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Immunoglobulin M and G Antibody Response to Type- and Subtype-Specific Antigens After Primary and Secondary Exposures of Mice to Influenza A Viruses

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A mouse model of influenza infection was studied to help define parameters that may affect serodiagnosis of human infections by immunoassays. Antibodies to both type- and subtype-specific influenza A antigens were measured by a solid-phase immunofluorometric assay. Dilute mouse sera were added to purified influenza virus that had been covalently bound to polyaminostyrene microbeads, and the bound antibody was detected by fluorescein isothiocyanate-labeled isotype-specific antisera. Results were consistent in that upon exposure of mice by either infection alone or by vaccination after infection, both immunoglobulin M (IgM) and IgG antibodies reactive with newly encountered subtype specific viral antigens were measured. IgG antibody was usually detectable by the solid-phase immunofluorometric assay several days before it could be detected by a hemagglutination inhibition test. Increased levels of antibody of the IgG1, IgG2a, IgG2b, and IgG3 subclasses were also measured during influenza infection. Surprisingly, response to type-specific viral antigens was of the IgG class in primary as well as in secondary exposure. The results suggest that for serodiagnosis of influenza infections by detection of specific IgM antibody, the assay should use subtype-specific antigens.

Improvements in the serological diagnosis of viral infections may be made possible by the introduction of immunoassay procedures to replace biological assays such as complement fixation and hemagglutination inhibition (HI). An advantage of immunoassays is the ability to use reagents specific for different classes or subclasses of antibodies, potentially providing an early diagnosis by detection of immunoglobulin M (IgM) class antibody in convalescent-phase sera.

This may be complicated for influenza, however, because individuals are likely to experience numerous infections during the course of their lives with viruses having common type-specific antigens but diverse subtype-specific hemagglutinin (HA) and neuraminidase (NA) antigens. Thus, it might be hypothesized that (i) an IgM response always would be elicited to both the type- and subtype-specific antigens of influenza virus whenever first experienced; and (ii) during subsequent infections or vaccinations later in life, the serum antibody response to the type-specific antigens would be restricted to IgG, whereas new subtype-specific antigens might elicit a mixture of primary (IgM) and secondary (IgG) responses, depending upon the degree of antigenic drift or shift that occurred between the new virus and the previous strains.

To our knowledge these hypotheses have not previously been tested directly in either humans or an experimental animal model. Therefore, to provide information that may be helpful for the development of improved serological tests for human infection we have examined the class and subclass of antibody responses with a mouse model system for type- and subtype-specific antigens of influenza virus by using the immunofluorometric assay (IFMA). The IFMA uses as a solid phase whole viruses that have been covalently bound to plastic microbeads. This solid-phase antigen binds antibodies, which are detected with fluorescein isothiocyanate (FITC)-conjugated isotype-specific reagents and quantified by means of a filter fluorometer. An IFMA system has previously been used to evaluate the specificity of commercially available reagents directed toward human or mouse immunoglobulins of different isotypes (15; N. G. Gonchoroff, T. W. Wells, D. J. Phillips, C. M. Black, A. P. Kendal, and C. B. Reimer, submitted for publication). Thus FITC-conjugated anti-

mouse IgM and IgG reagents were available for this study which were of previously determined specificity.

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MATERIALS AND METHODS

Influenza viruses. The type A influenza strains used in this study, A/PR/8/34 (H1N1), A/USSR/90/77 (H1N1), and A/Ann Arbor/6/60 (H2N2), contain related type-specific nucleoprotein (NP) and matrix (M) protein antigens. The H1N1 strains contain distinguishable but related subtype-specific HA and NA antigens, but these antigens are not known to cross-react with the H2N2 antigens of A/Ann Arbor/6/60.

The viruses used to infect mice, or as antigens in the IFMA, were grown in 10- to 12-day-old hen eggs by standard procedures (5). The harvested allantoic fluid used for solid-phase antigen was clarified by low-speed centrifugation and was stored with 0.1% (wt/vol) NaN_3 at 4°C. The virus used for infecting mice or cell cultures was stored at −70°C without preservatives.

Virus used for immunization of mice was grown in roller bottles of MDCK cells which, after infection, were incubated for 36 h in serum-free Eagle minimal essential medium containing gentamicin and 1 μg of tolylsulfonyl phenylalanyl chloromethyl ketone trypsin per ml. Cell culture fluid was clarified by low-speed centrifugation and was stored at 4°C with thimerosal (0.01%, wt/vol) until concentrated.

Concentration of virus. Influenza virus used for preparation of the solid-phase antigen was purified by differential and density gradient centrifugation, similarly to a previously described procedure (8).

Virus for immunizing mice was prepared by pelleting the virus grown in MDCK cell culture for 60 min at about 100,000 × *g*. This concentrate was adjusted to contain 6 mg of total protein per ml. A dose-response curve was constructed for irradiation of MDCK cell culture-grown virus with ^{60}Co. It was found that 1.40×10^6 rads resulted in complete inactivation of infectivity, but with minimal destruction of the virus as measured by a hemagglutination assay.

Solid-phase antigen. Solid-phase antigen was prepared by covalently binding purified influenza viruses to polyaminostyrene beads (17) by diazotization (15) at a concentration of 16 to 23 mg of virus protein per 25×10^{10} beads which saturated the beads. One lot of A/Ann Arbor/6/60 solid-phase antigen was treated with Triton N-101 and used to detect type-specific antibodies as previously described (14).

Infection and vaccination of mice. Large groups of 5- to 6-week-old, male ICR-CDC mice (received from the Veterinary Services Branch at the Centers for Disease Control) were lightly etherized and infected intranasally with 10^2 50% median infective doses (equivalent to 10^{-1} 50% median lethal dose) of egg-grown A/PR/8/34 virus. Subsets of the A/PR/8/34-infected mice were injected intraperitoneally with 0.8 mg of concentrated MDCK cell-grown virus on the day 29 after infection.

Mouse sera were collected and stored at −20°C until used for serological assays. Sera from groups of five animals were pooled in equal volumes for each data point.

IFMA. The IFMA was performed by previously published methods (8a, 14). Mouse serum dilutions were prepared in bulk volumes by using phosphate-buffered saline containing 0.25% trypsin-KIO_4-treated normal goat serum.

The IFMA titer of a serum was defined as the reciprocal of the dilution (interpolated graphically) that yielded a relative fluorescent intensity value 2 times that obtained in a test with correspondingly diluted serum from an uninfected animal. This (2× background) cutoff value was approximately 10 standard deviations above the mean relative fluorescent intensity from uninfected mouse sera ($P < 0.01$). In some cases a single serum dilution was used to conserve reagents, and fluorescent intensity values were expressed as the number of standard deviations above background obtained with normal mouse serum.

Anti-mouse Ig conjugates. Evaluation of the potency, specificity, and labeling of the FITC conjugates used in this study will be described in detail separately (Gonchoroff et al., in preparation). Goat or rabbit anti-mouse sera were purchased from U.S. Biochemicals, Cleveland, Ohio (goat anti-mouse IgG Fc specific); or Litton Bionetics, Kensington, Md. (goat anti-mouse IgG1, IgG2a, and IgG3 and also rabbit anti-mouse IgG2b). IgG fractions were isolated and labeled with FITC. FITC-conjugated goat anti-mouse IgM was purchased from Research Plus Laboratories, Denville, N.J. All conjugates were tested against a panel of 14 individual solid-phase mouse myeloma antigens representing all four subclasses of mouse IgG, IgM, IgA, and kappa and lambda Bence-Jones proteins by using the method of Phillips et al. (15). The IgG (Fc-specific) conjugate was thus shown to have less than 2.0% cross-reaction with either mu or alpha chains compared with the reaction with gamma chain. The IgM-specific conjugate had less than 5.0% cross-reactivity with alpha heavy chains. The conjugate had 0.09% cross-reaction with one IgG2b K myeloma, but 10% unwanted cross-reaction to a different IgG2b K myeloma (MOPC145). The IgM conjugate, when used at the working dilution, had a relative fluorescent intensity value of 0.420 when reacted with homologous IgM solid-phase antigen beads containing a standard amount of myeloma protein, whereas the IgG (Fc-specific) conjugate had an average relative fluorescent intensity value of 0.540 relative fluorescent intensity units against IgG solid-phase antigens. The IgG subclass specific conjugates used had less than 5.0% cross-reactivity with heterologous IgG, IgM, or IgA solid-phase antigens. Molar fluorescein/protein ratios were between 3.0 and 4.0 for these conjugates.

The conjugates were diluted to their working titers with 0.25% trypsin–KIO_4-inactivated normal serum of the same species as that from which the conjugate was derived.

HI tests. HI tests were performed according to established procedures (5) with mouse serum treated with trypsin–KIO_4 to inactivate nonspecific inhibitors. Antigens were Centers for Disease Control reference antigens (A/PR/8/34 and A/USSR/90/77) or virus of the same passage level used as a vaccine (A/Ann Arbor/6/60).

Monoclonal antibodies. Ascites fluids containing monoclonal antibodies produced by hybridomas se-

creting antibody specific for influenza type A (M and NP) were provided by R. G. Webster, St. Jude Children's Hospital for Research, Memphis, Tenn.

RESULTS

Specificity of antibodies detected by solid-phase influenza antigens by IFMA. Four solid-phase antigens were prepared, and their specificities were evaluated in an indirect IFMA using NP- and M-specific hybridoma antibodies to determine whether type-specific antigens were exposed (Table 1). As expected, the A/PR/8/34 and untreated A/Ann Arbor/6/60 solid-phase antigens reacted poorly with both NP- and M-specific antibodies in the IFMA. Triton N-101 treatment of the solid-phase antigen significantly ($P < 0.01$) increased the reaction of the A/Ann Arbor/6/60 antigen with both antibodies. The A/USSR/90/77 solid-phase antigen reacted quite well with the NP- and M-specific antibodies. The reason for availability of exposed type-specific antigens in the A/USSR/90/77 solid-phase preparation is unknown. Electron microscopy, however, demonstrated the presence of spontaneously disrupted virus particles with free nucleocapsids in the virus remaining after reaction with polyaminostyrene beads (unpublished data), which has not been seen with other strains.

Primary influenza response as measured by the IFMA. Pools of sera from infected animals and controls were tested in the HI and IFMA by using three antigens (Table 2). IgG antibody to A/PR/8/34 virus was observed on day 7 by the IFMA. Significant rises in HI titers and moderate elevation of IFMA-IgM antibody were seen on day 10. Low levels of antibody were also detected with A/USSR/90/77 antigen and may have represented a combination of antibody binding to cross-reactive (shared) determinants on the H1N1 surface glycoproteins, and to exposed type-specific antigen. Antibody to the type-specific antigens measured by the IFMA with Triton N-101–treated A/Ann Arbor/6/60 virus reacted as IgG class and was first detectable on day 10.

TABLE 1. IFMA results of influenza solid-phase antigen with anti-M and anti-NP hybridoma antibodies[a]

Solid-phase antigen	Anti-NP	Anti-M
A/PR/8/34	800	430
A/USSR/90/77	25,600	6,400
A/Ann Arbor/6/60	1,300	1,600
A/Ann Arbor/6/60 (Triton N-101 treated)	34,100	25,000

[a] IFMA reciprocal dilution at an endpoint twice background.

The presence of IgM immune antibody was verified by sucrose density gradient zonal centrifugation of serum collected from mice 11 days after infection (Fig. 1). IgM antibody to influenza virus, measured by the IFMA, occurred primarily in the fast-moving 19S zone, whereas IgG activity occurred in the slower-migrating zone. Binding with anti-IgG (Fc-specific) conjugate occurred only in the slower-migrating peak, validating the class specificity of this reagent.

The IgG subclass antibody response of this same set of mice is shown in Table 3. Titers were not determined because of limited amount of subclass-specific conjugates and mouse sera. By day 10, antibodies of all isotypes of IgG were detected. The highest levels (in terms of fluorescent intensity measurements) were seen for mouse IgG2a and IgG2b.

Antibody class and specificity after secondary stimulation. A group of mice were infected intranasally with egg-grown A/PR/8/34 virus. Then they were inoculated intraperitoneally with MDCK-cell culture grown antigen, thereby minimizing any possible response to host-specific antigens on the viruses.

The mice that were primed and boosted with the same (A/PR/8/34) antigen responded with increased HI titers to A/PR/8/34 (Table 4). This effect was seen by the IFMA to result from increased levels of IgG antibody measured with homologous A/PR/8/34 solid-phase antigen. No significant change in IgM antibody was detectable from that produced by the primary infection and present at the time of boosting.

Significant rises in IgG antibody were also seen with the Triton N-101–treated A/Ann Arbor/6/60 (H2N2) antigen which measures antibody to the type-specific proteins as well as to A/USSR/90/77 solid-phase antigen which detects type- and subtype-specific responses. Lack of production of antibody detectable by HI with A/USSR/90/77 suggests that most of the antibody detected in IFMA with this antigen was reactive with type-specific viral components.

When A/USSR/90/77 MDCK cell-grown antigen was injected into A/PR/8/34-primed mice, a classical example of "original antigenic sin" was observed. HI titers indicated that the antibody detected by that assay was largely directed to the virus used for the primary infection (A/PR/8/34), with a lower level response to the specific determinants on the A/USSR/90/77 antigen used for the secondary stimulation. The IFMA showed the antibody reactive with A/PR/8/34 to be primarily of the IgG class. Significant titer rises in both IgM- and IgG-class antibodies to A/USSR/90/77 solid-phase antigen and in IgG antibodies to Triton N-101–treated A/Ann Arbor/6/60 solid-phase antigen were measured. These observations are consistent with the production of

TABLE 2. Antibody response to primary A/PR/8/34 (H1N1) infection in mice, measured with a solid-phase IFMA and by HI

Serum or day postinfection[a]	Antibody response								
	A/PR/8/34 (H1N1)			A/USSR/90/77 (H1N1)			A/Ann Arbor/6/60[b] (H2N2)		
	HI	IgM[c]	IgG[d]	HI	IgM	IgG	HI	IgM	IgG
Prebled serum	*[e]	*	*	*	*	*	ND[f]	*	*
2	*	*	*	*	*	*	ND	*	*
4	*	*	*	*	*	*	ND	*	*
5	*	*	*	*	*	*	ND	*	*
6	*	*	*	*	*	*	ND	*	*
7	*	*	12[g]	*	*	*	ND	*	*
8	*	*	18	*	*	*	ND	*	*
9	*	*	55	*	*	*	ND	*	*
10	40	16	190	*	*	12	ND	*	60
11	80	50	225	*	*	14	ND	*	70
12	80	17	380	*	*	17	ND	*	60
15	80	20	480	*	*	110	ND	*	170
18	160	27	600	*	*	190	ND	*	290
21	160	14	400	*	*	70	ND	*	180
24	320	*	450	20	*	140	ND	*	240

[a] Sera from five animals were pooled in equal volumes for each data point.
[b] Solid-phase A/Ann Arbor/6/60 antigen treated with Triton to expose type-specific antigens.
[c] IFMA response measured with an IgM heavy chain-specific conjugate.
[d] IFMA response measured with an IgG heavy chain-specific conjugate.
[e] *, Endpoint ≤1:10.
[f] ND, Not done.
[g] Reciprocal dilution IFMA titers at 10 standard deviations above mean background (2× background).

IgM and IgG antibodies reactive with strain-specific antigenic determinants on the A/USSR/90/77 HA and NA antigens as well as with production of IgG antibodies reactive with type-specific M and NP antigens.

The A/PR/8/34-primed mice, when boosted with A/Ann Arbor/6/60, exhibited an early (4-day) HI response associated with IFMA IgM and IgG antibody response, to the A/Ann Arbor/6/60 antigen. A large rise in IFMA IgG antibody

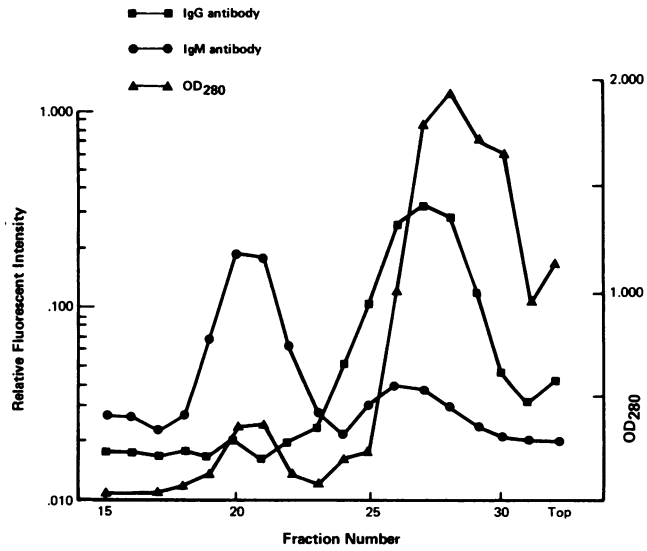

FIG. 1. Sucrose rate zonal centrifugation separation of IgM and IgG antibodies to A/PR/8/34. Pooled serum (400 μl) collected from mice 11 days after infection with A/PR/8/34 was layered onto a 10 to 38% (wt/vol) sucrose (in phosphate-buffered saline with 0.1% NaN_3) gradient. The sample was centrifuged at 110,000 × g in a T-27 rotor (Beckman Instruments, Palo Alto, Calif.) for 24 h. Fractions (1 ml) were collected and assayed by IFMA by using A/PR/8/34 solid-phase antigen.

TABLE 3. IgG subclass response in mice infected with A/PR/8/34 virus, measured with IgG subclass-specific conjugates

Day post-infection	IFMA response			
	IgG1	IgG2a	IgG2b	IgG3
2	0.00	−1.00	−1.11	−2.66
4	0.00	1.00	−1.11	−4.66
5	1.70	−0.50	−1.11	−2.66
6	1.70	1.50	1.11	−2.66
7	3.70	3.00	3.33	−0.67
8	1.70	4.00	5.55	−0.67
9	2.70	6.50	14.44	−0.67
10	7.70	28.00	34.44	4.66
11	14.70	41.00	57.77	14.00
12	14.70	17.00	58.88	8.67
15	25.00	67.00	135.55	26.00
18	46.70	88.00	191.11	44.00
21	24.70	74.50	115.55	12.00
24	45.70	86.00	156.66	21.33

[a] Values given are the numbers of standard deviations for the fluorescence intensity with serum samples from infected animals above the mean fluorescence intensity obtained with sera from a group of uninfected animals. Sera were tested at a 1:30 dilution. Data are from duplicate determinations run on the same day.

was also seen when measured with A/USSR/90/77 solid-phase antigen that has been shown above to detect type-specific antibodies. In addition a slight rise in IgM and IgG antibody to A/PR/8/34 solid-phase antigen was observed in these mice that had been infected with A/PR/8/34 and boosted with A/Ann Arbor/6/60. The antigenic specificity of these antibodies is not clear.

DISCUSSION

To measure antibodies reacting with either the surface glycoproteins, HA and NA, or the type-specific NP and M antigens of influenza A virus, we used different preparations of solid-phase viruses. Based on previous observations with monoclonal antibodies, solid-phase antigens prepared with purified whole virions were expected to react predominantly through their surface glycoproteins. For two of the solid-phase antigens, A/PR/8/34 and (untreated) A/Ann Arbor/6/60, this was substantiated by demonstrating that the antigens exhibited very low binding of monoclonal antibodies specific for NP or M. Triton N-101 treatment of the A/Ann Arbor/6/60 antigen increased the reactivity with NP- and M-specific monoclonal antibodies, consistent with a previous study (14). A third antigen, A/USSR/90/77, for unknown reasons (but probably due to the unusual lability of purified virions) had substantial reactivity with NP and M protein-specific monoclonal antibodies without Triton N-101 treatment. In most cases the combined use of the different solid-phase antigens enabled us to distinguish between the presence of subtype (i.e., HA or NA)-specific antibodies versus type (i.e., NP or M)-specific antibodies in sera of infected and immunized mice. Although it would be preferable to prepare solid-phase antigens with isolated, purified, subvirion components, such an approach greatly increases the expense and decreases the practicality of the assay for routine use and was not considered justified for the present evaluation of mouse antibody response.

Results were consistent in that, upon exposure of mice by either infection alone or infection followed by vaccination, both IgM and IgG antibody responses were elicited to the subtype-specific antigens of a virus strain when first encountered. The IgM response was not detected earlier than an IgG response in either infected or vaccinated animals. This might reflect either a relative difference in the sensitivity of the assays for quantitating IgM and IgG or, alternatively, might reflect the time sequence of synthesis of IgM and IgG antibodies under the circumstances of immunization that we used.

Antibody response in mice infected with A/PR/8/34 was detected by IFMA several days before detection of antibodies by HI, confirming that, in general, immunoassay procedures may have a higher level of sensitivity for low levels of antibody than do HI tests (1, 9, 13). Similarly, when A/PR/8/34-primed mice were vaccinated with A/USSR/90/77, antibody response to A/USSR/90/77 was also detected earlier by IFMA than by HI. Vaccination with A/Ann Arbor/6/60 (H2N2) rapidly produced detectable HI and IFMA antibodies against this virus, and no ad-

TABLE 4. Antibody response after secondary stimulation

Virus used as boosting antigen[a]	Day post-vaccination[b]	Antibody response to: A/PR/8/34 (H1N1)			A/USSR/90/77 (H1N1)			A/Ann Arbor/6/60 (H2N2)			A/Ann Arbor/6/60 (H2N2)[c]		
		HI	IgM[d]	IgG[e]	HI	IgM	IgG	HI	IgM	IgG	HI	IgM	IgG
A/PR/8/34 (H1N1)	1	320[f]	33	680	*[g]	*	135	ND[h]	ND	ND	ND	*	380
	4	1,280	42	4,000	*	11	700	ND	ND	ND	ND	12	1,200
	7	1,280	28	4,200	*	*	1,500	ND	ND	ND	ND	*	4,000
	10	1,280	38	8,000	*	*	1,700	ND	ND	ND	ND	*	2,500
	20	640	*	4,000	*	*	1,200	ND	ND	ND	ND	*	1,700
A/USSR/90/77 (H1N1)	1	40	12	400	*	*	60	ND	ND	ND	ND	*	185
	4	640	38	1,700	*	100	1,700	ND	ND	ND	ND	*	2,700
	7	1,280	32	3,200	*	56	3,333	ND	ND	ND	ND	*	4,800
	10	640	40	4,000	*	56	5,000	ND	ND	ND	ND	*	6,800
	20	640	20	3,333	20	*	4,350	ND	ND	ND	ND	*	3,000
A/Ann Arbor/6/60 (H2N2)	1	160	*	400	*	*	110	*	*	40	ND	ND	ND
	4	80	12	480	*	*	800	160	415	600	ND	ND	ND
	7	160	17	700	*	*	500	160	356	5,000	ND	ND	ND
	10	160	33	1,600	*	*	1,700	160	180	5,500	ND	ND	ND
	20	160	20	1,400	*	*	2,200	320	75	6,500	ND	ND	ND

[a] A group of 30 mice were inoculated intranasally with diluted allantoic fluid and then boosted intraperitoneally with uninfected MDCK cells. The protein content of this control was adjusted so that it was the same as contained in the virus vaccine; in no case could we detect an antibody response ≥10.

[b] Mice were inoculated intraperitoneally with antigen on day 29 after infection with A/PR/8/34. Sera from five animals were pooled in equal volumes for each data point.

[c] Solid-phase A/Ann Arbor/6/60 antigen treated with Triton N-101 to expose type-specific antigen.

[d] IFMA response measured with an IgM heavy chain-specific conjugate.

[e] IFMA response measured with an IgG heavy chain-specific conjugate.

[f] Reciprocal dilution.

[g] *, Endpoint ≤1:10.

[h] ND, Not done.

vantage in either test for early detection of antibodies was demonstrated. It is not clear why such a rapid antibody response to the A/Ann Arbor/6/60 antigen should occur in mice that have been primed with heterologous H1N1 virus, but the results are consistent with the view that heterotypic immunological interactions can be demonstrated between influenza virus strains containing HA and NA antigens classified as being of different subtype (6, 10, 11, 16, 18, 19).

One surprising observation was the failure to detect a primary IgM antibody response to the type-specific NP and M antigens as shown, for example, in immunoassays with Triton N-101–treated A/Ann Arbor/6/60 to measure antibody response to infection with A/PR/8/34. We cannot totally exclude the possibility that the lack of detection of IgM class NP- or M-specific antibody is an artifact due to the properties of the antigen on the Triton N-101–treated solid-phase virus, as might occur by blocking with excess HA- and NA-specific antibodies.

As predicted, antibody response upon secondary exposure to influenza A viruses comprised IgG antibody to type-specific NP and M antigens and IgM- and IgG-specific antibody to the subtype-specific HA and NA antigens. These results with the mouse model suggest that for maximum sensitivity in the early detection of influenza infections by measurement of IgM in convalescent-phase serum samples, the assays should be rendered specific for antibodies directed against the surface glycoproteins. Under these circumstances, provided that a significant degree of antigenic drift (or antigenic shift) has occurred between the HA of the currently infecting virus strain and previously circulating virus strains to which the host has been exposed, it may well be possible to detect an IgM antibody response to the most recently encountered virus strain. Such a result has been obtained by Murphy et al. (12), who applied an immunoassay with isolated HA subunits for detection of IgM-class antibody response to influenza infections in young adults who had almost certainly been previously infected with influenza A virus strains.

Traditional methods of measurement of IgM response after vaccination, i.e., sucrose density gradient centrifugation of serum samples and detection of antibody in the fractions by HI tests, have also been shown previously to detect IgM antibody in persons infected with H3N2 viruses several years after their appearance (2–4). Additional experience must now be gained with appropriately collected specimens from human cases later in the era of the H3N2 subtype and with influenza B viruses to validate the general diagnostic usefulness of IgM-specific assays with influenza. Application of the solid-phase IFMA procedure is more practical and efficient than the sucrose density gradient method for detection of IgM response in such cases. Non-specificity due to rheumatoid factors must be considered in both methods. Our results show that appropriate solid-phase antigens prepared with intact, whole viruses are adequate in the IFMA to measure IgM-specific response to the viral HA and NA antigens. This suggests a specific advantage for this IFMA assay over solid-phase enzyme immunoassays in which isolated HA and NA subunits are apparently needed to confer subtype specificity when antigen is adsorbed to microtiter plates (7). However, for maximum usefulness to measure antibody to subtype-specific determinants, solid-phase whole-virus antigen beads should be standardized for their lack of reactivity with type-specific antigens, for example, by testing them with monoclonal or specific polyclonal antibodies to M and NP as done in this study.

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