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# Liposomes Containing Anti-Idiotypic Antibodies: an Oral Vaccine To Induce Protective Secretory Immune Responses Specific for Pathogens of Mucosal Surfaces

SUSAN JACKSON,[1]* JIRI MESTECKY,[1] NOEL K. CHILDERS,[2] AND SUZANNE M. MICHALEK[1]

*Departments of Microbiology[1] and Community and Public Health Dentistry,[2] University of Alabama at Birmingham, Birmingham, Alabama 35294*

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**By using a gnotobiotic rat model system to study the induction of protective immune responses by anti-idiotype (anti-id) vaccines specific for antibodies directed at the cariogenic microorganism *Streptococcus mutans*, it was shown that administration of such an anti-id vaccine provided partial protection against dental caries after challenge with virulent microorganisms. Protective effects were first demonstrated by direct parenteral administration of the anti-id vaccine into salivary gland regions, as determined by reductions in microbial colonization and caries scores. Subsequently, the anti-id was incorporated into liposomes and administered by gastric intubation. Immunization by this regimen also resulted in a significant reduction in caries as well as *S. mutans* colonization of the oral cavity, with concomitant increases in salivary immunoglobulin A anti-*S. mutans* antibodies. These data provide evidence that anti-id vaccines specifically targeted at the secretory immune system can induce protective immune responses to pathogens of mucosal surfaces.**

The idea that a given immune response can be regulated by a network of idiotype (id)–anti-id interactions was proposed by Jerne in 1974 (8). This theory holds that id–anti-id reactions control the response to a given antigen by either positive or negative feedback mechanisms. Many studies have provided data that support this hypothesis by implicating id networks in the regulation of immune responses to numerous types of antigens and haptens (for a review, see references 1 and 23). Depending on the given system under study, the injection of anti-id prior to antigen exposure can result in either suppression of a given id-positive antigen-specific response or, in the context of this report, increased expression of id and antigen-specific antibody. The induction of immune responses by anti-id reflects the fact that between id and anti-id there exists a complementary structural relationship that is especially noteworthy when the id is within or adjacent to the antigen-binding site. Anti-id antibodies related to the antigen-binding site (usually termed Ab2β) often represent the internal image of the antigen and provoke an immune response because they are seen by the immune system as structurally related to antigen. Anti-id antibodies that recognize determinants outside the antigen-binding site (framework) are called Ab2α and are thought not to induce protective antigen-specific responses (for a review, see references 3 and 24).

Numerous studies have provided evidence that anti-id reagents can induce protective immunity against a variety of infectious agents and may therefore represent possible vaccine candidates for pathogenic organisms. For example, anti-id vaccines have been used to induce protective immunity to hepatitis virus, schistosomiasis, trypanosomiasis, and pneumococcal and *Escherichia coli* infections (5, 9, 13, 26, 27). Other experiments have shown the induction of specific immune responses by the administration of anti-id (e.g., rabies virus and poliovirus) (22, 28), but no data on the protective effect of such responses are available yet. All of these studies relied on parenteral injection of the anti-id vaccine. However, most pathogens infect the host through mucosal surfaces of the gastrointestinal or respiratory tract. Ample evidence supporting the existence of a common mucosal immune system (one that can be stimulated at remote sites by the introduction of antigen into any mucosa-associated lymphoid tissue) is available (15). Therefore, the development of protective vaccines that can be easily administered into mucosal tissues would be desirable. In this paper, we provide evidence for the first time that introduction of an anti-id vaccine into the mucosal immune system can provide protective immunity.

Dental caries is a multifactorial infectious disease that stems from a variety of interactive elements. These factors include the resistance of the host, nutritional status and diet (sugar intake), and the presence of cariogenic microflora. Many studies of recent years have lent strong support to the role of the organism *Streptococcus mutans* as a human pathogen in the establishment of caries (6). The identification of caries as a truly infectious disease caused by an identifiable microorganism led to the anticipation that the production of a protective vaccine would be possible. However, for a variety of reasons such a vaccine has not been used in the general population, despite numerous promising animal experiments (for a review, see reference 11). Further, the production of a suitable vaccine has been hampered by the inability to prepare large amounts of antigen which are both safe and cost-effective (11).

Caries is a disease localized at tooth surfaces and therefore is assumed susceptible to salivary immunoglobulin A (IgA) antibodies directed at *S. mutans*. In this regard, a number of the animal studies were aimed at the induction of a protective immune response via the oral administration of antigen. The currently accepted concept of a common mucosal immune system has provided an impetus to utilize the gut to induce protective responses to a number of mucosal pathogens, including *S. mutans* (14). Further, because of the apparent compartmentalization of the systemic and secretory IgA systems (7, 16), parenteral immunization usually does not stimulate an IgA response in external secretions (7,

* Corresponding author.

20, 21), observations which would tend to contraindicate the use of a parenteral caries vaccine. Thus, the induction of protective immunity to dental caries via the mucosal introduction of vaccine remains highly desirable. At the same time, the current studies provide a useful animal model system to assess the development of protective immunity following delivery of an anti-id vaccine directly into the mucosal immune system as well as a potential vaccine approach to the effective induction of protective immune responses against a variety of infectious diseases.

## MATERIALS AND METHODS

**Animals.** Germfree Fischer rats [CD F(344)GN/Crl BR], original breeders obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass., were used for these experiments. These animals were bred and maintained in Trexler plastic isolators at the University of Alabama at Birmingham Gnotobiotic Rat Facility. This strain has been shown to be an excellent model for the study of immune responses to *S. mutans* and dental caries (12, 17). Conventional rats were used to produce anti-*S. mutans* antibodies (id). Outbred rabbits (New Zealand White) were used to produce anti-id antibodies.

**Bacterial antigen.** *S. mutans* MT8148 (serotype c) was cultured and treated for vaccine preparation under previously described conditions (19). Filtered supernatants of these cultures were used as crude antigens for detection of precipitating antibodies by double diffusion in agar.

**Production of anti-id antibodies (anti-anti-*S. mutans* antibodies).** Conventional rats were hyperimmunized with whole Formalin-killed *S. mutans* cells in complete Freund adjuvant (~200 μl of organisms [optical density, 89.5] plus an equal volume of complete Freund adjuvant) by using multiple intramuscular injections. After 2 weeks, the animals were given booster doses of antigen in incomplete Freund adjuvant and this procedure was repeated until precipitating antibodies were detected as described above. At this point the rats were exsanguinated, and serum IgG was isolated by ion-exchange chromatography. The IgG preparation pooled from 23 animals was then incubated with packed whole *S. mutans* cells at 4°C overnight. After this procedure, the suspension was centrifuged and washed extensively, and the specific anti-*S. mutans* antibodies (id) were eluted by using 3 M potassium thiocyanate and dialyzed against phosphate-buffered saline. This antibody preparation was subsequently absorbed on a packed suspension of group B streptococci to remove any residual reactivity to irrelevant streptococcal antigens. The preparation was then tested by enzyme-linked immunosorbent assay (ELISA) and shown to bind to whole *S. mutans* cells as well as to purified protein antigen I/II (25) derived therefrom. At the same time, there was no detectable binding to the group B streptococcal antigen, a useful control antigen to exclude the presence of antibodies to lipoteichoic acid. After determining that the polyclonal id preparation retained its antigen-binding capabilities after affinity purification, it was subsequently used to immunize rabbits.

For rabbit immunizations, we used 100 μg of affinity-purified anti-*S. mutans* in complete Freund adjuvant injected intramuscularly, with repeated biweekly booster doses of the same antigen in incomplete Freund adjuvant. When precipitating antibodies directed against the immunogen (anti-*S. mutans*) were observed (by using double diffusion in agar), serum IgG was isolated by ion-exchange chromatography from the hyperimmune rabbit serum (pooled after multiple bleedings from three animals). The IgG was then absorbed on a column of insolubilized normal rat IgG until there was no further reactivity with this preparation, as determined by ELISA (sensitivity, ~1 ng/ml). At the same time, the putative anti-id reagent was shown to react with the original immunogen (id); by using an indirect standardization method based on quantification of total rabbit IgG, it was calculated that the serum pool contained about 40 μg of anti-id per ml. At this point, the preparation was assumed to contain partially purified anti-id antibodies, which were further purified by specific elution from a column of insolubilized rat hyperimmune (anti-*S. mutans*) IgG. It is important to emphasize that this column contained only hyperimmune total rat IgG (not previously affinity purified on *S. mutans* organisms) in order to avoid potential contamination of the anti-id preparation with components of the original antigen (*S. mutans*). In this regard, it should be noted that there was no reactivity of the anti-id with *S. mutans*, again determined by ELISA. By these procedures, approximately 8 mg of anti-id that did not react with normal rat IgG (absorbed on *S. mutans*) at the same time that it was shown by ELISA to bind to rat anti-*S. mutans* IgG was prepared.

**Liposome incorporation of anti-id and NRIgG.** Preparation of liposomes was accomplished by the methods that we have previously described (2). Briefly, dipalmitoylphosphatidylcholine, cholesterol, and dicetyl phosphate (all from Sigma Chemical Co., St. Louis, Mo.) were dissolved in chloroform and subsequently evaporated. Following the addition of antigen (anti-id or normal rabbit IgG [NRIgG]) in carbonate buffer, the formed multilamellar liposomes were sonicated by using a Densco sonifier (Crest Ultrasonics, Trenton, N.J.) to generate single unilamellar liposomes.

**Immunization of germfree rats with anti-id.** Two general types of immunization protocols were used. First, we employed parenteral immunization directly into the regions of the salivary glands of germfree rats (25 μg per animal; six to eight animals per group), using the schedule and procedures described previously (12). Second, germfree animals were gastrically intubated and subsequently challenged according to the schedule that we have previously followed (19). Briefly, two groups of 19-day-old germfree rat pups were intubated with liposomes containing anti-id or NRIgG. Five days later, all animals were infected with freshly cultured *S. mutans*. On days 30 and 44 the intubations were repeated, and on day 52 the animals were removed from the isolator. The immunogen preparation consisted of a volume of 0.25 ml of liposomes containing 10 μg of anti-id. Control animals in all experiments received identical immunizations with NRIgG. Following the collection of samples of serum and saliva, the animals were sacrificed and the mandibles were removed for microbiologic analyses and caries scoring.

**Serum and saliva samples.** Freshly obtained clotted blood samples were centrifuged to separate cells and serum. Serum was portioned and stored at −20°C. Rat saliva was collected from animals anesthetized with sodium pentobarbital after pilocarpine stimulation, as described previously (4). These samples were also portioned and stored at −20°C.

**Immunologic reagents.** A goat reagent specific for rat IgG was prepared in our laboratory. A monoclonal antibody specific for rat IgA was purchased (Zymed, San Francisco, Calif.).

**ELISAs.** Measurement of rat salivary IgA or serum IgG anti-*S. mutans* antibodies was done on polyvinyl chloride plates coated with whole Formalin-killed organisms. These procedures have been described in detail elsewhere (18). Specificity controls for these assays consisted of wells

coated with equivalent numbers of group B streptococci. Solid-phase assays for the determination of anti-id utilized affinity-purified anti-*S. mutans* IgG (coated at a concentration of 2 μg/ml) and control wells coated with rat IgG previously absorbed on packed *S. mutans* cells. Additional control wells were coated with whole Formalin-killed organisms or, in some cases, purified antigen I/II.

***S. mutans* cultures and caries scoring.** To determine the efficacy of immunization in protecting animals against colonization by *S. mutans*, mandibles were removed aseptically and defleshed after the animals were sacrificed with sodium pentobarbital. One mandible from each rat was used for enumerating the number of viable *S. mutans* cells, as previously reported (12). For caries scoring, both mandibles were then stained with murexide and hemisectioned with the aid of a dental drill. Buccal, sulcal, and proximal caries lesions were scored by the method of Keyes (10), as reported previously (12). All data regarding colony counts and caries scores were obtained and calculated without any knowledge by the investigator of which group of animals (test or control) was being analyzed.

**Statistics.** Significance was determined by Student's *t* test of anti-id-immunized versus control animals in each experiment, using square root transformation of data to stabilize variances.

## RESULTS

**Parenteral administration of anti-id induces anti-*S. mutans* antibodies and results in reduction of caries and bacterial colonization.** A pilot experiment was performed on a small group of rats injected intramuscularly with either anti-id or NRIgG in complete Freund adjuvant in order to determine if the anti-id preparation would indeed elicit the induction of anti-*S. mutans* antibodies. The animals were bled, and the serum samples were assayed by ELISA for anti-*S. mutans* activity. This preliminary experiment indicated that the anti-id reagent did indeed induce synthesis of serum anti-*S. mutans* antibodies. Therefore, a second study was initiated to confirm these results and to examine the question of potential protection by the anti-id-induced immune response. In this study, rats were immunized parenterally by direct injection into the regions of the salivary glands. Serum and saliva samples were assayed for the presence of anti-*S. mutans* antibodies. No statistical differences in salivary IgA antibodies between the two groups were noted; however, serum anti-*S. mutans* antibodies were elevated in the anti-id-immunized group at all dilutions assayed (Table 1). Further, when the protective effect of anti-id immunization into salivary gland areas was examined (as determined by *S. mutans* colonization and caries scores), the results indicated that protective effects were demonstrable (Table 2). Thus, in these studies, significant differences in animals immunized with an anti-id reagent specific for anti-*S. mutans* antibodies were noted, with respect to the subsequent ability of virulent *S. mutans* to colonize tooth surfaces and induce dental caries, despite the fact that there were no differences in salivary IgA antibodies in the groups under study.

**Gastric administration of anti-id–liposomes results in partial protection and induces salivary IgA anti-*S. mutans* antibodies.** Since the results reported above clearly indicated that the anti-id preparation used as an immunogen efficiently induced an anti-*S. mutans* response that could be protective, we initiated studies to determine whether mucosally administered anti-id was equally immunogenic. These experiments utilized anti-id incorporated into liposomes and placed directly into the gastrointestinal tract.

TABLE 1. Induction of anti-*S. mutans* antibodies by parenteral injection of anti-id

| Immunogen | Serum dilution | Mean $OD_{414}$ (SE) and range for anti-*S. mutans*[a] |
|---|---|---|
| Anti-id | 1:200 | 0.80 (0.17), 0.32–1.55 |
| Anti-id | 1:400 | 0.61 (0.10), 0.26–0.91 |
| Anti-id | 1:800 | 0.43 (0.11), 0.06–0.78 |
| NRIgG | 1:200 | 0.26 (0.05), 0.10–0.50[b] |
| NRIgG | 1:400 | 0.23 (0.05), 0.07–0.36[b] |
| NRIgG | 1:800 | 0.15 (0.05), 0.00–0.29[b] |

[a] $OD_{414}$, Optical density at 414 nm. Data are for each group of six animals.
[b] $P$ = 0.0066, 0.0033, and 0.0224, respectively, for comparative data between the two groups on each consecutive dilution.

After gastric intubation of liposome-borne anti-id, there were no differences in serum anti-*S. mutans* antibodies between the two groups of animals (data not shown). On the other hand, when levels of salivary IgA antibodies were determined, the results shown in Table 3 were obtained. These data indicated that a salivary IgA response could be elicited by gastric intubation of liposome-bound anti-id. As in the earlier experiment that employed injection of anti-id into salivary glands, protection against the induction of caries was assayed in two ways—by *S. mutans* colonization in the oral cavity and by caries scores. The abilities of virulent *S. mutans* both to colonize tooth surfaces and to induce dental caries were reduced by the intragastric immunization regimen (Table 4).

## DISCUSSION

In recent years, a number of investigations have centered on the establishment of protective immune responses by administration of anti-id vaccines. These efforts have been made for theoretical as well as practical reasons. The early experiments were aimed mainly at determining whether the compelling notion of producing such responses was truly feasible and have led to the generally accepted view that protection of the animal against a virulent virus, bacterium, or parasite can indeed be achieved by anti-id immunization. However, attempts to induce protection against pathogens of mucosal surfaces by using anti-id vaccines targeted at the secretory immune system have not been reported. This should be considered an important objective, since many disease-causing microorganisms have specific tropism for these tissues. In this report, we have demonstrated that such a goal may be achievable.

Using injection into salivary gland regions (an essentially parenteral immunization aimed specifically at the oral cavity), we first demonstrated that administration of anti-id reagents specific for anti-*S. mutans* antibodies could induce an immune response which was protective, indicating that our anti-id preparation contained the internal image of the antigen, or Ab2β antibodies. Interestingly, this did not appear to be attributable to salivary IgA antibodies specific for *S. mutans*. Although immunized animals did exhibit significantly elevated levels of serum antibodies, it is not clear whether these antibodies contributed to the observed protective effects. However, since the type of immunization employed (antigen-adjuvant emulsion) induced local granuloma formation and probable exudation of serum proteins, it is likely that the serum anti-*S. mutans* response was primarily responsible for the reduction in both *S. mutans* colonization and caries scores, as suggested by others (for a

TABLE 2. Summary of caries scores and colonization of *S. mutans* in the oral cavities of rats after salivary gland injection of anti-id

| Immunogen | Mean score (SE) for[a]: | | | | Mean colony count (SE) and range for *S. mutans* ($10^6$)[b] |
|---|---|---|---|---|---|
| | Smooth surface | | Sulcal surface | | |
| | Enamel | Dentin | Enamel | Dentin | |
| NRIgG | 11.16 (0.70) | 3.5 (0.62) | 18.5 (0.50) | 3.66 (0.71) | 125 (13.5), 63–161 |
| Anti-id | 8.5 (1.49)[c] | 2.33 (0.61)[d] | 15.33 (0.33)[e] | .67 (0.33)[f] | 51 (18.6), 19–124[g] |

[a] Data for each group of six rats. Enamel scores represent the incidence of lesions, whereas the dentin scores represent the severity of the lesions which occurred on smooth (buccal and proximal) and sulcal (pit and fissure) molar surfaces.
[b] Data for each group of six animals.
[c] −24%, $P = 0.1327$.
[d] −33%, $P = 0.2309$.
[e] −17%, $P = 0.0004$.
[f] −82%, $P = 0.0031$.
[g] $P = 0.009$.

review, see reference 11). IgG antibodies in crevicular fluid might also be expected to contribute to the observed protection. It is important to note that only partial protection was obtained and that the most significant differences were noted in the severity of lesions of sulcal surfaces rather than in the actual incidence of caries at any surface.

The most significant data that we report are those showing that a mucosally administered anti-id vaccine induced a protective immune response, as determined by microbial colonization and caries scores assessed by blind analysis. We have demonstrated that gastric intubation with very low doses of liposome-containing anti-id resulted in reduced incidence and severity of caries in germfree rats after infection with *S. mutans*. Again, we emphasize that the induced protection against caries was not complete and was most apparent when the severity of lesions rather than the incidence was assessed. Although the amount of protection observed in this study was less than that observed when animals were given an oral vaccine consisting of liposomes containing purified antigens of mutans streptococci (18), we assume that subsequent experiments using an increased antigen dose and possibly an oral adjuvant such as cholera toxin will demonstrate more efficient protection.

TABLE 3. Levels of salivary IgA anti-*S. mutans* antibodies induced by intragastric administration of anti-id[a]

| Immunogen | Saliva dilution | Mean $OD_{414}$ (SE) and range for IgA anti-*S. mutans* |
|---|---|---|
| Anti-id | 1:40 | 0.61 (0.12), 0.28–1.14 |
| Anti-id | 1:80 | 0.53 (0.12), 0.24–1.06 |
| Anti-id | 1:160 | 0.36 (0.08), 0.12–0.75 |
| NRIgG | 1:40 | 0.32 (0.08), 0.16–0.72 |
| NRIgG | 1:80 | 0.28 (0.10), 0.11–0.75 |
| NRIgG | 1:160 | 0.22 (0.07), 0.08–0.56 |

[a] Data for each group of eight animals. Although antibody levels were higher at each dilution in the anti-id-immunized group, there were no significant differences between groups. $OD_{414}$, Optical density at 414 nm.

Oral immunization regimens generally utilize considerably higher levels of antigen than those we have employed, introducing the likelihood (in the case of soluble protein immunogens) of inducing systemic tolerance (15). The attractiveness of using liposomes to deliver antigen to the gut is based on the known efficiency of these preparations in inducing immune responses with low antigen doses, compared with conventional oral immunizations (18). Because a very limited amount of purified anti-id was available for these studies, we did not examine the efficacy of oral anti-id immunization without incorporating it into liposomes. However, on the basis of our previous work demonstrating that 10 μg of liposome-incorporated antigen was much more effective in inducing protective immune responses to *S. mutans* antigens than 10-fold-higher amounts of the same antigens administered alone (18), we feel it is reasonable to assume that it is highly likely that such a regimen would prove inferior or even ineffective compared with those employed in the present experiments.

The potential use of anti-id vaccines offers several obvious advantages that have been previously discussed by numerous authors. Some of these benefits would include (i) the ability to produce "immortal" anti-id reagents in very large quantities as monoclonal antibodies, (ii) obviation of the

TABLE 4. Summary of caries scores and colonization of *S. mutans* in the oral cavities of rats after gastric intubation with liposome-containing anti-id

| Immunogen | Mean score (SE) for[a]: | | | | Mean colony count (SE) and range for *S. mutans* ($10^6$)[b] |
|---|---|---|---|---|---|
| | Smooth surface | | Sulcal surface | | |
| | Enamel | Dentin | Enamel | Dentin | |
| NRIgG | 11.57 (0.53) | 4.57 (0.43) | 18.57 (0.62) | 5.42 (0.43) | 147 (27.0), 44–200 |
| Anti-id | 9.62 (0.42)[c] | 2.12 (0.40)[d] | 16.5 (0.63)[e] | 2.37 (0.46)[f] | 77 (12.6), 44–120[g] |

[a] Data for each group of six rats. See Table 2, footnote *a*, for explanation of caries scores.
[b] Data for each group of eight animals.
[c] −17%, $P = 0.0118$.
[d] −54%, $P = 0.0068$.
[e] −11%, $P = 0.0355$.
[f] −56%, $P = 0.0005$.
[g] $P = 0.0001$.

possibility of inclusion of infectious antigen in the vaccine preparation, and (iii) avoidance of the obstacles encountered because some immunogens (e.g., certain parasites) are not amenable to either preparation in large quantities or recombinant DNA technology. With specific respect to dental caries, the potential of an anti-id vaccine is particularly appealing because of the evidence suggesting that antibodies directed at whole *S. mutans* organisms cross-react with human heart antigens (for a review, see reference 11). Thus, the goal of producing anti-id vaccines to prevent infectious diseases is exciting. We have provided evidence that it is possible to expand this approach to specifically focus on those diseases in which the primary target is a mucosal tissue, such as the lung or gut, by using oral vaccine preparations which offer their own advantage of ease of administration.

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