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Active Synthesis of Hemagglutinin-Specific Immunoglobulin A by Lung Cells of Mice That Were Immunized Intragastrically with Inactivated Influenza Virus Vaccine

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Intragastric inoculation with whole-virion vaccine of inactivated influenza virus resulted in production of hemagglutinin (HA)-specific immunoglobulin A (IgA) and IgG both in lung lavage fluids and in serum samples of mice. HA-specific IgA was the predominant isotypic antibody secreted in the lung lavage fluids (average IgA/IgG ratio, 13:1), whereas HA-specific IgG was the major antibody class in serum (average IgA/IgG ratio, 0.3:1). These responses were similar to the antibody responses stimulated by intranasal infection with live influenza virus. In vitro cultures of lymphoid cells from lungs and Peyer's patches, but not from spleens, in the presence of homologous antigen, from mice vaccinated intragastrically synthesized mostly HA-specific IgA. Mice immunized parenterally with inactivated influenza virus produced only IgG in lung lavage fluids and sera. Cultures of lymphoid cells from their spleens, but not their lungs, synthesized HA-specific IgG upon antigenic stimulation in vitro; neither synthesized IgA. These in vitro cell culture results, as well as the inverse relationship of IgA/IgG ratios in lung lavage fluids and sera, demonstrated that the IgA antibody in lung lavage fluids was actively synthesized locally in the lungs of intragastrically immunized mice. This finding was consistent with the migratory distribution of antigen-primed lymphoid cells from Peyer's patches to distant lymphoid tissue such as lung. Intragastric vaccination conferred protection against intranasal challenge with a lethal dose of virulent virus.

Hemagglutinin (HA) is responsible for attachment of influenza virus to cell surface receptors (11, 34). Antibodies to HA neutralize viral infectivity (15). Serum antibody to HA elicited by parenteral injection of inactivated influenza virus protects mice from severe pneumonitis induced by intranasal influenza virus challenge (26). However, resistance to respiratory viral infections may depend more on levels of secretory immunoglobulin A (IgA) in bronchial secretions than on levels in serum (10, 17, 24). Production of antibodies, primarily IgA, in pulmonary secretions is induced by immunization with live or inactivated influenza virus intranasally (7–9, 14, 17, 31–33) or orally (1, 5, 16). Secretory IgA is detected in saliva, tears, and nasal secretions of human subjects immunized orally with inactivated influenza virus vaccine. No systemic antibody response was obtained by using this approach (5, 16). We report predominant production of viral HA-specific IgA in lung secretions following oral administration of inactivated whole virion of influenza virus to mice. A systemic antibody response was also detected. The IgA was synthesized locally by lung lymphoid cells and might contribute to protection against virulent virus challenge.

MATERIALS AND METHODS

Animals. BALB/c female mice were purchased from Charles River Laboratories, Inc., Stone Ridge, N.Y. Mice were immunized at 12 to 20 weeks of age.

Antigens and immunization methods. Influenza A/Bangkok/1/79 (H3N2) virus was grown in allantoic fluid of 9- to 11-day-old eggs. This virus pool had an average titer of $10^{6.5}$ median egg infective doses per ml. Intranasal infection was accomplished by instilling 0.1 ml of the live virus into nostrils of mice anesthesized with ether. Formalin-inactivated whole virus, influenza A/Philippines/2/82/X-79 (H3N2), was provided by Connaught Laboratories, Swiftwater, Pa. For subcutaneous immunization, 0.2 or 5 μg of HA of inactivated influenza A/Philippines/2/82/X-79 virus was used; each mouse received two injections 3 weeks apart. Oral vaccination was conducted by pretreating mice with an intramuscular injection of Tagamet (Smith Kline & French Laboratories, Philadelphia, Pa.) (1.2 mg per mouse). One hour later, 0.1 ml of aluminum hydroxide gel antacid (Wyeth Laboratories, Inc., Philadelphia, Pa.) was delivered intragastrically with a blunt-end animal feeding needle (Popper & Sons, Inc., New Hyde Park, N.Y.). Blood collection and lung lavage procedures were performed 7 days after the booster immunization. This regimen was selected after preliminary studies that demonstrated its immunogenicity for unprimed mice. Then, 0.2 ml of inactivated virus was administered by the same method. Four consecutive daily doses were administered initially, and a booster dose was given 3 weeks later.

To evaluate whether this method might deliver some antigen intratracheally, trypan blue solution was inoculated intragastrically by the same technique used for vaccine administration. At different times after dye ingestion, both lungs and gastrointestinal tracts were examined for dye uptake. The blue dye was only found in the intestinal tract and not in the respiratory system in three independent trials.

Tissue specimens. Mice were bled from the orbital venous plexes with glass capillary tubes. Serum of individual mice was stored at −20°C. To obtain pulmonary secretions, lungs were lavaged with 0.7 ml of phosphate-buffered saline according to published methods (27), with modifications. An animal feeding needle was inserted intratracheally and fixed in place by tying with suture material. Lavaged fluids were

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TABLE 1. HA-specific isotypic antibodies in lung wash fluids and serum samples of infected or parenterally vaccinated mice

Immunogen	Inoculation route	Geometric mean antibody titer[a] (no. of responders/total no. of mice) or ratio					
		Lung lavage fluid			Serum		
		IgA	IgG	IgA/IgG	IgA	IgG	IgA/IgG
Infectious virus	Intranasal	26 (5/5)	7 (4/5)	3.7	243 (5/5)	1,280 (5/5)	0.2
HA (5 μg) of killed virus	Subcutaneous	3 (3/10)	61 (10/10)	0.05	<10 (0/10)	11,762 (10/10)	0.0003
HA (0.2 μg) of killed virus	Subcutaneous	<5 (0/9)	3 (5/9)	0.7	<10 (0/9)	470 (7/9)	0.006

[a] Specimens were collected 7 days after the booster dose of antigen or the infection. Titers were anti-HA responses measured by ELISA. Values of <5 and <10 were considered negative responses for lung lavage fluid and serum, respectively. Values of 2 and 3 were assigned for the respective tissue fluids for calculation purposes. Both responders and nonresponders were included in the calculations.

clarified by centrifugation (15,000 × *g* for 2 min) and stored at −20°C.

In vitro immunoglobulin production. Single-cell suspensions were prepared from lungs (28), Peyer's patches (13), and spleens (6) according to published methods. The cells were suspended in medium consisting of RPMI 1640, 5% heat-inactivated fetal bovine serum, 10^{-5} M 2-mercaptoethanol, 10 mM nonessential amino acids, 1 mM sodium pyruvate, 0.03% L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μg of gentamicin sulfate per ml. Cell suspensions were adjusted to 1×10^6 or 2×10^6 cells per ml, and 160 μl was inoculated into each well of round-bottom microtiter plates (Costar, Cambridge, Mass.). Inactivated influenza A/Philippines/2/82/X-79 virus was added in 10-μl portions to each well to yield a final concentration of 1 ng of HA per ml. The cell cultures were incubated at 37°C in a 5% CO_2–air humidified atmosphere for 12 days.

Virus challenge studies. Influenza A/Philippines/2/82/X-79 virus was adapted to mice by 12 consecutive lung passages in weanling mice. The virus pool obtained from lung tissues contained $10^{4.3}$ 50% lethal doses per ml and $10^{7.2}$ median egg infective doses per ml. Mice were anesthesized with ether and challenged intranasally with five 50% lethal doses of virus in a 0.1-ml volume.

ELISA. Quantitative determination of HA-specific IgG and IgA was by two-step enzyme-linked immunosorbent assay (ELISA) based on published methods (21), with modifications. Purified HA (25) of influenza A/Philippines/2/82/X-79 virus in carbonate buffer, pH 9.6, was coated onto wells in Immulon I plates (Dynatech Laboratories, Inc., Alexandria, Va.). The other sides of the plates were coated with carbonate buffer without antigen and served as control wells. This was used to detect nonspecific reactivity of samples. Lung lavage fluids and cell culture supernatants were prediluted 5-fold, sera were diluted 10-fold, and then 3-fold serial dilutions were carried out. Isotype-specific goat anti-mouse IgA or IgG (Southern Biotechnology Associates, Inc., Birmingham, Ala.) and alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin were subsequently incorporated. The substrate, 4-methylumbelliferyl phosphate in 1 M Tris buffer containing 3 mM $MgCl_2$, pH 9.8, was inoculated to react for 30 min at room temperature. The reaction was terminated by adding an equal volume of 0.02 M EDTA, pH 12.0, and the intensity of fluorescence was read by a Micro-Fluoro reader (Dynatech). To determine the endpoint, eight reference wells coated with purified HA were added with alkaline phosphatase conjugated to rabbit anti-goat immunoglobulin and with substrate as described above. The endpoint titer of each sample was determined as the highest dilution for which the fluorescence intensity exceeded the geometric mean reading plus 3 standard deviations of the reference wells.

RESULTS

Antibody responses to infection and parenteral vaccination. The results of one representative experiment are shown in Table 1. Most of the mice infected intranasally with live influenza virus responded with antibody production in both serum and lung. The lung lavage fluid contained a significantly higher titer of HA-specific IgA than IgG antibody, with a mean IgA/IgG ratio of 3.7. On the contrary, HA-specific IgG was the predominant antibody class in serum in all of the infected mice. Subcutaneous immunization with 5 μg of HA caused a few mice (3/10) to respond with marginally detectable levels of IgA in lung wash fluids, whereas none had measurable titers of IgA in serum. After subcutaneous immunization, all mice responded with HA-specific IgG in lung wash fluid and serum with mean IgA/IgG ratios of 5×10^{-2} and 3×10^{-4}, respectively. The lower dose of antigen elicited lower antigen-specific IgG responses and no IgA responses.

Antibody responses to intragastric administration of inactivated vaccine. All of the mice receiving daily oral doses of inactivated influenza virus containing 43 μg of HA per dose produced HA-specific antibodies in both lung lavage fluids and serum samples (Table 2). In lung lavage fluids, the predominant antibody isotype was IgA, with a mean IgA/IgG ratio of 13:1. On the contrary, an IgA/IgG ratio of 0.3:1 was obtained in serum samples. Graded lower dosages of antigen induced correspondingly lower antibody titers of both isotypes in both fluids. The inverse relationship between serum samples and lung lavage fluids in the ratios of IgA to IgG remained the same.

In vitro immunoglobulin production. Lymphoid cell suspensions from lungs of mice infected intranasally with live influenza A/Philippines virus synthesized HA-specific IgA when restimulated with homologous antigen in vitro (Table 3). The IgA/IgG ratio was 640:1. Specific IgG antibody was not detected. Similarly, lymphoid cell suspensions from Peyer's patches produced detectable levels of IgA but no

TABLE 2. Induction of HA-specific isotypic antibodies in lungs and serum samples of mice vaccinated intragastrically with various doses of inactivated vaccine

Amt (μg) of HA in vaccine[a]	Geometric mean antibody titer (no. of responders/total no. of mice) or ratio[b]					
	Lung lavage fluid			Serum		
	IgA	IgG	IgA/IgG	IgA	IgG	IgA/IgG
43	373 (7/7)	27 (7/7)	13	8,395 (7/7)	33,605 (7/7)	0.3
14	180 (6/6)	4 (4/6)	45	2,032 (6/6)	8,110 (6/6)	0.3
5	66 (7/7)	5 (5/7)	12	951 (7/7)	1,413 (7/7)	0.7

[a] Each dose of inactivated influenza A/Philippines whole-virus vaccine containing the specified quantity of HA was administered for 4 consecutive days at both primary and secondary vaccinations.

[b] Specimens were collected 5 days after the last booster dose. Anti-HA titers were measured by ELISA.

TABLE 3. In vitro production of HA-specific antibodies by various lymphoid cells of mice infected intranasally, injected subcutaneously, or inoculated intragastrically with inactivated influenza virus[a]

Immunization route	Geometric mean antibody titer or ratio[b]								
	Lung			Peyer's patch			Spleen		
	IgA	IgG	IgA/IgG	IgA	IgG	IgA/IgG	IgA	IgG	IgA/IgG
Intranasal infection	1,280	<5	640	20	<5	10	<5	<5	—[d]
Subcutaneous injection	<5	<5	—[d]	5[c]	20[c]	0.3	5[c]	20[c]	0.3
Intragastric ingestion	1,280	<5	640	320[c]	<5[c]	160	<5	<5	—[d]

[a] Immune lymphoid cells were collected 14 days, 5 months, and 6 days after intranasal infection, subcutaneous injection (5 μg of HA in each of two doses), and intragastric ingestion (43 μg of HA daily for 4 consecutive days; booster immunization 3 weeks later), respectively. Samples (160 μl) of viable cell suspensions at 10^6 cells per ml were incubated with 1 ng of HA of inactivated influenza virus in each microtiter well.

[b] Titers were anti-HA responses measured by ELISA. Values of <5 were considered negative responses, and a value of 2 was assigned for calculation of mean titers. Cultures without antigen restimulation gave a negative value.

[c] A viable cell concentration of 2×10^6/ml was used.

[d] —, The ratio could not be calculated.

IgG. Lymphoid cell cultures from spleens did not synthesize either isotype of HA-specific antibody. Cells from spleens and Peyer's patches of mice vaccinated subcutaneously synthesized predominantly HA-specific IgG antibody with a marginal titer of IgA. The IgA/IgG ratio was 0.3:1. Lung cells, however, did not produce measurable levels of either isotype of antibody.

Lymphoid cells from lungs and Peyer's patches of mice immunized orally with a daily dose of inactivated virus containing 43 μg of HA synthesized exclusively HA-specific IgA. These IgA/IgG ratios were 640:1 and 160:1, respectively. Spleen cell cultures did not produce detectable antibody.

Virulent virus challenge. Infection of 17 unimmunized mice with five 50% lethal doses of virulent influenza virus by intranasal challenge caused all of them to develop clinical signs of severe illness, including tachypnea, ruffled fur, hunched back, and weakness. A mortality rate of 88% (15/17) was observed within the 14-day observation period (Table 4). None of the 16 mice immunized by daily oral feeding with inactivated influenza virus vaccine containing 43 μg of HA showed any clinical signs of illness, and all remained alive at the end of the observation period. Similarly, mice immunized intranasally or subcutaneously with live or inactivated influenza virus containing 5 μg of HA in both primary and booster doses, respectively, survived after challenge.

DISCUSSION

This study demonstrated that intragastric delivery of inactivated influenza virus induced production of HA-specific IgA and IgG antibodies in lung lavage fluids and sera of mice. The predominant class of antigen-specific antibody in lung wash fluid was IgA, whereas the major antibody isotype in serum was IgG. This vaccination scheme protected mice against challenge with virulent influenza virus. The inverse relationship of IgA to IgG in lung lavage fluid and serum suggested that the IgA detected in lung wash fluid was due to active synthesis of antibodies at the mucosal surface. Further substantiation of this interpretation was found in experiments in which various lymphoid tissues from mice immunized intragastrically were cultured in vitro in the presence of homologous influenza virus antigen. Lung and Peyer's patch cells actively synthesized HA-specific IgA but not IgG, whereas spleen cells produced neither. Thus, HA-specific IgA was synthesized locally in lung tissue and was not simply transudated from serum to lung fluid.

TABLE 4. Protective efficacy of oral vaccination against challenge with homologous, virulent influenza A virus[a]

Immunization route	Mean (±SD) serum IgG titer at challenge[b]	No. of deaths/ total no. of mice (%)[c]
None	Negative	15/17 (88)
Oral	506 ± 5	0/16 (0)
Subcutaneous	21,878 ± 3	0/16 (0)
Intranasal	972 ± 4	0/16 (0)

[a] Approximately five 50% lethal doses of mouse lung-adapted virus was inoculated intranasally per mouse.

[b] Titers were anti-HA responses measured by ELISA.

[c] These data were collected during a 14-day observation period.

Antigen sensitization of lymphoid cells in gut-associated lymphoreticular tissues, commonly termed Peyer's patches, results in migration of sensitized cells to distant mucosal sites (18, 20, 33). Our in vitro antibody synthesis study indicated that lymphoid cells from lungs and Peyer's patches of mice immunized intragastrically produced HA-specific IgA. This result favored the hypothesis that HA-specific IgA-producing plasma cells in lung tissue originated from Peyer's patches and subsequently synthesized and secreted IgA at pulmonary surfaces.

In our study, both parenteral and intragastric immunizations induced protective immunity. The efficacies of the two methods may or may not be equivalent. Immunity induced by parenteral vaccination protects against complications of infection but probably does not prevent infection. Fasekas de St. Groth and Donnelley (10) demonstrated that intranasal stimulation with live or inactivated virus is associated with an efficacy of approximately 3×10^6 or 35- to 120-fold greater than that of parenteral inoculation with inactivated vaccine. This difference may be due to induction of IgA secretion in lung fluid. Liew et al. (17) demonstrated that protection against respiratory challenge with influenza virus relied on the presence of virus-specific IgA in lung but not antibodies or cytolytic lymphocytes in blood circulation. Since our intragastric immunization evoked local humoral immunity which was similar to that caused by intranasal immunization, it is possible that oral vaccination which induced lung IgA may offer more effective protection than immunity stimulated by the parenteral route. We have not as yet studied the effects of intragastric immunization on virus replication in lung postchallenge.

The close correlation between the antibody profiles in tissue fluids and those produced by lymphoid cell cultures from immunized mice indicated the usefulness of the in vitro antibody biosynthesis system. This methodology offers an

opportunity to understand how cellular and soluble components of the immunological system, as well as genetic factors, regulate induction of mucosal responses.

Current immunoprophylaxis against influenza virus infection uses parenteral administration of inactivated influenza virus vaccines. Antibody titers in serum that are induced by these vaccines correlate with protection against infection (19; D. Hobson, A. S. Beare, and A. Ward-Gardner, Proc. Symp. Live Influenza Vaccines, Yugoslav Acad. Sci. Arts, p. 73–83, 1972). Annual immunization of adults reduces absenteeism from work (30). Vaccination of persons in nursing homes significantly reduces the severity of illness and prevents hospitalization, pneumonia, and death (2). The efficacy of the vaccine in preventing illness in the elderly is sometimes relatively low, with some prospective studies reporting an efficacy of about 30% (2). Annual immunization of school children may not affect the incidence of influenza infection over a 5-year period (12). Thus, protection offered by current vaccination is less than complete. There is, therefore, a need to develop a better method to induce protective immune responses.

Since the respiratory tract is the primary target site for influenza virus infection, the presence of neutralizing antibody at that site may offer more effective control of viral invasion (17). Numerous studies of alternative approaches to influenza vaccination have been reported. Inactivated influenza virus vaccine administered intranasally induces secretion of antigen-specific antibodies in the respiratory tract (32). This method of vaccination is, however, practically difficult to perform. Induction of antibodies in the respiratory tract by intranasal inhalation (17, 26, 27) or ingestion of live influenza virus (3, 4) has been documented. Cold-adapted, attenuated live influenza virus vaccines are genetically stable and are being studied extensively (7, 8, 22). Development of suitable live, attenuated influenza B viruses, tolerance of live, attenuated viruses in unprimed individuals, and their immunogenicity in individuals with moderate levels of antibodies are difficult outstanding issues which must be resolved if live, attenuated vaccines are to be considered superior to the current inactivated vaccines. Inactivated influenza vaccine in an enteric coated capsule has been given to human volunteers and induced significant secretory immune responses (5, 16). Ease of vaccine administration makes this approach attractive. Based on these results, our studies are of potential relevance to human vaccine development. At present, this vaccination regimen requires multiple doses of vaccine with a higher antigenic mass than that for parenteral immunization. An adjuvant system to potentiate the immune response would reduce and simplify the immunization schedule. Some other investigators have found that oral immunization induces antigen-specific antibodies in distal mucosal sites (16, 20). However, others have reported less success. Ogra and Karzon (23) failed to detect IgA in the nasopharynges of children with colostomies who were immunized intracolonically with poliovirus vaccine. Similarly, ingestion of enteric capsules with live adenovirus vaccine does not result in detectable levels of IgA in nasal secretion (29). It is possible that the site of antigenic stimulation in the intestinal tract may influence the outcome of immunization.

We have thus developed a murine model in which secretory antibodies were actively synthesized at a distant mucosal site when antigen was delivered intragastrically. Lymphoid cell cultures produced antigen-specific antibodies. These systems will allow us to further understand immunoregulatory mechanisms affecting mucosal immune responses and to study new approaches to immunization so as to develop more efficacious vaccines against pulmonary pathogens.

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