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Research Article

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Selective Induction of an Immune Response in Human External Secretions by Ingestion of Bacterial Antigen

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ABSTRACT Ingestion of capsules which contained killed *Streptococcus mutans* by four healthy human subjects led to the appearance of specific antibodies in external secretions. Salivary and lacrymal antibodies were detected within 1 wk of ingestion and continued to increase throughout a 14-day immunization period, with a gradual decline during the 2 ensuing months. A second period of immunization resulted in a pronounced increase of specific antibody levels which occurred earlier than in the primary immunization period and reached peak levels by day 10. No change was detected in serum antibody levels throughout either immunization period. The antibody activity in all secretions was associated with the immunoglobulin A class, as determined by immunochemical analyses. These data indicate that ingestion of bacterial antigens selectively stimulates the immune response in secretions.

INTRODUCTION

Secretory IgA (s-IgA)[1] is the prevalent class of immunoglobulin on mucous membranes. Produced locally by plasma cells that are distributed in the secretory glands and in the lamina propria of intestinal and respiratory tissues, s-IgA is selectively transported into external secretions (1). However, the mechanisms involved in the stimulation of the immune response in these secretions remain obscure. Parenteral immunization results in the production of serum antibodies that are principally associated with the IgG class, but is nevertheless ineffective in stimulating the secretory immune system (2–6). Only trace amounts of immunoglobulins derived from serum reach external secretions, and their lack of resistance to various proteolytic enzymes makes them unsuitable to function in the mucosal environment. Furthermore, evidence is available to suggest that the serum-derived IgG antibodies may prevent the mucosal absorption of an antigen used in systemic immunization, although the absorption of unrelated antigens is enhanced (7). A secretory immune response, restricted to the site of application of antigen, has been elicited by antigens either locally applied to mucous surfaces (2, 4, 8, 9) or injected into secretory glands (10–15). The appearance of s-IgA-associated antibodies to locally administered antigens (2, 4, 9) suggested that the induction of a secretory immune response is the result of local penetration of antigens with subsequent stimulation of immunocompetent cells in secretory tissues (2, 4, 8, 16). Although effective in stimulating a secretory immune response, injections of antigens into secretory glands are of questionable acceptance because of the anatomical inaccessibility of some glands, the possible requirement of adjuvants, the impairment of normal function of the gland, and the concomitant stimulation of IgG-associated antibodies (15).

The demonstration that oral administration of antigens (in man and animals) induces the appearance of s-IgA antibodies in mammary secretions (6, 17, 18) and the presence in saliva, milk, and colostrum of naturally occurring s-IgA-associated antibodies to bacterial antigens (*Escherichia coli* [19], *Streptococcus pneumoniae* [17], and *Streptococcus mutans* [20]) suggest that local stimulation of the gland may not be required for this induction. We report that ingestion of a bacterial antigen induced the selective appearance of antibodies of the IgA class in the external secretions of human salivary and lacrymal glands, without a serum antibody response.

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[1] *Abbreviations used in this paper*: CFU, colony-forming units; H chains, heavy chains; S-IgA, secretory IgA.

METHODS

Subjects. Four individuals (one female and three males, 30–35 yr old) volunteered for this study. Unstimulated samples of parotid saliva were collected with the use of a plastic intraoral cup designed in this laboratory (21). This device also allowed for the simultaneous collection of separate samples of submandibular-sublingual salivas without the admixture of parotid secretion. This permitted the comparison of the titers of antibodies in secretions of anatomically distinct salivary glands. Whole saliva, which contains secretions of the major and minor salivary glands as well as contributions from the crevicular fluid, was collected by having the subject drool into a sputum cup packed in ice. Lacrymal secretions were collected after irritation of the conjunctiva with a mist of oil expressed from lemon rind. Blood specimens obtained by venipuncture were collected into heparinized tubes; leukocyte and differential counts were determined.

Preparation of antigen. The candidate immunogen was selected through screening of saliva and serum samples by microtitration (20) for agglutinin antibodies to over 40 different strains of the indigenous oral bacterium, *Streptococcus mutans*. *S. mutans* OMZ-176 (sterotype d), to which none of the four subjects demonstrated significant serum or salivary antibody activity, was selected for this study.

To prepare a large amount of bacterial antigen for immunization, 10 liters of dialyzed medium (22) was inoculated with a log phase culture of *S. mutans* OMZ-176 and incubated at 37°C for 16 h. During the last 4 h of culture, the acid formed was neutralized by sterile 1 N NaOH. Cells were harvested by centrifugation at 10,000 *g* for 30 min, washed three times with phosphate-buffered saline (pH 7.0), and resuspended in 0.5% formalin-saline at a concentration of 5×10^9 colony-forming units (CFU)/ml. After 3 days, sterility was tested by culturing in sodium thioglycolate broth and on blood agar. The cells were extensively washed with sterile distilled water and then lyophilized.

Gelatin capsules (no. 000, Parke, Davis & Co., Detroit, Mich.) were filled with 100 mg of desiccated cells (representing approximately 1×10^{11} equivalent CFU). To avoid the contact of extraneous antigen with the oral mucosa, the capsules were repeatedly rinsed with tap water and swallowed with a liberal amount of water; the oral cavity was then thoroughly rinsed. Capsules were taken daily at specified times. In the initial immunization series, capsules were taken for 14 consecutive days; in the second series, for 7 consecutive days. Saliva, tears, and plasma samples were collected at specific intervals.

Immunoglobulin and antibody measurement. Serum immunoglobulin levels were measured by single radial immunodiffusion using commercially obtained immunoplates and standards (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.). IgG and IgM in external secretions were measured with the use of single radial immunodiffusion plates specifically prepared with commercial antisera (Behring Diagnostics) for determining low levels of these immunoglobulins. Dilutions of commercial preparations of IgG and IgM were used as standards. Polymeric s-IgA purified from a pool of human colostrum by ammonium sulfate precipitation, Sephadex G-200 and Sepharose-6B gel filtration (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), and DEAE-cellulose chromatography (23) was used as the standard for single radial immunodiffusion measurements of s-IgA. The concentration of polymeric IgA was standardized spectrophotometrically using an extinction coefficient of $E^{1\%}_{1\ cm\ 280\ nm} = 13.8$. Since the collection of saliva and tears was not quantitative, s-IgA levels in these secretions were related to the concentration of total protein (24) (in micrograms of IgA per milligram of total protein) rather than to flow rates.

To determine the distribution of the antibody activity in saliva and tears, selected samples (first precipitated with ammonium sulfate, then redissolved and dialyzed against 1% ammonium bicarbonate buffer pH 7.0) were applied to a column of Sephadex G-200 (K9/70 cm) equilibrated in the same buffer. Protein concentrations, immunoglobulin levels, and antibody activity were determined on pooled fractions.

Bacterial agglutinations were performed by microtitrations (20) in V-plates (Cooke Laboratory Products Div., Alexandria, Va.). 50 μl of formalinized *S. mutans* OMZ-176 (2×10^8 CFU/ml) was added to serial, twofold dilutions of the sample in barbital buffer (pH 7.4). The plates were incubated for 2 h at 37°C and allowed to stand for 18 h at 4°C. Titers were determined by reading agglutination patterns. When related to the absolute levels of IgA in selected samples of secretions, agglutinin titers displayed similar dynamics to unadjusted samples. In an effort to determine the immunoglobulin class responsible for antibody activity, attempts were made to enhance or inhibit the agglutinin titers with antisera specific for heavy (H) chains of immunoglobulins (IgA, IgM, and IgG). The agglutinated and sedimented bacteria were washed three times with buffer in the microtiter plates. 50 μl of an optimal concentration of H-chain-specific antisera (Behring Diagnostics) was added and the plates were incubated for 2 h at 37°C. After 18 h at 4°C, agglutination patterns were read. Enhancement of titers with H-chain-specific antisera suggested the class of Ig responsible for antibody activity. The inhibition of the antibody activity was performed in a manner similar to that of titer enhancement, except that anti-H-chain or anti-secretory component (25) sera were added to the selected samples of saliva and tears and allowed to incubate for 3 h at 37°C before the antigen was added.

To further confirm the Ig class responsible for agglutinin activity, a suspension of *S. mutans* OMZ-176 was heat-fixed on microscope slides and thoroughly washed with chilled 0.15 M carbonate-bicarbonate buffer, pH 8.5. The smears were incubated for 45 min at ambient temperature with selected samples of saliva, tears, or serum and washed extensively with carbonate-bicarbonate buffer. The slides were incubated for an additional 45 min with appropriate dilutions of fluorescein isothiocyanate-labeled antisera specific for H chains of human IgM, IgG, and IgA, produced either in this laboratory (26) or obtained from a commercial source. Then the slides were washed with buffer, mounted in glycerol, and examined with a fluorescence microscope equipped with a vertical illuminator according to Ploem (Orthoplan, Leitz, Wetzlar, W. Germany). The light source was a mercury lamp (Osram HBO 100 W) equipped with a filter system for narrow-band excitation.

Selected samples of plasma or serum were tested for C-reactive protein, rheumatoid factor, and anti-streptolysin O by the Department of Clinical Pathology at the University of Alabama in Birmingham. Since it has been suggested that *S. mutans* may share antigenic determinants with the human heart, selected serum and saliva samples were assayed for the presence of heart-reactive antibodies by the indirect immunofluorescence techniques of Zabriskie and Freimer (27). Fresh sections of human heart obtained at autopsy were imbedded in OCT compound (Ames Co., Div. of Miles Lab., Inc., Elkhart, Ind.), frozen in liquid nitrogen, and stored at −80°C until sectioning. Desiccated 4-μm sections were incubated with saliva or serum for 1 h at ambient temperature, washed in phosphate-buffered saline

(0.01 M, pH 7.2), and stained with fluorescein isothiocyanate-labeled, rabbit antihuman immunoglobulin (Behring Diagnostics). All sections were examined with a Leitz Orthoplan fluorescence microscope equipped with a vertical illumination and filter system appropriate for narrow-band excitation.

RESULTS

Measurement of the immune response. After the 1st wk of immunization, significant agglutinin titers were observed in external secretions (Fig. 1 a–d). These titers continued to rise throughout the immunization period and reached their highest levels 10–25 days from the initial ingestion of antigen. During the ensuing 50 days, the antibody titers slowly declined but did not decrease to preimmunization levels. 66 days after the termination of the primary immunization, a second (7-day) period of antigen ingestion was begun. This secondary challenge resulted in an earlier, more pronounced increase in the levels of specific antibodies. Parallel dynamics of antibody appearance and titers were observed in parotid, submandibular-sublingual, and whole salivas, as well as tears, after both primary and secondary stimulations. Augmentation of agglutination with H-chain-specific antisera (Table I) revealed that agglutinin titers of parotid saliva and tears could be specifically enhanced when the washed and redispersed agglutinates were incubated with antisera specific for the α-chain; no effect was evident with antisera to μ- and γ-chains. The agglutinin activity in selected samples of saliva and tears could be abolished or substantially reduced by the addition of anti-α-chain-specific antiserum to these samples before titration by agglutination. Likewise, agglutinin activity could be inhibited by pretreatment of samples with anti-secretory component serum. Examination of bacterial smears that had been incubated with samples of secretions and then washed and reacted with fluorescein isothiocyanate-labeled antisera to H-chains corroborated the IgA nature of the induced antibodies. Also, when selected samples of secretions were fractionated on Sephadex G-200, the antibody activity was detected in a fraction that corresponded to the elution position of human s-IgA (Fig. 2). The s-IgA character of the antibodies was confirmed by anti-H-chain enhancement and anti-secretory component inhibition of agglutination and by indirect immunofluorescence on bacterial smears. With the exception of an apparently negligible increase in serum agglutinins in subject 1 (Table I), no changes in serum titers were observed during either immunization period. Serum agglutinins to this strain of *S. mutans* that were evident before, during, and after the oral immunization (Table I) belonged primarily to the IgM and IgG classes as ascertained by the enhancement of the agglutination with μ- and γ-chain antisera, but not α-chain antisera. The induced antibodies were specific for the immunizing strain because there was no comparable increase in agglutinin titers to other non-cross-reacting serotypes of *S. mutans* (Table II). A slight increase in agglutinin titers was observed with *S. mutans* 6715 (serotype g), which may be explained by the presence of common antigens between serotypes d and g (28).

No alterations in body temperature, in the leukocyte counts and differentials, or in serum immunoglobulin levels were observed after ingestion of large doses of *S. mutans* OMZ-176 (2.1 g). Likewise, C-reactive protein, anti-streptolysin O, and rheumatoid factor titers were uninfluenced by ingestion of this bacterial antigen. An apparent rise was noted in salivary IgA levels and agglutinin titers (0.82 correlation). Human heart-reactive antibodies could not be detected by indirect immunofluorescence in saliva and serum samples collected before, during, and after immunization.

DISCUSSION

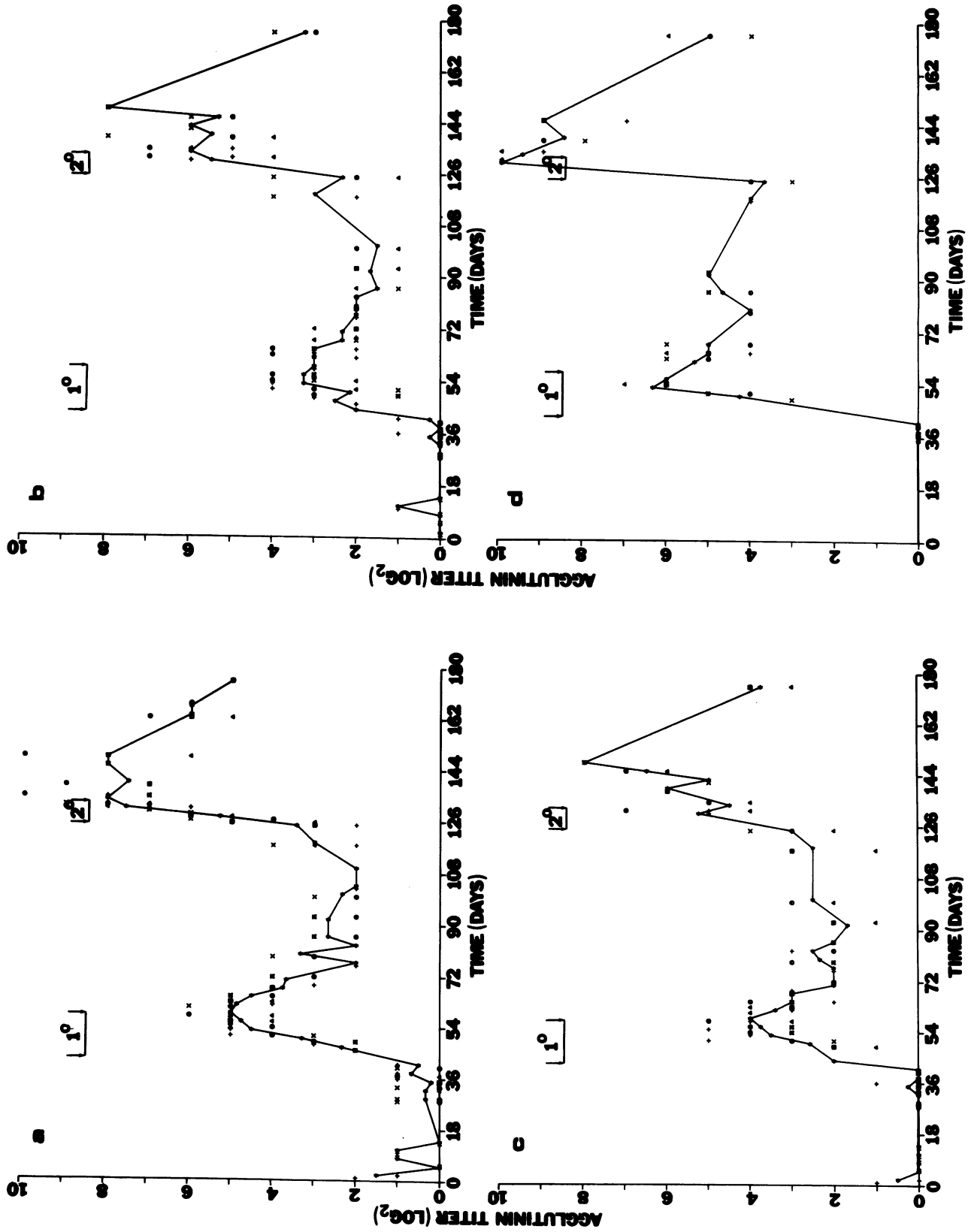
The results of this study indicate that ingestion of bacterial antigen by humans resulted in the selective induction of a humoral immune response that appeared simultaneously in secretions of salivary and lacrymal glands. The dynamics of antibody production observed after the second series of antigen ingestion

TABLE I

Anti-S. mutans OMZ-176 Agglutinin Titers of Selected Samples of Parotid Saliva, Tears, and Serum after Augmentation with H-Chain-Specific Antisera (Anti-α, Anti-γ, and Anti-μ)

	Subject 1			Subject 2			Subject 3			Subject 4		
Period	Parotid	Tears	Serum	Parotid	Tears	Serum	Parotid	Tears	Serum	Parotid	Tears	Serum
Preimmunization	1(2,1,1)*	<1(1,<1,<1)	1(1,2,2)	<1(1,<1,<1)	<1(1,<1,<1)	2(2,4,2)	1(2,1,1)	1(1,<1,<1)	2(2,4,3)	1(2,<1,<1)	1(2,1,1)	2(1,4,4)
Peak primary	5(8,5,5)	5(9,5,5)	2(2,4,4)	4(7,4,4)	6(8,6,6)	1(1,3,2)	3(8,5,5)	6(8,6,6)	2(2,4,4)	6(9,6,6)	7(8,7,7)	2(2,4,4)
Postprimary	3(5,3,3)	2(6,2,2)	3(3,4,4)	3(5,3,3)	3(6,3,3)	2(2,4,3)	2(4,3,3)	3(5,3,3)	2(2,4,4)	3(5,3,3)	4(6,4,4)	2(3,4,4)
Peak secondary	10(12,10,10)	9(12,9,9)	2(2,4,5)	7(9,7,7)	11(12,11,11)	2(2,3,3)	8(11,8,8)	9(11,9,9)	3(3,5,4)	9(12,9,9)	10(>12,10,10)	2(2,4,4)
Postsecondary	5(8,5,5)	4(7,4,4)	1(1,3,2)	3(6,3,3)	6(8,6,6)	2(2,3,3)	5(7,5,5)	5(9,5,5)	2(2,4,4)	5(8,5,5)	8(12,8,8)	3(3,4,5)

* Log_2 agglutinin titer determined by microtitrations with 2×10^8 CFU/ml of formalin-killed *S. mutans* OMZ-176. Numbers in parentheses represent the $\log_2$ agglutinin titers after washing and reacting with H-chain-specific antisera (anti-α, anti-γ, and anti-μ, respectively).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144
162
180

implied that an anamnestic response could be induced in secretions. The presence of these antibodies raises a question as to the mechanisms involved in their induction.

Several possible explanations may be offered for the appearance of these antibodies in glandular secretions remote from the apparent site of antigenic stimulation. An ingested antigen could be absorbed from the gastrointestinal tract, enter the circulation, and after deposition in secretory tissues, stimulate immunocompetent cells. This explanation is unlikely because circulating antigens should also induce a pronounced serum antibody response; this had not been observed in our experiments or in investigations performed in other laboratories (6, 17, 19, 29). Direct introduction of the antigen into circulation by i.v. immunization induced an excellent response in serum but stimulated no s-IgA antibodies in secretions (5, 6, 17). Further, if an immunogen is carried to secretory tissues by the circulation, serum antibodies should actually prevent the antigen from reaching the secretory tissues and inhibit a secretory immune response. Montgomery and co-workers (6) have recently demonstrated that high titers of antibodies in serum of systemically (intravenously) immunized animals did not prevent the induction of a secretory response by subsequent oral immunization with the identical antigen. The deposition of antigens (with or without adjuvants) into the parenchyma of secretory glands results in production of antibodies associated with IgA as well as IgG; the IgG class is usually more prevalent (15).

A more probable explanation for the occurrence of antibodies in glandular secretions, as a consequence of the ingestion of antigens, involves the selective seeding of committed precursors of IgA-producing cells from a central site of antigenic stimulation to remote secretory glands (6, 17–20, 29–32). This hypothesis is based upon several experimental observations. It has been demonstrated that the micropinocytotic activity of the epithelial cells covering gut (or bronchial-)-associated lymphoid tissues allows an active transport of both soluble and particulate antigens or their fragments to the underlying lymphoid cells (31, 33). Antigenic stimulation induces the differentiation and proliferation of bone marrow-derived (B) and thymus-derived (T) cells, followed by an exodus of these cells through ductus thoracicus into the general circulation. The committed cells subsequently

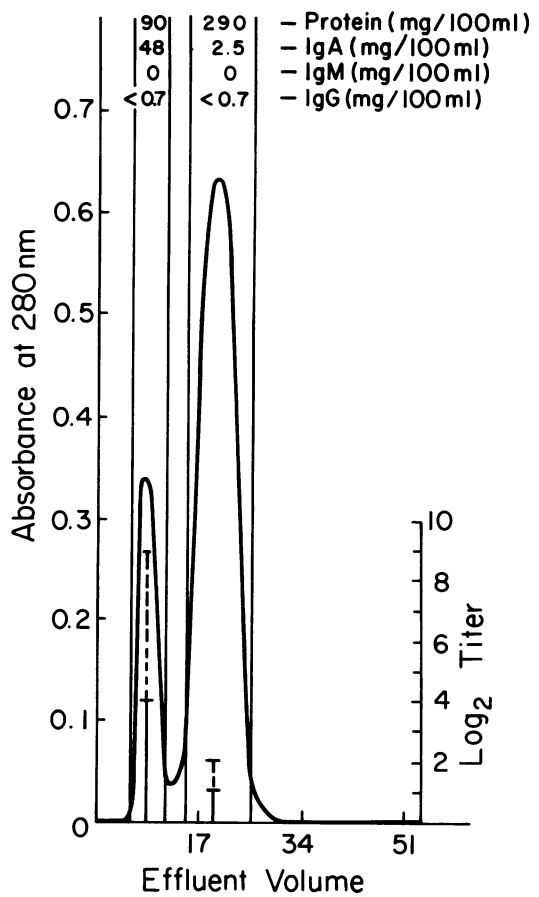

FIGURE 2 Sephadex G-200 filtration (column 1.6 × 100 cm) of ammonium sulfate-precipitated parotid saliva (after primary immunization) from one of the subjects. Solid lines under the absorbance profile represent the $\log_2$ anti-*S. mutans* OMZ-176 agglutinin titers of the pooled fraction. Broken lines indicate the titers of the same samples after augmentation with anti-α antisera, as determined by the microtitration technique. Levels of IgA, IgM, and IgG were determined by single radial immunodiffusion and protein concentrations by the Lowry method (24).

settle preferentially in the secretory tissue and differentiate into IgA-producing plasma cells. Gut- and bronchial-associated lymphoid tissues and mesenteric lymph nodes are indeed enriched sources of IgA precursor cells that exhibit a strong preferential homing for the intestinal and respiratory tracts as well as for secretory glands (32, 34–40). A convincing demonstration of the function of Peyer's patches as sites for antigen stimulation of IgA precursor cells was provided

FIGURE 1 Dynamics of the agglutinin antibody response to *S. mutans* OMZ-176 in glandular secretions after primary (1°) and secondary (2°) oral immunization: (a) parotid saliva; (b) submandibular-sublingual and minor gland saliva; (c) whole saliva; (d) tears. Individual agglutinin titers are represented by the following symbols: ○, subject 1; △, subject 2; +, subject 3; and ×, subject 4. The curve represents the mean $\log_2$ agglutinin titers for all samples collected per 3-day period.

TABLE II

Anti-S. mutans (Serotype a→e and g) Agglutinin Titers in Selected Parotid Saliva Samples after Oral Immunization with S. mutans OMZ-176

Subject	Period	*S. mutans* strain					
		AHT (a)	BHT (b)	10449 (c)	OMZ-176 (d)*	LM-7 (e)	6715 (g)
1	Preimmunization	6‡	8	7	1	9	9
	Peak secondary	7	8	7	10	8	10
2	Preimmunization	5	7	7	<1	8	5
	Peak secondary	5	6	6	7	7	7
3	Preimmunization	8	9	9	1	7	5
	Peak secondary	8	8	8	8	6	9
4	Preimmunization	6	—	6	1	—	7
	Peak secondary	5	—	5	9	—	7

* Immunization strain.

‡ Log_2 agglutinin titer determined by microtitrations with 2×10^8 CFU/ml of formalin-killed *S. mutans* a→e and g (20).

by Robertson and Cebra (41). When a single Peyer's patch in an isolated rabbit intestinal loop was stimulated with a bacterial antigen, antibodies were produced in other parts of the intestine. However, the introduction of the same antigen into an ileal loop, which lacked Peyer's patches, failed to induce an immune response in other portions of the gut. The appearance of s-IgA-associated antibodies in saliva and milk, but not in serum, as a consequence of natural (20) or artificial oral immunization (6, 17–19, 29, 30), indicates that this mechanism of s-IgA induction is also operational in secretory glands. The inability to induce s-IgA antibodies in secretions remote from the site where antigens were applied (2, 4, 8, 9) may be explained by the lack of involvement of lymphoid tissues such as Peyer's patches, which have the potential to seed distant secretory tissues with sensitized precursors of IgA-producing cells.

Although there is no direct evidence concerning the origin of IgA-precursors in salivary and lacrymal glands, a recent report indicates that precursors of IgA-producing plasma cells found in mammary glands originate from gut-associated lymphoid tissues and mesenteric lymph nodes (40). The suggestion that precursors of IgA-producing plasma cells of human salivary glands may indeed originate at distant sites is based on studies of patients undergoing extracorporeal cesium irradiation of blood. A dramatic decrease in salivary IgA levels was displayed, whereas the serum levels remained unchanged (42).

The parallel appearance of antibodies at anatomically remote secretory sites such as salivary and lacrymal glands (in the apparent absence of a serum response) as a consequence of the ingestion of bacterial antigen suggests the existence of a common mechanism for the induction of an immune response on mucosal surfaces. The reason for the absence of a pronounced serum response after oral immunization is not clear; however, the induction of a selective systemic hyporesponsiveness could be considered (43).

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