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Subclass Distribution and Molecular Form of Immunoglobulin A Hemagglutinin Antibodies in Sera and Nasal Secretions After Experimental Secondary Infection with Influenza A Virus in Humans

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Serum and nasal wash specimens from 13 human volunteers undergoing experimental secondary infection with influenza A/Peking/2/79 (H3N2) wild-type virus were examined for the molecular form and subclass distribution of immunoglobulin A (IgA) antibodies to the viral hemagglutinin (HA). Nasal IgA antibodies were polymeric and did not bind radiolabeled secretory component, indicating that they were secretory IgA antibodies. Both IgA1 and IgA2 antibodies were detected; however, IgA1 accounted for most of the rise in IgA anti-HA levels seen after infection. In serum virtually all of the IgA HA antibodies were of the IgA1 subclass. Furthermore, the serum antibodies were predominantly polymeric and were capable of binding radiolabeled secretory component. These results suggested that the serum IgA antibodies to HA were of mucosal origin and that influenza A virus HA preferentially stimulates an IgA1 response.

Infection with influenza virus in humans results in the appearance of antibodies to the hemagglutinin (HA) or neuraminidase or both in both serum and nasal secretions (for reviews, see references 8 and 31). Although antibodies found in nasal secretions are primarily of the immunoglobulin A (IgA) isotype, antibodies of the IgM, IgG, and IgA isotypes are detectable in serum (3, 24).

Human IgA is represented by two subclasses (IgA1 and IgA2) and two molecular forms (polymeric and monomeric) with characteristic distributions in various body fluids. Serum IgA occurs predominantly in the monomeric form and has a relatively larger proportion of IgA1 molecules than do external secretions, which contain almost exclusively polymeric IgA and have a nearly equal distribution of IgA1 and IgA2 (5, 10, 12, 32, 33). Unlike the IgG subclasses (22, 25), the distribution of specific antibodies within the IgA subclasses has not been extensively studied. Furthermore, the distribution of antibody activity with respect to polymeric and monomeric IgA has not been established. The kinetics, magnitude, and persistence of an IgA-associated immune response in serum and secretions depends on the route of immunization and the form of the antigen, i.e., soluble versus particulate or living versus killed.

The study of a virus that infects mucosal surfaces provides a model which can be used for the elucidation of the probable origin(s) of serum and secretory IgA antibodies and may ultimately be helpful in determining the most efficient routes of immunization. Although serum antibodies can be correlated with protection against infection with influenza virus, the presence of secretory IgA antibodies in secretions of the upper respiratory tract (24), particularly those directed towards the HA determinants, is thought to be a major determinant of immunity (3, 4, 7, 8, 23, 24, 31). Therefore, we investigated the distribution of serum and nasal IgA antibodies to HA with respect to their IgA subclasses and molecular forms in individuals before and after infection with influenza virus.

MATERIALS AND METHODS

Clinical specimens. Sera and nasal washes were obtained from adult volunteers experimentally infected with a live, cloned suspension of influenza A/Peking/2/79 (H3N2) wild-type virus applied intranasally. Samples were stored at −20°C until assayed. Each of the 13 volunteers whose serum and nasal wash specimens were analyzed in the present study had been infected with virus, as indicated both by recovery of virus (average duration of virus shedding, 4.2 days) and by development of an immune response. The vaccine trial protocols were approved by the Clinical Research Subpanel of the National Institute of Allergy and Infectious Diseases, Bethesda, Md., and the Human Volunteers Research Committee at the University of Maryland, Baltimore. Clinical details as well as total HA-specific IgA, IgG, and IgM titers for these subjects have been published previously (3). All volunteers had preinfection HA inhibition titers in serum of ≤1:8 against A/Peking/2/79 (H3N2) but had preinfection titers of ≥1:8 against the A/Aichi/2/68 (H3N2) prototype virus, indicating that these volunteers were undergoing at least a second infection with an influenza A virus belonging to the H3N2 subtype (3).

Radioimmunoassay for HA-specific IgA1 and IgA2 antibodies. For each subject, serum samples from days 0, 21, and 28 and nasal washes from days 0, 14, and 21 or 28 were analyzed for HA-specific IgA1 and IgA2 antibodies by a solid-phase radioimmunoassay.

HA, which was purified from the X73 reassortant virus A/Bankok/1/79 (H3N2), was kindly provided by M. Phelan, Bureau of Biologics, Food and Drug Administration, Wash-

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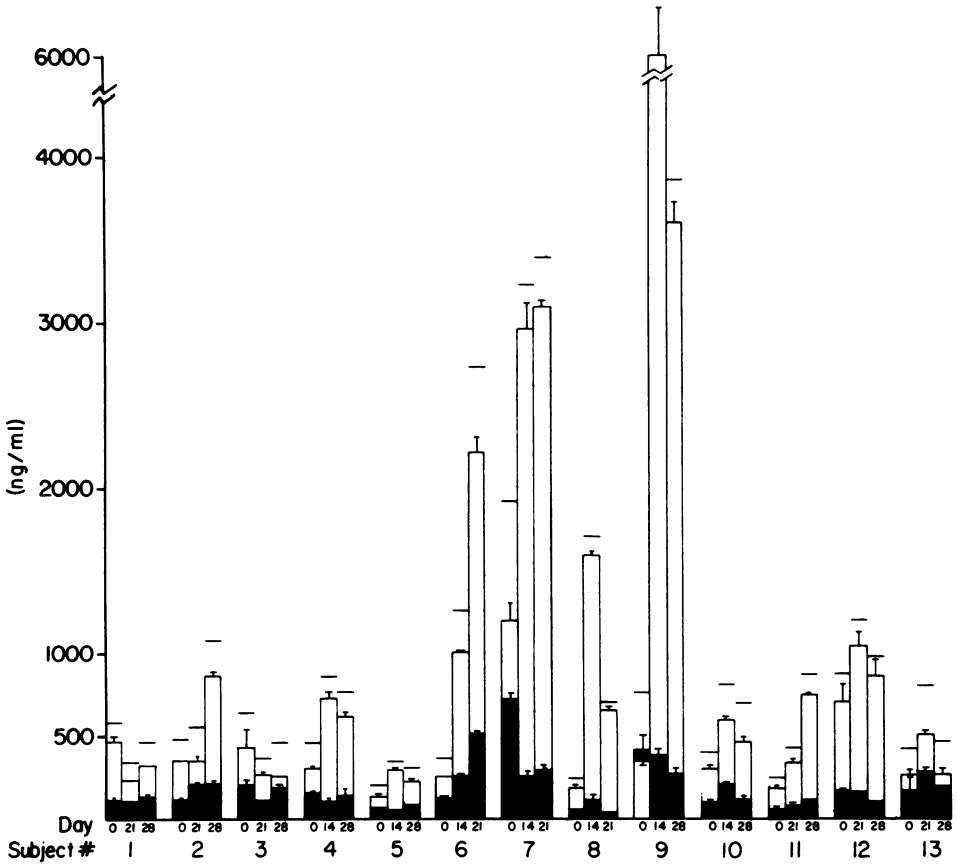

FIG. 1. Subclass distribution of IgA HA antibodies in nasal washes. Open bars, IgA1; solid bars, IgA2. Vertical lines at the tops of the bars indicate the standard deviations of assay replicates. Horizontal bars indicate the sum of IgA1 and IgA2. Each group of three bars represents one subject sampled on the days indicated.

ington, D.C. (28). The HA of this virus is closely related to that of the A/Peking/2/79 (H3N2) virus used as the infecting agent in this study (3). Human myeloma IgA1λ (Git) and IgA2λ (Fel) proteins and secretory component (SC) were isolated and purified as previously described (16). Mouse monoclonal human IgA subclass antibodies were labeled with ^{125}I by the lactoperoxidase method (20).

Strips of polystyrene wells (Immulon I; Dynatech Laboratories, Inc., Alexandria, Va.) were coated overnight with HA at 0.1 μg/ml in 0.05 M carbonate-bicarbonate buffer (pH 9.6). The assay was performed as previously described (2) except that 1% bovine serum albumin in 0.01 M phosphate-buffered saline (pH 7.4) was used for blocking, for washing, and as the diluent. The fractions were applied to HA-coated and uncoated blocked wells, followed by ^{125}I-labeled affinity-purified mouse monoclonal human IgA1 or IgA2 antibodies. These antibodies have been shown to be specific for human IgA1 and IgA2, respectively, regardless of light-chain type or IgA2 allotype (9).

Because of the lack of purified human anti-HA IgA1 and IgA2 standards, a method of standardization which most closely mimicked the antigen-binding assay was used. Wells were coated with rabbit anti-human λ chain, followed by the application of purified human myeloma IgA1λ (Git) or IgA2λ (Fel) protein. The standards were then developed with the labeled monoclonal subclass antibodies. The values reported for fractionated and nonfractionated samples are relative to these standards. The lower limit for the reliable detection of IgA1 or IgA2 antibodies was ca. 1 ng/ml.

Fractionation of samples. Selected serum and nasal wash specimens were fractionated by high-pressure liquid chromatography on a size exclusion column (300 by 7.5 mm) of Bio-Sil TSK-250 (Bio-Rad Laboratories, Richmond, Calif.) by elution with 0.05 M sodium sulfate in 0.02 M sodium phosphate (pH 6.8) at a flow rate of 1 ml/min. A 10-μl portion of an undiluted sample was injected, and 5-drop fractions (~142 μl) were collected starting 5 min after injection. Samples were applied directly to HA-coated wells for analysis by radioimmunoassay. The column was standardized with a mixture of polymeric and monomeric IgA myeloma proteins.

IgA1 protease. IgA1 protease was prepared by the method of Higerd et al. (13) from *Haemophilus influenzae* HK50 (obtained from Mogens Kilian, The Royal Dental College, Aarhus, Denmark) grown on chocolate agar plates.

RESULTS

Subclass distribution of IgA HA antibodies. In nasal washes, both IgA1 and IgA2 antibodies to HA were detected. Significant rises in IgA HA antibody levels, defined as a rise of greater than 2 standard deviations above day-0 levels, were seen in all subjects except subjects 1 and 3 (Fig. 1). Subjects with poor IgA responses did have significant IgG responses in serum, as shown previously (3). When rises in IgA antibody over preinfection levels were seen, the largest contribution appeared to be from the IgA1 subclass. In many cases IgA2 anti-HA levels either remained the same or

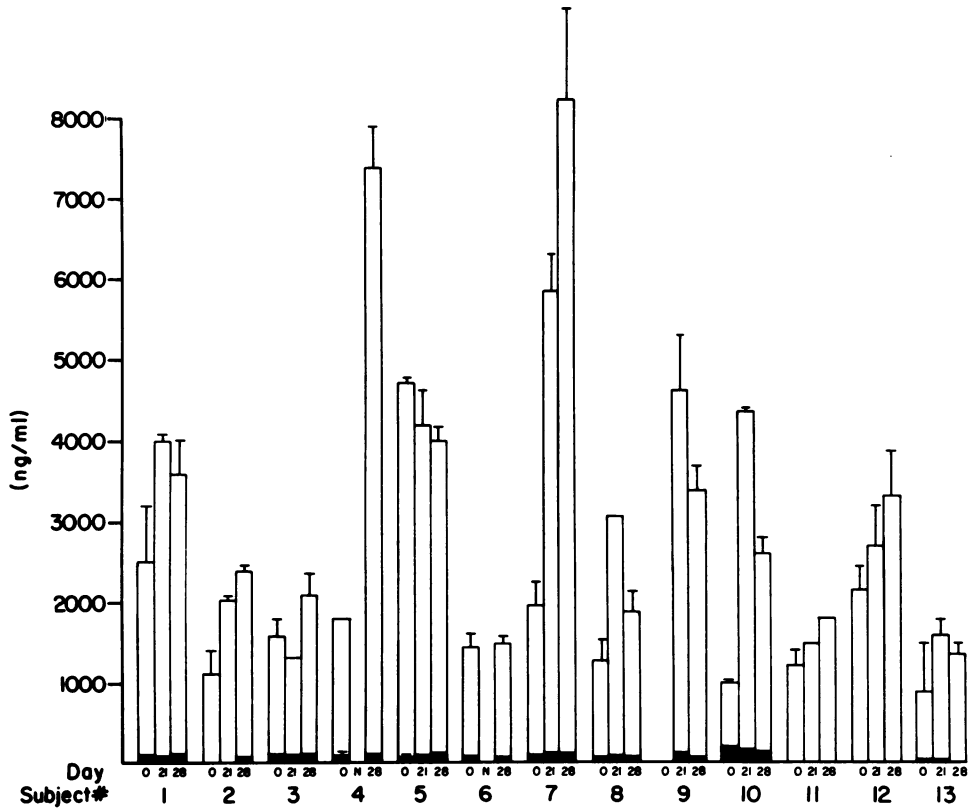

FIG. 2. Subclass distribution of IgA HA antibodies in serum. Open bars, IgA1; solid bars, IgA2. Vertical lines at the tops of the bars indicate the standard deviations of assay replicates. Each group of three bars represents one subject sampled on the days indicated. N, No sample available. Subject 9 had no detectable IgA HA antibodies on day 0.

actually decreased slightly, even in the presence of a large increase in IgA1 antibody levels.

In serum (Fig. 2), the levels of IgA2 HA antibodies were surprisingly low. The previously reported data concerning the distribution of total IgA1 and IgA2 in normal human serum indicate an average ratio of about 80% IgA1 to 20% IgA2 (5, 10, 12, 32). These findings indicate a selective increase of IgA1 HA antibodies rather than a proportional increase of both subclasses.

Molecular form of IgA HA antibodies. Nasal wash and serum samples were fractionated by high-pressure liquid chromatography on a size exclusion column and assayed by radioimmunoassay. Both the IgA1 and IgA2 antibody activities in nasal wash specimens were composed entirely of polymers and appeared to be secretory IgA, as there was no binding of radiolabeled SC (Fig. 3). SC is known to bind in vitro to polymeric J-chain-containing IgA but not to secretory IgA (19, 26, 30). These results were not unexpected because ca. 95% of the total IgA in human secretions, such as colostrum, is polymeric and contains SC (16, 33). In contrast, normal human serum IgA is predominantly monomeric (85 to 90%) (10, 16, 26, 33). However, the profile of serum IgA1 revealed a disproportionately large amount of polymeric IgA (Fig. 4, closed circles). IgA2 antibody activity could not be determined on high-pressure liquid chromatographic fractions of serum owing to its initial low concentration and the dilution (1:100) which occurred during chromatography. To determine whether the IgA1 antibody was a true polymer and not aggregated protein, we refractionated and developed the same sample with ^{125}I-SC; polymeric but not monomeric IgA bound SC (Fig. 4, open circles). This indicated that the putative polymer was in fact J-chain-containing polymeric IgA, as nonspecifically aggregated monomeric IgA does not bind SC (19, 30). Because these sera did contain IgM HA antibody (3), some of the SC binding may have been due to IgM HA antibody, which would also be bound to the HA-coated plate. To determine the contribution of IgM to the observed SC binding, we incubated a sample of serum with an equal volume of IgA1 protease from *H. influenzae* HK-50 for 18 h at 37°C, fractionated it, and assayed it for IgA1 HA antibody activity and SC binding. The control consisted of serum incubated with an equal volume of phosphate-buffered saline. Digestion with IgA1 protease virtually eliminated HA-binding activity as detectable by anti-IgA1 (Fig. 4, triangles), whereas SC-binding activity now eluted exclusively in the void volume, corresponding to the elution position of IgM (Fig. 4, plus symbols). At least one serum sample from each of the 13 subjects was fractionated and analyzed for IgA HA antibodies. Although the proportions of polymer to monomer varied, there was always a higher proportion of polymer to monomer in the postinfection samples than one would expect from the ratios of total polymer to monomer found in normal human serum (10, 16, 26, 33).

DISCUSSION

The importance of secretory IgA and serum IgG, IgA, and IgM antibodies for immunity to influenza virus is well recognized (3, 4, 7, 8, 23, 24, 31). Mucosal immunization with nonreplicating antigens generally induces both a local immune response at the site of immunization and a disseminated response at locations remote from the site of

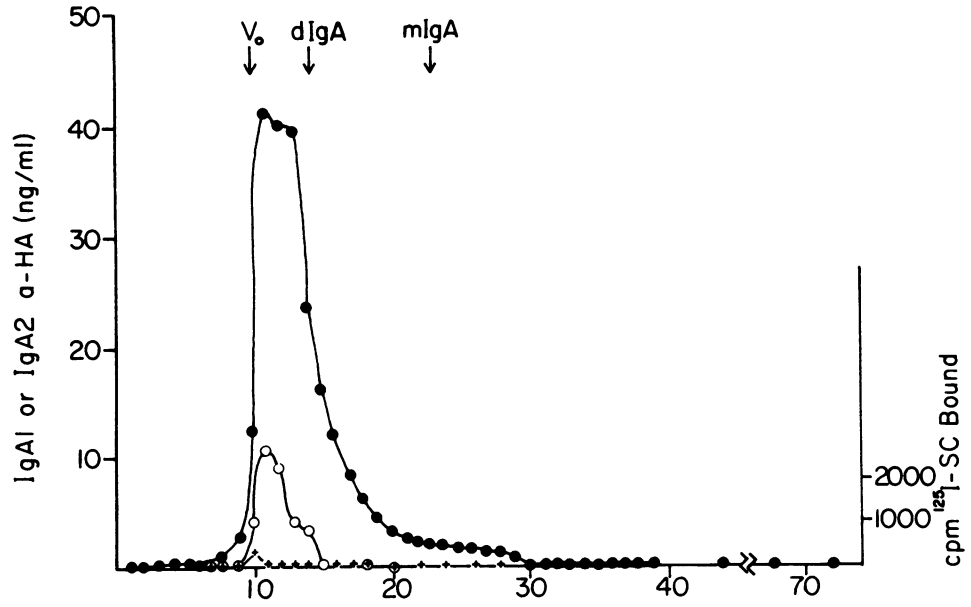

FIG. 3. Fractionation and analysis on a Bio-Sil TSK-250 column of HA antibodies (a-HA) in nasal washes obtained from subject 7 on day 21. Fractions were applied to HA-coated wells and assayed for IgA1 (●), IgA2 (○), and SC binding (+). Arrows indicate the void volume (V_o) and the elution positions of dimeric (dIgA) and monomeric (mIgA) human myeloma IgA standards. Numbers on the abscissa represent fraction numbers.

antigen encounter when bronchus- or gut-associated lymphoid tissues are stimulated (for a review, see reference 21). In humans, infection with a replicating antigen such as influenza virus antigen also leads to a systemic immune response manifested by the presence of serum antibodies of the IgM, IgG, and IgA isotypes (3, 24). IgA antibodies in human secretions originate mostly from submucosal plasma cells and are selectively transported by an SC-mediated mechanism. In the absence of inflammation, only a small proportion of IgA in secretions is derived from the circulation (11; L. F. Bertoli, H. Kubagawa, W. J. Koopman, J. Mestecky, and M. D. Cooper, Fed. Proc. 42:840, 1983). Because the IgA HA antibodies detected in nasal secretions from volunteers examined in this study were polymeric and failed to bind ^{125}I-SC, we conclude that they represent true SC-containing secretory IgA.

The origin of IgA HA antibodies in serum is less obvious. The parallel rise in IgA antibodies in serum and secretions observed in individuals undergoing primary as well as secondary infections with influenza A virus (3, 24) has been taken as evidence for a common mucosal origin of both serum and secretory IgA antibodies (3). Human serum IgA, which is of predominantly monomeric form, originates primarily from tissues such as bone marrow, spleen, and lymph nodes (17, 18). The polymeric form of serum IgA antibodies to HA further suggests that these antibodies detectable in the circulation were derived from secretory tissues known to produce polymeric IgA (1, 15) and escaped the transport process operational in secretory glands. The true polymeric nature of the IgA was demonstrated by the elution position of the IgA antibodies on a standardized size exclusion column and by the ability of the high-molecular-weight fractions to bind SC. The only serum proteins capable of binding SC are polymeric IgA and IgM (19, 26, 30), which interact with SC through their Fc regions. The cleavage of IgA1 HA antibodies with IgA1 protease abolished the detection of specific IgA1 antibodies with radiolabeled monoclonal anti-IgA1 and diminished the binding of ^{125}I-SC. The residual SC binding was presumably due to IgM as ascertained by the elution position on a standardized column, its antibody activity (as evidenced by its ability to bind to an HA-coated plate), and the resistance of IgM to IgA1 protease cleavage. The polymeric IgA antibodies detected in serum could have originated in the nasal mucosa or in remote mucosal tissues seeded by cells derived from bronchus- or gut-associated lymphoid tissues or in both. Previous studies have indeed shown that human milk contains antibodies to influenza virus (27), and oral ingestion of virus leads to the appearance of specific IgA antibodies in nasal secretions (34). We cannot exclude the possibility that HA might have the unique property of stimulating the production of only polymeric IgA even in tissues which are known to produce predominantly monomeric IgA.

Although serum antibodies to the HA antigen were almost exclusively of the IgA1 subclass, analyses of nasal washes from infected individuals revealed that the HA antibodies in those washes were of both IgA subclasses. However, as in serum, there was a selective increase in the IgA1 subclass. In other recent reports, serum IgA antibodies to cytomegalovirus (17) and phosphorycholine, β-lactoglobulin, and tetanus toxoid (6) were detected in both IgA subclasses; external secretions were not examined. It has recently been demonstrated that the nature of the antigen may also influence the subclass distribution of IgA antibodies. For example, salivary antibodies to microbial protein and carbohydrate antigens are predominantly of the IgA1 subclass, whereas those to lipoteichoic acid or lipopolysaccharides are predominantly IgA2 (T. A. Brown and J. Mestecky, Infect. Immun., in press). It is apparent from the present study that a different IgA subclass distribution of specific antibodies may be found when external secretions are examined in addition to serum.

Bacteria that colonize the upper respiratory tract, such as *H. influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*, are capable of producing proteases specific for the hinge regions of human IgA1 proteins (14, 29). If the

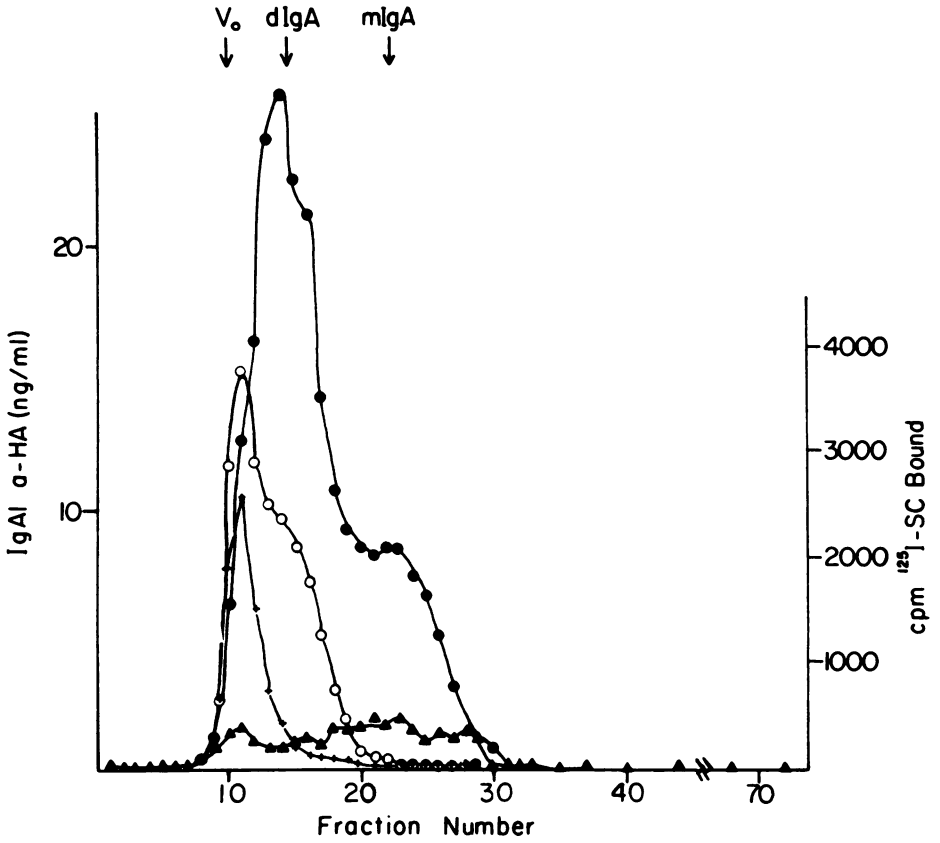

FIG. 4. Fractionation and analysis on a Bio-Sil TSK-250 column of HA antibodies (a-HA) in serum obtained from subject 7 on day 21. Fractions were applied to HA-coated wells and analyzed for IgA1 in an undigested sample (●), IgA1 in a sample digested with IgA1 protease before fractionation (▲), SC binding in undigested serum (○), and SC binding in digested serum (+). Arrows indicate the void volume (V_o) and the elution positions of dimeric (dIgA) and monomeric (mIgA) human myeloma IgA standards.

anti-HA response, which is thought to be the major determinant of protection against influenza A virus, occurs primarily in the IgA1 subclass, there is a greater potential for interference with local immunity by such proteases. Significant levels of antibodies to HA in the protease-resistant IgA2 subclass would therefore be desirable to provide compensatory protection. Although IgA2 constitutes ca. 15 to 20% of total serum IgA (5, 10, 12, 32) and although we were able to demonstrate HA antibodies of the IgA2 subclass in nasal washes, the levels of IgA2 antibodies to HA in serum were lower than expected. It is possible that polymeric IgA2 produced locally in secretory tissues does not enter the circulation in significant quantities, is preferentially transported into secretions, or is catabolized faster than polymeric IgA1 or monomeric serum IgA of both subclasses. Studies are in progress to determine the molecular form and subclass of IgA HA antibodies in volunteers receiving a parenteral, inactivated vaccine which, assuming HA does not uniquely stimulate polymer production, would more likely lead to the appearance of monomeric IgA antibodies in serum.

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