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Influenza Type A Virus M Protein Expression on Infected Cells Is Responsible for Cross-Reactive Recognition by Cytotoxic Thymus-Derived Lymphocytes

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M protein of influenza A virus was detected with rabbit antiserum by both indirect immunofluorescence and by antibody plus complement-mediated cytolysis on the cell surfaces of both productively and nonproductively infected cells. In contrast, antiserum to nucleoprotein failed to react with unfixed infected cells, but did bind to fixed infected cells, especially in the perinuclear area. Incorporation of antiserum to M protein in a T-cell-mediated cytotoxicity assay produced almost complete abrogation of lysis of *H-2*-compatible cells infected with an influenza A virus of a subtype which differed from that used to elicit the cytotoxic T cells. However, the antibody did not significantly block ^{51}Cr release from cells infected with the homotypic type A influenza virus. These observations are in accord with the hypothesis that the cross-reactive cytotoxic T-cell responses seen with cells infected by heterotypic influenza A viruses are due to recognition of a common M protein.

In recent years, in vitro assays have been developed which detect, at a cellular level, *H-2*-restricted, T-cell-mediated cytotoxic responses in mice after immunization with influenza viruses (4, 8, 10, 12, 18; C. S. Reiss and J. L. Schulman, submitted for publication). There is disagreement about the specificity of the cytotoxic T-lymphocyte (CTL) response; some investigators have observed cross-reactivity with target cells infected with any type A influenza virus (4, 10, 18; Reiss and Schulman, submitted for publication), whereas others have found the cytotoxic recognition limited to target cells expressing a homotypic hemagglutinin (8, 12). It has been suggested that the heterotypic recognition might be due to the expression of a common M protein on the surface of cells infected with influenza type A viruses (1, 2, 5). However, the expression of M protein on target cells commonly used in these assays has been explained as an artifact of using nonproductively infected cell lines (11). Nucleoprotein (NP) has also been detected outside infected cells (17).

In these studies, we observed M protein expression early after infection on both productively and nonproductively infected cells of three species. We were able to abrogate the cross-reactive, but not the homotypic, virus recognition by CTLs by using antibody to M protein.

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MATERIALS AND METHODS

Mice. Female C3H/StHa mice (*H-2*^{k}) were purchased from West Seneca Labs, Inc. (Buffalo, N.Y.), and were used between 8 and 20 weeks of age.

Viruses. Influenza viruses A/PR/8/34 (H0N1) (PR8 virus), A/Japan/305/57 (H2N2) (Japan virus), and B/Lee/40 (B/Lee virus) and recombinant virus X-7 [influenza A/NWS/33 (H0)-A/RI/5$^+$ (N2)] (13) were obtained from laboratory stocks and grown in the allantoic cavities of 10- to 11-day embryonated chicken eggs. Viruses were stored at −70°C.

Tissue culture cell lines. L-929 murine fibroblasts (*H-2*^{k}) were the generous gift of Peter Doherty, Wistar Institute (Philadelphia, Pa.). Bovine kidney cells (MDBK) and canine kidney fibroblasts (MDCK) were obtained from Microbiological Associates (Bethesda, Md.).

Immunization of mice. C3H mice were inoculated parenterally with 100 hemagglutination units of virus. Six days later their spleens were asceptically removed, and a single-cell suspension of splenic lymphocytes was made in Hanks balanced salt solution supplemented with 5% fetal bovine serum.

Cell-mediated cytotoxicity assay. CTL assays were performed as previously described (Reiss and Schulman, submitted for publication). Briefly, 10^5 L-929 cells were infected in 35-mm tissue culture dishes with a multiplicity of infection of approximately 10 50% egg infective doses. Uninfected dishes were mock infected. After adsorption, dishes were washed, ^{51}Cr pulsed, and incubated for an additional 5 h. Dishes were washed before addition of splenic lymphocytes at an effector/target ratio of 100:1 in 5% serum con-

taining Dulbecco minimal essential medium. A further incubation period of 12 h preceded sampling of supernatants for ^{51}C release determination. In general, four replicates were used. For antiserum blocking, 10% immune rabbit serum or 10% normal rabbit serum was added with the effector cells. Percent immune release was determined by the formula 100 × [(counts released by sensitized lymphocytes − counts released by control lymphocytes)/(total 5% Triton X-100-releasable counts − counts released by control lymphocytes)]. Statistical significance was determined by Student's *t* test.

Preparation of antisera. Rabbit antiserum to sucrose gradient-purified PR8 virus was obtained from laboratory stocks. Antisera to internal influenza virus proteins were the generous gift of Doris Bucher. M protein and NP were isolated from X-7 virus (H0N2) by sodium dodecyl sulfate disruption and electrophoresis on polyacrylamide gels, first under nonreducing and then under reducing conditions (6, 7). The isolated proteins were judged chromatographically pure by the presence of only one Coomassie blue-staining band on analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Rabbits were inoculated subcutaneously with 50 μg of protein dissolved in phosphate-buffered saline with complete Freund adjuvant; 6 weeks later, the rabbits were restimulated with 50 μg of the same antigen in aqueous suspension. Sera were collected 1 week later and stored at −20°C.

Adsorption of anti-M protein antiserum with X-7 virus. Preliminary experiments showed significant immunofluorescence in the presence of antibody to M protein. To determine whether this antiserum (made to M protein isolated from X-7 virus) contained antibody recognizing other determinants expressed on the surface of the virus, we used intact X-7 virus purified by sucrose gradient centrifugation to remove such contaminating antibody. One-half milliliter of the serum was adsorbed with 2 mg of purified virus at 4°C for 3 h. The virus was removed by ultracentrifugation and then by adsorption of the serum with 0.5 ml of packed human erythrocytes.

Indirect immunofluorescence. Three tissue culture cell lines were used in these studies: L-929 cells, which are abortively infected with most influenza A viruses (15); MDBK cells, in which most influenza A viruses produce noninfectious particles in the absence of exogenous proteases (16); and MDCK cells, which in the presence of trypsin are productively infected with most influenza A viruses. Cells were seeded at 10^5 cells per 2 ml of culture medium supplemented with serum on Lab Tex culture slides; 24 h later the cells were washed with serum-free medium and infected at a multiplicity of infection of 50 to 100 50% egg infective doses of PR8 virus per cell. After 60 min of adsorption, cells were washed extensively and incubated for 6 h with serum-free medium at 35°C. Cells were washed, incubated for 60 min at 4°C with normal rabbit serum or with one of the rabbit antisera (anti-M protein, anti-NP, anti-PR8), washed, and incubated at 4°C for 60 min with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories, Downingtown, Pa.). Slides were washed, air dried, fixed with ethanol, dried, and mounted in 9:1 glycerol–phosphate-buffered saline. Fluorescence was observed with a Zeiss research microscope, using a Zeiss mercury arc lamp and dark-field condenser, fluorescein isothiocyanate excitation, and Zeiss barrier filters. Cells were examined both under dark-field conditions and with the filters in place. Fluorescence was scored as 0 (none) or from ± (dull) to ++++ on the basis of brightness. Polaroid type 57 film was used.

In one experiment with anti-NP antiserum, cells were fixed with acetone for 10 min before incubation with anti-NP, to assess the ability of anti-NP to detect internal antigens.

RESULTS

Expression of M protein on influenza A virus-infected cells. We undertook immunofluorescence studies to determine whether the cross-reactivity we and others have observed in the cell-mediated cytotoxicity of influenza A virus-infected cell targets was due to the expression of a common M protein. In these experiments we infected three cell lines with PR8 virus and incubated the infected cells with antiserum to whole virus or to isolated M or NP proteins. Infection in all three cell lines was verified by the expression of hemadsorption. Both productively infected (MDBK and MDCK) and abortively infected (L-929) cells expressed M protein accessible to specific antiserum on the surface (Table 1). Furthermore, upon the addition of anti-M protein antiserum and complement, lysis of infected cells could be demonstrated by ^{51}Cr release in all three cell lines (data not shown). To exclude the possibility that the antiserum possessed antibodies to components other than M protein expressed on the surface of X-7 virus,

TABLE 1. *Surface and internal immunofluorescence of A/PR/8/34 virus-infected cell lines*

Assay conditions	Fluorescence with:		
	L-929[a]	MDCK[b]	MDBK[c]
Uninfected cells with:			
Normal rabbit serum	0	0	0
Anti-PR8 virus	0	0	0
Infected cells with:			
Normal rabbit serum	0	0	0
Anti-PR8 virus	++++	+++	+++
Anti-NP	±	±	ND[d]
Fixed cells[e] + anti-NP	++	++	++
Anti-M protein	++	++	+
Anti-M protein adsorbed with X-7 virus	+	+	ND

[a] Murine fibroblasts; nonpermissive infection.
[b] Canine kidney cells; permissive, infectious particles.
[c] Bovine kidney cells; permissive, noninfectious particles.
[d] ND, Not done.
[e] Cells were acetone fixed and then exposed to antiserum.

the antiserum was extensively adsorbed with intact virus. Subsequent examination of immunofluorescence of infected cells with the adsorbed antiserum resulted in some reduction in the intensity of fluorescence, but bright fluorescence was still evident (Fig. 1). From these findings we concluded that it is likely that M protein is present on the external cell surface of infected cells, but probably not on the virus. In contrast, only dull fluorescence was detected when infected cells were treated with antiserum to NP. No lysis of infected cells was detected in the presence of anti-NP and complement (data not shown). However, when cells were fixed before exposure to antiserum, bright fluorescence, especially in the region of the nucleus, was observed.

Blocking of cell-mediated cytotoxicity.

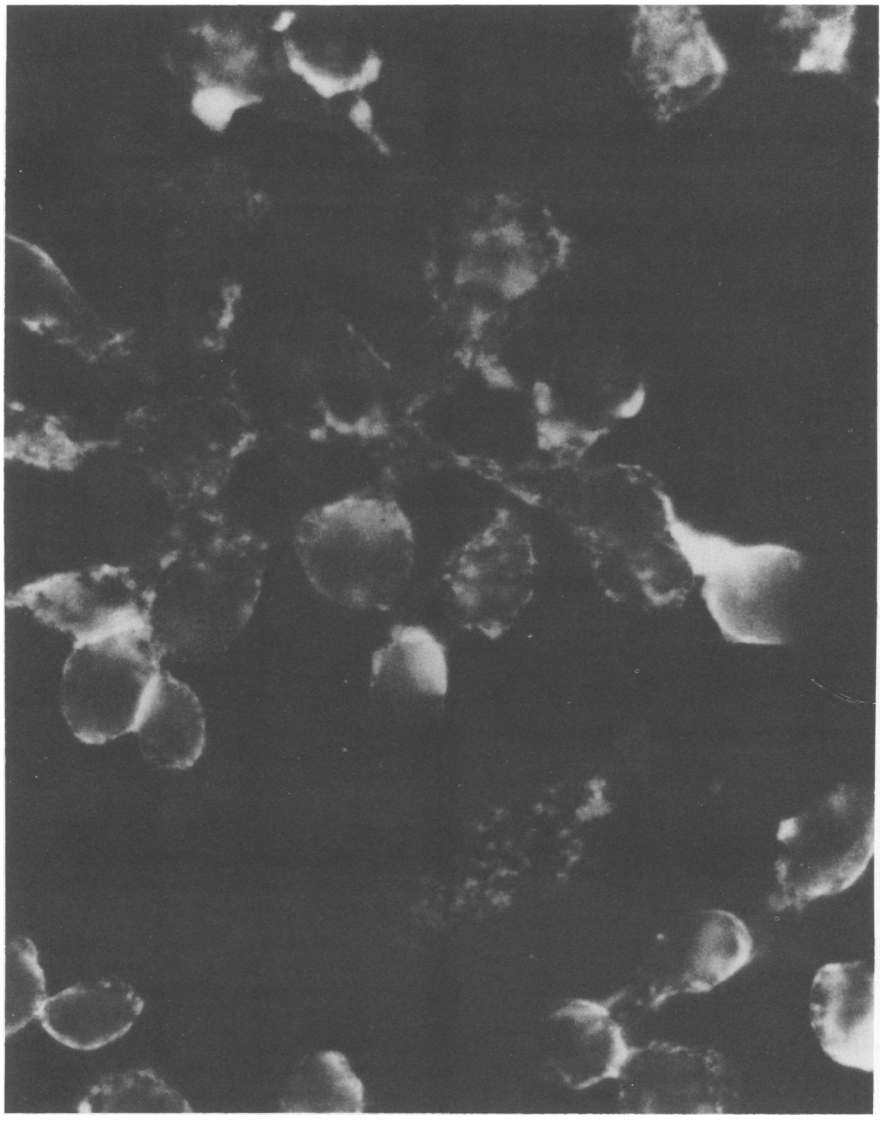

FIG. 1. *Immunofluorescence of infected L-929 cells with anti-M antibody. L-929 cells were infected with PR8 virus (multiplicity of infection, ten 50% egg infective doses) and 6 h later were incubated with rabbit anti-M antibody (previously adsorbed with X-7 virus) for 60 min at 4°C, washed, and incubated at 4°C for 60 min with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. After washing, cells were fixed, dried, and mounted in glycerol-phosphate-buffered saline (9:1) prior to examination. (× 250; Polaroid 57 film.)*

Experiments were conducted to determine whether antibody to M protein could block the cross-reactive CTL response. Spleen cells from PR8 virus-sensitized mice were tested with target cells infected with homotypic PR8 (H0N1) virus and with cells infected with heterotypic Japan (H2N2) virus. Simultaneous addition of anti-M protein antibody and sensitized lymphocytes resulted in a significant reduction in the cytotoxic activity on cell targets infected with heterotypic virus, but caused only a slight decrease in the activity against cells infected with homotypic virus (Table 2). These results suggest that the cross-reactive killing is in part due to the presence of a common M protein on the surfaces of infected cells.

DISCUSSION

Blocking by antiserum of determinant(s) recognized by CTLs has been demonstrated in a number of other systems, including MHC antigens (9, 14) and minor histocompatibility antigens (K. Fisher-Lindahl and H. Lemke, Eur. J. Immunol., in press). In viral studies, Finberg and his colleagues (R. W. Finberg, S. J. Burakoff, H. Weiner, and B. N. Fields, submitted for publication) have recently observed blocking of reovirus type-specific CTLs by antiserum specific for the S1 gene product. Effros et al. have reported blocking of influenza virus strain-specific, but not cross-reactive, CTLs from the thoracic duct with monoclonal antibodies directed at the hemagglutinin (R. B. Effros, M. E. Frankel, W. Gerhard, and P. C. Doherty, J. Immunol., in press). Blanden et al. observed that monoclonal antibodies directed at MHC antigens block CTL recognition of one virus, but not another (3). A synergistic effect of monoclonal antibodies to *H-2*[k] and to the influenza virus hemagglutinin in inhibiting cross-reactive CTL activity has been described by Askonas and Webster (B. A. Askonas and R. G. Webster, Eur. J. Immunol., in press). The latter finding implies that some of the cross-reactive lysis of targets infected by influenza viruses of different subtypes might be due to recognition of cross-reactive determinant(s) on the hemagglutinin molecule.

TABLE 2. *Blocking effect of antiserum to M protein on cell-mediated cytotoxicity with PR8 virus-sensitized lymphocytes*[a]

Target L-929 cells infected with:	% SIR[b] with:		% Reduction in cytotoxicity[c]	P
	Normal rabbit serum[d]	Anti-M protein[e]		
PR8 virus	24.3	22.4	7.7	>0.05
Japan virus	36.0	10.1	71.9	<0.01

[a] Mice were sensitized 6 days previously with 0.2 ml of A/PR/8/34 virus at a hemagglutination titer 1:1,024. The ratio used was 100 effectors:1 target cell.

[b] SIR, Specific immune response.

[c] $100 \times \{[SIR(NRS) - SIR(anti\text{-}M)] \div SIR(NRS)\}$

[d] 10% normal rabbit serum in Dulbecco minimal essential medium during the 12-h incubation period with lymphocytes and ^{51}Cr-pulsed target cells.

[e] 10% rabbit antiserum to M protein.

Cross-reactive CTL recognition of influenza virus-infected target cells, to a great extent, may be due to the presence of M protein on infected cell membranes, as has been suggested by the findings of other investigators (1, 2, 5). In the studies described above, we demonstrated that cross-reactive but not strain-specific CTLs could be blocked by antiserum to M protein. There are two possible interpretations to these findings: (i) a common M protein, expressed on the surfaces of influenza virus-infected cells, is recognized by a portion of the virus-specific CTL population, and (ii) the cross-reactivity observed is due to a common determinant(s) present on the viral hemagglutinin. Our data support the conclusion that a portion of the CTL response might be due, in part, to M protein; however, we do not rule out the possibility that cross-reactive recognition might be due in part to recognition of other virus-associated proteins. The interpretation of the present results depends on the purity of the antiserum used in these studies. Although this reagent had no detectable reactivities with other viral proteins, we cannot exclude the possibility that it contained contaminating antibodies to other viral proteins. Although we observed some loss of fluorescence when X-7 virus was used to adsorb the antiserum, there are two possible explanations: (i) the antiserum was specifically adsorbed with M protein from within the virion which was exposed due to loss of integrity of the viral envelope during viral isolation procedures, or (ii) the antiserum contained a specificity which reacted with the exterior of the virus. In particular, it is possible that some of the HA2 subunit copurified with the M protein. However, the M protein used to elicit the antibody was obtained from X-7 (H0N2) virus, which contains a hemagglutinin very closely related to that of PR8 (H0) virus and much more distantly related to the hemagglutinin of Japan (H2) virus. Consequently, if contaminating antibody to HA2 were present, one might expect to observe greater reactivity of the antiserum with PR8 virus-infected cells than with Japan virus-infected cells. It is difficult to explain the results observed if antibody to other viral proteins contaminated the anti-M protein reagent. Whereas PR8 virus-infected cells express both strain-specific and cross-reactive determinants, Japan virus-infected cells express

only cross-reactive determinants. It is not surprising that we did not see a decrease in lysis of these targets; a cell can only be killed once. Strain-specific effector T cells are equally effective on their targets in the absence of cross-reactive CTLs (blocked by antibody). Our observations of blocking cross-reactive CTL recognition with anti-M protein antibody are consistent with data obtained by Braciale with 2-deoxyglucose (5). Serologically detectable viral glycoproteins were not expressed on the surface of infected cells, but the targets were still susceptible to lysis by cross-reactive CTL effectors. In addition, Effros and her colleagues were able to block only virus-specific, and not cross-reactive, lysis by using a panel of monoclonal antibodies to the viral hemagglutinin (Effros et al. J. Immunol., in press). These findings strongly support our conclusion that M protein is recognized by a portion of the cross-reactive CTL elicited by influenza virus immunization.

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