Oral Immunization of Rats with Proteinoid Microspheres Encapsulating Influenza Virus Antigens

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Influenza virus antigen microspheres were prepared by a pH-dependent process using a protein-like polymer (proteinoid) made by thermal condensation of amino acids. The efficacy of these preparations to induce specific IgG responses when used as oral vaccines in rats was evaluated. A single enteric dose of M1 entrapped in proteinoid microspheres was able to induce a significant IgG response to M1 as early as 2 weeks postdosing, while rats dosed orally with the same M1 total dose (no microspheres) showed no detectable antibody response. An unencapsulated hemagglutinin and neuraminidase (HA-NA) preparation induced a moderate anti HA-NA IgG response. A single enteric dose of HA-NA spheres induced a response in 33% of the rats; this response was up to eight times higher than that observed in the rats dosed with unencapsulated antigen.

KEY WORDS: oral immunization; microspheres; proteinoid; influenza antigens.

INTRODUCTION

Current vaccination against influenza virus infection consists of parenteral administration of formalin-inactivated virus vaccines. The aim of these vaccines is to induce neutralizing antibodies active against the hemagglutinin (HA)[4] of the currently circulating influenza virus strains. These antibodies have been correlated with protection against infection (1). Protection offered by current vaccines, however, is less than complete; the efficacy of the vaccine in preventing illness in the elderly has been reported to be as low as 30% (2). The phenomenon of antigenic drift, together with the fact that the vaccine produces only short-lived immunity, makes annual vaccination necessary.

Oral immunization offers many practical advantages over parenteral immunization. Not only is this route of administration more acceptable to patients, but the reduction in the need for highly trained personnel and refrigerated storage results in simpler logistics for mass immunization. In addition, oral immunization has been shown in various systems to induce a vigorous immune response in mucosal surfaces, which are the most common sites of entry of infectious agents (3,4). Oral delivery systems based on microencapsulation have recently been tested due to their expected ability to deliver high local enteric concentrations of antigen; the encapsulating matrix would also be expected to provide protection against the low pH of the stomach and the proteolytic enzymes of the gut (5). Oral administration of polylactide polyglycolide microparticles has been shown to potentiate the induction of secretory IgA antibody response to a model poor immunogen (ovalbumin) after a series of booster immunizations (6). An oral vaccine which could protect against all type A influenza viruses (including those which have not as yet emerged in the human population) and which could engender long-lasting immunity and produce a good cellular immune response would be exceptionally valuable.

We have been able to induce a significant antibody response against influenza virus HA-NA and M1 antigens by separate oral administrations of the purified antigens in a novel oral delivery system. This system utilizes thermally condensed amino acids that undergo a phase transition (microencapsulation) at specific pH values. The antibody response elicited against the influenza virus antigens resulted from the administration of a single dose. These results have important implications for vaccine development in general and influenza vaccines in particular.

MATERIALS AND METHODS

Purification of Antigens. M1 was isolated from an unused batch of swine influenza vaccine donated by the Drug Directorate, Health Protection Branch, Bureau of Biologics, Ottawa, Ontario, Canada. The vaccine was prepared with the high-yielding recombinant strain X-53a, which derives its HA and NA from the parent strain A/NJ/11/76 (H1N1) and its internal proteins, including M1, from the parent strain A/PR/8/3411 (7,8). The virus was inactivated with β-propiolactone in the course of vaccine production. M1 was isolated as described by Khan *et al.* (9). Briefly, the vaccine material was centrifuged at 90,000*g* for 60 min and the resulting pellet was dissolved in 10% sodium dodecyl sulfate (SDS) by sonication and recentrifuged at the same speed. The supernatant was then fractionated on a BioGel A-5M column with 0.2 *M* Tris–HCl buffer (pH 7.4) containing 0.1% SDS and the M1-containing fractions were recycled to achieve a high purity. M1 purity was checked by SDS–polyacrylamide gel electrophoresis (PAGE).

The HA-NA preparation was isolated according to the procedure of Gallagher *et al.* (10). Influenza virus (A/PR8/34) was centrifuged at 90,000*g* for 60 min. The viral pellet was then solubilized with 0.05 *M* sodium acetate buffer (2.0 m*M* NaCl, 0.2 m*M* EDTA, pH 7.0) containing 7.5% octylglucoside and recentrifuged under the same conditions. The resulting supernatant was ca. 90% HA and 10% NA as confirmed by SDS-PAGE.

Microspheres. A protein-like polymer ("proteinoid") was used for making the M1 microspheres. This polymer was synthesized by a thermal condensation of a combination of

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[4] *Abbreviations used:* CTL, cytotoxic T lymphocytes; ELISA, enzyme-linked immunosorbent assay; FCA, Freund's complete adjuvant; HA, hemagglutinin; NA, neuraminidase; M1, M1 protein; NP, nucleoprotein; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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aromatic and acidic amino acids following the synthetic procedure described by Steiner and Rosen (11). M1 microspheres were prepared as follows. Dry proteinoid was dissolved in deionized water to a concentration of 100 mg/mL. The M1 solution, 2 mg/mL in Tris/SDS, was acidified by adding anhydrous citric acid (Aldrich) to a concentration of 1.7 *N* and gum arabic (Aldrich) to a concentration of 10 mg/mL (pH 2). Both solutions were equilibrated to 40°C in a water bath for 20 min. The proteinoid solution was then decanted into the citric acid/M1 solution and rapidly mixed. The resulting microsphere suspension was then immediately removed from the 40°C water bath and allowed to return to room temperature. Formation of microspheres occurred within seconds and was confirmed by microscopic examination. Final concentration of M1 in this suspension was 1.0 mg/mL.

HA-NA microspheres were prepared following the same protocol but substituting a HA-NA solution in PBS in place of the MI solution. Final concentration of the HA-NA was also ca. 1.0 mg/mL. "Empty" microspheres were prepared following the same procedure described for the M1 microspheres, with the only modification being that a 1.7 *N* citric acid/gum solution was used in place of the M1/citric acid/gum solution. The "empty" sphere cargo was thus only a citric acid/gum solution. Microspheres were monitored by microscopic examination and particle size distribution was examined in a Horiba 500 laser diffraction particle size distribution analyzer.

Unencapsulated Antigens. M1 was diluted in 1.7 *N* citric acid, 10 mg/mL gum arabic to the same final concentration as the M1 microspheres (1 mg/mL). HA-NA antigen was diluted in the same manner.

Solutions for Subcutaneous Control. M1 in Tris (no SDS) was diluted to a concentration of 167 μg/mL. An equal amount of Freund's complete adjuvant (FCA; Sigma) was added and the mixture was thoroughly homogenized. The final concentration of M1 in the mixture was 83.3 μg/mL. HA-NA solutions for subcutaneous administration were prepared in the same manner except for the buffer: 0.01 *M* phosphate-buffered saline (PBS) (pH 7.2) replaced Tris–SDS.

M1 Experiment. Male Sprague Dawley rats (ca. 350 g) were used in this experiment. Oral dosage was by gavage. For the experiment with M1, four groups of five rats each (the subcutaneous control group had four) were dosed as follows: Group 1 was dosed orally with 1 mg of M1 in microspheres/rat in 1.0 mL, Group 2 was dosed orally with 1 mL/rat of "empty" spheres, Group 3 was dosed with 1 mg of unencapsulated M1/rat in 1 mL, and Group 4 was dosed SC with 25 μg/rat of M1 in 0.3 mL. Blood samples (300 μL) were taken from each rat by tail bleeding before dosing and at 1, 2, 3, and 4 hr postdose (to assay for antigen) and at 14, 28, and 42 days postdose (for antibody assay).

The same immunization and bleeding schedule was followed when dosing with HA-NA spheres, with the following modifications: all rats received an oral booster with HA-NA spheres (250 μg/rat) 42 days after the first oral dose and blood samples were again taken 14 days after the booster dose. No samples were taken for antigen measurements. Since in the M1 study significant titers were present as early as 2 weeks postdose, in the HA-NA study an additional blood sample was taken 7 days postdose. All blood samples were centrifuged in a Beckman microfuge B for 5 min and the serum was harvested. Serum samples were stored at 20°C until assayed.

Assay for Anti-M1 and Anti-HA-NA Specific Serum IgG. Serum IgG specific for M1 was assayed by an ELISA technique performed as described by Khan *et al.* (9). Anti-HA-NA specific IgG responses were assayed by the same technique. Titers were determined as the serum dilution at which the absorbance was three times the background absorbance obtained with control wells that had all the reagents, except antibody.

RESULTS

We were able to manufacture proteinoid microspheres successfully with both HA-NA and M1 antigens of influenza virus type A. These microspheres, when examined microscopically, showed a size range of ca. 0.1 to 5.0 μm. The "empty" microspheres used as a negative control exhibited similar shape and size distribution (Fig. 1). Microspheres made with this proteinoid have been examined by scanning electron microscopy and found to be fenestrated structures. The proteinoid used in this study formed microspheres which are stable when incubated in simulated gastric fluid. When exposed to a pH greater than 5, they disassemble rapidly, releasing their contents. The microsphere preparation used in this study included both encapsulated and unencapsulated antigens.

Plasma samples from rats dosed orally with "empty" microspheres showed no significant antibody titer against either M1 or HA-NA antigens when assayed in ELISA (see Table I). The very low titers that were observed at 42 days in some of the animals are the result of non-specific absorption and are not significant. As expected, rats dosed with 25 μg of either M1 or HA-NA antigen (with FCA) subcutaneously (run as positive controls) developed a vigorous antibody response with titers that ranged from 54,000 to 330,000 in the case of M1 and 176,750 to 909,000 in the case of HA-NA (Table I).

Plasma samples from three of the five rats dosed with M1 microspheres showed a very significant primary re-

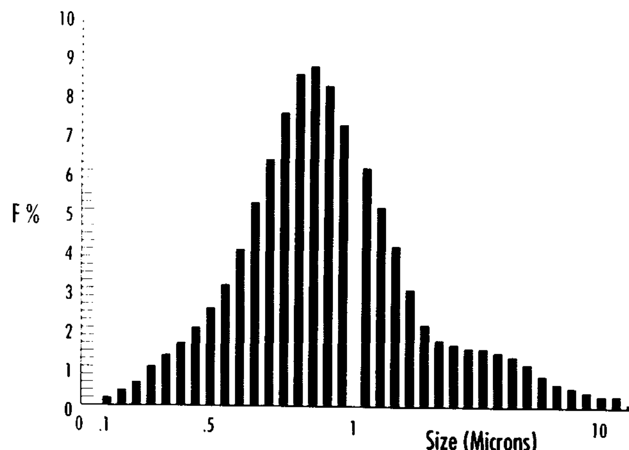

Fig. 1. Particle size distribution histogram of proteinoid microspheres measured in a Horiba 500 laser diffraction particle size distribution analyzer.

Table I. Anti-M1 Protein Antibody Titers in Serum from Rats Dosed with M1 Protein Spheres *vs* Controls

Dosing	Rat no.	Titer 14 days	Titer 28 days	Titer 42 days
Oral M1 protein, unencapsulated, 1 mg/rat	197	<30	<30	<30
	198	<30	<30	<30
	199	<30	<30	<30
	200	<30	<30	35
	201	<30	<30	56
Empty spheres	203	<30	<30	82
	204	<30	<30	70
	205	<30	<30	60
	206	<30	<30	86
	207	<30	<30	45
M1 protein spheres, 1 mg/rat total	209	<30	<30	64
	210	2,150	820	5,200
	211	860	430	1,150
	212	760	1,850	3,000
	213	<30	<30	62
sc control, 0.025 mg/rat in FCA	215	40,000	62,000	330,000
	217	34	8,000	54,000
	218	430	8,000	125,000
	219	270	6,600	78,000

sponse to M1 antigen. All three rats had titers ranging from 760 to 2150 as early as 14 days postdosing, compared to <30 in all the rats that received the same amount of unencapsulated M1 (Table I). Titers in the group that received microspheres increased to 1150–5200 by 42 days.

Four of six rats immunized with unencapsulated HA-NA did show a moderate anti-HA-NA IgG response, with titers of 3400–17,675, while two of six rats dosed with HA-NA spheres showed a significant response (Fig. 2). The rats that did respond, however, reached titers at least eight times higher than those obtained in the controls. Although several rats showed higher titers after the oral booster with HA-NA spheres given 42 days postdose, most did not show a significant increase in titers.

DISCUSSION

The subject of immunity to influenza induced by parenteral or intranasal immunization has been extensively studied (12–18). Oral immunization of mice with inactivated influenza virus induced hemagglutinin-specific secretory IgA (S-IgA) in the lung and IgG isotype antibodies in the serum. This immune response resulted in protection against challenge with a lethal dose of the virus (17). We have been able to induce a significant primary antibody response to M1 antigen from influenza type A virus with a single enteric dose of M1 entrapped in proteinoid microspheres. A vigorous response, but in a lower percentage of animals, was also generated with HA-NA spheres. The significant primary IgG response observed may be the result of uptake of antigen-loaded microspheres into Peyer's patches and eventual delivery of antigen into the circulation. Thus, the spheres may protect the antigen and target it to mucosally associated lymphoid tissues (19). This may not, however, be the only mechanism by which proteinoid microspheres facilitate transport of proteins to the bloodstream. We have demonstrated delivery to the bloodstream of proteins with no known oral bioavailability, such as monoclonal antibodies of the IgG subtype (20).

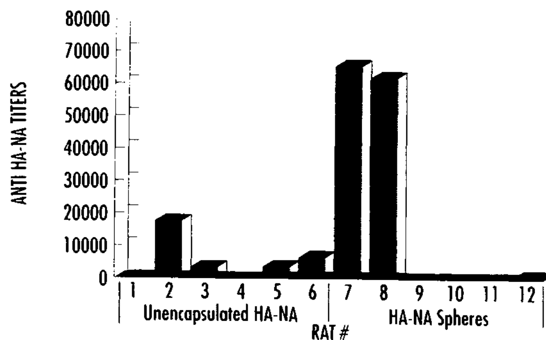

Fig. 2. Peak anti-HA-NA titers of rats immunized orally with HA-NA microspheres vs. unencapsulated HA-NA.

The fact that rats dosed with unencapsulated HA-NA do show some response, while those dosed with unencapsulated M1 show no response, is of interest. The techniques used for the preparation and purification of HA-NA allow it to retain its lectin properties and thus bind to mucosal cells, possibly facilitating its transport and allowing it to induce a systemic IgG response. M1, being an internal component and lacking these properties, would not be able to be transported by this mechanism. Thus M1 was able to induce an IgG response only when dosed in microspheres.

Two of the five animals dosed with M1 microspheres did not show a significant increase in antibody titers, as did four of the rats dosed with HA-NA spheres. This might reflect interanimal variability in the response or variability in physiologic factors such as gastric emptying time and intestinal pH and motility. Titers obtained with SC immunization were much higher than those obtained with the entrapped M1;

however, it must be noted that the SC immunization involved the use of a very strong adjuvant (FCA). Important parameters that were not examined were the local mucosal immune response or the generation of cellular immune responses. Both of these responses could be highly relevant in protection from influenza infection. For example, secretory IgA antibodies are reported to have antiviral activity in the absence of complement and may act as an immunological barrier to the entry of respiratory viruses (17,18). Several groups have found an enhanced local immunity and, in some cases, maximal extended protection, by orally administering antigens as compared to subcutaneous or intraperitoneal administration (4,12,21,33). In addition, while the role of M1 antibodies in providing protection or aiding recovery from influenza virus infection is unknown, cytotoxic T lymphocytes to internal antigens of influenza appear to play a role in reducing the severity of infection (24). Studies done in mice have demonstrated that the antibody response to hemagglutinin can be enhanced by T cells primed by the internal components of the virus (25). Both the local mucosal immune response and the cellular response will be carefully examined in future experiments.

Other groups have shown enhanced immunogenicity of encapsulated material administered orally. Eldridge and coworkers (26) have made microspheres with biodegradable polymers. The resulting microspheres have a size range of 1–10 μm and degrade during a period of ca. 6 weeks following intramuscular administration. This same group has shown that microspheres with a size of less than 5 μm are specifically taken up into Peyer's patches throughout the gastrointestinal tract and are carried within macrophages to the mesenteric lymph nodes and spleen.

Moldoveanu and collaborators (12) have immunized mice with influenza virus by oral and systemic routes using these polylactide polyglycolide microspheres as a delivery system. They found that microencapsulated vaccine given by the oral route induced a higher titer of antibodies than a similar dose of nonencapsulated antigen. In contrast to mice immunized with free antigen, influenza-specific IgA and IgG antibodies remained elevated in mice orally immunized with microencapsulated material. Most of these responses were observed only after the mice were boosted by additional oral doses of microencapsulated antigen.

In most studies oral administration of microspheres induced a significant immune response only after repeated exposures over a period of weeks or as a booster after parenteral immunization (6,12,19,26). To our knowledge, we are the first to demonstrate a vigorous primary response as early as 2 weeks postdose with a single oral dose. Experiments that are now in progress seek to examine dosing strategies that will optimize the response (level of dose, dosing schedule), proteinoid compositions and/or excipients that will maximize encapsulation, and resistance to pathogenic challenge in orally dosed animals.

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