ANTIBODY TO INFLUENZA VIRUS MATRIX PROTEIN DETECTS A COMMON ANTIGEN ON THE SURFACE OF CELLS INFECTED WITH TYPE A INFLUENZA VIRUSES*

By WILLIAM E. BIDDISON, PETER C. DOHERTY, and ROBERT G. WEBSTER

(From the Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104, and St. Jude Children's Research Hospital, Memphis, Tennessee 38101)

Cytotoxic thymus-derived lymphocytes (T cells)[1] generated by immunization with type A influenza viruses cross-react extensively when assayed on target cells infected with type A, but not with type B, influenza viruses (1, 2). No correlation is found between the magnitude of cell-mediated lysis and the extent of serologically defined similarity between the hemagglutinin (H) and neuraminidase (N) subunits expressed on the surface of both the virion and the virus-infected cell (1). For example, immunization of mice with A/PR/8/34, HON1 (PR8) or A/Hong Kong/8/68-X-31 [H3N2] (HK) results in clonal expansion of at least two populations of T cells: the one tends to be specific for the virus used to immunize, while the other is highly cross-reactive for all type A viruses which had been tested (1, 2). Sera obtained from such mice neither cross-neutralize (between PR8 and HK), nor bind to cells infected with these two viruses (1).

The existence of these cross-reactive T-cell populations is somewhat surprising, since all available evidence indicates that the discriminatory capacity of T-cell recognition is at least equivalent to that found for antibody-forming cells (B cells) (3–5). Perhaps these cross-reactive influenza-immune cytotoxic T cells recognize a common determinant which does not stimulate B cells during the infectious process. Possible candidates are the common internal matrix (M) protein and ribonucleoprotein (NP) components of the influenza virion (6). Evidence that the M protein may be expressed on the cell surface is presented here.

Materials and Methods

Viruses. The influenza type A viruses PR8 (A/PR/8/34 [HON1]), Bel (A/Bellamy/42 [HON1]), JAP 305 (A/Japan/305/57 [H2N2]), HK (A/Hong Kong/8/68-X-31 [H3N2]), and an influenza type B virus (B/Lee/40) were supplied and grown in embryonated eggs as previously described (1).

Preparation and Specificity of Antiserum to Matrix Protein. M protein was prepared from

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[1] *Abbreviations used in this paper:* B cell, antibody-forming cell; H, influenza virus hemagglutinin; HK, A/Hong Kong/8/68-X-31 [H3N2]; M, influenza virus matrix protein; N, influenza virus neuraminidase; NP, influenza virus ribonucleoprotein; T cell, thymus-derived lymphocyte.

purified influenza virus (A/NWS/33 [HO]-equine/Prague/1/56 [Neq1]) as described by Laver and Webster (7). The isolated M protein was boiled in detergent for 2 min and antisera were prepared in goats (8). The specificity of the anti-M serum was demonstrated in gel diffusion tests (9). The anti-M serum gave a single line of precipitation with disrupted influenza A viruses but failed to react with influenza B viruses; anti-M serum also failed to neutralize the infectivity of influenza A viruses (9).

The above information taken together with the finding that all of the other major viral proteins of influenza virus (H, N, and NP) are irreversibly denatured by boiling (10) suggests that the antiserum contained antibodies to M protein only.

Antibody-Mediated Complement-Dependent Serum Cytotoxicity Assay. The capacity of antibody directed against cell surface antigens to mediate complement-dependent lysis of ^{51}Cr-labeled target cells was assayed in microtiter plates as described by Goldstein et al. (11). All manipulations were performed in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 200 U/ml penicillin, and 100 μg/ml streptomycin. Tissue culture-grown P815 mastocytoma cells (H-2^d) were infected with influenza virus (1), labeled with $Na_2{}^{51}CrO_4$, and used as target cells (1.5×10^4 cells per well). Fresh-frozen guinea pig serum (50 μl of 1:8 dilution per well) was utilized as a source of complement. Results are expressed as mean percent specific lysis for quadruplicate determinations, and were calculated by the formula: (exp-GPS) $\times$ 100/W-GPS, where exp is the experimental release, GPS the release in the presence of guinea pig serum only, and W the release in the presence of distilled water.

Results

Hyperimmune goat antiserum to M protein of type A influenza virus (anti-M) was assayed on normal P815 cells, and on P815 cells that had been infected with HK (H3N2) or with B/Lee influenza viruses. This anti-M serum mediated lysis of the HK-infected cells, but no activity was detected on the normal or B/Lee-infected targets (Fig. 1). Preimmune serum from the same goat caused no demonstrable cytotoxicity. A second experiment (Table I) showed that the anti-M serum reacted with all of the type A influenza virus-infected cells which were tested, and confirmed the lack of lytic capacity for type B-infected and normal P815 cells. However, both control targets (B/Lee-infected and normal) were lysed when exposed to appropriate antisera, specific for type B virus and alloantigens, respectively.

The anti-M serum apparently detects a common antigenic determinant which is expressed on the surface of cells infected with different type A viruses since prior incubation of the anti-M serum with HK-infected cells removed all the reactivity for each type A virus-infected target which was assayed (Fig. 2). In contrast, absorption with normal or B/Lee-infected cells caused no diminution in lytic titer.

Although it has been demonstrated that the M protein is not expressed on the surface of the influenza virion (6), it could be that the cell surface antigen(s) detected is present as a result of breakdown of the virus inoculum at the plasma membrane. The M protein constitutes 47% of total virus protein, forms close associations with lipids, and thus might be expected to adsorb readily to the cell membrane (9, 11). However, a kinetics study established that only minimal amounts of antigen are demonstrable by 3 h after infection, and maximal expression does not occur for a further 2–4 h (Fig. 3). Thus, it seems likely that synthesis of new virus protein must occur before this antigen(s) appears on the cell surface.

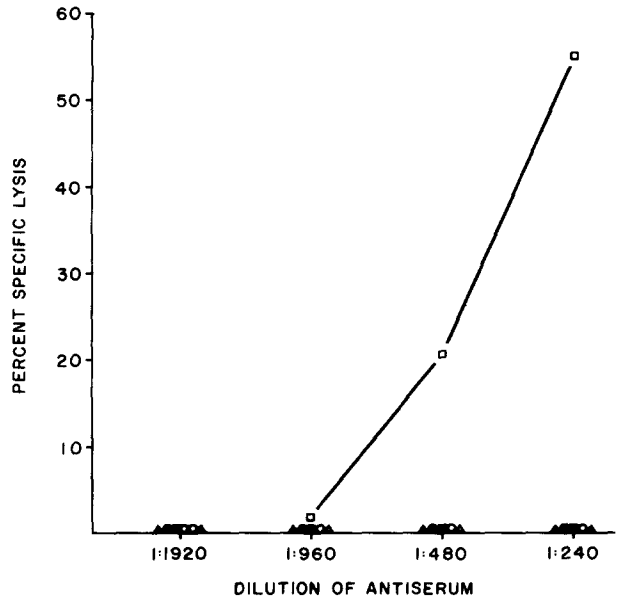

FIG. 1. P815 cells were labeled with ^{51}Cr and infected for 7 h with HK (□, ■) influenza A virus, B/Lee (△, ▲) influenza B virus, or no virus (○, ●). These target cells were then assayed with various dilutions of goat anti-M serum (□, △, ○) or preimmune goat serum (■, ▲, ●) in the serum cytotoxicity assay.

Discussion

The antiserum probably binds to M protein on the cell surface, although this is not yet rigorously established. The mode of antigen expression may be analogous to that which occurs in the oncornavirus system, where a glycosylated analogue of an internal protein (p30) is expressed on the cell membrane (12). The fact that the M protein is boiled in detergent before immunization excludes most of the other viral components (H, N, and NP are irreversibly denatured under these conditions, 10), although the involvement of some unknown carbohydrate moiety is not excluded. The carbohydrate chicken host component which is shared between egg-grown type A influenza viruses is also present on type B viruses (13), but these viruses do not cross-react either in this system or in the cytotoxic T-cell assay (1, 2).

The essential point is that a common serologically detectable entity is present on the surface of cells infected with type A, but not type B, influenza viruses. In addition, the kinetics of appearance of this component on the cell membrane are comparable to those found for the expression of antigens which are recognized by influenza-immune T cells.[2] This time-course data also indicates that antigen expression is dependent upon synthesis and incorporation into the membrane rather than attachment to the cell surface of antigen contained in the virus inoculum.

Influenza A and B viruses initiate an abortive cycle of replication in P815

[2] J. Bennink and P. C. Doherty. Manuscript in preparation.

TABLE I

Reactivity of Anti-M Serum against Type A and B Influenza Virus-Infected Cells

P815 cells infected with	Anti-M serum						Control sera			
	1:80	1:160	1:320	1:640	1:1,280	1:2,560	BALB/c* anti-CBA	CBA* anti-BALB/c	A/J‡ anti-PR8	BALB/c‡ anti-B/Lee
N.1	0.8 ± 0.1§	0	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0	0.1 ± 0.0	59 ± 2	0.2 ± 0.0	0.3 ± 0.0
B/Lee	3 ± 0.2	4 ± 0.2	3 ± 0.1	0	8 ± 2.8	−2 ± 0.2	−0.6 ± 0.0	77 ± 1.0	5 ± 0.2	40 ± 2.2
PR8 (HON1)	70 ± 0.7	63 ± 0.9	56 ± 0.8	29 ± 0.9	6 ± 0.3	5 ± 0.2	0.8 ± 0.0	72 ± 0.4	68 ± 0.8	9 ± 0.6
Bel (HON1)	65 ± 2.6	55 ± 1.3	49 ± 1.1	33 ± 2.4	5 ± 0.4	1 ± 0.0	1.0 ± 0.0	58 ± 1.9	0.4 ± 0.0	5 ± 0.1
JAP 305 (H2N2)	60 ± 3.9	69 ± 1.5	66 ± 1.2	52 ± 2.1	16 ± 0.7	5 ± 0.3	0.6 ± 0.0	65 ± 1.6	13 ± 1.0	8 ± 0.3
HK (H3N2)	81 ± 0.4	73 ± 1.7	78 ± 1.2	70 ± 2.5	32 ± 1.3	8 ± 0.3	2 ± 0.1	84 ± 2.5	2 ± 0.0	−0.5 ± 0.0

* Alloantisera (diluted 1:50) were prepared by multiple intraperitoneal inoculation of spleen cells into allogeneic recipients.

‡ Antisera against PR8 and B/Lee viruses (diluted 1:25) were prepared by multiple intraperitoneal inoculation of live virus into A/J or BALB/c recipients.

§ Mean percent specific lysis ± standard error of the mean for quadruplicate determinations.

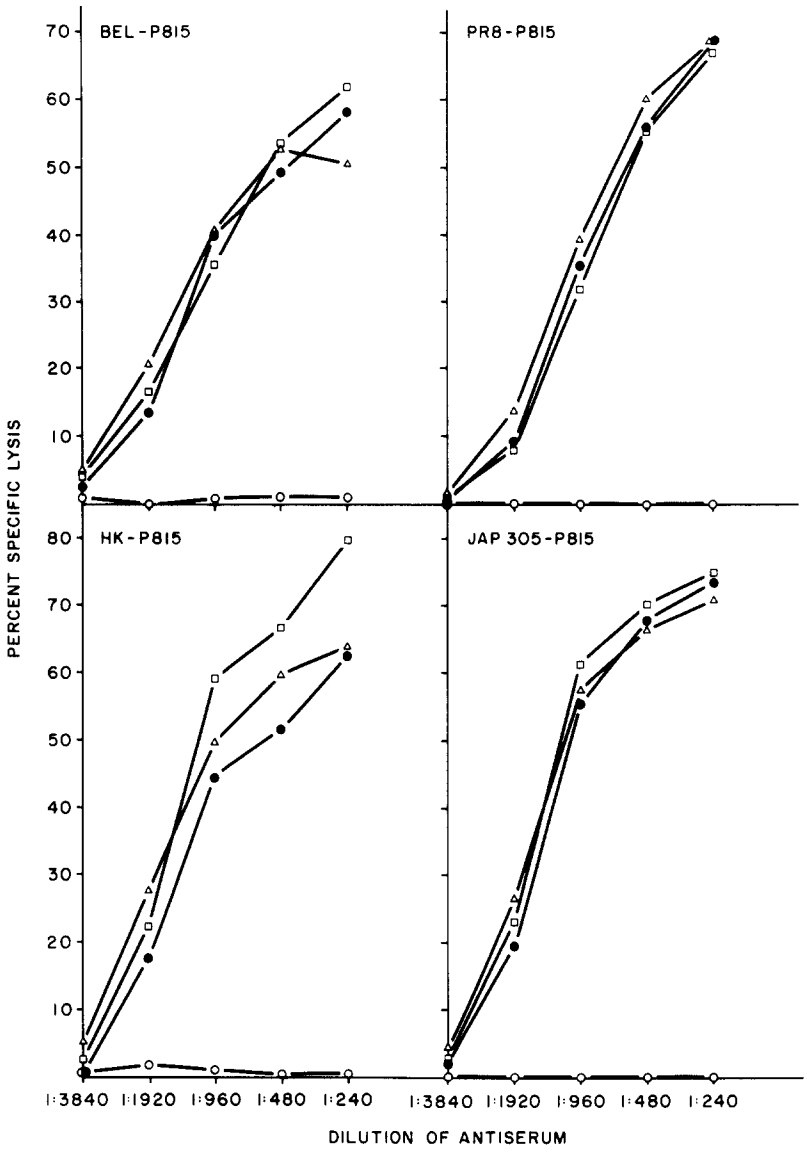

FIG. 2. Goat anti-M serum (1 ml of a 1:50 dilution) was absorbed for 3 h at room temperature with 30×10^6 normal P815 cells (△) or P815 cells which had been infected for 7 h with HK virus (○) or B/Lee (□) virus. These absorbed sera were then compared with the unabsorbed serum (●) for their reactivity against P815 target cells infected with either Bel, PR8, HK, or JAP 305 type A viruses.

mastocytoma cells (R. G. Webster, unpublished results), with the production of influenza virus particles that hemagglutinate but have low levels of infectivity. We do not yet know whether M protein is expressed on the surface of cells infected with influenza virus during permissive replication and future studies will establish this point. However, since influenza A virus-infected P815 cells are

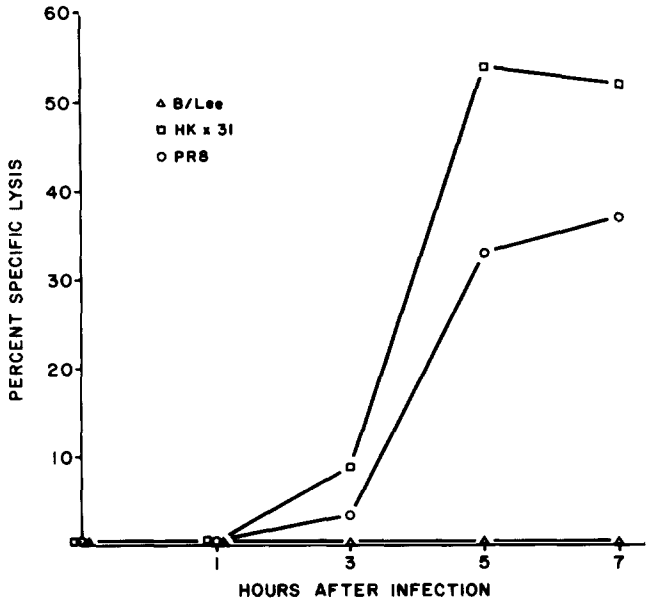

FIG. 3. P815 cells were assayed for reactivity with goat anti-M serum (1:320) plus complement at various times after infection with HK (□), PR8 (○), or B/Lee (△) influenza viruses.

susceptible targets for the murine cross-reactive T-cell populations generated by in vivo immunization (2), the in vitro findings of serological and cell-mediated cross-reactivity are important in this system and point the way for future studies in man.

Mice are able to generate an antibody response to the M protein when immunized with the antigen in Freund's complete adjuvant (9). Therefore, to explain the cross-reactivity between influenza A virus-infected cells, it is not necessary to consider that the cross-reactive T cells recognize determinants on the cell membrane that are unable to stimulate B cells, or that immunoglobulin molecules and the T-cell receptors have grossly different affinity characteristics. Furthermore, mice primed with M protein in adjuvant develop a delayed-type hypersensitivity response when challenged with M protein (9), which presumably reflects reactivity of at least one class of T cells.

The cytotoxic T-cell response that is generated during influenza infection is, as with other systems of this type, restricted to H-2 compatible interactions (1, 2). The implication that the M protein, rather than the H or N antigens, may be preferentially recognized by virus-immune cytotoxic T cells raises some interesting possibilities. The influenza H and N antigens exist as independent structures on the cell and virion membranes. Both stimulate antibody production and require the involvement of helper T cells (14), which presumably operate via the *I* region of the *H-2* gene complex (15, 16). The M protein does not normally induce an antibody response during primary infection (17). Is the M protein representative of a class of molecules which mainly stimulates cytotoxic rather than helper T-cell function? Does this reflect preferential association with *H-2K* or *H-2D* (rather than with *H-2I*) gene products (18)?

Summary

Antisera to the type-specific internal influenza virus matrix (M) protein of a type A influenza virus were produced in goats. In the presence of complement, anti-M serum was cytotoxic for target cells which were infected with a variety of serologically distinct type A influenza viruses, but did not react with type B influenza virus-infected cells. Absorption experiments indicated that anti-M serum detected a common antigen(s) on the surface of type A-infected cells. This serological cross-reactivity parallels the cross-reactivity observed for the cytotoxic T-cell response to type A viruses.

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