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# Alveolar Bone Loss in Rats After Immunization with *Actinomyces viscosus*

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We investigated a possible cause-and-effect relationship between sensitization against *Actinomyces viscosus* Ny1 and destructive periodontal disease in RIC-Sprague-Dawley rats. Germfree rats (66) were either immunized with *A. viscosus* Ny1 (day 20) or orally infected with *A. viscosus* Ny1 (days 38 and 39) or both. We measured alveolar bone loss in maxillary and mandibular molars, in vitro T-lymphocyte responsiveness, and serum antibody titers. In immunized and monoassociated rats bone loss in both jaws progressed rapidly between days 37 and 72, whereas the rate of further resorption decreased until day 100. In monoassociated rats, development of bone loss was much slower, and the maximum resorption measured was, at best, half of the bone loss compared with the former group. However, no amplification of bone loss by immunization was observed in a second experiment using 63 conventional rats kept in relative gnotobiosis. Antibody titers to *A. viscosus* Ny1 in gnotobiotic monoassociated rats were higher in immunized animals, whereas no difference was found in the respective groups of the relative gnotobiotic experiment. The fact that immunization more than doubled alveolar bone loss in gnotobiotic monoassociated rats confirms the allergic nature of the disease. The lack of such an effect under conventional conditions points to suppressor mechanisms whose decrease might convert stable periodontal lesions into progressive ones.

Immunopathological mechanisms evoking gingival inflammation and destruction of alveolar bone have gained increasing attention again. Decades after Hulin (18) reported periodontal disease to be the host's allergic response to orally presented antigens, antibodies to oral microorganisms were shown in human periodontal disease (15), immunoglobulin-bearing lymphocytes and plasma cells were demonstrated in periodontal lesions (29, 33), and many reports have correlated the degree of in vitro lymphocyte stimulation by antigens from oral microorganisms with the severity of human gingivitis and periodontal disease (2, 20, 28, 35). In contrast to these studies in humans, in gnotobiotic rats monoinfected with *Eikenella corrodens*, measurement of in vitro lymphocyte stimulation suggested that cell-mediated immunity in periodontal disease was "protective" rather than "destructive" (22).

We have recently overcome the problem of measuring cell-mediated immunity to *Actinomyces viscosus* Ny1 with rodent lymphocytes, caused by the inherent B-cell mitogenicity of *Actinomyces* sp. fractions (8), by culturing purified T lymphocytes instead of nonseparated spleen cells (5, 6). Consequently, we reported the sensitization by oral infection with *A. viscosus* Ny1 of gnotobiotic rats and showed that progress of bone loss paralleled both the increasing serum agglutinin titers and the increasing sensitization of T-cells (Burckhardt et al., J. Periodontal Res., in press).

Here we examined a possible cause-and-effect relationship between sensitization against *A. viscosus* Ny1 and destructive periodontal disease. We show the allergic nature of the disease because immunization against *A. viscosus* Ny1 more than doubled the amount of bone loss in gnotobiotic rats, but had no effect in animals in relative gnotobiosis (25). This difference in severity of destruction between gnotobiotic and conventional rats was well reflected by antibody titers but not by the results of the in vitro lymphoproliferative assays.

## MATERIALS AND METHODS

**Animals.** Inbred RIC-Sprague-Dawley rats were used. In the gnotobiotic experiment, 66 germfree rats (average age, 19 days) from six litters were weaned and transferred to screen-bottom stainless-steel cages in three plastic isolators. Diet 2000s, sterilized by gamma radiation (3 Mrad; Eidgenössische Forschungsanstalt, CH-8820 Wädenswil), and autoclaved fluoride-free tap water were available ad libitum.

The following treatments were randomly distributed among the isolators: (A) immunization with *A. viscosus* Ny1 on day 20, germfree control; (B) sham immunization, monoassociation with *A. viscosus* Ny1 on days 38 and 39; and (C) immunization as for group A and association as for group B. At 37, 58, 72, and 100 days of age, five or six rats were removed from each treatment, weighed, exsanguinated by decapitation, and splenectomized.

The body weights of the animals have been published together with plaque extent and extent and severity of smooth-surface caries (7).

In the second experiment in relative gnotobiosis (25), 63 conventional rats of the same strain were grouped and treated as above, but the controls in group A were sham immunized. To suppress the indigenous gram-positive flora, all animals from 34 to 37 days of age were treated with 2 g of penicillin (Novo Industry A/S, Bagsvaerd, Denmark) per liter in the drinking water, and animals 37 days of age and older received, by the same route, 0.2 g of erythromycin (Ilotycin, Gluceptate, Dista Products Co. Ind.) per liter. This provided favorable conditions for the orally associated erythromycin-resistant strain *A. viscosus* Ny1E.

**Bacterial strain, culture conditions, and preparation of bacterial antigens.** *A. viscosus* Ny1 was obtained from J. S. van der Hoeven, University of Nijmegen, The Netherlands. The strain was grown in a defined medium (3) when used for immunization and to prepare a broken cell supernatant (BCS) antigen fraction as previously described (5). Briefly, strain Ny1 cells were disintegrated, the particulate fraction was removed by high-speed centrifugation, and the supernatant was concentrated by precipitation with 80% saturated ammonium sulfate. The erythromycin-resistant mutant strain Ny1E was selected by continuously culturing the strain in actinomyces broth (BBL Microbiology Systems) with increasing amounts of the antibiotic up to 1 g/liter.

**Immunization.** On day 20, germfree rats of groups A and C were immunized with 2 mg of lyophilized cells of strain Ny1 emulsified in 0.1 ml of equal parts of water and incomplete Freund adjuvant (Difco Laboratories) as previously described (7). Rats of group B were sham immunized omitting the bacterial cells. In the experiment in relative gnotobiosis, the 21 rats of group C were immunized as above, and the rats of groups A and B were sham immunized.

**Oral association of *A. viscosus* Ny1.** On days 38 and 39, germfree rats of group B and C were monoassociated with 24-h cultures of strain Ny1 grown in actinomyces broth as previously described (17). The rats in relative gnotobiosis were orally infected with strain Ny1E on days 37 and 38.

**Evaluation of bone loss.** The area defined by the cementoenamel junction and the alveolar bone crest and five vertical distances were measured with a planimeter on prints enlarged from standardized radiographs of defleshed maxillae and mandibles. The detailed methodology will be described elsewhere (Gaegauf-Zollinger et al., manuscript in preparation). The vertical distances between the cementoenamel junction and the alveolar bone crest or deepest point of bone resorption were measured at the distal furcation of the first molars and the furcation of the second and third molars. Interdentally, between the first and second and the second and third molars, the distance was measured perpendicularly from a line drawn between the cementoenamel junction of the two adjacent molars to the deepest point of the interdental pocket. Statistical differences were calculated by using Student's *t* test and one degree of freedom per animal.

**T-lymphocyte cultures and antibody titers.** Spleen cell suspensions, one of each group of rats, were prepared, and T-lymphocyte populations were obtained by filtration through Degalan rat immunoglobulin-anti-immunoglobulin G columns as previously described (5, 6). T-cells, $5 \times 10^5$ per culture in flat-bottomed microtiter plates, were kept in Eagle high-amino acid medium (10) supplemented with 1% syngeneic serum. Cell proliferation induced by concanavalin A (ConA) or BCS antigens was measured by the uptake of [*methyl*-$^3$H]thymidine (NET 027A; 2 Ci/mmol; lot 1014-79; New England Nuclear Corp., Boston, Mass.) after 4 days of culture as previously described (5).

Serum antibody titers were determined by agglutinating strain Ny1 cells in microtiter plates as previously described (Burckhardt et al., in press).

## RESULTS

**Alveolar bone loss.** At 37, 58, 72, and 100 days of age we withdrew five or six gnotobiotic rats from each of the three isolators, exsanguinated them by decapitation, and removed their spleens. We determined areas and vertical bone loss on radiographs of longitudinally hemisectioned jaws (Fig. 1, 2, 4, and 5). Figure 3 illustrates the extent of maxillary bone loss in immunized and orally infected rats at 72 days of age.

Because the third molars had not erupted consistently in all animals at 37 days of age, Fig. 1 and 2 show, from day 37 on, areas of bone loss of first and second molars only, whereas Fig. 4 and 5 display vertical bone loss of all molars, but starting at 58 days of age.

Note that in the two infected groups of the gnotobiotic experiment, immunization more than doubled the amount of bone loss compared with the immunized germfree control.

In the second experiment, the relative gnotobiotic rats were killed at an average age of 70 days. Table 1 summarizes their body weights. We determined areas of bone loss and vertical bone loss as described above and included the results in Fig. 1, 2, 4, and 5. All measurements revealed that the two infected groups experienced significantly ($P < 0.001$) more alveolar bone loss than did the noninfected controls.

In contrast to the gnotobiotic experiment, however, immunization before oral infection failed to amplify bone loss. Measurements of areas and vertical distances in groups A and B

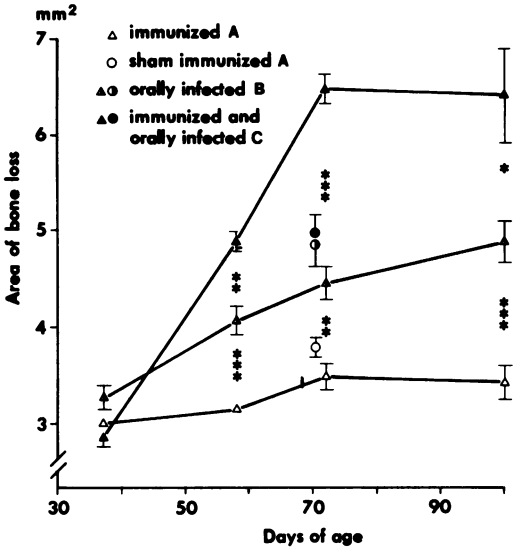


FIG. 1. *Areas of bone loss of the first and second maxillary molars. Triangles, Gnotobiotic rats; circles, rats in relative gnotobiosis (specified in the text). Statistically significant differences are indicated for gnotobiotic rats as follows: *, 0.01 < P < 0.05; **, 0.001 < P < 0.01; ***, P < 0.001. Mean values ± standard error; the standard error is not shown if it is smaller than the symbol used.*

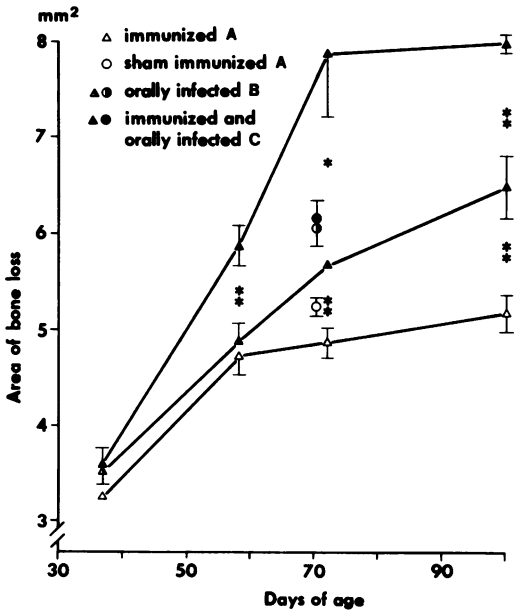


FIG. 2. *Areas of bone loss of the first and second mandibular molars. Triangles, Gnotobiotic rats; circles, rats in relative gnotobiosis. Values and statistically significant differences are as in Fig. 1.*

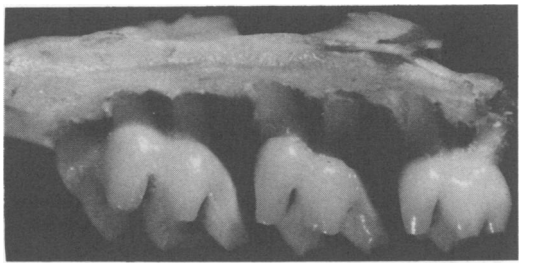

FIG. 3. *Bone loss in the right maxilla of an immunized and monoassociated gnotobiotic rat at 72 days of age.*

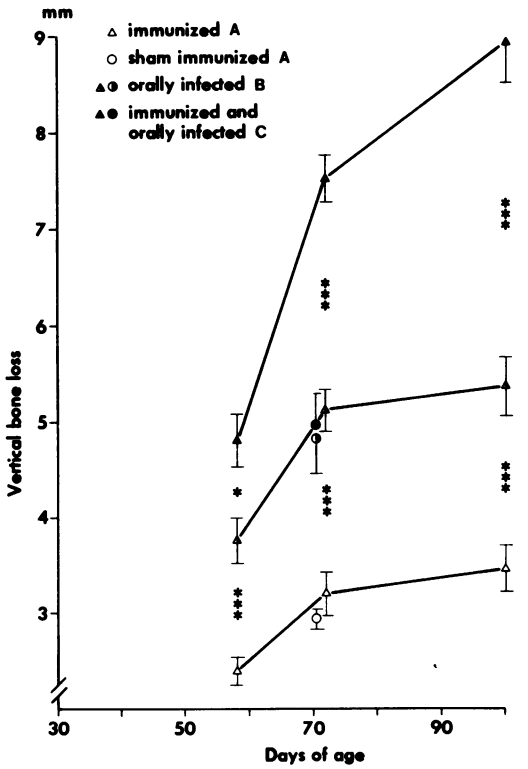


FIG. 4. *Vertical bone loss of all maxillary molars. Triangles, Gnotobiotic rats; circles, rats in relative gnotobiosis. Values and statistically significant differences are as in Fig. 1.*

were almost equal in the two experiments (Fig. 1, 2, 4, and 5).

**In vitro response of T lymphocytes.** T lymphocytes were cultured in microtiter plates containing either ConA at concentrations ranging from 5.0 to 0.15 μg/ml or dilutions of the *Actinomyces* antigen preparation, BCS, ranging from 500 to 0.05 μg/ml (final concentrations). These conditions measured, after 4 days of culture, the maximum anamnestic response to strain Ny1 antigens by T-cells from rats immu-

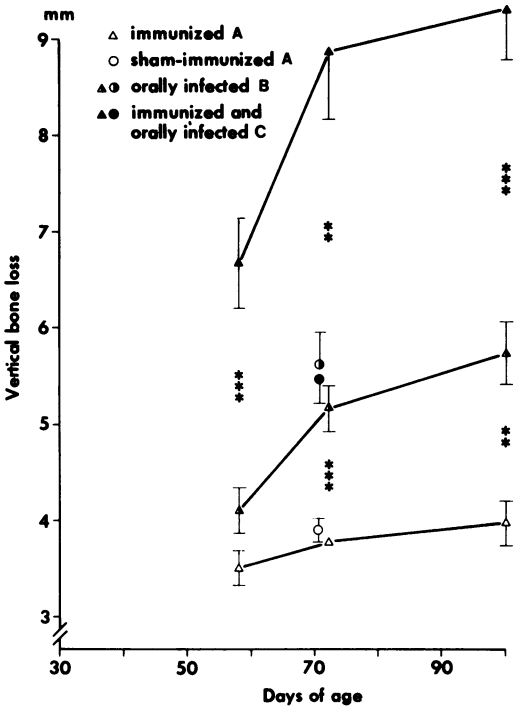


FIG. 5. *Vertical bone loss of all mandibular molars. Triangles, Gnotobiotic rats; circles, rats in relative gnotobiosis. Values and statistically significant differences are as in Fig. 1.*

TABLE 1. *Body weights of rats in relative gnotobiosis[a] at 70 days of age (average)*

| Group | Treatment[b] | Avg body weights of rats | |
|---|---|---|---|
| | | Females | Males |
| A | Control | 147 ± 7.1 (10) | 210 ± 12.2 (11) |
| B[c] | Orally infected | 147 ± 10.3(9) | 211 ± 14.2 (12) |
| C[d] | Immunized and orally infected | 149 ± 9.3 (10) | 200 ± 14.4 (11) |

[a] See text for treatment with antibiotics.

[b] Body weights given in grams ± standard deviation, with the number of rats given in parentheses.

[c] Rats were orally infected with *A. viscosus* Ny1E on days 37 and 38.

[d] Rats were immunized subcutaneously with *A. viscosus* Ny1 in incomplete adjuvant on day 20.

nized with strain Ny1 (5) and from gnotobiotic rats orally infected with strain Ny1 (Burckhardt et al., in press). Table 2 summarizes the peak lymphocyte activation of T-cells from gnotobiotic rats. In addition, we observed a plateau-type reaction particularly in group C. On day 100, 0.5 μg of BCS, 100-fold less than the maximum, provoked a [$^3$H]thymidine uptake of 84 ± 3 kdpm compared with 55 ± 1 kdpm in group B and 47 ± 0.5 kdpm in group A.

TABLE 2. *Activation of T-cell cultures from germfree (A) and monoassociated (B and C) rats by Actinomyces antigens (BCS) or ConA as measured by the uptake of [$^3$H]thymidine[a]*

| Days of age | [$^3$H]thymidine uptake (kdpm) after activation by BCS (50 μg ml$^{-1}$) in groups | | | [$^3$H]thymidine uptake (kdpm) after activation by ConA (5 μg ml$^{-1}$) in group: | | |
|---|---|---|---|---|---|---|
| | A | B | C | A | B | C |
| 37 | 109 ± 5[b] | 9 ± 1 | 67 ± 3 | 451 ± 19 | 423 ± 29 | 381 ± 17 |
| 58 | 36 ± 7 | 0 ± 0 | 5 ± 1 | 210 ± 4 | 25 ± 7 | 243 ± 13 |
| 72 | 60 ± 4 | 14 ± 2 | 22 ± 2 | 381 ± 21 | 376 ± 27 | 433 ± 9 |
| 100 | 97 ± 4 | 97 ± 9 | 94 ± 2 | 374 ± 11 | 354 ± 26 | 441 ± 25 |

[a] Groups A and C were immunized subcutaneously with *A. viscosus* Ny1 in incomplete adjuvant on day 20, and groups B and C were monoassociated with *A. viscosus* Ny1 on days 38 and 39.

[b] Mean values ± standard error of triplicates; the mean values of unstimulated control cultures ($n$ = 15) varied between 0.4 and 7.4 kdpm (mean, 2.1).

T-cell cultures from rats in relative gnotobiosis were set up at 70 days of age, and Table 3 shows the peak values of lymphocyte activation. Whereas the response to ConA was very similar within the three groups and slightly lower compared with that of the gnotobiotic rats, there was an unexpected response to BCS in group A and a marked difference between groups B and C.

**Serum antibodies to *A. viscosus* Ny1.** Serum antibody titers were estimated by agglutinating strain Ny1 cells in microtiter plates with sera of germfree nonimmunized animals as negative controls. Figure 6 shows the results. In separate experiments, we confirmed the antibody nature of the agglutinins with fluorescein isothiocyanate-labeled anti-rat immunoglobulin antibodies (not shown). Clearly, in the gnotobiotic experiment, oral infection amplified antibody titers induced by immunization. In group C titers reached the maximum of $\log_2$ 13 at 58 days of age and slowly decreased thereafter, whereas in group B titers continuously increased and attained comparable values at 100 days of age. In contrast, associated rats in relative gnotobiosis had a mean titer of $\log_2$ 10 at 70 days whether they had been immunized or not.

## DISCUSSION

Our results demonstrate a cause-and-effect relationship between sensitization to *A. viscosus* Ny1 and destructive periodontal disease. Germfree rats immunized with strain Ny1 before monoassociation with the same bacterium lost twice the amount of alveolar bone compared with sham immunized orally infected animals. In conventional rats, however, immunization failed to amplify bone loss (Fig. 1, 2, 4, and 5). No dis-

crepancy in physical condition accompanied this difference of immune reactivity since the body weights of rats in relative gnotobiosis (Table 1) matched the weights of rats in the isolators at a similar age (7).

The rate of bone loss in first and second molars decreased after 72 days of age, particularly in immunized and infected rats (Fig. 1 and 2). We expected a reduction considering the substantial defects present at that time (Fig. 3). Further bone loss was primarily observed in the interdental spaces between the second and third molars and at the third molars (Fig. 4 and 5). Plotting vertical bone loss at the first and second molars and areas of bone loss of all molars resulted in similar curves (data not shown). In our experiments the overall substantial amounts of bone loss in general and the localized crater-like interdental destruction in particular can hardly be explained only by cessation of bone production (19). Note that resorption in maxillary molars preceded that in mandibular molars which, in sham immunized orally infected rats, failed to reach statistically significant differences with uninfected controls at 58 days of age (Fig. 2 and 5).

TABLE 3. *Activation of T-cell cultures from rats in relative gnotobiosis at 70 days of age by Actinomyces antigen (BCS) or ConA as measured by the uptake of [$^3$H]thymidine*

| Group[a] | [$^3$H]thymidine uptake (kdpm) after activation by: | |
|---|---|---|
| | BCS (50 μg ml$^{-1}$) | ConA (5 μg ml$^{-1}$) |
| A | 64 ± 2[a] | 300 ± 15 |
| B[b] | 95 ± 3 | 272 ± 30 |
| C[b,c] | 170 ± 3 | 305 ± 7 |

[a] Mean values ± standard error of triplicates; the mean values of unstimulated control cultures ($n$ = 15) varied between 4.7 and 7.7 kdpm.

[b] Rats were associated with *A. viscosus* Ny1E on days 37 and 38.

[c] Rats were immunized subcutaneously with *A. viscosus* Ny1 in incomplete adjuvant on day 20.

These results differ from reports using other strains of *A. viscosus* (4, 19, 23), or other species of *Actinomyces* (12, 13, 19), in which little or no mandibular bone loss occurred. It should be pointed out that we compared bone loss in monoassociated rats with the fictitious bone loss in germfree animals. In the latter case, the distance between the cementoenamel junction and the alveolar bone crest also increased during rapid growth because the rate of tooth eruption was faster than the rate of concomitant bone apposition (discussed in reference 1).

Note that measurements of bone loss in rats in relative gnotobiosis at an average age of 70 days correspond well with those in gnotobiotic

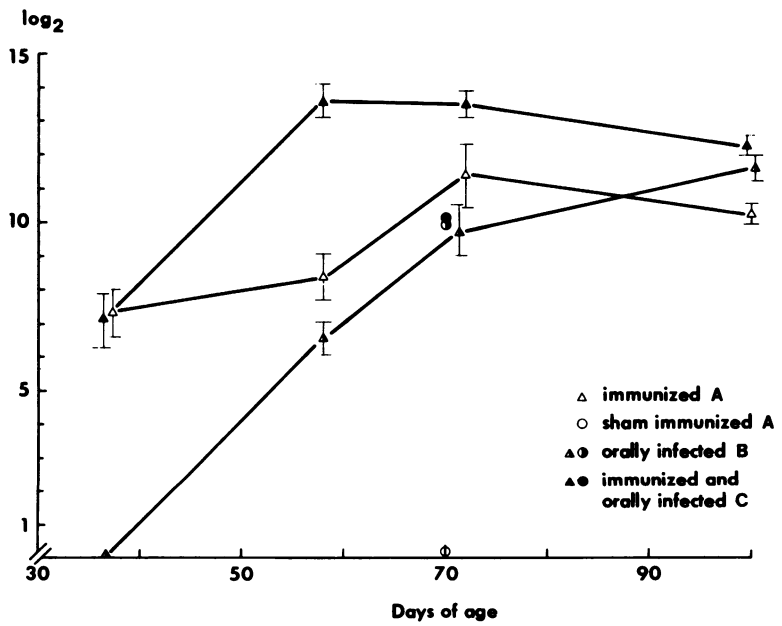


FIG. 6. *Serum antibody titers to A. viscosus Ny1 determined by agglutinating A. viscosus cells in microtiter plates. Triangles, Gnotobiotic rats; circles, rats in relative gnotobiosis. Mean values ± standard error (n = 5 or 6).*

rats, except that in the former, immunization did not affect the disease (circular symbols in Fig. 1, 2, 4, and 5).

The crucial point of discussion is: how are the reactivity of infiltrated cells and their destructive potential at the site of chronic inflammation (14, 17) correlated with the measured parameters of cell-mediated and humoral immunity? Table 2 shows a considerable anamnestic response to strain Ny1 antigens of T-cells from immunized, but not from sham immunized, rats at 37 days of age. After oral infection, on day 58, the T-cell response to both antigen and mitogen was much lower, presumably reflecting the induction of suppressor macrophages (9, 24, 30, 31). We have recently shown that the T-cell response to strain Ny1 antigens requires some silica-sensitive macrophages, usually present after filtration through Degalan columns (6). But the lower thymidine uptake in cultures from group A at day 58 suggests that other factors besides monoassociation caused suboptimal T-cell stimulation (Table 2). However, the anamnestic response at the end of the experiment was in the range of previous ones (Burckhardt et al., in press). If the time course of the T-cell response (Table 2) is compared with the development of bone loss (Fig. 1, 2, 4, and 5), it is evident that most bone was lost while the cell-mediated immune response, as measured in vitro, was low. The latter could be due either to the presence of suppressor cells (discussed by Burckhardt et al., in press) or to a depletion of antigen-reactive T lymphocytes from the spleen to the site of inflammation. Johnson et al. (22) reported a similar coincidence of decreased in vitro splenocyte proliferation and increased periodontal disease in rats monoinfected with *E. corrodens*. In contrast to their results, suggesting that a lack of protective immunity caused the disease, we here report a cause-and-effect relationship between allergy to *A. viscosus* and degree of bone loss. The enhancement of bone resorption in immunized gnotobiotic rats paralleled the increased serum antibodies to strain Ny1. Neither parameter was increased in immunized rats in relative gnotobiosis which, however, attained higher T-cell activation by antigen. Because group A of this experiment, neither immunized nor associated with strain Ny1 or Ny1E, showed a considerable T-cell response to BCS, other factors might have influenced the results; this suggests that these values (Table 3) should be considered with caution.

The fact that antibody titers to strain Ny1 in gnotobiotic, immunized, and monoassociated rats reached a plateau at 58 days of age (Fig. 6) points to a feedback inhibition by anti-idiotype antibodies which has been implicated in the network of the immune system (21, 27). In rats in relative gnotobiosis increased macrophage activity, caused by the indigenous flora at the time of immunization, suppressed the immune response (30; Burckhardt, unpublished data). This might explain why immunization of these animals failed (i) to amplify the humoral immune response to strain Ny1 (Fig. 6) and (ii) to exacerbate bone resorption (Fig. 1, 2, 4, and 5).

Finally, we emphasize the predominant role of antibodies, regulated by thymus-dependent lymphocytes (16), in the pathogenesis of destructive periodontal disease (recently reviewed in references 32 and 34) for the following reasons: (i) drug-induced suppression by cyclosporin A of the T-cell-dependent allergic response hardly influenced bone loss in gnotobiotic rats (Guggenheim et al., J. Periodontal Res., in press); (ii) in vitro cell-mediated cytotoxicity for rat fibroblasts requires antigens from strain Ny1 but, surprisingly, no prior sensitization of lymphocytes (Gaegauf-Zollinger et al., in preparation); (iii) lymphoid cells of the B-cell lineage, capable of antibody production, were shown in periodontal lesions in rats (17) and humans (15, 29, 33); and (iv) antibodies to oral microorganisms have, at least, two possible modes of action, first, by facilitating colonization and increasing the amount of dental plaque deposits (data not shown) and, second, by complex formation with antigen and elicitation of local immune complex disease (11).

In conclusion, we show that the allergic response to *A. viscosus* Ny1 in rats amplifies destructive periodontal disease during growth, and we present evidence for natural inhibition and suppression of the allergic response. A loss of suppressor cells with increasing age was shown in certain autoallergic diseases in mice (26). By analogy, we consider a loss of suppressor function with age to convert stable periodontal lesions in humans into progressive ones.

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