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Immunoglobulin G subclass antibody responses of mice to influenza virus antigens given in different forms

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Summary

Total IgG and IgG subclass antibody responses in mice were studied after infection with virulent and non-virulent influenza viruses, and after vaccination with inactivated whole virus or purified surface glycoproteins (HANA-flu). Infection induced high IgG2a, low IgG1 and IgG2b, and very low IgG3 levels of antibody in serum. Whole virus vaccine induced high IgG2a, moderate IgG2b, and very low IgG1 and IgG3 levels of antibody. In marked contrast, HANA-flu preparations induced high IgG1, low IgG2a, and very low IgG2b and IgG3 levels of antibody. Booster doses of whole virus and HANA-flu significantly elevated serum antibody levels, but the relative distribution of anti-influenzal antibody among the IgG subclasses was unchanged. Mice primed with HANA-flu prior to infection with mouse-adapted virus, produced high IgG2a, moderate IgG1, and low IgG2b and IgG3 levels of serum antibody. These data indicate that the physical form in which viral protein antigens are presented to the immune system can influence the subclass distribution of antibodies produced during primary immune responses and that once priming has occurred, responses to antigen presented in a different form are altered.

Influenza; Vaccine; IgG subclasses

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Introduction

Studies of human sera have shown that serum antibodies to viruses, such as hepatitis B virus (Morell et al., 1983) herpesviruses (Linde et al., 1983; Sundqvist et al., 1984) poliovirus (Beck, 1981) and rubella (Linde, 1985), are not equally distributed among the IgG subclasses. Also, it is known that the relative murine IgG subclass antibody response to some bacterial antigens can be altered by physical changes in the form of the immunizing antigen (Karch and Nixdoroff, 1983; Karch et al., 1983).

Immunoglobulin G antibodies in the mouse can be divided into 4 subclasses (G1, G2a, G2b and G3) based on their biological, immunological and physiochemical properties (Nisonoff et al., 1975). Only antibodies in the G2a and G2b subclasses have demonstrated the ability to activate the complement pathway (Spiegelberg, 1984). Murine macrophages and lymphocytes also differ in their capabilities to bind immunoglobulins of the various IgG subclasses (Diamond et al., 1981) and these differences have an effect on antibody dependent cellular cytotoxic mechanisms.

Antibody responses of mice to influenza virus have been well studied and IgG antibody has been shown to play an important role in the prevention of influenza virus infection (Couch et al., 1984; Ramphal et al., 1979; Virelizer et al., 1979). However, little data are available in regard to the IgG subclass specific antibody response to influenza virus. The influence of HANA-flu priming on secondary responses induced by infection was also evaluated.

Materials and Methods

Virus source, growth and purification

Influenza A/Hong Kong/68-like (H3N2) viruses (A/HK) were used throughout the study. The X-31 high yield recombinant virus (Kilbourne, 1969), containing the genes coding for hemagglutinin (HA) and neuraminidase (NA) proteins of A/HK virus and the remaining genes from influenza A/Puerto Rico/34 (H1N1) virus, was obtained from Dr. Edwin Kilbourne, Mount Sinai Hospital, New York, NY. Pools of mouse-adapted, lethal and nonlethal A/HK viruses were obtained from Dr. Philip Wyde, Baylor College of Medicine, Houston, TX (Wyde et al., 1978). Purified A/Bangkok/1/79 (H3N2) virus (X-73) and purified HA protein derived A/HK for use in antibody assays were obtained from Connaught Laboratories and Dr. Doris Bucher (Mt. Sinai, N.Y.) respectively.

The X-31 virus was propagated in 10-to 11-day-old embryonated hen's eggs. After 48–72 h of incubation at 35°C, virus pools were concentrated from allantoic fluids and purified by selective low and high speed centrifugation (Balkovic and Six, 1986). The final virus pellets were resuspended in phosphate buffered saline (PBS) pH 7.2, at one-hundredth the original volume. Purified virus pools were stored at 4°C in PBS with 0.01% sodium azide. SDS-polyacrylamide gel electrophoresis of the X-31 virus preparations revealed the expected viral protein bands.

Immunogen Preparation

Purified X-31 virus was inactivated by addition of formalin to a final concentration of 1:4000 and incubation for 48 h at 4°C. Excess formalin was removed by dialysis against PBS before inoculation into mice.

HANA-flu surface proteins were purified after disruption of the virus (3–5 mg protein/ml) by addition of octylglucoside to a final concentration of 2% w/v. The preparation was allowed to stand at room temperature for 30 min and insoluble materials were removed by centrifugation at 100,000 × *g* for 120 min at 4°C. The HANA-flu subunits were removed from the supernate by centrifugation through a 35% sucrose cushion at 104,000 × *g* for 24 h at 4°C. The pellets were resuspended in PBS with 0.1% sodium azide and they were stored at 4°C. SDS-polyacrylamide gel electrophoresis of the preparation revealed protein bands at the molecular weights expected for HA and NA and examination of negatively stained preparations in an electron microscope showed protein aggregates characteristic of HANA-flu preparations.

Mice

Female CBA/CaJ mice were obtained from Jackson Laboratories, Bar Harbor, ME and female C3H/HeN mice were obtained from Charles River Breeding Laboratories, Wilmington, MA. The mice were used at 6–8 wk old. The mice were maintained in plastic cages with barrier filters and they were allowed food and water ad libitum.

Sample Collection

Anesthetized mice were bled through the retro-orbital plexus. Blood specimens were allowed to clot and serum was separated by centrifugation at 2000 × *g* for 5 minutes at 4°C. Serum samples were removed and stored at −20°C until tested.

Antisera

Cappel Laboratories, West Chester, PA was the source of affinity-purified rabbit anti-goat IgG antibodies, IgG fractions of goat antisera to mouse IgG (heavy chain specific). Goat antisera against murine IgG1, IgG2a, and IgG2b were obtained from Meloy Laboratories, Springfield, VA. Goat antiserum against mouse IgG3 was obtained from Gateway Immunosera Co., St. Louis, MO.

Anti-Influenza Monoclonal Antibodies

Ascites fluids containing monoclonal antibodies specific for the HA of A/USSR/90/77 (H1N1) virus were provided by Dr. Robert Webster, St. Jude's Children Hospital, Memphis, TN (Kendal et al., 1981) and monoclonal antibodies specific for the HA of A/HK virus were provided by Dr. Walter Gerhard, Wistar Institute, Philadelphia, PA (Laver et al., 1979). Monoclonal antibodies specific for the nucleoprotein and matrix protein of type A influenza viruses were obtained from Dr. Maurice Harmon, Centers for Disease Control, Atlanta, GA (Walls et al., 1986).

Antibody Determinations

Total IgG and IgG subclass antibody responses in the serum were determined by a solid phase radioimmunoassay (SPRIA) described previously (Balkovic and Six, 1986). When A/HK and A/Bangkok/1/79 whole viruses were used as the test antigens, the wells were coated with a solution containing a hemagglutination titer of 32. Purified HA was added at a concentration of 20 ng per well. These quantities of coating antigen were determined to be optimal for detection of monoclonal antibodies specific for the HA. Serial dilutions of mouse serum or monoclonal antibodies were added to individual wells of the plate. After 3 h at 37°C, plates were washed and goat antibodies specific for murine IgG heavy chain or IgG subclasses were added. The goat anti-mouse IgG3 was used at a 1:3200 dilution; all other goat antisera were used at 1:10,000 dilution. After incubation for 3 h at 37°C the plates were washed and affinity-purified ^{125}-I labeled rabbit anti-goat antibodies (20,000 cpm) were used to quantitate immune complexes. Antibody titers were expressed as the dilution of serum that bound 2000 cpm of labeled antibody. This endpoint was determined by extrapolation from a plot of the cpm bound versus the sample dilution and it represented a point on the binding curves that was more than 20 standard deviations above the binding observed with 1:100 dilution of sera from unimmunized mice. Antibody titers for the H1 specific monoclones were determined by the same procedure except A/USSR/77 whole virus was used as the test antigen in these instances.

Results

Specificity of goat antisera to murine IgG subclasses

To assess their specificity goat antisera were tested against a panel of anti-HA specific IgG monoclonal antibodies of known isotype (Table 1). Slight crossreac-

TABLE 1

Specificity of goat antisera for detection of murine IgG subclass antibodies

Goat antisera to murine	Murine monoclonal antibodies[a]			
	IgG1	IgG2a	IgG2b	IgG3
IgG1	140,000	<2,000	10,000	3,300[b]
IgG2a	<2,000	33,000	8,000	<2,000
IgG2b	<2,000	<2,000	50,000	<2,000
IgG3	<2,000	<2,000	<2,000	66,000
IgG	150,000	136,000	95,000	80,000

[a] Monoclonal antibodies specific for the HA protein of influenza A virus were used. The IgG1, IgG2a and IgG2b antibodies were monoclones A-21, A-18 and A-20 (Laver et al., 1979). The IgG3 preparation was monoclone 110-1 (Kendal et al., 1981).

[b] The IgG3 preparation is known to contain small quantities of IgG1 antibody (Kendal et al., 1981) the low titer observed here probably does not represent crossreactivity of the goat anti-mouse IgG1 antiserum.

tivity was noted with the G1 and G2a antisera against a G2b monoclonal antibody, but the G2b and G3 subclass antisera were highly specific. Comparison of the antibody titers determined with subclass specific antisera to those determined with an antiserum reactive to all subclasses indicated that the sensitivities for detection of G1 and G3 antibodies were comparable, but that the G2a and G2b antibody levels were underestimated by 76 and 47%, respectively. The subclass antibody titers presented below have been adjusted to compensate for the differences in sensitivity and specificity of the subclass antisera. Specifically, 20 and 16% of the G2b antibody titer were subtracted from the G1 and G2a antibody titers, respectively. The G2a and G2b antibody titers were multiplied by factors of 4.1 and 1.9, respectively, to compensate for the low sensitivity of the goat anti-sera. Because antibodies of the G2b subclass were a minor component of the total IgG response following all 3 modes of immunization, the only significant adjustments were to correct for the low sensitivity of the G2a and G2b reactions.

Serum IgG subclass antibody responses

The IgG subclass antibody responses of groups of CBA/CaJ mice that received influenza virus antigens in different forms are presented in Table 2. Infection with nonlethal A/HK virus induced an antibody response in all 4 IgG subclasses, but the major portion of these antibodies were in the G2a subclass. Immunization with inactivated whole virus also induced antibodies in all 4 IgG subclasses and antibodies of the G2a subclass were selectively produced. Moderate levels of G2b were also observed. Significant rises in antibody were detected in all 4 subclasses after the second dose of whole virus vaccine and the relative distribution for anti-virus antibodies among the 4 subclasses was comparable to the primary response. In contrast, mice immunized with purified HANA-flu proteins produced high levels of G1 antibody and low levels of G2a antibody. None of the mice demonstrated a detectable G3 antibody response. Antibody rises were seen in all animals in 3 of the 4 IgG subclasses after a second dose of the HANA-flu preparation but only 2 of 5 mice developed a G3 antibody response. Again anti-influenza antibodies were found predominantly in the G1 subclass. A similar experiment was performed in C3H/HeN mice and the distribution of IgG subclass antibodies following these different modes of immunization were comparable to those observed in CBA/CaJ mice (data not shown).

To determine if priming of the mice with HANA-flu proteins would alter the distribution of IgG subclass antibody during a subsequent virus infection, mice were immunized with HANA-flu and 8 wk later they were challenged with 1 MLD_{50} of lethal A/HK virus (Table 3). Immediately prior to virus challenge all 7 animals tested possessed moderate levels of G1 antibody and low levels of G2a. Two of 7 had detectable levels of G2b antibody and G3 antibody was not detected in any of the sera. Six of 16 animals survived the challenge with lethal virus and all 6 animals developed an antibody response in all 4 IgG subclasses. Relatively high levels of both G1 and G2a antibodies were present and low levels of G2b and G3 were observed. In contrast, unprimed animals produced G2a antibodies predominantly. While an antibody response in the other 3 subclasses was evident in all the mice

TABLE 2

Subclass and total IgG serum antibody responses of mice to influenza virus antigens given in different forms[a]

Immunogenic form	No. of doses	Geometric mean antibody titer ($\times 10^{-3}$) ± SEM in the indicated immunoglobulin subclass and class				
		IgG1	IgG2a	IgG2b	IgG3	Total IgG
Infection	1	15.0 ± 5.5	113.2 ± 16.5	16.4 ± 2.8	0.7 ± 0.2	107.4 ± 16.4
Inactivated virus[c]	1	1.0 ± 0.5	38.7 ± 8.6	10.0 ± 2.1	0.4 ± 0.1	43.2 ± 8.3
	2	12.9 ± 6.3	591.4 ± 55.7	90.2 ± 42.7	5.9 ± 1.9	495.8 ± 10.7
HANA-flu[c]	1	9.1 ± 1.3	1.8 ± 0.2	0.9 ± 0.2	0.4 ± 0.1	8.0 ± 0.8
	2	289.8 ± 38.8	101.1 ± 32.9	28.4 ± 4.6	1.8 ± 0.3	266.7 ± 44.7

[a] Groups of 5 female CBA/CaJ mice were used.

[b] Mice received 500 median infectious doses of nonlethal mouse-adapted A/HK virus intranasally.

[c] Mice were given 5 μg of viral protein intramuscularly. Booster dose of 5 μg of viral protein was given intramuscularly 4 wk after initial immunizing dose.

TABLE 3

Subclass and total IgG serum antibody responses of unprimed and HANA-Flu primed mice following challenge with influenza virus

Groups of mice	No. of specimens	Geometric mean antibody titers ($\times 10^{-3}$) ± SEM in the indicated immunoglobulin subclass and class				
		IgG1	IgG2a	IgG2b	IgG3	Total IgG
HANA-flu[a] before challenge	7	1.03 ± 0.3	0.2 ± 0.03	0.1 ± 0.02	0.0 ± 0.0	1.4 ± 0.15
HANA-flu[b] following challenge	6	17.6 ± 3.8	55.5 ± 16.0	4.9 ± 1.7	0.7 ± 0.4	80.8 ± 17.0
Controls[c] following challenge	6	1.6 ± 0.4	34.2 ± 11	2.6 ± 0.7	0.3 ± 0.07	24.8 ± 4.9

[a] Sixteen female C3H/HeN mice were immunized with 5 μg of HANA-flu protein antigen, 8 wk later serum samples were collected from 7 mice to assess their prechallenge antibody status.

[b] Sixteen immunized mice were challenged with 1 median lethal dose of influenza A/HK virus; 4 wk later serum specimens were collected from the 6 surviving animals.

[c] Twelve unimmunized mice were also challenged and serum specimens were collected 4 wk later from the 6 surviving animals.

TABLE 4

Serum immunoglobulin G antibody responses to A/Hong Kong/68 and A/Bangkok/1/79 H3N2 influenza viruses following immunization with A/Hong Kong/68 virus antigen given in different forms[a]

Immunogenic form	No. of specimens	Geometric mean antibody titers ($\times 10^{-3}$) ± SEM to the indicated antigen	
		A/Hong Kong/68	A/Bangkok/1/79
Infection[b]	5	94.7 ± 17.1	7.4 ± 2.1
Inactivated virus[c]	5	22.2 ± 4.5	0.6 ± 0.2
HANA-flu[c]	5	2.3 ± 0.9	0.0[d]

[a] Groups of 5 female C3H/HeN mice were immunized and 4 wk later serum samples were collected for assessment of antibody status.

[b] Mice received 500 median infectious doses of nonlethal mouse-adapted A/HK virus intranasally.

[c] Mice were given 5 μg of viral protein intramuscularly.

[d] Antibody was not detected in any specimen at the lowest dilution of serum tested, 1:100.

the antibody levels were of a low magnitude. The distribution of IgG subclass antibody following infection with nonlethal virus (Table 2) was comparable to that observed in unprimed survivors of lethal virus challenge (Table 3).

As shown in Table 4, antibodies elicited by all 3 forms of immunization were highly specific for the A/Hong Kong/68 virus. When A/Bangkok/1/79 virus was used as the test antigen, the antibody titers were more than ten-fold lower following infection and immunization with inactivated virus. Serum specimens from animals receiving the HANA-flu preparation failed to react with the A/Bangkok/1/79 antigens. Since the 2 viruses used as test antigens contained the same internal proteins (matrix and nucleoprotein), antibodies to these antigens constituted a small portion of the immune response measured under these assay conditions. Titrations with monoclonal antibodies specific for the nucleoprotein and matrix protein (monoclones A1 and A2, Walls et al., 1986), established that insufficient quantities of internal antigens were exposed in the microtiter wells to efficiently detect responses to them (data not presented). These data suggested that most of the antibodies detected by the SPRIA were directed to the surface glycoproteins. To confirm this result, 2 serum specimens from each immunization group were examined using the A/HK virus and purified HA protein derived from it; the antibody titers obtained with the 2 antigens were comparable for each specimen. Thus, these assays conditions detected preferentially antibodies directed to the HA protein.

Discussion

The manner in which influenza virus antigens are presented to the immune system of animals can influence the IgG subclass of the antibody response. In the present study, antibodies to the HA protein were induced with 3 different preparations and each induced a distinctive profile of isotypic serum antibodies. The distribution of antibody among the 4 IgG subclasses was not related to the mag-

nitude of the response because booster doses with the same antigen preparation did not alter the pattern. However, the antigenic form first encountered by the immune system was apparently important.

HANA-flu preparation induced antibodies predominantly of the G1 subclass and animals primed by this method produced approximately ten-fold higher levels of G1 upon exposure to infectious virus than did unprimed animals. Whether antibodies of different subclasses afford comparable levels of protection to influenza virus has not been systematically evaluated. Our failure to observe protection in HANA-flu immunized mice may reflect the low levels of antibody that were present at the time of virus challenge. Although the inability of G1 antibodies to activate complement and to participate in antibody dependent cellular cytotoxicity reactions may have importance when antibody levels are low. On the other hand, mice bearing an X-linked defect that reduced their capacity to produce G1 and G3 antibodies but had no effect on G2a and G2b antibody production were not more susceptible to infection than nondefective mice (Reale et al., 1985; Balkovic and Six, unpublished observation). The factors regulating the distribution of specific antibodies to the different heavy chain isotypes have not been well defined. It has been known for many years that synthesis within the immunoglobulin classes was under T-lymphocyte control and recent evidence suggests that this is also true for IgG subclasses. A T-cell derived lymphokine that enhances secretion of G1 immunoglobulin and suppresses secretion of G3 immunoglobulin has recently been defined (Isakson, 1986). Since antibody responses were observed infrequently in the G3 subclass following immunization with HANA-flu it is interesting to speculate that this lymphokine may have been selectively induced by immunization with this preparation. Whether other lymphokines with specificity for different IgG subclasses exist is not known, but it is clear that the subsets of T-lymphocytes stimulated by influenza virus infection are different from those stimulated by nonreplicating antigens (Leung and Ada, 1982; Mills et al., 1986; Morrison et al., 1986). Also, a recent study has shown that T-helper lymphocytes specific for matrix protein can provide the lymphokines necessary for a T-dependent antibody response to the HA (Scheile and Gerhard, 1986). Thus, T-helper lymphocytes with specificity for different virus proteins may be operative in development of anti-HA antibodies when the antigen is given in complex forms such as inactivated whole virus or infectious virus.

Little information is presently available on the subclass distribution of influenza antibodies in humans. A recent study of acute and convalescent sera from 10 patients naturally infected with H3N2 viruses suggested that 78% of the total antivirus antibody was of the G1 subclass (Julkunen et al., 1985). Low level responses in the G3 and G4 subclasses were also observed in some sera but none of the patients produced G2 antibodies. Whether these patients were experiencing primary or subsequent infections was not given, but the data closely resemble our findings in mice because antibodies of the G1 and G3 subclasses in humans are most active in complement fixation.

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Footnotes

Since submission of this manuscript, Coutelier et al. (J. Exp. Med. 165, 64–69, 1987) have shown that IgG2a antibodies are preferentially elicited by infections with a wide variety of viruses, but immunization with soluble proteins induced antibodies of the IgG1 subclass. Moreover, Snapper and Paul (Science 236, 944–947, 1987) have shown that addition of recombinant interferon γ to activated murine B cells stimulates the production of IgG2a immunoglobulin but suppresses production of IgG1, IgG2b and IgG3 immunoglobulins.

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