QUANTITATION OF INFLUENZA VIRUS ANTIGENS ON INFECTED TARGET CELLS AND THEIR RECOGNITION BY CROSS-REACTIVE CYTOTOXIC T CELLS*

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Cytotoxic T cell (T_c)[1] lysis of influenza virus-infected target cells is restricted by the major histocompatibility locus and requires the presence of viral antigen on the infected cell surface (1, 2). A major characteristic of the T_c response to influenza viruses in both mouse and man is its cross-reactivity with serologically distinct type-A strains (3–5). It has been suggested that viral matrix (M)-protein, which is indistinguishable between different type-A viruses, could be responsible for recognition by cross-reactive T_c. This antigen was detected on the surface of infected cells by sera from animals hyperimmunized with purified M-protein (6, 7); the assertion from quantitative studies (8) was that M-protein constituted a high proportion of the viral antigens expressed on abortively infected (P815 mastocytoma) cells. Because these findings were based upon the use of heterogenous antisera, which reacted with M-protein, but the specificity of which was not rigorously determined, we have selected a hybridoma that secretes monoclonal antibody to A-virus M-protein for quantitation of M-protein on infected cells. In our initial experiments (9) we found that M-protein could be detected on the surface of infected cells, but only in very low amounts; i.e., a maximum of 2×10^3 sites/P815 cell infected for 16 h with different type-A viruses.

In this study we have further examined the M-protein content of infected cell membranes as well as the kinetics of its appearance. We find that although the total M-protein content of solubilized, isolated plasma membranes is low, virus replication is required for its appearance. Approximately 100 times more hemagglutinin than M-protein is detected on abortively infected cells 16 h postinfection. Neither the monoclonal anti-M nor the anti-M-protein from hyperimmunized rabbits blocks T cell killing of infected target cells alone or in combination with anti-H-2. Our results are discussed in relation to virus protein recognition by T_c.

Materials and Methods

Viruses. Influenza virus strains X-31 (H3N2) = A/Hong Kong/1/68 × A/PR/8/34, A/WSN (H0N1), A/Japan 305/57 (H2N2), A/USSR/90/77 (H1N1), and B/Hong Kong (B/

* Supported in part by grant DRG-207-F from the Damon Runyon–Walter Winchell Cancer Fund; Senior International Fellowship F06-TW00249 from the Fogarty International Center, National Institutes of Health; and by research grants AI 08831 and AI 42510 from the National Institute of Allergy and Infectious Diseases.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; e.r., endoplasmic reticulum; HA, hemagglutinin(ation); K_a, binding affinity constant; M, viral matrix; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RIP, radioimmunoprecipitation; RPMI/10, RPMI-1640 medium that contained 10% heat-inactivated fetal calf serum; T_c, cytotoxic T cell(s).

J. Exp. Med. © The Rockefeller University Press · 0022-1007/80/05/1014/12 $1.00
Volume 151 May 1980 1014–1025

HK) were cultivated in embryonated chicken eggs, and virus-containing allantoic fluid was titrated for hemagglutination (HA) activity before use.

T_c: Generation and Assay. CBA T_c were generated after intranasal priming of mice with A/X-31 and a secondary in vitro culture with A/USSR-infected lymphoblasts as previously described (10). CBA lymphoblasts stimulated for 2 d with *Escherichia coli* lipopolysaccharide served as target cells after a 5-h infection with A/X-31 virus (for virus infection of cells, see below). ^{51}Cr-labeled target cell lysis was assayed in flat microplates at various ratios of killer: target cells (11) for 3 h. Antisera were absorbed with B/HK-infected P815 cells before incubation with the target cells. All wells contained the same volume (0.22 ml).

Monoclonal Antibodies. Hybrid cell line 174, which forms antibodies to M-protein, was selected after fusion of myeloma cells P3/X-63-Ag8 with immune spleen cells according to the method of Köhler and Milstein (12). The spleen cell donor was a CBA mouse that was immunized with two intramuscular injections of purified M-protein from A/USSR virus (13) in complete Freund's adjuvant. Fusion was carried out 4 d after an intraperitoneal boost injection (150 μg of M-protein). The hybridoma cells were cloned and grown as solid tumors in (CBA × BALB/c)F_1 mice. The specificity of the antibody for influenza and M-protein was established by immunoprecipitation of ^{35}S-labeled virus proteins and polyacrylamide gel electrophoresis.

Virus Infection of Cells. P815 mastocytoma cells were maintained by passage in (CBA × DBA)F_1 mice. In vitro culture done was in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) that contained 10% heat-inactivated fetal calf serum (RPMI/10). 10^7 P815 cells were infected with 800 HA U of influenza virus in 1 ml of RPMI-1640 medium. After 1 h at 37°C, the cells were washed and incubated at 2×10^6/ml in RPMI/10 at 37°C for up to 6 h or at 33°C for 16 h (10).

Virus replication was prevented in certain experiments by actinomycin D (14): cells were preincubated with actinomycin (1 μg/ml) for 1 h, then infected with A-virus in the presence of the inhibitor at 1 μg/ml. Virus was inactivated by UV light: virus (1.5 ml of X-31-containing allantoic fluid) was treated for 4 min at 25 cm from a Philips 25-W germicidal bulb (Philips Electronic Instruments, Inc., Mahwah, N. J.). An infectivity check of this UV-treated virus by passaging twice through embryonated chicken eggs showed greatly reduced viral growth; however, slight infectivity could still be detected.

Plasma Membrane Preparations. The methods of Crumpton and Snary (15) and Bridgen et al. (16) were used. 16-h-infected (X-31) P815 cells (10^9 cells) were fractionated by differential centrifugation and discontinuous sucrose density gradients after disruption with a Stansted pump (Stansted Fluid Power Ltd., Essex, England). Control cells (5×10^8 cells) were infected with X-31 virus 1 h before disruption. While endoplasmic reticulum (e.r.) was pelleted, plasma membranes were recovered at the 25–36% sucrose-band interface after centrifugation at 22,000 rpm for 18 h in a Beckman SW41 rotor (Beckman Instruments Inc., Spinco. Div., Palo Alto, Calif.). Membrane fractions were washed and solubilized in 0.25 ml 2% sodium deoxycholate, 10 mM Tris, pH 8.2, with phenylmethylsulfonyl fluoride present. M-protein content in the lysed membrane fractions was determined by direct, solid-phase microradioimmunoassay.

Solid-Phase Radioimmunoassay. For the detection and quantitation of M-protein or antibodies to M-protein flexible plastic plates (Cooke Engineering Co., Alexandria, Va.) were coated with purified M-protein, detergent-solubilized membranes, or disrupted virus according to the general procedure of Rosenthal et al. (17). Antigens were dried onto the wells overnight at 37°C, then briefly fixed with methanol. Indirect binding assays used the appropriate ^{125}I-labeled goat anti-rabbit immunoglobulin or rabbit anti-mouse antiserum. Direct assays were with the IgG2 fraction of ^{125}I-labeled monoclonal anti-M antibody. Radiolabeled antisera were allowed to react for 15 h at 4°C. The plates were washed, and the wells were separated to count the radioactive antibody bound.

The M-protein content of purified membranes was quantitated by direct solid-phase radioimmunoassay. Binding of ^{125}I-monoclonal anti-M to serial dilutions of the membrane lysates (in phosphate-buffered saline [PBS]/1% bovine serum albumin [BSA]) and of the purified M-protein standard (from 1 mg/ml) dried into wells was compared. The M-protein content was estimated on the basis of the standard M-protein antibody-binding curve. Three sample dilutions showed a ≤11% SD of the mean. Conversion from the concentration of M-

protein (milligrams per milliliter) detected in the solubilized membrane samples to the number of molecules per cell was done by the following equation:

$$\text{M-protein molecules per cell} = \text{M-protein detected } (\mu g/ml) \times \frac{\text{volume solubilized membranes (ml)} \times \text{Avogadro's number}}{\text{M-protein } (\mu g/mol) \times \text{No. cells extracted}}$$

Antibody-binding Studies. The IgG2 fraction of the monoclonal 174 anti-M antiserum was purified on a staphylococcal protein-A-Sepharose column (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) according to Ey et al. (18) and ^{125}I-labeled as previously described. 28% of the ^{125}I-antibody bound to M-protein on plastic dishes and wells that were coated with detergent-disrupted type-A viruses. Quantitative binding of antibody to cells was assayed as described before (19). P815 cells at 2×10^5/0.1 ml (virus-infected or uninfected controls) were incubated for 90 min at 20°C with increasing concentrations of radiolabeled antibody (2.5×10^{-3}–1×10^1 μg/0.1 ml) in Hepes-buffered RPMI-1640 that contained 1.7% BSA and 0.1% NaN_3. Cells were washed twice, and radioactivity in the cell pellets was counted.

Binding data obtained with monoclonal antibody were evaluated by the Scatchard equation as described in a previous report (19). A graph of moles of antibody bound per moles of cells vs. moles of antibody bound per moles of free antibody × moles of cells yielded linear plots with a slope equal to the binding affinity constant (K_a) and the x-intercept equal to the number of antigenic sites per cell. All quantitation of viral antigens on infected cell surfaces was done by Scatchard analysis.

Expression of HA on infected cells was assayed by monoclonal antisera directed against H1 (clone W18/1) or H3 (clone 3/1) of type-A viruses (20, 21). After fractionation on a protein-A-Sepharose column, the active IgG2a fractions (determined by HA inhibition titration) were radiolabeled and used in equilibrium binding studies as above.

Complement-mediated Lysis of Virus-infected Cells. ^{51}Cr-labeled virus-infected cells were incubated with diluted antibody for 30 min at 4°C. The cells had been infected for 16 h with A/X-31 or B/HK virus. After one wash they were resuspended in 1:1 RPMI-1640 medium with 1% fetal calf serum guinea pig complement (absorbed with agarose, diluted 1:3 in PBS) and incubated at 37°C for 1 h. Cells were then pelleted by centrifugation, and aliquots of the supernates were counted for radioactivity. All antisera used had been previously absorbed with B/Hong Kong-infected target cells.

Preparation of Labeled Virus Antigens. Primary chick embryo fibroblasts were infected with A/WSN/33 (H0H1) influenza virus. The virus was adsorbed for 30 min and then replaced with minimal essential medium that contained 70 μCi of [L-^{35}S]methionine (1,270 Ci/mmol) (Amersham Corp., Arlington Heights, Ill.) per milliliter. The cultures were incubated at 37°C for 48 h, and the labeled virus was purified by velocity centrifugation. Labeled virus was lysed in 0.05 M Tris-HCl (pH 7.5), 0.6 M KCl, and 0.5% Triton X-100 (Rohm and Haas, Philadelphia, Pa.) for 30 min at 25°C. Protein aggregates were removed by centrifugation at 150,000 g for 45 min.

Immunoprecipitation. 200 μl of hybridoma tissue culture fluid was added to 100 μl of normal mouse serum (1/400) in radioimmunoprecipitation (RIP) buffer (0.05 M Tris-HCl [pH 8.0.], 1 mM EDTA, 0.15 M NaCl, and 0.25% BSA) with 100 μl of labeled viral antigens diluted to 50,000 cpm/100 μl. The reaction mixture was incubated for 2 h at 25°C and then centrifuged at 800 g for 15 min. This was necessary to prevent labeled M-protein from adhering nonspecifically to precipitates. 50 μl of a 1/5 dilution of a rabbit anti-mouse Ig mixture was incubated for 2 h at 25°C, then overnight at 4°C. Precipitates were washed three times with RIP buffer and dissolved in 25 μl of polyacrylamide gel electrophoresis (PAGE) sample buffer that contained 10% 2-mercaptoethanol. Immunoprecipitates were analyzed by 8% sodium dodecyl sulfate-PAGE as described by Laemmli (22) for 15 h at 70 V. The gels were processed for fluorography and exposed to Kodak XR-5 film (Eastman Kodak Co., Rochester, N. Y.) for 20 h.

Results

Specificity of Monoclonal Antibodies to M-Protein. The monoclonal anti-M (174) is an IgG2 antibody and can be efficiently fractionated on staphylococcal protein-A-

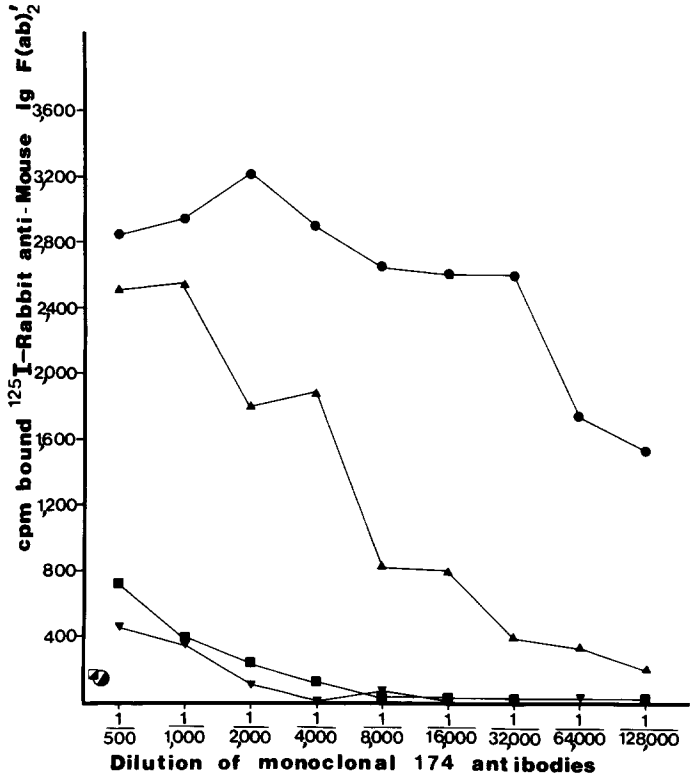

FIG. 1. Fractionation of monoclonal anti-M-protein antiserum on staphylococcal protein-A-Sepharose. Immunoglobulin fractions sequentially eluted from the protein-A column were titrated for anti-M-protein activity by indirect solid-phase radioimmunoassay. Bound monoclonal antibody was detected by ^{125}I-labeled rabbit anti-mouse F(ab')$_2$. Antibody binding to purified A-virus M-protein: (●) unfractionated 174 serum; (▼) 174 serum components not adsorbed to protein-A column (fall through); (▲) IgG2 fraction from 174; (■) IgG1 fraction from 174; (◪) normal mouse serum, 1/100 dilution. Antibody binding to disrupted B/Hong Kong virus: (◕) 174 serum.

Sepharose columns (Materials and Methods). The resulting antibody fraction interacts with M-protein of influenza virus A/X-31, but not with that of B/HK (Fig. 1). It has equally high reactivity with all other type-A viruses tested (USSR, A/Jap/Bel, WSN, and Jap 305; data not illustrated). The antibody specificity was verified by immunoprecipitation of ^{35}S-labeled viral proteins and PAGE. Fig. 2 shows that reaction of the monoclonal antibody is restricted to M-protein alone.

Quantitation of M-Protein in Solubilized Membranes Purified from A/X-31 Virus-infected P815 Cells. Because our antibody is monoclonal it was important to ascertain that the low number of M-protein sites detected on the surface of infected cells could not simply be attributed to the possibility that the antibody interacts with a part of the M-protein molecule that is poorly accessible on intact cells. For this reason we purified plasma membranes and e.r. from P815 cells infected 16 h with X-31 virus and estimated the content of membrane-associated M-protein in detergent lysates, which would include both surface and internal M-protein. Cells in contact with the virus 1 h, without further virus replication, served as control. Radioimmunoassay of the solubilized fractions (Fig. 3) shows that membranes isolated from 16-h-infected cells bind small but significant amounts of ^{125}I-monoclonal anti-M, whereas control cell

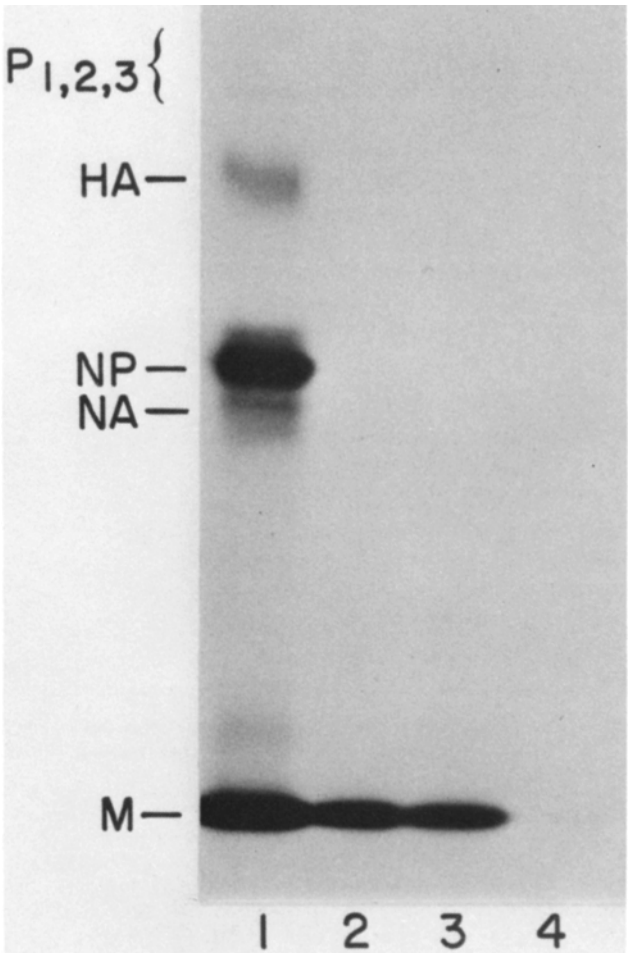

FIG. 2. Specific immunoprecipitation of M-protein by monoclonal antibody 174. The ^{35}S-labeled A/WSN virus proteins resolved in this fluorogram are: (lane 1) radiolabeled whole virus used in immunoprecipitation; (lanes 2 and 3) precipitate by culture supernates of hybrid cells 174; (lane 4) precipitation with parental P3X63-Ag8 culture supernate only. $P_{1,2,3}$, polymerases; NP, nucleoprotein; NA, neuraminidase.

membranes bind about the same low level as disrupted type-B virus. By comparing several dilutions of the membrane samples (1/5, 1/10, 1/40 for the e.r.; 1/10 and 1/20 for plasma membrane) with corresponding points on the standard M-protein titration curve, we estimate that there is a total content of ~1.3 μg of M-protein in the e.r. fraction and 0.8 μg of M-protein in plasma membranes derived from 10^9 16-h-infected cells. This corresponds to 1.9×10^4 molecule/cell in the plasma membrane and 3.1×10^4 molecule/cell in the e.r. (see Materials and Methods for calculation).

Is Virus Replication Required for the Appearance of Cell Surface M-Protein? We studied the kinetics of cell surface M-protein in relation to virus replication (Table I). Whereas the binding of ^{125}I-anti-M-protein to cells infected overnight with A/X-31 gave the usual result of showing ~1.6×10^3 M-protein site/cell, cells treated overnight with A/X-31 inactivated by UV irradiation, or with infectious A/X-31 in the presence of

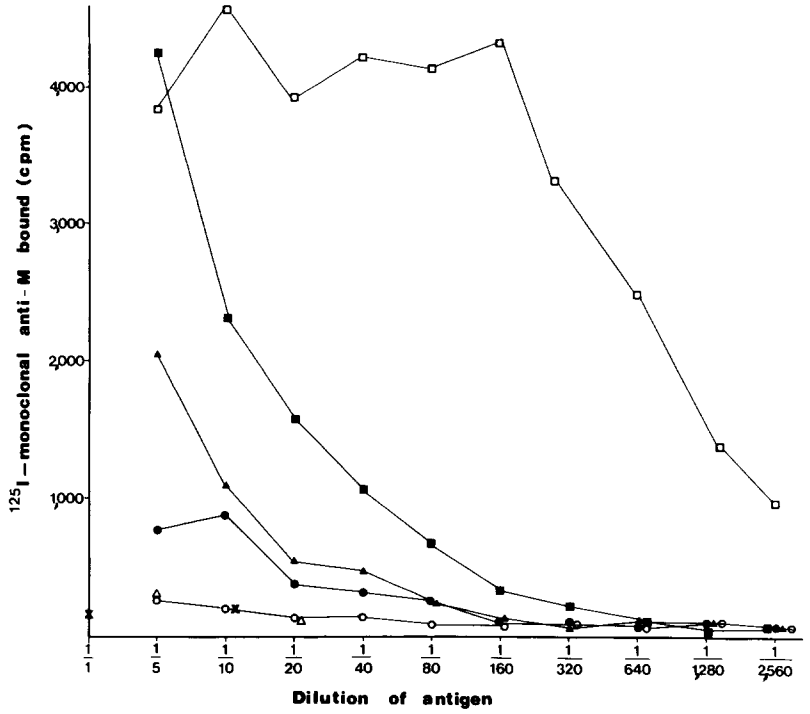

FIG. 3. Quantitation of M-protein in lysates of X-31 virus-infected P815 cell membranes by solid-phase radioimmunoassay. Membranes were purified from 16-h infected cells and control cells (infected 1 h before disruption) and solubilized in deoxycholate. Wells were coated with the lysates, and the amount of M-protein was determined by direct binding of ^{125}I-monoclonal anti-M antibody. Radioactivity of antibody (cpm) bound to: (●) plasma membrane, 16-h-infected cells; (▲) e.r., 16-h-infected cells; (○) plasma membrane, control cells; (△) e.r., control cells; (□) purified M-protein standard; (■) solubilized A/X-31 virus (600 HA U/25 μl); (×) solubilized B/Hong Kong virus (300 HA U/25 μl).

actinomycin D to prevent virus replication (14), showed far less antibody binding. The transient low binding of anti-M-protein soon after cell treatment (which suggested < 100 M-protein sites) can probably be assigned to rupture of virus, because after 16 h of incubation there was no detectable specific binding of anti-M. Although the number of M-protein sites on the surface of cells infected for 16 h is only $\sim 10^3$, virus replication is required for the appearance of M-protein, and it does appear to be synthesized by the host cell.

Expression of HA on Virus-infected Cells. We measured viral HA on 16-h infected cells to confirm that our conditions of infection and incubation permitted normal expression of virus proteins on the cell surface. Quantitative binding studies with radiolabeled monoclonal antibodies (Table II) show a high number of HA sites on cells infected with different type-A viruses. The 2.75×10^5 molecules/cell seen in USSR-infected P815 cells and 1.2×10^5 sites/X-31-infected cell are approximately two orders of magnitude higher than the amount of M-protein detected on the cell surface in similar infections.

Does Antibody to M-Protein Block T Cell-mediated Cytotoxicity? If M-protein were recognized by cross-reactive T_c we would expect inhibition of cell-mediated lysis of

TABLE I

Virus Replication is Required for the Appearance of Cell Surface M-Protein

Infection	Time of incubation	Surface M-protein
	h	*site/cell*
Infectious A/X-31 virus	0.5	0*
	3	100‡
	16	1,600§
UV light-inactivated A/X-31 virus	0.5	0
	3	100‡
	16	0
Actinomycin D and infectious A/X-31 virus	0.5	80‡
	3	0
	16	0

* Indicates no binding of ^{125}I-anti-M above background binding to uninfected cells.

‡ The very low values are derived from the amounts of ^{125}I-antibody specifically bound per cell at saturation, where binding below saturation level was insufficiently above background for evaluation by Scatchard analysis.

§ M-protein sites are assayed by binding of ^{125}I-monoclonal antibody to M-protein on infected P815 cells over a wide range of antibody concentrations; the number of sites obtained by extrapolation of Scatchard plots of the binding data (9).

TABLE II

Expression of Viral HA by P815 Cells Infected for 16 h with Type-A Influenza Virus

Infection	^{125}I-Labeled monoclonal antibody	HA
		*molecule/cell**
A/X-31	3/1 (anti-H3)	1.2×10^5
A/USSR	W18/1 (anti-H1)	2.7×10^5

* Estimate from Scatchard plots of ^{125}I-antibody-binding data.

influenza virus-infected target cells by an excess of antibody. Neither the monoclonal nor the heterogeneous antibodies to M-protein blocked T cell cytotoxicity either alone or in the presence of monoclonal antibodies to H-2 (30R3) (kindly donated by Dr. Lemke, University of Cologne, Cologne, Federal Republic of Germany) (Table III). The monoclonal anti-H-2 on its own partially inhibits cytotoxicity, but this inhibition is not enhanced by anti-M.

Complement-mediated Lysis of A-Virus-infected Cells by Antibodies to Viral Proteins. P815 cells were infected overnight with A/X-31, and complement-mediated lysis by monoclonal and heterogeneous antisera from rabbits hyperimmunized with M-protein was assayed. Cell lysis was observed with rabbit antiserum to M-protein, even after absorption of the serum with B/HK-infected P815 cells. On the other hand, the monoclonal anti-M-protein failed to effect lysis of infected cells, although the antibody 174 is an IgG2a, which should fix complement (Fig. 4). This finding does not permit final conclusions. It could be a result of the low number of M-protein sites on infected cells or of the fact that monoclonal antibodies do not necessarily lyse cells with complement. We obtained similar results with monoclonal anti-HA antibodies. IgG2a

TABLE III

Antibody to M-Proteins Does Not Inhibit T Cell Killing of Virus-infected Targets

Treatment of target cells	Percentage of control cytotoxicity	
	4*	8*
—	100	100
Normal mouse serum (1/100)	96	98
Anti-nucleoprotein (clone 150/7) (1/100)	97	98
174 anti-M-protein (1/100)	100	97
174 anti-M-protein (1/50)	98	108
30 R3-anti-H-2 (1/100)	68	77
30 R3-anti-H-2 and 174 anti-M (1/100)	67	75
Rabbit anti-M (1/50)	97	103
Rabbit anti-M and 30 R3 (1/100)	65	78

* 3-h ^{51}Cr-release assay yielded 25 and 39% virus-specific killing at killer:target cell ratios of 4 and 8, respectively. CBA cytotoxic cells induced in vitro with A/USSR-infected lymphoblasts (4). CBA lymphoblasts infected with A/X-31 virus served as target cells. Target cells were pretreated with antisera for 30 min before addition of cytotoxic cells.

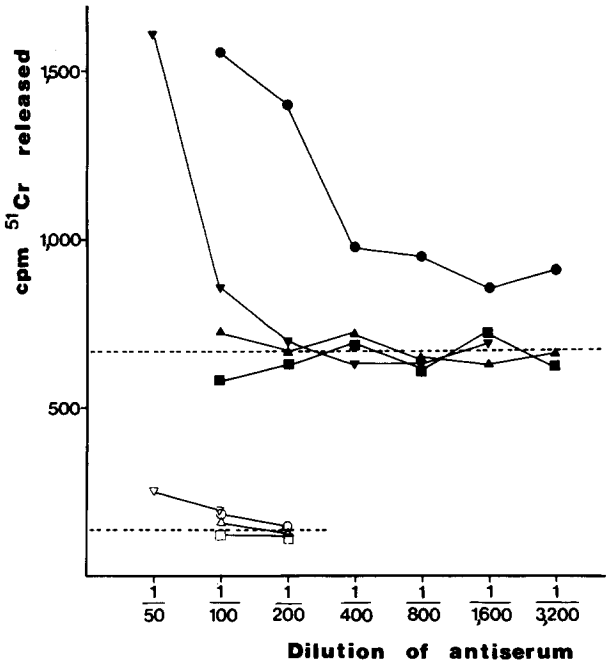

FIG. 4. Lysis of virus-infected P815 cells by antibody to viral proteins and complement. Cells were infected for 16 h with A/X-31 virus or B/HK and labeled with ^{51}Cr. The dotted line indicates the level of background ^{51}Cr release. Lysis (radioactivity released) by the following antibodies: A/X-31-infected target cells: (●) 74/6 anti-HA H3(IgM); (▲) 3/1 anti-HA H3(IgG2a); (▼) hyperimmune rabbit anti-M; (■) monoclonal anti-M 174 (IgG2). B/HK-infected cells: (○) 74/6; (△) 3/1; (▽) rabbit anti-M; (□) monoclonal anti-M 174.

monoclone 3/1 does not lyse infected cells in the presence of guinea pig complement, although ^{125}I-labeled 3/1 antibody does bind to A/X-31-infected cells. An IgM monoclonal antibody to HA (74/6) was efficient in complement-mediated lysis. Howard and Corvalan (23) also found that not all monoclonal antibodies to rat histocompatibility antigens, though able to fix complement, will lyse cells.

Discussion

Accurate quantitation of M-protein on influenza virus-infected target cells is important for assessment of the role of that antigen in recognition by cross-reactive T_c. We have recently selected a hybridoma secreting monoclonal anti-M to pursue this question. The monoclonal anti-M-protein antibody 174 has the advantage of monospecificity, which could not be fully ascertained with heterogeneous hyperimmune antisera used in the past. The monoclonal serum shows no cross-reactivity with other A-virus proteins or with cells infected with type-B influenza viruses. In addition, the high-titer hybridoma serum can be readily purified to yield an IgG2 fraction of high antibody content and a K_a of 1–2 × 10^9 liter/mol (9), thereby facilitating quantitative binding studies.

In our preliminary studies (9) we detected very low levels of specific binding of the monoclonal anti-M to P815 cells infected up to 16 h with different type-A viruses. Only when nonspecific binding was deducted could saturation of M-sites by the antibody become evident. Scatchard plots of the binding data showed that a maximum of 2–3 × 10^3 M-protein molecules appear on each P815 cell surface infected for 16 h with different type-A viruses. This is much lower than the 10^5–10^6 sites estimated by Ada and Yap (8). Our figure does not result from any generally reduced protein synthesis under our infection conditions, because high levels of HA expression (>10^5 molecule/surface of each cell) were readily obtained; and, typically, 65–75% of the infected P815 cells can be lysed specifically by T_c (10).

Another possibility, that the epitope on the M-protein detected by our monoclonal anti-M is poorly accessible on the cell surface, is ruled out by studies with solubilized, purified cell membranes. In the lysates, all M-protein molecules (internal and external) should be available to the antibody, yet we do not see the two to three order of magnitude increase that would be suggested by the values of Ada and Yap (8). Instead, radioimmunoassay detected the equivalent of ~1.9 × 10^4 molecule/cell in the plasma membrane lysate, and 3.1 × 10^4 molecule/cell in the e.r. fraction. Adjustment of these values for estimated recovery of total membrane protein increases the figures to ~3.8 × 10^4 and 6.2 × 10^4 molecule/cell in these respective fractions, assuming a 50% yield as suggested by the data of Crumpton and Snary (15) with <10^{10} Bristol-8 lymphoblastoid cells. Our samples (5 × 10^8 and 10^9 cells) were disrupted and purified according to their procedures. The 10- or 20-fold higher M-content of lysed plasma membranes compared with infected cells is consistent with the view (24) that M-protein is localized principally on the cytoplasmic side of the host cell plasma membrane. M-protein is also found in internal membrane fractions (24), as is the case in our e.r. fraction. Because only a small fraction of the M-protein associated with plasma membranes is found on the surface of the P815 cells even after a long period of infection, we examined the possibility that the surface M-protein might have originated from ruptured input virus that was exposed on the cell membrane as incubation progressed. Our findings with actinomycin D treatment and

UV-inactivated virus show that virus replication is indeed required for cell surface M-protein appearance. This, and evidence from kinetic studies of M-protein appearance on infected P815 cells (9), indicate that the external M-protein detected in these studies is synthesized within the host cell. However, we have no direct evidence that M-protein is expressed as a membrane protein in the same sense as for the viral glycoproteins. The possibility remains that some slight amount of virus budding or rupture of partially assembled virions could be the source of the surface M-protein.

The study of complement-mediated lysis of infected target cells with monoclonal antisera of viral protein was inconclusive. Monoclonal anti-M-protein did not promote lysis, even though it is of the IgG2 class and should be capable of fixing guinea pig complement. Although we know that $>10^5$ HA molecules are expressed on the cell surface, the monoclonal anti-HA 74/6 (IgM) was effective, whereas the 3/1 anti-HA (IgG2a) killed only very slightly, if at all (Fig. 4). It is clear that complement-dependent lysis is less sensitive than antibody-binding studies in detection of cell surface antigens, that monoclonal antibodies, in particular, vary in their ability to cause complement-mediated cell lysis as reported by Howard and Corvalan (23). In confirmation of earlier results by Biddison et al. (6), our studies with hyperimmune rabbit anti-M (which had been preabsorbed by B/Hong Kong-infected P815 cells) showed that it could bind to and promote complement-mediated lysis of 16-h A-virus-infected P815 cells. However, because of the heterogeneous nature of this antiserum, lysis of infected targets is not adequate proof that M-protein is integrated as a membrane protein in the host cell surface. The use of such a heterogeneous serum is the most likely explanation of the estimates of Ada and Yap (8), which differ so markedly from ours. Because the specificities of heterogeneous antibodies are not well defined (and might not be detected by immunodiffusion tests), possible low cross-reactivity with other virus components or antigens on infected cells cannot be completely ruled out. Ada and Yap (8) reported it difficult to purify antibody from the hyperimmune antisera (anti-M-protein or anti-HA) and therefore found <2% of added radioactivity bound to infected cells. In contrast, our radiolabeled monoclonal probes all showed a minimum of 20% binding under conditions of antigen excess. Finally, our quantitation of surface viral antigens is by extrapolation of linear Scatchard plots of the antibody-binding data to saturation, which is an analysis that requires monoclonal antibody (19).

The central question regarding the possible recognition of M-protein by T_c cross-reactive for different type-A influenza viruses is not yet resolved. P815 cells are excellent target cells for T cell lysis after a 3- or 16-h infection period. After 3 h, only $\sim 10^2$ M-protein sites can be detected on infected P815 cells (9). We do not know how many antigenic sites are required to induce T cell killing. On the other hand, our findings concerning the low numbers of M-protein sites exposed on cells and the lack of inhibition of T cell cytotoxicity by antibodies to M-protein even in the presence of anti-H-2 leave in doubt that it is the M-protein that is recognized by T_c. Other evidence is accumulating that suggests that the cross-reactive T_c may have specificity for the homologous part of the HA molecules. Effros et al. (26) have provided evidence of the inhibition of T_c specific for the different subtypes of A-virus by antibodies to HA. We find that some (but not all) monoclonal antibodies to HA of type-A virus in combination with monoclonal antibodies to H-2 synergistically inhibit T cell-mediated lysis of infected target cells (25).

Summary

Monoclonal antibody to type-A influenza virus matrix (M)-protein was used to quantitate the appearance of M-protein on abortively infected P815 cells. After 16 h of infection with different type-A viruses, only a low amount of M-protein appears on the surface of infected cells ($\sim 10^3$ site/cell) in contrast to $\sim 10^5$ hemagglutinin molecules on each cell surface. However, virus replication is required for M-protein appearance. Analysis of solubilized membranes purified from 16-h-infected cells shows $\sim 10^4$ M-protein molecule/cell in the plasma membrane, a content that is consistent with the observed low surface expression, and that indicates that most of the M-protein is localized internally. We found no evidence that cross-reactive cytotoxic T cells could recognize M-protein; neither monoclonal antibody nor hyperimmune anti-M-protein antiserum could inhibit T cell killing, either alone or in combination with monoclonal anti-H-2 antibody. Taken together, the low level of M-protein appearance and lack of T cell blocking by anti-M-protein antibody leaves doubt that M-protein is the antigen recognized by cross-reactive cytotoxic T cells.

We thank A. Williams for $F(ab)_2$ of pure rabbit anti-mouse Ig. Dr. Hackett is grateful to the Damon Runyon–Walter Winchell Cancer fund for a fellowship.

Received for publication 2 January 1980.

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