled P815 cells were infected with influenza virus and after the period of virus adsorption (30 min) were transferred into medium with or without 5 mM 2-DG.<sup>3</sup> The cells were incubated at 37°C for 6.5 h to allow viral antigen expression. At that time <sup>51</sup>Cr release was measured in the presence of specific antibody and complement. A/WSN-infected cells in the presence of 2-DG are susceptible to lysis by anti-matrix antibody and complement (Fig. 3). On the other hand, A/JAP-infected are rendered almost completely insusceptible to lysis by antiserum to the A/JAP hemagglutinin when maintained in 2-DG (Fig. 4). While these findings are in agreement with the predicted effect of 2-DG, (Fig. 3 also shows that 2-DG does somewhat decrease in the susceptibility of A/WSN-infected cells to lysis by anti-matrix antibody. This effect is also seen with A/JAP and A/PR/8-infected cells, and probably reflects an inhibitory effect of 2-DG

<sup>3</sup> The 2-DG concentration of 5 mM was chosen on the basis of data of Jackson et al. (17).

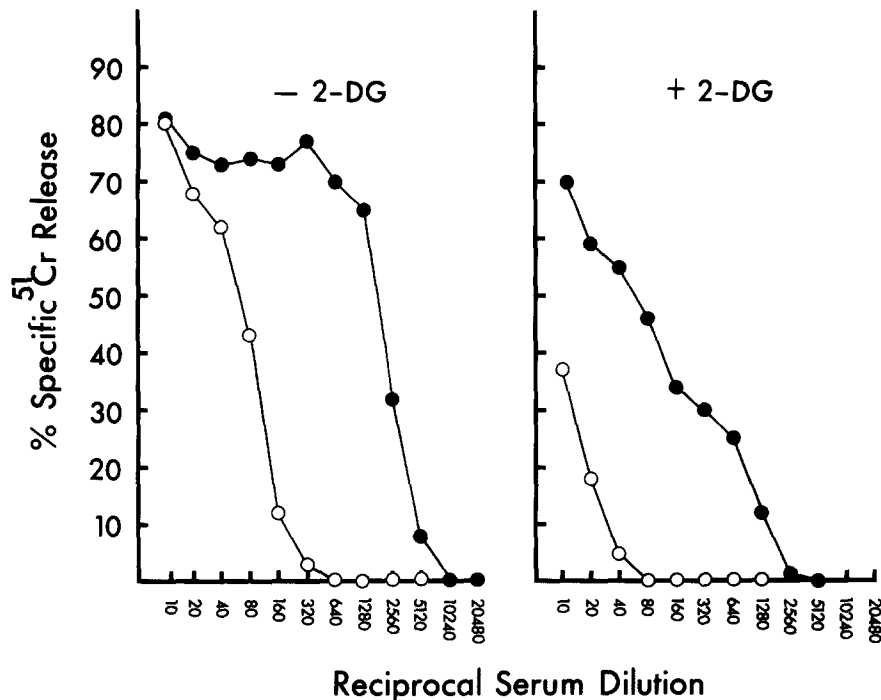


FIG. 3. Effect of 2-DG on the complement-dependent lysis of influenza A/WSN-infected P815 cells by anti-A matrix protein antiserum. Symbols as in Fig. 1. Details in Materials and Methods and text.

on overall protein synthesis (16). Although anti-hemagglutinin antisera specific for other virus strains were not available, the inhibitory effect of 2-DG on viral hemagglutinin expression could be assayed by another technique, that is, the capacity of virus-infected cells to specifically adsorb fowl erythrocytes (5). Up to 10 h after infection (a time when 90% or more cells can hemadsorb) P815 cells infected with A/WSN, A/JAP, or A/PR/8 and maintained in 2-DG showed no hemadsorption (data not shown).

The above results provide direct evidence that 2-DG acts preferentially to inhibit the expression of influenza glycoprotein on the infected cell surface but not matrix protein expression. The effect of 2-DG on lysis of virus-infected targets by cytotoxic T cells was then examined. Spleen cells from mice previously immunized with A/JAP virus were stimulated *in vitro* with A/JAP-infected stimulator cells.<sup>2</sup> After 5 days in culture, the cytotoxic activity of the cultured spleen cells was analyzed by cold target competition on <sup>51</sup>Cr-labeled homologous (A/JAP-infected) targets or target cells infected with influenza virus of a different subtype (i.e., A/WSN) in the presence or absence of 2-DG. As shown previously,<sup>2</sup> in the absence of 2-DG, the cytotoxic activity of JAP effectors tested on homologous targets is inhibitable by JAP cold targets but not by WSN or influenza B/Lee cold targets (Fig. 5 A). In the presence of 2-DG, however, both A/JAP and A/WSN cold targets inhibit killing of A/JAP targets by A/JAP effectors (Fig. 5 C). Furthermore, inhibition by A/JAP and A/WSN cold targets



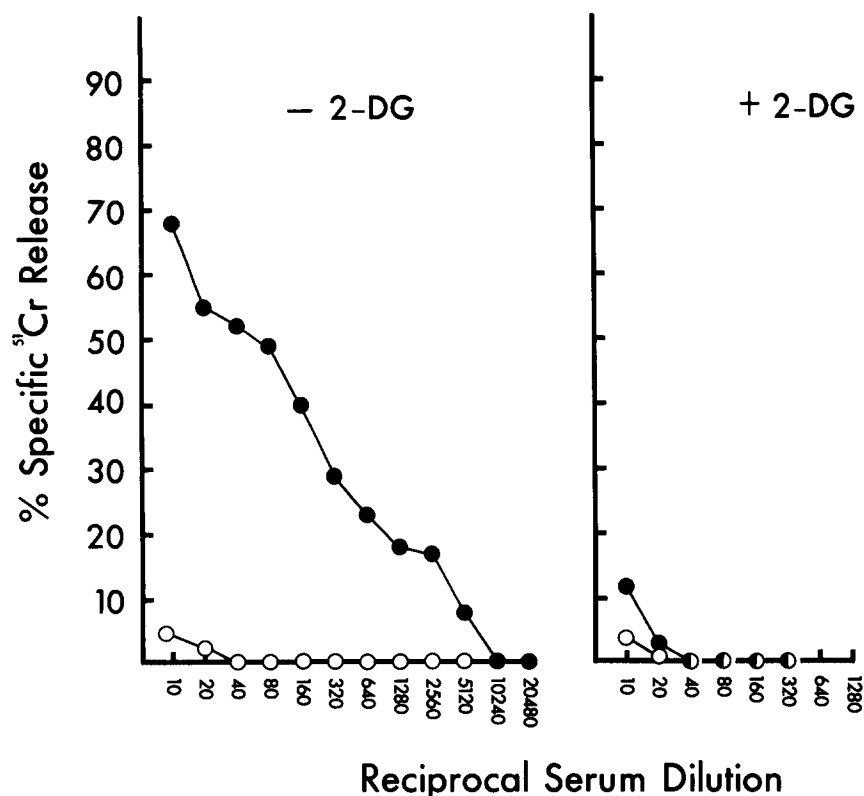


FIG. 4. Effect of 2-DG on the complement-dependent lysis of influenza A/JAP-infected P815 cells by antiserum to the A/JAP hemagglutinin. Symbols as in Fig. 1. Details in Materials and Methods.

of killing of <sup>51</sup>Cr-labeled heterologous (A/WSN) targets by JAP effectors is not altered by 2-DG treatment (Fig. 5 B and D). The most likely explanation for these data is that JAP-infected targets treated with 2-DG, which lack viral glycoproteins, are not recognized by the virus strain-specific subpopulation of cytotoxic T cells but are lysed by the cross-reactive T-cell subpopulation. Presumably, these latter T cells recognize a nonglycosylated viral antigen on the infected cell surface, such as M protein.

Several features of the data in Fig. 5 suggest that other effects of 2-DG on cellular function, e.g., inhibition of glycolysis (17) are unlikely to be relevant. First, the overall lytic activity of cytotoxic T cells was not inhibited by 2-DG. Second, the susceptibility of <sup>51</sup>Cr-labeled targets to lysis was unaltered in the presence of 2-DG. Third, the effect of cold competitors on killing of A/WSN targets by A/JAP effectors was unchanged in the presence of 2-DG (Fig. 5 B and D).

Further evidence of the specificity of the 2-DG effect comes from experiments with cross-reactive cytotoxic T cells. As demonstrated in a previous report,<sup>2</sup> stimulation of primed spleen cells with type A influenza of a different subtype results in specific enrichment of the cross-reactive T-cell subpopulation. When

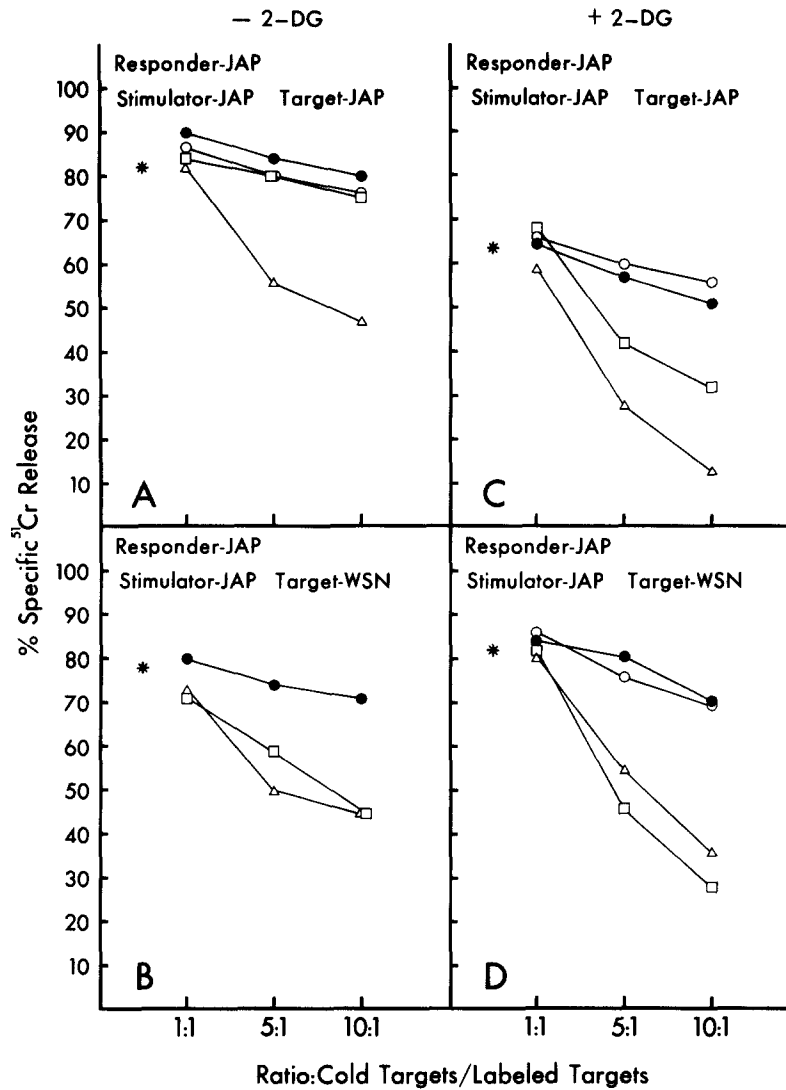


FIG. 5. Effect of 2-DG on the inhibition of homologous secondary cytotoxic T cells by unlabeled cold competitors. Spleen cells from BALB/c mice previously immunized with influenza A/JAP (Responder) were cultured *in vitro* with stimulator cells infected with influenza A/JAP (Stimulator) as previously described.<sup>2</sup> After 5 days in culture, the spleen cells were tested for cytotoxicity on <sup>51</sup>Cr-labeled infected P815 target cells in the presence of increasing numbers of unlabeled cold competitors. Cytotoxicity assays were carried out in parallel in the absence (-2-DG) or presence (+2-DG) of 5 mM 2-DG. The ratio of effectors to targets is 2.5:1 in assays carried out in the absence of 2-DG and 5:1 for assays in the presence of 5 mM 2-DG. The ratio of unlabeled competitors to labeled targets is as indicated. Unlabeled competitors are P815 cells infected with influenza A/WSN (□-□), A/JAP (Δ-Δ), B/Lee (●-●) or uninfected (O-O). Values are the means of four individual assay wells. Standard errors, less than ± 3% in all cases, are omitted for clarity. Asterisk (\*) indicates the percent specific <sup>51</sup>Cr release in the absence of competitor cells.

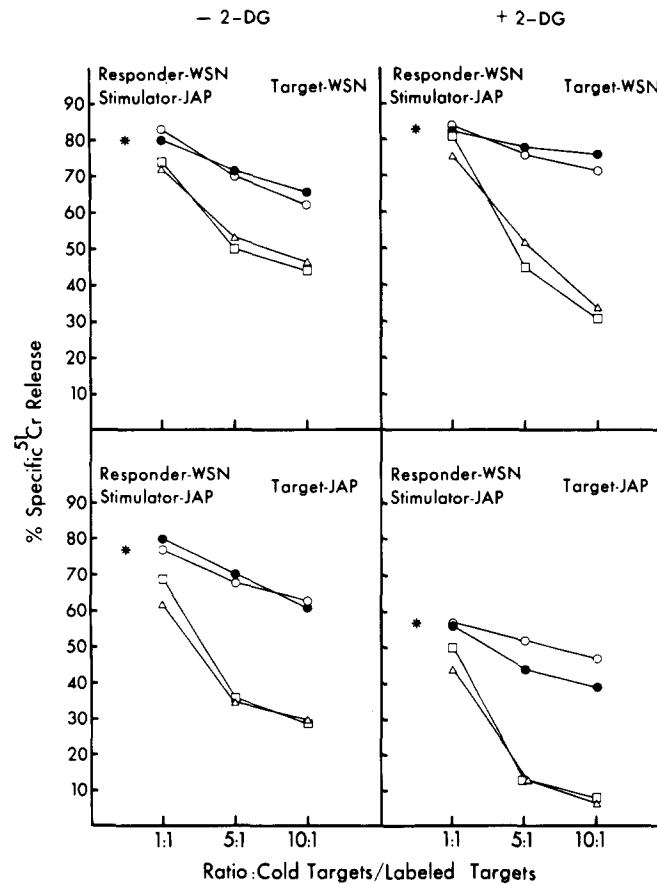


FIG. 6. Effect of 2-DG on the inhibition of heterologous secondary cytotoxic T cells by unlabeled cold competitors. Spleen cells from BALB/c mice previously immunized with influenza A/WSN (Responder) were cultured *in vitro* with stimulator cells infected with influenza A/JAP (Stimulator) and tested for cytotoxic activity after five days of culture. Other information as in Fig. 5.

A/WSN-primed spleen cells stimulated *in vitro* with A/JAP-infected stimulator cells are examined for lytic activity by cold target competition, the pattern of competition by cold targets is unaffected by 2-DG (Fig. 6).

### Discussion

Immunization of mice with type A influenza virus results in the generation of two populations of cytotoxic T cells, one of which is specific for the target cells infected with the immunizing virus, the other of which cross-reacts with target cells infected with type A influenza viruses of different subtypes (18).<sup>2</sup> In some cases, influenza A strains of different subtypes (e.g. A H0/N1, A H2/N2) have both serologically unrelated hemagglutinin and neuraminidase antigens. Since these two virion surface antigens are also thought to be the only viral antigens expressed on the surface of the infected cell (3), the existence of a cross-reactive cytotoxic population was unexpected. The purpose of this report was to deter-

mine if the reactivity patterns exhibited by these two cytotoxic T-cell populations could be accounted for by known viral antigens. Experiments presented here indicate: (a) that influenza matrix protein which is an internal antigen in the virion appears to be expressed on the surface of P815 cells infected with type A influenza viruses; (b) that inhibition of viral glycoprotein expression but not matrix protein expression on the target cell surface by 2-DG renders infected cells unsusceptible to lysis by virus strain-specific cytotoxic T cells, but does not affect killing by cross-reactive cytotoxic T cells.

Evidence for the internal location of influenza matrix protein has for the most part come from electron microscopic observations which demonstrate an electron dense layer on the inner surface of the lipid bilayer of the virion and on the inner surface of the cytoplasmic membrane of the infected cell at virus budding sites (3, 19). The lack of reactivity of anti-matrix antibody with intact virions in standard serologic tests (20) as well as studies with subviral particles (21, 22) adds further support for the internal location of the matrix protein at least in the virion, although experiments with protease-treated virus particles suggest that the molecule may be embedded in the lipid bilayer of the virion (23). Data presented here, however, strongly suggest that influenza A matrix protein is expressed during the course of infection on the surface of influenza-infected cells. Rabbit antiserum prepared against purified matrix protein from egg-grown virus specifically lysed, in the presence of complement, P815 target cells infected with type A influenza of three different subtypes, but did not lyse influenza B/Lee-infected or uninfected targets. This pattern of cross-reactivity exhibited by the anti-matrix antiserum is consistent with the known serologic cross-reactivity of influenza A matrix protein from viruses of different subtypes (24). Nonspecific adsorption of matrix protein onto the surface of infected cells appears not to be an important factor in the observed lysis of infected cells by anti-matrix antibody, as cell mixing experiments failed to demonstrate enhanced cytotoxicity of  $^{51}\text{Cr}$ -labeled unrelated targets (B/Lee-infected or uninfected) in the presence of an excess unlabeled susceptible cells. It is also unlikely that the cytotoxic activity of the anti-matrix antisera is due to contaminating antibodies to other viral antigens since among the major viral antigens only antibody to the RNP complex would be expected to show cytotoxicity for the targets infected with type A viruses of different subtypes. Antiserum directed to the RNP complex, however, was not specifically cytotoxic. Furthermore, the specificity of the assay as a method of detecting viral antigens is supported by the fact that antiserum directed to the A/JAP (H2/N2) hemagglutinin is cytotoxic only for A/JAP-infected targets.

Although the experiments presented here have dealt exclusively with influenza-infected P815 cells, Ada and Yap<sup>4</sup> have recently shown that a newly synthesized polypeptide with the molecular weight and electrophoretic properties of matrix protein can be precipitated from the outer surface of influenza-infected L929 cells with purified anti-matrix antibody. Thus two different murine cell lines both of which are susceptible to lysis by cross-reactive cytotoxic T cells when infected with type A influenza virus (18)<sup>2</sup> appear to express matrix protein on their cell surfaces. Finally, there is a precedent for the expression of

<sup>4</sup> Ada, G. L., and K. L. Yap. Manuscript in preparation.

internal virion antigens on the cell surface in the oncornavirus model where the virion core antigen P<sup>30</sup> is detectable on the surface of transformed cells (8, 25, 26).

The infection of both P815 and L cells with influenza virus appears to be abortive, that is, infectious virus particles are not produced by the infected cell. This fact raises the possibility that expression of matrix protein on the cell surface may be a feature of abortively infected cells, but not productively infected cells. This possibility cannot be excluded from the data presented here. Experiments are now in progress to test this possibility.

Experiments with the inhibitor of glycoprotein synthesis 2-DG directly implicated the influenza glycoprotein(s), hemagglutinin, and/or neuraminidase as the target of the virus strain-specific cytotoxic T-cell population, since inhibition of glycoprotein synthesis in the target cells renders the target unsusceptible to lysis by this T-cell population. Evidence in favor of matrix protein at the target of the cross-reactive cytotoxic T-cell population is at present circumstantial, i.e. 2-DG-treated target cells express matrix protein on their surfaces and are susceptible to lysis by the cross-reactive T-cell population. Support for this concept comes also from the fact that among the influenza-specified polypeptides only H, N, and matrix protein can be detected in plasma membrane preparations of infected cells (27). Furthermore, recent results in the oncornavirus system suggest that cytotoxic T cells specifically recognize the internal virion antigen P<sup>30</sup> on the surface of virus-transformed target cells (28). Direct evidence for the role of matrix protein in cross-reactive killing by cytotoxic T cells may come from blocking experiments with anti-matrix antibody. Such experiments are now in progress.

The phenomenon of heterotypic immunity, in which prior infection with type A influenza of one subtype increases resistance to challenge with virus of another subtype (29), has not been adequately explained. In this connection Webster and Hinshaw have recently demonstrated that parenteral immunization of mice with matrix protein enhances the clearance of influenza virus from the lungs of these mice when subsequently challenged by intranasal inoculation with infectious virus.<sup>5</sup> Both of these phenomena could be explained on the basis of findings reported here. If epithelial cells of the respiratory tract, when infected with influenza virus express matrix protein on their surfaces, they would in theory be susceptible to lysis by cytotoxic T cells and/or antibody generated in response to matrix protein either from prior infection with virus of a different subtype or from prior immunization with this antigen. Of greatest interest in this regard is the possibility of influencing the course of influenza infection in humans via the immune response to influenza matrix protein.

Two mechanisms have been proposed to explain the specific recognition of virus-infected cells by cytotoxic T cells and the concomitant *H-2* restriction of this recognition. On one hand there is the possibility that cytotoxic T cells have two independent classes of receptors which recognize *H-2 K* or *D* gene products and viral antigens respectively (dual recognition) (30-32). In this instance the *K* or *D* gene products and the viral antigens can be considered to be discrete

<sup>5</sup> Webster, R. G., and V. S. Hinshaw. 1977. Matrix protein from influenza A virus and its role in cross protection in mice. In press.

independent entities on the surface of the infected cell. On the other hand, there is the possibility that cytotoxic T cells possess a single receptor with specificity for both viral and *H-2*-coded products on the cell surface. In this case, either viral antigens and *H-2* gene products must be intimately associated on the infected cell surface forming a new interaction antigen (1) or viral antigens must be specifically altered by *K* or *D* gene products to be recognized as self (altered self) (33). The results presented in this and a previous report<sup>2</sup> suggest that the two subpopulations of cytotoxic T cells generated in response to influenza virus recognize different influenza virus antigens on the surface of virus-infected histocompatible cells. These results are compatible with the dual recognition model of cytotoxic T-cell recognition as outlined above. Furthermore, if, as the present results suggest, influenza matrix protein and one or both of virion surface glycoproteins (hemagglutinin and neuraminidase) are the targets of different cytotoxic T-cell subpopulations, then the receptor on the T cell which recognizes viral antigens must have at least the same degree of specificity as anti-matrix and anti-hemagglutinin (or anti-neuraminidase) antibody. This conclusion is consistent with current evidence for sharing of idiotypes (immunoglobulin  $V_H$  regions?) between T cells and antibody (34). Explanation of the present results by an altered self model for cytotoxic T-cell recognition has certain limitations. It has been proposed that viral antigens are specifically glycosylated by batteries of glycosyl transferase enzymes encoded in the *K* or *D* regions of the *H-2* locus and that the pattern of carbohydrate on the viral antigens dictate the *H-2* specificity of cytotoxic T-cell recognition (33). The finding in this report that inhibition of glycoprotein synthesis in the target cell by 2-DG inhibits lysis of target cells by one subpopulation of cytotoxic T cells but not the other argues against this model. Furthermore, if influenza matrix protein which is a nonglycosylated protein at least in the influenza virion (3) is the target for the cross-reactive cytotoxic T cells, then glycosylation of viral polypeptides would appear to be irrelevant to cytotoxic T-cell recognition. Recent studies with mice bearing mutations in the *K* and *D* regions of the *H-2* locus also argue against this model (35).

Another model for altered self recognition is the formation of a new interaction antigen as a result of association between viral antigens and *H-2* *K* or *D* products on the infected cell surface. Since influenza is a clean virus, that is, host proteins are not incorporated into the virion, it is unlikely that the viral-*H-2* association is covalent. However, it is possible that covalent association could occur in areas of the cell surface where virus budding is not occurring. Whether this association is covalent or noncovalent, the results presented here would imply that influenza antigens (i.e., the viral glycoproteins and matrix protein) with different physical and chemical properties (3) are equally capable of associating with *H-2* specified polypeptides.

The experiments presented here have dealt with the structures on the surface of virus-infected cells which are recognized by cytotoxic T cells. These experiments suggest that distinct influenza antigens expressed on the surface of infected cells are recognized independently by influenza-specific cytotoxic T cells. At issue still is the nature of the recognition structure(s) on the cytotoxic T cell. While no definite conclusions can be drawn from the above results, influ-

enza virus may prove to be a useful probe in the study of the receptor(s) on cytotoxic T cells.

### Summary

Two distinct subpopulations of cytotoxic T cells are generated in the primary or secondary response of mice to type A influenza viruses. One subpopulation is specific for the immunizing virus strain. The other subpopulation shows a high degree of cross-reactivity for heterologous type A virus of a different subtype. This report examines the possibility that distinct influenza virus antigens, expressed on the surface of the infected cell, are recognized by the different subpopulations of influenza-specific cytotoxic T cells. Data are presented which demonstrate that influenza A matrix protein, an internal virion antigen, is detectable on the surface of target cells infected with influenza A viruses of different subtypes. Since this viral antigen is type specific, i.e., serologically cross-reactive among all type A influenza viruses, it could serve as the target for cross-reactive cytotoxic T cells. To further examine the specificity of the two cytotoxic T-cell subpopulations, experiments were carried out by using the inhibitor of glycoprotein synthesis - 2-Deoxy-D-Glucose 2-DG. These experiments examine first the effect of 2-DG on the expression of influenza matrix protein and viral glycoprotein on the infected cell surface and second, the susceptibility of 2-DG-treated target cells to lysis by cytotoxic T cells. 2-DG inhibits the expression of the viral hemagglutinin glycoprotein on the cell surface but does not inhibit the expression of the nonglycosylated matrix protein. Furthermore, inhibition of glycoprotein synthesis in infected target cells abrogates the reactivity of infected target cells to lysis by virus strain-specific but not cross-reactive cytotoxic T cells. These findings suggest that the influenza glycoproteins (hemagglutinin and/or neuraminidase) and the nonglycosylated matrix protein are the targets for the virus strain-specific and cross-reactive cytotoxic T cells, respectively. These results are discussed in the light of available information on influenza virus structure and the biology of influenza infection and in terms of current models for cytotoxic T-cell recognition of virus-infected cells.

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