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# Periodontal Bone Loss and Immune Characteristics of Congenitally Athymic and Thymus Cell-Reconstituted Athymic Rats

HIROMASA YOSHIE,[1] MARTIN A. TAUBMAN,[2]* JEFFREY L. EBERSOLE,[2] DANIEL J. SMITH,[2] AND CAROLYN L. OLSON[2]

*Department of Immunology, Forsyth Dental Center, Boston, Massachusetts 02115,[2] and Department of Periodontology, Niigata University School of Dentistry, Niigata, Japan[1]*

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**We investigated the ability to reconstitute the T-cell deficiency in congenitally athymic (nude; *rnu/rnu*) Rowett rats by intravenous injection of $10^7$ heterozygous normal (*rnu/+*) rat thymus cells. The thymus cell-reconstituted animals showed essentially normal lymphocyte proliferation to phytohemagglutinin P and concanavalin A, normal spleen and lymph node T-lymphocyte distribution, and normal serum immunoglobulin G levels. These rats were used to study the effect of T-cell deficiency on periodontal disease. Animals with minimal oral flora were divided into three groups: nude, thymus cell-reconstituted nude, and normal rats. Immune response, gingival inflammation, and periodontal bone loss were examined in these animals. Less than 40% of the T cells recovered from the normal rat gingiva could be recovered from the nude rat gingiva. In contrast to the situation in normal or thymus cell-reconstituted rat gingiva, B cells were vastly predominant in the nude rat gingiva, with approximately four B cells for each T cell. There was increased periodontal bone loss in the nude rats compared with that in the normal rats. Thymus cell reconstitution of the nude rats was associated with decreased bone destruction. It is suggested that T cells have a regulatory function in relation to the potentially excessive B-cell response to abundant antigen and polyclonal activators in the oral cavity.**

The immune response can play a major role in the genesis of periodontal disease. Its protective or destructive influence has been a controversial matter. For example, active immunization can interfere with the development of gingival inflammation (10, 29). There are also reports which suggest that the immune response can contribute to periodontal destruction (5, 7, 18, 20, 34). Immunization with a soluble antigen (ovalbumin) or *Actinobacillus actinomycetemcomitans* induced more periodontal bone loss in germfree or gnotobiotic rat systems (9, 10, 34, 35, 38). The balance between humoral and cell-mediated immunity seemed to be important in the outcome of periodontal destruction. Therefore, much attention has been focused on the cell populations in the periodontal tissues (4, 22, 23, 31–33). Gingivitis has been characterized by a thymus-dependent lymphocyte cell infiltrate, while B cells and plasma cells are predominant in periodontal lesions (22, 23).

Seemingly contradictory results have recently been reported with respect to the role of T lymphocytes in experimental periodontal disease (33). Cyclosporin A (a T-cell-suppressive agent)-treated animals showed a tendency toward less bone loss in the rat model (17). Cyclophosphamide, an immunosuppressive agent, induced more bone loss in rats (30). The injection of antithymus globulin into dogs did not greatly influence the development or maintenance of gingivitis (25, 26). We believe that many of these observations can be unified by relating the development of periodontal disease to an imbalance among T-lymphocyte subpopulations. To examine this hypothesis, we have been studying experimental periodontal disease and the immune capabilities of congenitally athymic nude rats (36, 39). These rats have been shown to be grossly deficient in functionally (1) mature T cells, and their immunoglobulin levels and immune responses indicate an imbalance of functional T lymphocytes (6, 36, 40). In this study, we examined the effects of reconstitution of congenitally athymic rats with a balanced T-cell population on periodontal inflammation and alveolar bone loss. Such reconstitution has not been previously performed in nude rats.

## MATERIALS AND METHODS

**Animals.** Fifty-six male and female Rowett homozygous nude (*rnu/rnu*) and heterozygous normal (*rnu/+*) rats (13) were used in this study. These rats were obtained from the MRC Laboratory Animals Centre (Carshalton, Great Britain) and bred in our animal facility in plastic Trexlar-type isolators. Preliminary studies indicated that these rats harbored at least the following organisms: *Streptococcus* spp., *Bifidobacterium* spp., and *Pseudomonas* or *Proteus* species. The colony was expanded by mating nude males with normal (*rnu/+*) females. Animals were fed presterilized L-356 diet (Teklad Mills, Madison, Wis.) and were provided autoclaved distilled water ad libitum.

**Experimental protocol.** Thirty-day-old animals were taken from plastic isolators and put into filter-topped cages with raised bottoms to prevent impaction of bedding material. Thymuses were aseptically excised from 2- to 3-day-old normal Rowett rats. Thymocytes were prepared by expressing the thymic tissue through stainless-steel mesh. After being washed twice with RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.), viable thymocytes were counted with ethidium bromide and acridine orange (14). Approximately $10^7$ viable (95 to 97% viability) cells from one thymus were suspended in 1 ml of the medium and injected into the tail vein of one 30-day-old nude rat.

In preliminary studies to examine the genetic homogeneity of the rats, tail skin was grafted (2) to the tails of nonlittermate Rowett rats. These grafts ($n$ = 12) survived for more than 4 months, at which time the experiment was terminated. We also monitored thymocyte-reconstituted rats for potential graft-versus-host reactions. The spleen weight and ratio of spleen weight to body weight for each thymus

* Corresponding author.

cell-reconstituted rat and nude rat was measured. The mean weight ± the standard error of nude rat spleens ($n$ = 20) was 494 ± 26 mg, and that of thymus cell-reconstituted rat spleens ($n$ = 16) was 431 ± 25 mg. The ratios ([spleen weight/body weight] × $10^3$) were 2.03 ± 0.10 and 1.80 ± 0.11, respectively. Thus, there was no evidence of graft-versus-host reaction (16) in the thymus cell-reconstituted nude rats. These combined findings suggested that no major histoincompatibilities existed between the donor cells and the recipient animals. Consequently, data support the probability of survival of the transferred cells. The rats were divided into the following groups: (i) homozygous nude rats ($n$ = 21; 10 male, 11 female), (ii) thymus cell-reconstituted nude rats ($n$ = 17; 7 male, 10 female), and (iii) heterozygous normal littermate rats ($n$ = 18; 8 male, 10 female).

The rats were exsanguinated at 120 days of age after collection of saliva. Gingival tissues were excised for cell counts. Spleen, cervical, and mesenteric lymph nodes were removed for cell cultures and immunofluorescence analyses. Finally, the rat heads were defleshed for measurement of bone loss (10).

**Lymphocyte preparation and blastogenesis assay.** Spleen and cervical lymph nodes were aseptically removed, and lymphocytes were prepared for culture by expressing tissues through stainless-steel mesh. Viable cells ($5 \times 10^5$) were incubated in microculture plates (Costar, Rochester, N.Y.) with 200 μl of RPMI 1640 medium containing 10% normal rat serum, $5 \times 10^{-5}$ M 2-mercaptoethanol (15), and 12.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. An optimal concentration of 10 μg of concanavalin A (ConA, A grade; Calbiochem-Behring, La Jolla, Calif.) per ml and 1:1,000 diluted phytohemagglutinin P (PHA; Difco Laboratories, Detroit, Mich.) were added to separate test cultures. All cells from individual rats were tested at least in triplicate with each stimulus. The cultures were incubated at 37°C in an atmosphere of 5% $CO_2$ in air for 3 days (Hotpack, Philadelphia, Pa.). Twenty-four hours before harvesting, 0.2 μCi of tritiated thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to each well. The cells were harvested on glass fiber filters, dried, and counted in a liquid scintillation counter (Beckman LS 100C) with a xylene-based scintillation fluid (Scintilene; Fisher Scientific Co., Pittsburgh, Pa.). All results are reported as mean counts per minute ± standard error and mean stimulation index (mean counts per minute of stimulated culture/mean counts per minute of unstimulated culture).

**Immunofluorescence assay of splenocytes and mesenteric lymphocytes.** Splenocytes and mesenteric node lymphocytes ($5 \times 10^5$) were washed and suspended in 0.05 ml of phosphate-buffered saline containing 5% bovine serum albumin with 0.05 ml of appropriately diluted anti-T and anti-Fab reagent (T reagent, 1:4 to 1:8; B reagent, 1:4) in a manner to eliminate cytophilic immunoglobulin and binding via Fc-bearing cells (34). Duplicate cell preparations were made for each reagent and for all preparations with a cytocentrifuge (Shandon Southern, Sewickley, Pa.). Slides were observed with a Leitz fluorescence microscope equipped with a Ploem vertical illuminator, XB075 Xenon lamp, and excitation filters BG 38 and BG 12. The cells were identified as lymphocytes by morphological criteria, and those showing rim or more diffuse surface staining were considered to be positive T or B cells. Percentages of positive-staining lymphocytes were determined by counting at least 100 cells (34).

**Determination of immunoglobulin level in serum and saliva.** Blood was obtained from the retro-orbital venous plexus, and immunoglobulin levels were determined by radial immunodiffusion (24) with monospecific rabbit antiserums directed to γ, α, or μ chains as previously described (12).

**Gingival cell preparation and immunofluorescence assay.** Gingival tissue was prepared as previously described (34). Briefly, the gingival tissues were separated by cutting approximately 1 mm from the gingival margin under a stereomicroscope equipped with a reticule eyepiece. After all visible blood was removed by washing, these segments were incubated at 37°C for 30 min in 1.5 mg of Dispase (protease, neutral, grade II; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 1 ml of RPMI 1640 medium. The Dispase-treated segments were expressed through 60-mesh stainless-steel screens and through an 80-mesh copper sieve to obtain a single-cell suspension in 2 ml of phosphate-buffered saline. The gingival cells were counted with a hemacytometer, and the percentage of lymphocytes relative to total gingival cells was determined from Wright-Giemsa-stained smears after counting at least 300 cells. The cell suspension was then washed and directly stained with anti-T and anti-Fab reagents, using the same procedure as previously described for the immunofluorescence assay of splenocytes and mesenteric lymphocytes (34).

**Horizontal bone loss.** After the mandibular and maxillary jaws were defleshed, a reticule eyepiece was used to measure the distance from the cemento-enamel junction to the alveolar crest under 25× magnification. Recordings were made in the long axis of both buccal and lingual root surfaces of all molar teeth with careful superimposition of buccal and lingual cusps to ensure reproducibility, as previously described (34). There were six recordings for the first molar tooth, which has three roots, and four recordings for the second and third molars, each of which has two roots. The sum of the recordings for each tooth surface was used as a measure of the total bone loss on the particular surface. The bone loss was determined without prior knowledge of the group designation of the animals.

**Statistical analysis.** Data were analyzed by the unpaired Student's $t$ test, and differences were considered to be statistically significant at $P < 0.05$. Bone loss data were subjected to analysis by Dunnett's $t$ statistic (11, 41).

## RESULTS

**Proliferative responses of cervical lymphocytes and splenocytes to PHA and ConA.** Initially, we examined blastogenesis to PHA and ConA as T-cell test mitogens for cells from the three groups of animals (Fig. 1). No proliferative responses to PHA by cells from the nude group were observed, and responses to ConA were remarkably low in comparison with those of normal rats ($P < 0.001$). However, incorporation of thymidine by cells from nude rats was enhanced by exposure to ConA (stimulation index [mean ± standard error], 14.7 ± 4.9, cervical lymphocytes; 4.2 ± 0.9, splenocytes). Thymus cell-reconstituted nude rat lymphocyte and splenocyte responses to ConA and PHA (Fig. 1) were always significantly elevated ($P < 0.001$) relative to those in nude rats but were less than the responses in the heterozygous normal rats. Therefore, infusion of thymus cells into the nude rats resulted in partial restoration of mature T-cell function.

**T- and B-cell percentages in mesenteric lymph node and spleen.** The T- and B-cell percentages of lymphocytes in mesenteric lymph nodes and spleens from nude, thymus cell-reconstituted nude, and normal rats are shown in Fig. 2. The percentages of T cells from mesenteric lymph nodes and spleens of nude rats were 13 and 11%, respectively. These percentages were significantly below ($P < 0.001$) the per-

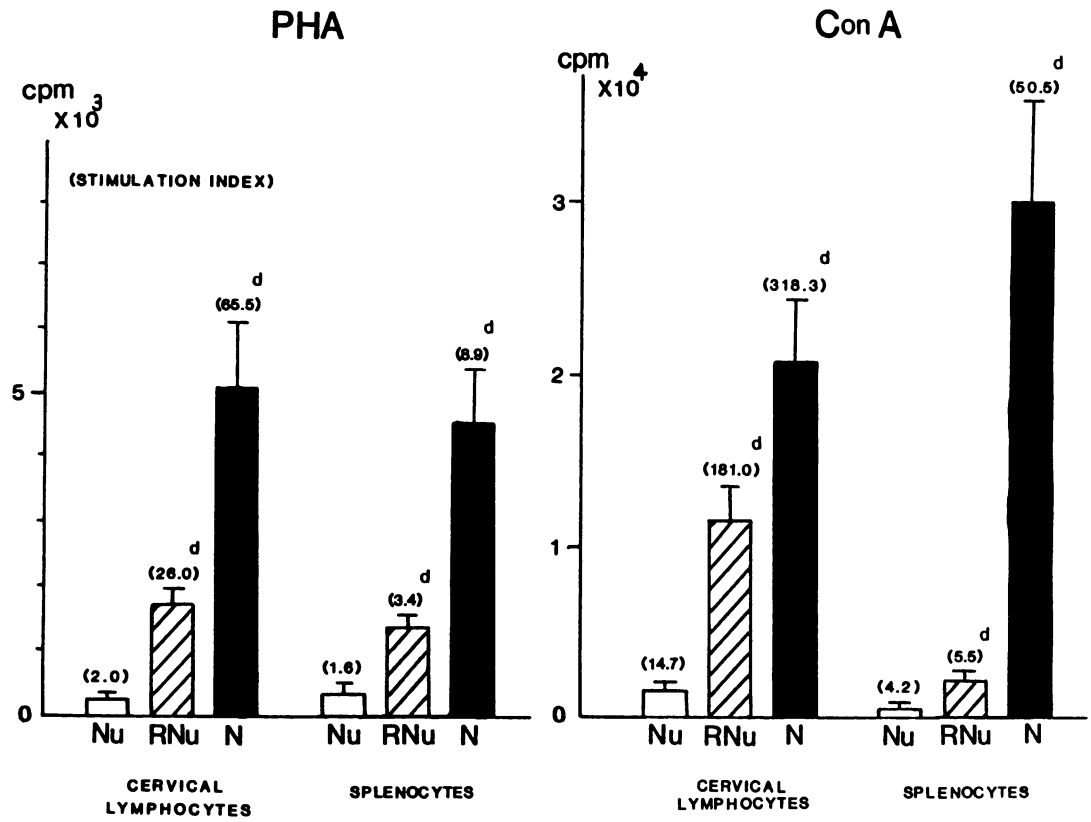


FIG. 1. Proliferative responses to PHA and ConA of cervical lymphocytes and splenocytes from nude (Nu; $n$ = 20), thymus cell-reconstituted nude (RNu; $n$ = 16), and normal (N; $n$ = 16) rats. Each column represents the mean counts per minute of tritiated thymidine incorporated after 3 days in culture, and the standard errors of the means are shown by the brackets (mean stimulation index). d, Significant difference at $P < 0.001$ in comparison with the values in nude rats.

centages of these cell types found in normal rats (65 and 43%, respectively). Thymus cell transfer to nude rats resulted in significantly elevated T-cell percentages in both organs, but these did not quite reach the levels in normal rats. Mesenteric lymph nodes from the nude rats contained 64% B cells, which was due to the diminished percentage of T cells. Percentages of mesenteric node B cells were significantly lower ($P < 0.001$) in thymus cell-reconstituted nude and normal rats (31%, reconstituted nude; 40%, nude). Percentages of B cells in the spleen were not greatly different among the three groups.

**Immunoglobulin levels in serum.** Serum immunoglobulin G (IgG), IgA, and IgM levels were examined in nude, thymus cell-reconstituted nude, and normal rats (Table 1). Serum IgG concentration in nude rats was nearly 50% lower ($P < 0.01$) than that demonstrated for the normal group. The concentration of IgG in thymus cell-reconstituted rat serum was not significantly different in comparison to the levels in the normal group. Serum IgA and IgM concentrations in nude rat serum were increased as compared with those of the thymus cell-reconstituted nude and normal groups. When IgA and IgG levels in nude rats were different from immunoglobulin levels in normal rats, thymus cell-reconstituted nude rat immunoglobulin levels also differed from those of the nude rats. This observation indicated that reconstitution with thymus cell restored normal T-cell-dependent B-cell function.

**Gingival cells.** The means of all gingival cell types (epithelial cells, fibroblasts, erythrocytes, polymorphonuclear leukocytes, lymphocytes, monocytes, and plasma cells) recovered from the rats in the three groups are shown in Fig. 3A. Although there was a tendency for the nude rats to demonstrate slightly lower gingival cell counts, there were no statistically significant differences among the three groups. The numbers of gingival lymphocytes were similar in the nude, thymus cell-reconstituted nude, and normal rats (Fig. 3B). The mean percentages of lymphocytes per total gingival cells in nude rats were higher than those in normal or thymus cell-reconstituted nude groups (lymphocyte percentage per total cells, 11.9 ± 0.8% in nude, 9.6 ± 1.0% in thymus cell-reconstituted nude, and 8.3 ± 0.9% in normal rats). The characterization of these gingival lymphocytes (relative counts of T and B cells) is shown in Fig. 3C and D. T-cell numbers in nude rat gingiva were 2.6 times lower than those in normal rat gingiva ($P < 0.005$). There were no significant differences in T-cell counts between the thymus cell-reconstituted nude and normal rat groups. In all three groups B cells were the most prominent lymphocyte type. This was most pronounced in the nude rat group, which had a mean B-cell/T-cell ratio of 3.7 which was significantly higher ($P < 0.005$) than thymus cell-reconstituted (1.4) or normal (1.5) ratios. Thus, the gingivae of nude rats are extremely rich in B cells, whereas the normal and thymus cell-reconstituted nude rat gingivae showed less dramatic B-cell prominence.

**Horizontal bone loss.** The mean value of total bone loss (Table 2) was greater in the nude rats than in the normal rats, as we have previously shown (39). The localization of bone loss was also examined (Table 2), and the scores on all buccal surfaces and buccal maxilla in the nude group were significantly greater ($P < 0.05$) than the scores of the normal

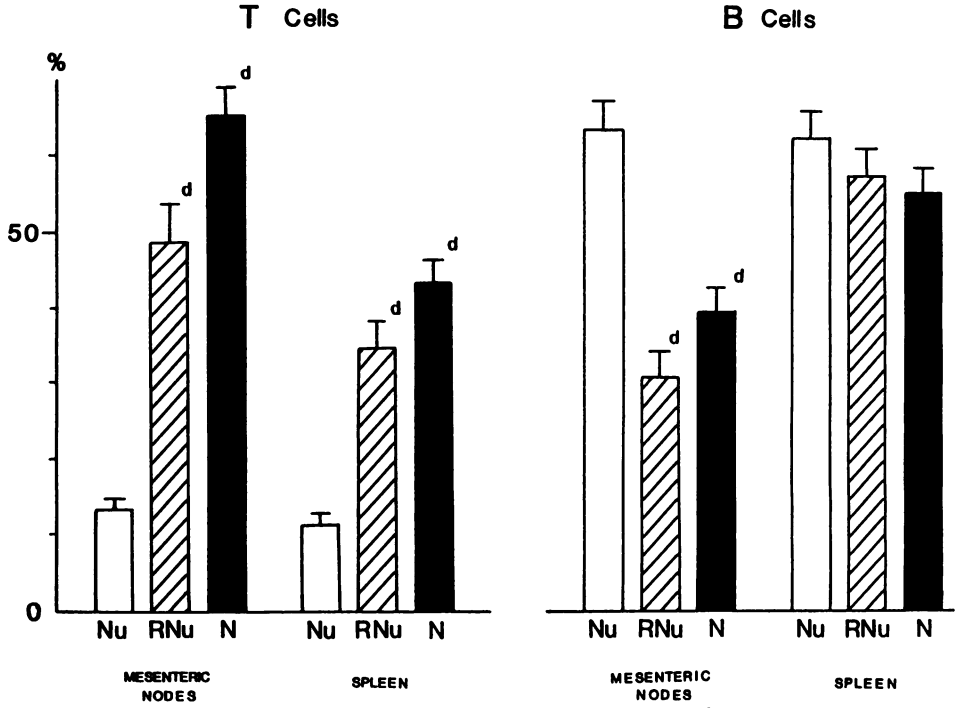


FIG. 2. T- and B-cell percentages in mesenteric lymph nodes and spleen in nude (Nu; $n = 19$), thymus cell-reconstituted nude (RNu; $n = 17$), and normal (N; $n = 16$) rats. Each column represents the mean percentage of T or B cells described as the percentage of lymphocytes, and the standard errors of the means are shown by the brackets. d, Significant difference at $P < 0.001$ in comparison with the values in nude rats.

group. Thymus cell-reconstituted nude rats did not demonstrate any differences in periodontal bone loss from the normal group, on any surface. Therefore, reconstitution of nude rats with thymocytes can restore a more normal bone loss pattern.

## DISCUSSION

We demonstrated a novel method of thymus cell reconstitution of athymic rats and described the effects of such a reconstitution on cell composition with particular emphasis on the periodontal tissues. The T-cell-deficient nude rat showed no proliferation of cervical lymphocytes and splenocytes to PHA and a weak response to ConA. It has been reported that immature T cells appear to be highly sensitive to ConA but do not respond to PHA, with mature T cells showing a reversed pattern (8). These findings support the presence of immature regulatory T cells in nude rats. Cells staining with fluorescein isothiocyanate-conjugated anti-thymus cell serum were found in the mesenteric lymph nodes (13.3%) and in the spleens (11.2%) of nude rats. Therefore, it is possible that immature T-lymphocyte populations exist in the nude rats which may be precursor T cells.

Serum IgG levels were significantly reduced in nude rats in comparison with those in normal rats. These observations were similar to our previous studies of the nude rat (36). Serum IgG concentration in thymus cell-reconstituted animals was increased as compared with the value in nude rats, and there was no significant difference between the thymus cell-reconstituted and normal groups. Thus, the T-cell percentages, immunoglobulin levels, and blastogenesis activity, described above, confirmed that the nude rats were grossly deficient in functional T cells. Thymus cell-reconstituted nude rats demonstrated recovery of T-cell functional balance, as has been demonstrated in nude mice (28).

We evaluated gingival inflammation of these rats on the basis of characteristics of the immune response in the three

TABLE 1. Immunoglobulin levels in serum of nude, thymus cell-reconstituted nude, and normal 120-day-old rats

| Group | Serum immunoglobulin level (mg/ml) (mean ± SE) | | |
|---|---|---|---|
| | IgG | IgA | IgM |
| Nude ($n = 20$) | 6.59 ± 0.76 | 0.19 ± 0.02 | 1.24 ± 0.08 |
| Thymus cell-reconstituted nude ($n = 17$) | 10.13 ± 0.85[b] | 0.13 ± 0.02[a] | 1.17 ± 0.09 |
| Normal ($n = 17$ to 18) | 11.64 ± 0.55[c] | 0.11 ± 0.01[c] | 0.78 ± 0.05[c] |

[a] Significant difference at $P < 0.01$ in comparison with the values in nude rats.
[b] $P < 0.005$.
[c] $P < 0.001$.

TABLE 2. Bone loss in nude, thymus cell-reconstituted nude, and normal 120-day-old rats

| Group | Horizontal bone loss (mm) (mean ± SE) | | |
|---|---|---|---|
| | Total | Lingual total | Buccal total |
| Nude ($n = 17$) | 37.5 ± 0.9 | 25.1 ± 0.5 | 12.4 ± 0.4 |
| Thymus cell-reconstituted nude ($n = 17$) | 36.7 ± 1.3 | 25.0 ± 0.8 | 11.6 ± 0.7 |
| Normal ($n = 18$) | 35.5 ± 1.1 | 24.6 ± 0.5 | 10.9 ± 0.6[a] |

[a] Significant difference at $P < 0.05$ in comparison with the value in nude rats when analyzed by Dunnett's $t$ statistic.

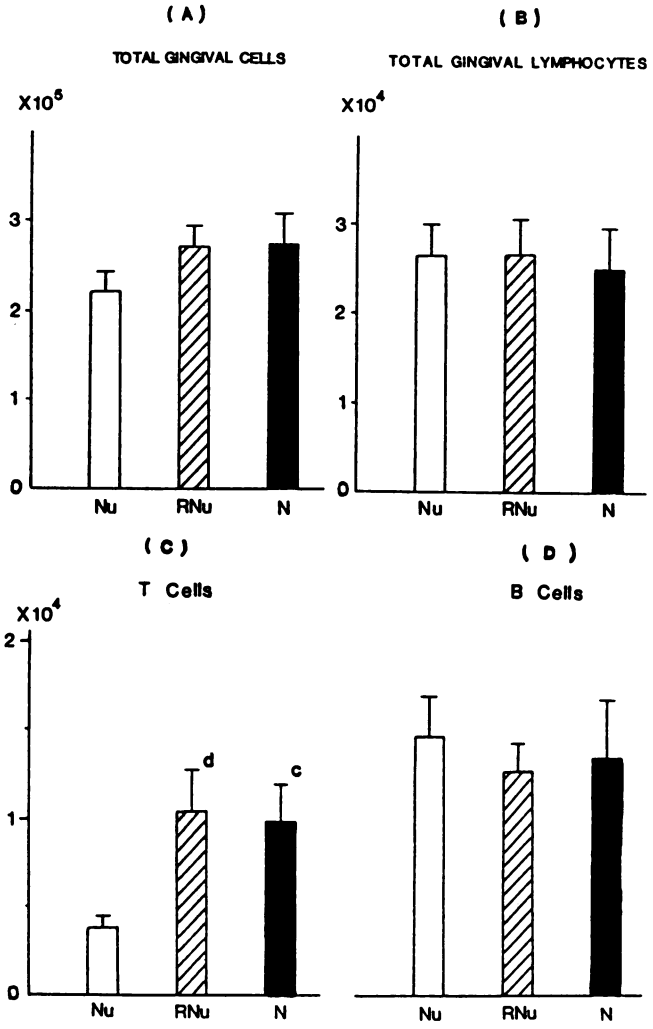


FIG. 3. (A and B) Total gingival cells and lymphocytes recovered from nude (Nu; $n = 20$), thymus cell-reconstituted nude (RNu; $n = 17$), and normal (N; $n = 16$) rats. Each column represents the mean counts per minute, and the standard errors of the means are shown by the brackets. (C and D) T- and B-cell numbers in the gingiva of nude, thymus cell-reconstituted nude, and normal rats. Each column represents the mean count, and the standard errors of the means are shown by the brackets. Significant difference at c, $P < 0.005$; and d, $P < 0.001$, in comparison with the values of in nude rats.

groups. There was a tendency for increased gingival lymphocytes in nude rats, but no significant differences were found. In normal and thymus cell-reconstituted rats inflammatory cells of the gingiva included T and B lymphocytes (B-cell/T-cell ratio, 1.4 to 1.5). However, in nude rats, B cells were predominant, and there were few T cells in the gingiva (B-cell/T-cell ratio, 3.7). These T cells may be immature and may lack regulatory capabilities. Therefore, the characteristic feature of inflammation in the nude rat gingiva is the predominance of B lymphocytes.

On the basis of the findings that bone formation and resorption in the nude rats occurred in a remodeling pattern which was quite similar to that seen in normal littermates (39), our data indicated that there was a tendency to increased total bone loss in nude rats and that bone loss on buccal aspects in nude rats was significantly increased in comparison with that in the normal rats. This observation confirmed and extended our previous findings (39) and those of others (3) showing significantly increasing bone loss in thymus-deficient rodents.

The effects on periodontal bone appear small. However, total bone loss, determined as previously described (9, 10, 34, 35, 38, 39) for rats, is a combination of the effects of passive eruption, which is greatest on lingual surfaces and diminishes after about 80 days of age (1), and of actual periodontal bone loss. Furthermore, it should be emphasized that the major differences in bone loss in these present experiments occurred on buccal surfaces where passive eruption is least (1). The differences seen only reflect differences in the actual bone loss component. Calculation of the passive eruption component (19, 39) for a rat the same age as used in the experiments (120 days) indicates that approximately 50% of the total measured bone loss may be attributed to passive eruption. Actual periodontal bone loss is appreciably less than the total bone loss shown. It should also be mentioned that these measurements are quite reproducible and that there is little variability among animals as indicated by the small error terms. Therefore, what appear to be small differences are statistically significant and meaningful.

The mean bone loss scores of thymus cell-reconstituted nude rats did not differ from those of the normal rats. These results suggested that T cells affect bone loss. These findings are consistent with the suggestions of Seymour and coworkers (31–33), who postulated that the pathogenesis of chronic inflammatory periodontal disease involves the conversion of a stable T-cell lesion to a progressive B-cell lesion (33). The maintenance of such a stable T-cell lesion in periodontal disease was attributed to the regulatory function of T lymphocytes (33). In the present study, it was found that serum IgG levels in nude rats were extremely low, suggesting an imbalance of T-cell function and confirming the possibility that T-cell regulatory function could influence alveolar bone loss.

Several studies have recently described protective or destructive aspects of T cells in periodontal disease in rodents (3, 17, 21, 25–27, 30). In each of these cases the T-cell regulatory balance was probably altered. We restored normal T-lymphocyte function to congenitally athymic animals and also restored a normal, reduced bone loss pattern. From our current data, and the reports mentioned above, it seems reasonable to suggest that T cells play a role in periodontitis, including tissue breakdown. This contention is strongly supported by our recent findings in humans showing that there are aberrations in T-cell subset distribution in periodontal disease tissue (37). We suggested that these alterations may represent a mechanism to regulate local, potentially excessive B-cell host response to abundant antigen and polyclonal activators in the gingival crevice area. Similar phenomena may be occurring in rodent systems. It is important to further assess the role of antigen-sensitized T cells and T-cell subsets on periodontal disease in humans and in rodent models.

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