Immunoglobulin-Specific Radioimmunoprecipitation Assays for Quantitation of Nasal Secretory Antibodies to Hemagglutinin of Type A Influenza Viruses

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Radioimmunoprecipitation (RIP) assays were developed to selectively quantitate class-specific antibodies to purified hemagglutinins (HA) of type A influenza virus in nasal secretions. Rabbit anti-human secretory piece of immunoglobulin A (IgA) and rabbit anti-human IgG were used as second antibodies. A third antibody, goat anti-rabbit IgG, was incorporated into the system to separate immune complexes formed between iodinated HA, nasal wash test specimen, and second antibody. The utilization of this reagent avoided the need for large quantities of IgA and IgG antibody-negative carrier secretions. Nasal wash specimens obtained from 14 adults immunized with an inactivated type A influenza virus vaccine were evaluated by RIP and viral neutralization assays. Significant homologous postvaccination secretory IgA and IgG antibody levels were demonstrable in 13 (93%) of individuals by RIP, whereas only 5 (36%) exhibited rises by viral neutralization tests. Moreover, the geometric mean IgA and IgG antibody levels were at least 20- and 37-fold greater than the neutralizing antibody titer. The pattern of heterologous immunoglobulin-specific antibody responses tended to be similar to those observed with the homologous HA subunit.

Infection or vaccination with type A influenza virus can be accompanied by the appearance of 11S immunoglobulin A (IgA) and 7S IgG antibodies in nasal secretions to the viral surface antigens, the hemagglutinin (HA) and neuraminidase (1, 2, 5, 7, 8, 12, 24). Information on the relative significance of these antibodies in host defense against infection has been obtained almost exclusively by neutralization (Nt) and hemagglutination-inhibition tests. However, these procedures are not only relatively insensitive for the determination of antibody titers, but also lack the capacity to distinguish the class of immunoglobulin associated with the response.

In this report, radioimmunoprecipitation (RIP) assays capable of independently measuring secretory IgA and IgG antihemagglutinin antibodies (AHAb) to type A influenza virus will be described. The principal feature of the methodologies was the utilization of a third heterologous antibody for the precipitation of immune complexes. As a means of comparing the sensitivity of the RIP assays to viral Nt tests for determining nasal secretory antibody titers, the immune responses to parenteral immunization with an inactivated vaccine were studied in normal adults.

MATERIALS AND METHODS

HA subunits. HA subunits of the H3N2 viruses A/Port Chalmers/73 (A/PC/73) and A/Hong Kong/68 (A/HK/68) were purified as previously described (13). In brief, the glycoproteins were recovered from sodium dodecyl sulfate (SDS)-treated virions after electrophoresis on cellulose acetate strips.

Iodination. The method described by Greenwood et al. (9) with a minor modification was employed to obtain iodinated preparations of HA subunits with specific activities of 6.7 to 9.6 $\mu\text{Ci}/\mu\text{g}$ of protein (21). Reagents were added to a siliconized glass vial at 25°C in the following sequence: 30 μl of 0.2 M phosphate buffer (pH 7.2), 20 μg of HA subunit (20 μl), 1 mCi of Na ^{125}I (10 μl) (Amersham/Searle Corp., Arlington, Heights, Ill.), and 50 μg of chloramine-T in 0.05 M phosphate buffer, pH 7.2 (10 μl). After 30 s, the labeling reaction was terminated by the addition of 100 μg of sodium metabisulfite in 0.2 M phosphate buffer, pH 7.2, (10 μl) followed by 100 μg of potassium iodide in water (100 μl). The labeled HA subunits were separated from free iodine by Sephadex G-100 chromatography. Fractions containing iodinated subunits were supplemented with 25% fetal calf serum and 2 mM ethylenediaminetetraacetic acid and used either directly or stored at -70°C. Frozen preparations were rechromatographed on a Sepharose 6B column prior to use in RIP assays.

Polyacrylamide gel electrophoresis. Iodinated

A/PC/73 HA subunits (10,000 cpm) in 0.5% SDS and 0.5% 2-mercaptoethanol were heated at 100°C for 2 min. After the addition of 25% sucrose, 0.1% 2-mercaptoethanol, and 0.05% bromophenol blue, the preparation was subjected to electrophoresis in 8% polyacrylamide gels which contained 0.1% SDS at 8 mA per gel (25). The apparent molecular weights of the HA1 and HA2 polypeptides were determined by inclusion of markers of known molecular weights in companion gels, and after complete migration of the tracking dye, gels were frozen, sliced into 1-mm fractions, and assayed for radioactivity.

RIP assays. A series of preliminary experiments were performed to establish conditions which would optimize determinations of class-specific AHAb. The reaction system contained the following reagents: 100 μ l of RIP buffer (phosphate-buffered saline, pH 7.2, containing 1 mg of bovine serum albumin per ml, 0.1 mM ethylenediaminetetraacetic acid, 0.3% Triton X-100, and 10% fetal calf serum), 50 μ l of a nasal wash (NW) dilution (serial fourfold dilutions of concentrated NW specimens), 50 μ l of iodinated HA subunits diluted to 10,000 cpm/50 μ l, 25 μ l of either rabbit anti-human secretory piece (SP) of IgA or 25 μ l of rabbit anti-human IgG (Bio-Rad Laboratories, Richmond, Calif.), and 25 μ l of goat anti-rabbit IgG. The concentration of the second antibody, either rabbit anti-human SP or anti-human IgG, was in slight excess of that required to effect maximal combination with the respective immunoglobulin in the lowest NW dilution used in the test. The third antibody, goat anti-rabbit IgG in equivalence with the rabbit anti-human reagents, was used to precipitate the immune complexes from the unreacted 125 I-labeled HA subunits. The primary and secondary antigen and antibody reactions were each incubated for 2 h at 37°C, and the final reaction with goat anti-rabbit IgG was held for 18 h at 4°C. After addition of 1 ml of RIP buffer, the reaction mixtures were centrifuged for 20 min at 3,000 rpm (4°C), and radioactivity in the precipitate and supernatant was counted for 1 min.

Prior to use in RIP assays, the rabbit anti-human SP and goat anti-rabbit IgG reagents were absorbed with human serum immunoglobulins covalently attached to Sepharose 2B. This procedure eliminated any low-level cross-reactivity of these reagents with human serum immunoglobulin which could compromise the specificity of the RIP assays.

NW specimens. Paired specimens from a group of adult volunteers who participated in an antigenicity evaluation of an inactivated monovalent A/PC/73 subunit vaccine were used in this study (26). The individuals received either 700 or 1,400 chicken cell agglutination units by the intramuscular route. The initial NW specimens were collected at the time of vaccination, and the second specimens were obtained 28 days later. After collection, the materials were homogenized in glass tissue grinders, centrifuged to remove insoluble materials, and reduced 10-fold in volume by Sephadex as previously described (18). Human IgA and IgG (low level) radial immunodiffusion plates (Meloy Laboratories, Inc., Springfield, Va.) were used to determine immunoglobulin concentrations. For RIP antibody determinations, NW specimens were treated with receptor-destroying enzyme

by a previously described method (10). Other test procedures were performed with untreated secretions.

Absorption of NW with class-specific antisera. Rabbit anti-human SP or rabbit anti-human IgG was added to NW specimens in quantities proportional to that used in the RIP assays. After incubation for 2 h at 37°C, goat anti-rabbit IgG was added in equivalence to facilitate separation of immune complexes. The reaction mixture was held for 16 h at 4°C and subsequently centrifuged at 3,000 rpm for 30 min. The absorption procedure was repeated with the supernatant fluids. Absorbed and unabsorbed samples, the latter of which were comparably diluted in saline and carried through the incubations, were centrifuged on sucrose gradients by a modification of previously described methods (15, 17). β -Galactosidase and purified human IgG were used as sedimentation markers. The gradients were centrifuged at 36,000 rpm in an SW-41 rotor for 16 h at 5°C, and the resulting fractions were independently assayed for secretory IgA and IgG anti-A/PC/73 HA antibody.

Agar-gel double immunodiffusion. Tests were performed as previously described (16) by using 1.5% agarose plates (Meloy Laboratories).

Nt test. NW specimens heated for 30 min at 56°C were assayed for antibody activity by the hemadsorption-inhibition method (2). The assays were performed with serial fourfold dilutions of concentrated NW specimens. An antigenic hybrid possessing the HA of A/PC/73 and the neuraminidase of A/equine 1/Prague/56 (Heq1Neq1) was used as the test virus.

RESULTS

The homogeneity of the labeled HA subunits and distribution of incorporated isotope were examined by SDS-polyacrylamide gel electrophoresis. Figure 1 shows the pattern obtained with an iodinated A/PC/73 preparation. The peaks corresponding to the two nonidentical polypeptide chains of the HA subunit contained greater than 90% of the radioactivity. The remainder of the isotope was recovered in the region of high-molecular-weight material and probably represents incompletely reduced HA. Similar results were observed with iodinated A/HK/68 preparations.

FIG. 1. Analysis of iodination pattern of A/PC/73 HA preparation by SDS-polyacrylamide gel electrophoresis.

The specificity of the rabbit anti-human SP and rabbit anti-human IgG sera used to develop the immunoglobulin-specific RIP assays was determined by two types of evaluations. NW specimens before and after absorption with each reagent were fractionated into immunoglobulin species of varying sizes by ultracentrifugation on sucrose gradients and analyzed for SP-associated IgA and IgG antibody to labeled A/PC/73 subunits. The results obtained with one specimen are presented in Fig. 2. With the unabsorbed specimen, rabbit anti-human SP dis-

played the capacity to detect antibody over a broad range of molecular species (Fig. 2A), while reactivity of the IgG reagent was limited to fractions primarily in the 7S range (Fig. 2B). Absorption of each NW specimen with either anti-SP or anti-IgG resulted in the complete reduction of antibody activity in only the corresponding RIP assay. Additional confirmation of the specificity of these reagents was provided by agar-gel double immunodiffusion tests as shown in Fig. 3. Reaction of anti-SP with NW and serum specimens resulted in the formation of a

FIG. 2. Distribution of secretory IgA (A) and IgG (B) antibodies in unabsorbed (O) and specifically absorbed (Δ) NW specimens after ultracentrifugation on sucrose gradients.

FIG. 3. Reactivity of rabbit anti-human SP (A) and rabbit anti-human IgG (B) reagents with NW and serum (S) specimens by agar-gel double immunodiffusion assays.

precipitin line that was common only to secretions (Fig. 3A). Both types of specimens, NW and serum, exhibited a line of identity in the anti-IgG analysis (Fig. 3B). The qualitative difference in precipitin band sizes of the two types of specimens may be indicative of the known absence of an IgG isotype and varying concentrations of IgG subclasses in nasal secretions (18).

Figure 4 illustrates representative antibody titration curves obtained with a postvaccination specimen by the immunoglobulin-specific RIP assays. All antibody titrations were performed in duplicate, and the mean of the highest dilution which precipitated 20% of the iodinated HA preparation was selected as the titration end point. This level of precipitation was always within the linear portion of the curve. Since individual titers varied less than 38% in repeat tests performed over a several-month interval, a twofold rise in a postvaccination specimen was considered to be a significant response.

A comparison of RIP and Nt homologous postvaccination antibody responses of 14 individuals immunized with A/PC/73 vaccine is presented in Table 1. Also shown are secretory IgA and IgG antibodies to A/HK/68 HA obtained by the RIP assays; sufficient quantities of three of the specimens were not available for A/HK/68 antibody assays. Before immunization, all vaccine recipients lacked measurable antibodies (<20) to A/PC/73 by the immunoglobulin-specific assays, although three subjects, 2, 9, and 12, had low levels of antibody by the Nt test. The latter three individuals and two others, 6 and 11, possessed antibody to the heterologous HA (A/HK/68) variant by the RIP-SP test. In postvaccination homologous assays, secretory IgA and IgG antibodies to A/PC/73-

TABLE 1. Homologous and heterologous postvaccination nasal secretory antibody responses among individuals vaccinated with A/Port Chalmers/73 (H3N2) vaccine

Vaccine recipient	Antibody titers by indicated assay ^a				
	A/PC/73		A/HK/68		Nt
	RIP-SP	RIP-IgG	RIP-SP	RIP-IgG	
1	<20 ^b	<20	<20	<20	<4 ^b
2	38	70	45	80	<4
3	48	103	106	243	<4
4	56	48	20	46	<4
5	68	211	211	ND ^c	<4
6	71	152	184	130	<4
7	143	160	80	271	<4
8	194	250	ND	ND	<4
9	243	599	320	735	12
10	246	632	320	788	12
11	343	859	905	1810	24
12	405	881	1688	1940	32
13	425	519	243	970	16
14	520	664	ND	ND	24

^a Titers are expressed as the reciprocal of NW dilutions.

^b For statistical calculations, titers of <20 and <4 were classified as 10 and 2, respectively.

^c ND, Not determined.

FIG. 4. Antibody titrations of NW specimen by immunoglobulin-specific RIP assays.

HA were demonstrable in 13 (93%) of the vaccinated persons. Determinations by the Nt procedure showed that only five (36%) responded to vaccination; one individual with preexisting antibody did not exhibit a rise in titer. This relative frequency of antibody conversion was significantly lower than those observed by the RIP assays ($P < 0.02$). While the geometric mean titer by the RIP-SP assay (200) tended to be lower than the level by the RIP-IgG procedure (368), the difference was not significant ($P > 0.20$). In each instance, however, the level of response was greater than the mean neutralizing antibody titer of 10 ($P < 0.001$). Concurrences between the titer in NW specimens by RIP and Nt tests were estimated by r , the Spearman rank coefficient. The analyses showed that there was a positive correlation between RIP secretory and RIP-IgG titers ($r = 0.09077$, $P < 0.001$). The same was true in comparisons between RIP-secretory or RIP-IgG and Nt levels ($r = 0.8713$ and $r = 0.8859$, respectively; in each case, $P < 0.001$). Among the 12 postvaccination NW specimens examined for heterologous AHAB, significant elevations were evident in 8 and 10 instances by the RIP-SP and RIP-IgG assays, respectively. In five individuals in which preexisting secretory antibody was detected, three rises were observed. Although the data are not sufficiently complete for any comparative anal-

yses, the results were generally similar to those obtained with the A/PC/73 HA subunits.

The relative immunoglobulin concentrations in the NW specimens are presented in Table 2. IgA and IgG levels in individual secretions collected before vaccination closely corresponded to those in day 28 samples (rank correlation test: $r = 0.5587$, $P < 0.05$ for IgA, and $r = 0.6547$, $P < 0.01$ for IgG). In postvaccination specimens there was no significant relationship between RIP secretory antibody titers and IgA concentration (rank correlation test: $r = 0.1824$, $P > 0.20$). A similar analysis for IgG, however, revealed a correlation between antibody and immunoglobulin levels ($r = 0.6557$, $P < 0.01$).

DISCUSSION

Nasal secretions contain relatively high concentrations of IgA and low levels of IgG (19). Base-line IgA values in an individual tend to be constant, although levels vary from one person to another. In contrast to the IgA pattern, the quantity of nasal IgG in a given individual shows no constancy on a day-to-day basis. Because of the aforementioned considerations, RIP systems for the quantitation of nasal secretory class-specific antibodies would require large quantities of IgA and IgG AHAb-negative carrier secretions. These requirements were obviated by the incorporation of a third antibody, goat anti-rabbit IgG, in equivalence with the second antibodies, rabbit anti-human SP and rabbit anti-human IgG, in the RIP assays to separate immune complexes.

TABLE 2. *Immunoglobulin concentrations in nasal secretions*

Vaccine recipient	Concn of immunoglobulin (mg%)			
	IgA		IgG	
	Day 0	Day 28	Day 0	Day 28
1	21.6	14.3	4.1	3.0
2	11.0	12.5	4.8	5.8
3	15.7	11.8	5.5	5.5
4	10.5	11.4	4.8	7.0
5	13.4	11.2	10.0	6.5
6	15.7	15.7	3.8	4.4
7	11.0	10.5	<3.0 ^a	5.8
8	10.5	14.5	<3.0	<3.0
9	13.2	15.9	7.0	10.0
10	11.0	9.1	6.5	12.0
11	15.7	17.5	6.6	14.5
12	11.4	11.1	6.0	10.5
13	15.7	19.5	6.5	5.0
14	17.4	39.4	ND ^b	42.0

^a For statistical calculations, a value of <3.0 was classified as 1.5.

^b ND, Not determined.

The anti-A/PC/73 IgA antibodies measurable in secretions by the RIP-SP assay were heterogeneous with respect to molecular size. The two major species had sedimentation coefficients of 11S and 7S. Findings by others that secretory component attached to various polymers, as well as to monomeric IgA, can be present in secretions supports the conclusion that antibody was of secretory origin (3, 17, 23).

The reason(s) for the somewhat higher anti-IgG A/PC/73 and A/HK/68 HA antibody titers demonstrated by the RIP assay was not examined in this study. A previous study by others has shown that IgG selectively accumulates in secretions by both transudation from serum and local synthesis (4). In this connection, a comparison between individual A/PC/73 titers in NW and serum specimens by RIP-IgG assays showed a positive correlation following vaccination. On the other hand, the magnitude of the NW IgA rises was not related to serum IgG titers (unpublished data). The latter observation is compatible with evidence which indicates that secretory IgA is a product of an independent immunological system (22).

The utilization of the RIP assays for the measurement of nasal secretory antibodies resulted in levels of detection not attainable by the Nt test. A possible explanation for the greater sensitivity of the RIP assays is simply that they are capable of reacting with fewer antibody molecules than the Nt test. In addition, the RIP assays may also recognize antibody specificities directed against antigens located on the lower portion of HA subunits which are usually inaccessible in the intact virion and, hence, not measurable by the Nt test. These considerations are supported by other studies (6, 21). Several lines of evidence support the contention that the relatively high RIP titers were attributable to specific antibody: the occurrence of significant rises in titer among vaccinees with comparable IgA and IgG concentrations in pre- and postvaccination specimens; the difference in levels of RIP antibody responses in persons with relatively comparable immunoglobulin concentrations; and the concordance of antibody responses to A/PC/73 by RIP and Nt tests.

Individual HA subunits of type A influenza viruses carry cross-reactive and variant-specific antigenic determinants (14). Our present knowledge of these in the immune response is limited to serological evaluations (11, 26). RIP assays using variants within the same antigenic subtype as competing antigens should provide additional insights into the nature of the nasal secretory response associated with an antigenic exposure to influenza virus.

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