INFECTION AND IMMUNITY, Feb. 1996, p. 665–667 Vol. 64, No. 2
0019-9567/96/$04.00+0

Persistence of Serum and Salivary Antibody Responses after Oral Immunization with a Bacterial Protein Antigen Genetically Linked to the A2/B Subunits of Cholera Toxin

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Received 15 August 1995/Returned for modification 4 October 1995/Accepted 14 November 1995

Primary oral immunization of mice with a bacterial protein antigen genetically coupled to the A2 and B subunits of cholera toxin induced specific secretory immunoglobulin A and serum immunoglobulin G antibodies that persisted at substantial levels for at least 11 months. A subsequent single booster immunization did not further enhance the antibody responses. Long-term antibody persistence may be especially important in infections caused by common pathogens for which continuous immunity would be advantageous.

We have recently developed a generally applicable oral immunization strategy by which the desired mucosal immunogen is genetically fused to the A2 subunit of cholera toxin (CT), which mediates association with the B subunit of CT, a potent immunoenhancing agent (3). The antigen we selected for evaluating the oral immunogenicity of such nontoxic CTA2/B-based constructs is the saliva-binding region (SBR) of the AgI/II adhesin from the oral bacterium *Streptococcus mutans*. The SBR that is genetically linked to CTA2/B, designated SBR-CT$^{\Delta A1}$, was found to be immunogenic by the oral route and elicited high levels of secretory immunoglobulin A (S-IgA) and serum IgG antibodies to AgI/II (3). Despite its great importance for mucosal defense, the S-IgA antibody response is often of relatively short duration, lasting from a few weeks in experimental animals to a few months in humans (6). The issue of whether the secretory immune system is capable of anamnestic immune responses has been debated, and recent studies with mice and humans have addressed the concept of immunological memory at the mucosal surfaces (11, 15). Immunological memory can be manifested as a long-lasting immune response or as a faster and more vigorous anamnestic response to reencounter with an antigen. A desirable vaccine characteristic is the induction of prolonged immune responses, especially when the pathogenic organism is frequently encountered at mucosal surfaces, in which case a continuing level of immunity may be necessary.

The aim of this study was to evaluate the duration of antibody responses to the AgI/II adhesin after oral immunization of mice with SBR-CT$^{\Delta A1}$ about 1 year earlier (3). One group consisted of five mice previously given three doses of 100 μg of SBR-CT$^{\Delta A1}$ together with 5 μg of intact CT as an adjuvant [except for animal 5 in panels A and C of both Fig. 1 and 2, the mice were given the dose adsorbed on $Al(OH)_3$, which was shown to enhance serum IgG antibody responses after oral immunization (3)]. A second group comprised three mice which were similarly treated with the exception that they were immunized in the absence of intact CT. A third group consisting of six mice which were sham immunized with buffer only were used as naive controls. Saliva and serum samples were collected 11 months after the last dose of the primary immunization, and all three groups of mice were subsequently given 100 μg of SBR-CT$^{\Delta A1}$ by gastric intubation (3). CT adjuvant (5 μg) was coadministered to those mice that had also received CT during the primary immunization and to half of the naive control animals. Samples of saliva and serum were collected again 7 days after the booster immunization, and antibody responses were evaluated by enzyme-linked immunosorbent assay on plates coated with native AgI/II and CT. Unknown antibody concentrations were calibrated against mouse immunoglobulin reference serum standards assayed simultaneously in the same microtiter plate (3). Results were evaluated by Student's *t* test by using the MultiStat program (Biosoft, Cambridge, United Kingdom) with a Macintosh computer. Differences were considered significant at a P value of <0.05.

Substantial levels of serum IgG (Fig. 1) and salivary IgA (Fig. 2) antibodies to AgI/II and CT persisted at least until day 357, although at lower concentrations than immediately after immunization (day 28), even in mice that did not receive an adjuvant dose of intact CT (panels B and D in both Fig. 1 and 2). During the same period, the six sham-immunized mice did not develop detectable serum or salivary antibody responses, except for two animals that showed trace levels of salivary IgA to AgI/II (0.15 and 0.12% of total IgA). The salivary antibody response of the sham-immunized group (0.05% ± 0.07% of total IgA) was significantly less ($P < 0.05$) than the salivary antibody responses in either of the immunized groups (0.82% ± 0.56% of total IgA [Fig. 2A] and 0.51% ± 0.27% of total IgA [Fig. 2B]). The prolonged duration of antibody responses might be explained by persisting antigen that provided a continuous low-level stimulation of memory cells (2). The mechanism of antigen persistence may involve follicular dendritic cells, which bind antigen-antibody complexes via cell surface Fc receptors and slowly release them over long periods (14). Alternatively, the existence of molecules cross-reacting with AgI/II (or cross-reactive enterotoxins in the case of CT) cannot be ruled out, although *S. mutans* is not a natural inhabitant of the murine oral cavity.

A recall response was not observed in serum after the oral booster immunization (Fig. 1), as the antibody responses to AgI/II and CT before and immediately after the booster immunization were not significantly different. However, the mouse (animal 3 in Fig. 1C) that had the lowest antibody levels

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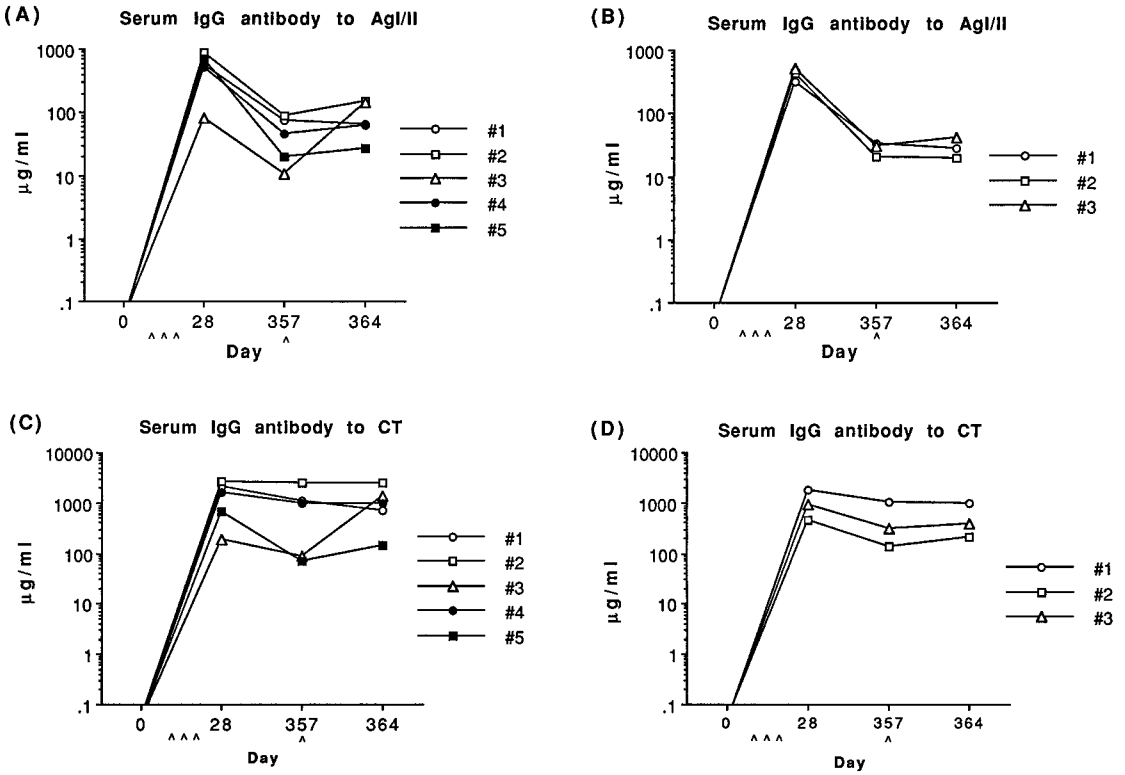

FIG. 1. Persistence of serum IgG antibody to AgI/II and CT after peroral immunization of mice with SBR-CT$^{\Delta A1}$ chimeric protein (∧∧∧) and a single booster immunization 11 months later (∧). Mice were given the immunogen in the presence (A and C) or absence (B and D) of CT adjuvant. Data are presented for each mouse individually.

to CT showed a remarkable 16-fold increase in the level of serum IgG, resulting in a higher final response than was observed shortly after the primary immunization. This mouse also showed an enhanced anamnestic IgG response to AgI/II which was 13 times higher than that observed immediately prior to the booster immunization (Fig. 1A). This finding suggests that anamnestic responses are not readily elicited in the presence of a relatively high persisting antibody response. As expected from previous studies (3), naive mice developed a poor IgG antibody response to AgI/II or CT upon challenge with one dose of SBR-CT$^{\Delta A1}$ (≈1 μg/ml; data not shown).

An enhanced salivary IgA anamnestic response was not observed in the mice following the oral booster immunization, even when CT was used as an adjuvant (Fig. 2A and C). Trace levels of salivary antibody to AgI/II observed in two of the six naive mice were not altered after the single booster immunization, while the remaining mice did not develop any salivary response (data not shown), as expected from previous studies (3). The lack of a typical recall response in saliva was also seen in previous experiments in which mice were perorally immunized with native AgI/II chemically coupled to CTB plus CT adjuvant and challenged 6 months later (13). However, CT has been shown to induce long-term memory to an unrelated antigen, keyhole limpet hemocyanin, as evidenced by an increased number of specific IgA antibody-producing cells in the gut lamina propria after booster immunization (15). It appears that, after peroral immunization, the anamnestic response in the salivary glands may depend on recruitment of memory cells from the Peyer's patches or other mucosal induction sites, whereas the gut lamina propria may possess an additional source of memory represented by local memory cells that differentiate into plasma cells upon in situ activation by antigen adsorbed through intestinal epithelial cells (10). This might result in the memory response being manifested more readily at the gut lamina propria than at a remote effector site such as the salivary glands. Rats immunized orally with polysaccharide-peptide conjugates in liposomes (8) did show enhanced anamnestic antibody responses in saliva against both components, but in this study both the primary and secondary responses were of short duration (about 30 days).

SBR represents an AgI/II adherence domain that mediates the binding of *S. mutans* to the saliva-coated tooth surfaces (1, 4). S-IgA antibodies to the whole AgI/II molecule inhibit *S. mutans* adherence in vitro (5) as well as *S. mutans* colonization and dental caries development in vivo (7). It is, therefore, expected that salivary S-IgA antibodies against the adherence domain of AgI/II will also confer protection, and we are currently testing this possibility in an experimental rat caries model. Since *S. mutans* infects more than 95% of the human population and caries is a common infectious disease, the continuous presence of salivary S-IgA as well as serum-derived IgG antibodies may be necessary to suppress an organism that is continually present in the oral cavity (9, 12). Our data show that induction of long-term antibody responses is possible upon primary immunization with the SBR-CT$^{\Delta A1}$ chimeric protein. This is further supported by the finding that AgI/II-responsive T cells persist in cervical and mesenteric lymph nodes for at least 6 months after immunization (13a). Application of this immunization strategy to other mucosal infections by linking identified candidate immunogens to CT$^{\Delta A1}$ may elicit similarly prolonged mucosal antibody responses.

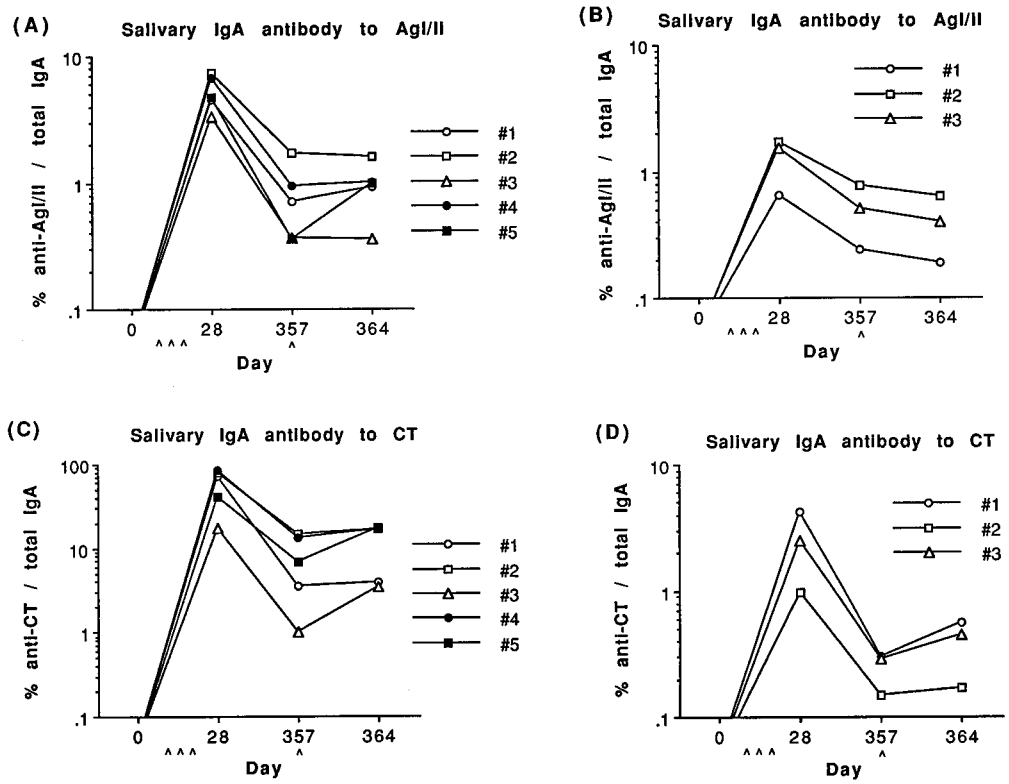

FIG. 2. Duration of salivary IgA antibody to AgI/II and CT following peroral immunization of mice with SBR-CT$^{\Delta A1}$ chimeric protein (∧∧∧) and a single booster immunization 11 months later (∧). Mice were given the immunogen in the presence (A and C) or absence (B and D) of CT adjuvant. Data are presented for each mouse individually.

We thank Hong-Yin Wu for valuable advice.

This work was supported by U.S. Public Health Service grants DE06746, DE08182, and DE09081.

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Editor: V. A. Fischetti