Comparison of an Adherence Domain and a Structural Region of *Streptococcus mutans* Antigen I/II in Protective Immunity against Dental Caries in Rats after Intranasal Immunization

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Previous studies have identified an N-terminal saliva-binding region (SBR) on *Streptococcus mutans* surface antigen I/II (AgI/II) and suggested its importance in the initial adherence of *S. mutans* to saliva-coated tooth surfaces and subsequent development of dental caries. In this study, we compared the SBR with a C-terminal structural region of AgI/II (AgII) in their abilities to induce protective immunity against caries in rats. When SBR, AgII, or the whole AgI/II molecule was administered intranasally as a conjugate with the B subunit of cholera toxin (CT), in the presence of CT adjuvant, substantial levels of salivary immunoglobulin A anti-AgI/II antibodies were induced. Evaluation of caries activity showed that the SBR, though not as protective as the parent molecule, was superior to AgII and thus can be further considered as a component in a multivalent caries vaccine.

Dental caries is an infectious disease in which *Streptococcus mutans* plays a major role. Although not life-threatening, this oral disease is among the most prevalent and costly in developing as well as industrialized countries (12, 19). An important first step in pathogenesis involves adherence of *S. mutans* to the saliva-coated tooth surfaces; adherence is mediated by a 185-kDa surface fibrillar protein referred to as surface antigen I/II (AgI/II), PAc, or P1 (2, 16). This adhesin has thus received attention as a target for immunological intervention against caries via salivary immunoglobulin A (IgA)-mediated inhibition of the initial adherence. In this regard, it has been demonstrated that human secretory IgA antibodies to AgII are capable of blocking the binding of *S. mutans* to saliva-coated hydroxyapatite (8). It has also been shown that intranasal (i.n.) immunization of rats with AgII, conjugated to the B subunit of cholera toxin (CT) for targeting and adjuvant action, induces specific salivary IgA antibodies and confers protection against caries development (10).

According to several reports from independent research groups (3, 6, 13), the functional domain of AgI/II responsible for initial adherence is localized on the N-terminal one-third of the molecule, which contains an alanine-rich repeat region. To render this saliva-binding region (SBR) immunogenic by mucosal routes of administration, we genetically linked the SBR with the A2 and B subunits of CT (SBR-CTA2/B) to construct a chimeric protein resembling CT in which the CT toxic A1 segment, namely AgII, which represents the C-terminal and cell-surface-proximal region of the adhesin (17). On the native AgI/II molecule, the SBR and AgII are well separated by a segment of approximately 50 kDa. AgII is comparable in size to the SBR (AgII, 48 kDa; SBR, 42 kDa), but unlike the SBR, it does not contain adhesion epitopes (6). Thus, the present study was designed to determine the comparative protective potential of salivary IgA responses directed against either an adherence domain (SBR) or a structural region without any known functional significance (AgII).

Groups of germfree, 19-day-old Fischer rats were immunized by the i.n. route three times at 14-day intervals with 50 µg of SBR-CTA2/B chimeric protein, AgII-CT B, or AgII/CT B chemical conjugates (see below) in the presence of an adjuvant amount (1 µg) of CT (List Biological Laboratories, Campbell, Calif.). These groups, as well as an immunized control group, were orally infected (on days 3, 4, and 5 after the initial immunization) with *S. mutans* UA130 and fed a cariogenic diet (10). The presence of *S. mutans* after infection was confirmed by microbiologic techniques (10). All groups consisted of five rats except for the AgII/CT B group, which contained four (however, one died before the termination of the experiment).

The immunogens used in this study were prepared as follows. SBR-CTA2/B was purified from *Escherichia coli* cell extracts by size-exclusion chromatography followed by anion-exchange chromatography, as previously described (5). Native AgII was chromatographically isolated from the culture supernatants of *S. mutans* IB162 (9), and part of the yield was digested with pronase to generate the protease-resistant AgII (17). Recombinant CT B (rCTB) was purified from periplas-
mic extracts of *E. coli* MTD9 (4) by galactose affinity chromatography (21). AgII and AgI were conjugated to rCTB by means of *N*-succinimidyl-(3-[2-pyridyl]-dithio)propionate (10).

To assess the antibody responses, serum and saliva samples were obtained 1 day before the initial immunization and at the termination of the experiment, i.e., 2 weeks after the last immunization (10). Moreover, at termination, mice were killed, and mandibles were removed, cleaned, stained with murexide, and hemisectioned for caries evaluation by the Keyes method, as previously described (10).

The levels of isotype-specific antibodies in serum and saliva and of total salivary IgA were determined by enzyme-linked immunosorbent assay on microtiter plates coated with native AgI/II, recombinant SBR (isolated from *E. coli* lysates by metal-chelation chromatography [20]), AgII, Gm ganglioside (Calbiochem, La Jolla, Calif.) followed by CT, or goat anti-rat IgA. Peroxidase-conjugated goat antibodies to the appropriate rat immunoglobulin isotype (IgG for serum samples and IgA for saliva samples) served as the developing reagents (all goat anti-rat antibodies were provided by Roger Lallone, Brookwood Biomedical, Birmingham, Ala.). The concentrations of antibodies and total IgA in test samples were calculated by interpolation on standard curves generated with a Fischer rat immunoglobulin reference serum (15), and data were expressed as geometric means ± standard deviations.

One-way analysis of variance in conjunction with the Bonferroni multiple-comparisons test (InStat program; GraphPad Software, San Diego, Calif.) was used for statistical evaluation of antibody response and caries activity data.

The requirement of intact CT as an adjuvant in this study was determined after a preliminary i.n. immunization experiment using rCTB conjugated with AgII or its SBR and AgII derivatives. Unexpectedly, rats responded very weakly or not at all in terms of serum and salivary antibodies to either the streptococcal antigens or rCTB. This unresponsiveness (at least by the Fischer strain of rats), which was reproduced in an additional experiment (data not shown), was somewhat surprising since rCTB is readily immunogenic by the i.n. route in mice (5, 21) and in humans (1). In a previous i.n. immunization study in which AgII/CT B conjugate was found to be immunogenic and protective in the Fischer rat model (10), CT B was obtained from a commercial source and contained traces of holotoxin. Therefore, to address the question of whether the SBR is also a protective immunogen against caries, we included 1 µg of CT adjuvant per dose for i.n. immunization of rats.

All immunized groups (SBR-CTA2/B, AgII-rCTB, and AgII/II-rCTB) generated salivary IgA and serum IgG responses to AgII (and CT) (Fig. 1A) at significantly higher levels (*P* < 0.01) than those seen in the unimmunized/infected control and in preimmune samples. Salivary IgA antibodies induced in the SBR-CTA2/B + CT immunized rats recognized SBR and the parent AgII molecule (specific antibody > 2% of total IgA) but not the AgII component (Fig. 1A). The converse was true after AgII-rCTB + CT immunization, i.e., salivary IgA antibodies reacted with the parent molecule and AgII, but essentially no reactivity to the SBR was seen (some background antibody activity is probably due to oral infection with *S. mutans*). The salivary IgA response induced by AgII/II-rCTB + CT was directed primarily to AgII rather than to SBR (Fig. 1A). Similar results were previously obtained in mice immunized with AgII/II-CT B chemical conjugates by the i.n. route. In that more than 60% of the salivary IgA anti-AgII antibody activity was directed against AgII and only about 10% was directed against the SBR (unpublished data). These findings are suggestive of antigenic competition, which might serve as a microbial protective mechanism whereby *S. mutans* directs the host response to a part of the AgII molecule that is not involved in adherence. Serum IgG responses to AgII and its components followed a pattern similar to that seen in saliva (Fig. 1B). AgII was more immunogenic than the SBR with regard to their respective abilities to induce serum IgG antibodies to the whole AgII or to themselves (*P* < 0.01). We then examined the effectiveness of the induced salivary IgA (and possibly serum IgG) antibodies in protection against *S. mutans*-induced caries, which should depend on both the magnitude and, more importantly, the epitope specificity of the response.

Rats immunized with AgII/II-rCTB + CT or SBR-CTA2/B + CT were significantly protected (*P* < 0.01) from dental caries compared with unimmunized controls (Fig. 2). This is in contrast to animals immunized with AgII-rCTB + CT, which gen-

**FIG. 1.** Levels of salivary IgA (A) and serum IgG (B) antibodies to SBR, AgII, AgII, and CT in unimmunized rats infected with *S. mutans* and in infected rats immunized by the i.n. route with SBR-CTA2/B, AgII-rCTB, or AgII/II-rCTB. Immunized animals were given three doses of the appropriate chimeric immunogen supplemented with CT adjuvant at 14-day intervals. Results are from samples collected two weeks after the last immunization and are expressed as the geometric means ± standard deviation.
Unimmunized rats and rats immunized by the i.n.
route with SBR, AgII, or AgI/II (linked to rCTB and with CT as an adjuvant) were infected with S. mutans UA130 and fed a cariogenic diet. Values are the mean caries scores for proximal enamel and sulcal dentinal slight lesions (18), as a component in a combined mucosal vaccine. Such combination may result in an additive or synergistic effect and in greater protection against this oral disease.

We thank Cecily Harmon and Pam Smith for excellent technical assistance, Vickie Barron for secretarial assistance, and Roger Lallone for providing antibody reagents.

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9. Hardy, L. N., K. W. Knox, R. A. Brown, A. J. Wicken, and R. J. Fitzgerald. Approximately in the middle of the molecule) also suppresses the binding of S. mutans to saliva-coated hydroxyapatite (14). Interestingly, Scatchard analysis of AgI/II binding to saliva-coated hydroxyapatite suggested the presence of more than one binding site (6).

The finding that the SBR was in general a more protective immunogen than AgII was not surprising because of the SBR’s postulated functional significance as an adhesion domain in S. mutans infection. Limited caries inhibition in rats immunized with AgII could possibly be explained by anti-AgII antibody-mediated aggregation and clearance of S. mutans from saliva. An additional mechanism of protection against caries might be opsonization of S. mutans, with subsequent phagocytosis by polymorphonuclear leukocytes in the area of the tooth that is bathed with gingival crevicular fluid (18). In this respect, IgG antibodies to AgII are less opsonic than antibodies to AgI (18), which contains the SBR and constitutes the N-terminal two-thirds of AgI/II. It is also noteworthy that early experiments on systemic immunization of rhesus monkeys revealed that animals immunized with AgI/II or AgI developed fewer caries lesions than those immunized with AgII (11).

In summary, it appears that the SBR, although more protective than AgII, may not be capable of substituting for the whole AgI/II molecule in a caries vaccine. However, AgI/II appears to contain potentially harmful epitopes, which cross-react with human IgG, located at the C-terminal part of the molecule (13). On the other hand, no such presumably deleterious epitopes have been identified within the SBR. Our present results indicate that the SBR should be considered, along with other virulence factors (including those important at a later stage of the infection [for a review, see references 12 and 19]), as a component in a combined mucosal vaccine against dental caries. Such combination may result in an additive or synergistic effect and in greater protection against this oral disease.

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