Influence of age and immunization on development of gingivitis in rats

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To study the effect of age and antigenic priming on the development of gingivitis, 33 healthy rats were placed in contact with *Streptococcus mutans*, *Actinomyces viscosus*, *Fusobacterium nucleatum*, and *Bacteroides gingivalis*. On days 0, 3, 7, and 14 after inoculation, the gingival condition was judged clinically and histologically, and serum antibody titers against the bacteria were measured. The rats were divided into three groups: 1 month old, 3 months old, and 3 months old immunized. None of the young rats developed gingivitis during the experiment, whereas half of the adult and all of the adult immunized rats bled on probing on days 7 and 14. In general, antibody titers against the bacteria were low in young rats, moderate in adult rats, and high in adult immunized rats. These results indicate that adult rats react stronger to plaque antigens than young rats and that previous contact with the antigens increases the reaction. □ *Animal models; host response; periodontal disease*

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Periodontal disease is rarer in children than in adults (1), and a longer period of time is needed for inflammatory changes to take place after discontinuance of oral hygiene measures (2, 3). With a given plaque score the intensity of gingival inflammation increases with age from early childhood to adult age (4–6). Histologically, gingivitis in children is dominated by T lymphocytes, with considerably less plasma cells than seen in adults (7, 8). The lesion resembles the early lesion of adult gingivitis, but apparently it does not progress to established or advanced stages of disease (7). Matsson (9) has suggested that these differences could be explained by differences in periodontal morphology, in composition of dental plaque, or in immunologic reactions. Although the microflora associated with gingivitis in children has been described as distinctly different from that of adult gingivitis (10), periodontal pathogens are frequently present in the mouth of young children (11, 12). Therefore, it still is tempting to believe that the increased reactivity in older individuals could be due to immunologic priming as a consequence of prolonged contact with dental plaque antigens.

In the present study we examined the local and systemic reactions to a known microflora in 1) healthy young rats, 2) healthy adult rats, and 3) healthy, but primed adult rats.

Materials and methods

Experimental design

Three groups of 11 SPF male and female RT1[1] LEW/Mol rats were used; 2 groups were 3 months old (B_1 and B_2), and 1 group 1 month old (A). One of the 3-month-old groups of rats (B_1) was immunized with *Fusobacterium nucleatum* ATH 101, *Streptococcus mutans* 6715, *Bacteroides gingivalis* ATCC 33277, and *Actinomyces viscosus* ATCC 19246 (two intraperitoneal injections with 10^9 formalin-killed cells of each micro-

organism when the rats were 4 and 6 weeks old).

To maintain clinically healthy gingiva, all animals were fed tetracycline in drinking water from weaning until the start of the experiment (2.5 g tetracycline and 4.33 g kalium sorbate added to 1 l of water, applied for periods of 14 days with 7 days off in between).

On day 0, 10^9 cells of each of the above-mentioned microorganisms were introduced into the mouths of the rats, and 5% sucrose was added to the drinking water throughout the experiment.

On days 0, 7, and 14 after the start of the experiment three rats from each group were killed (on day 3, two rats per group), and the variables below were evaluated without prior knowledge of the group designation of the animals.

Plaque and gingivitis scores

Immediately before they were killed, the occurrence of plaque and gingivitis was assessed on methohexital (Brietal®)-anesthetized rats. On upper and lower incisors, visible plaque index and gingival bleeding index were used (13). A score of 1 was recorded if visible plaque/gingival bleeding was present on one or more locations.

Antibody titer measurement

Blood samples were collected from axillary vessels and allowed to clot for 30 min at 20°C. After centrifugation serum was sampled and stored at −20°C. Colony material of all bacteria was plated on 14-cm^2 glass plates in a drop of sterile water and air-dried. After 15 min of fixation in 99% ethanol and being washed three times for 5 min in phosphate-buffered saline (PBS), 10 μl of the serum dilutions 1:1, 1:4, 1:16, 1:64, 1:256, and 1:1024 were dropped on the cells, which were then incubated in hygrophore for 30 min at 20°C. Control plates were incubated with PBS instead of serum. After renewed washing, the plates were incubated for 30 min with 1:20-diluted fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat Ig (anti-kappa and anti-lambda) (Dakopatts, Copenhagen, Denmark). Then the plates were washed again, mounted in non-fading medium (10 ml PBS containing 100 mg *p*-phenylenediamine + 90 ml glycerol) (14), and interpreted in a Zeiss fluorescence microscope using epi-illumination. The microscope was equipped with FITC interference filters (The Optical Laboratory, Lyngby, Denmark) and a 50-W xenon lamp. The serum antibody titer of each rat was defined as the highest dilution with a clearly positive fluorescence of bacteria.

Numeric density

Specimens of buccal gingiva from lower molar regions were fixed in Carnoy's solution for 6 h, dehydrated twice in absolute alcohol for 1 h, embedded in paraffin wax, cut in 5-μm sections, and stained with hematoxylin and eosin or methyl green and pyronin. Every specimen was stereologically analyzed in 40 sections, and the number of lymphocytes, plasma cells, and macrophages per 1 mm^3 was calculated (15, 16).

Immunocytochemistry

From each animal a piece of buccal gingiva from the lower molar area was excised, embedded in Tissue Tec R, and frozen in 2-methyl-butane liquid nitrogen.

Frozen sections were incubated for 60 min with the monoclonal antibodies OX6 (B lymphocytes; Serotec, Blackthorn, Oxon, U.K.) or W3/25 (T helper cells; Sera-lab, Crawley Down, Sussex, U.K.). The second layer was peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts, Copenhagen, Denmark). The peroxidase was shown by reaction with 3-amino-9-ethylcarbazole (Sigma). The slides were counterstained with hematoxylin. Control specimens were incubated with OKT4 (human helper cells) or OKT8 (human suppressor/cytotoxic cells) (both Ortho, Raritan, N.J., USA). These specimens as well as specimens without the first or second layer showed no reaction whatsoever.

Statistics

Numeric density data were expressed as arithmetic means ± standard error of the

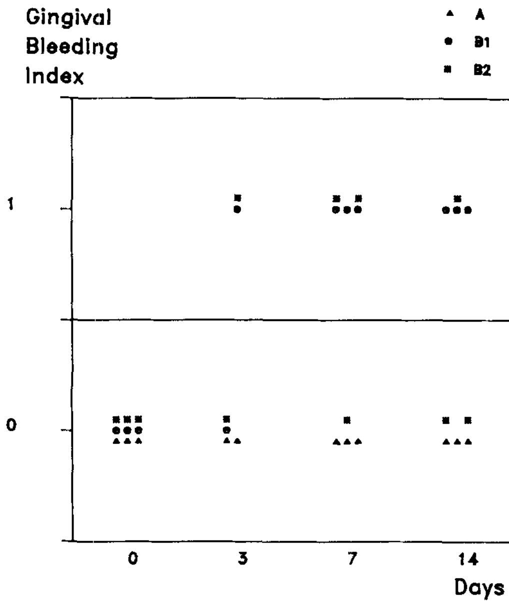

Fig. 1. Development of gingivitis in the three groups of rats during the experiment. Each symbol represents one animal.

mean. Other variables were demonstrated graphically. Because of the low number of animals per subgroup, no statistical tests were made. Instead, trends in the material will be described.

Results

Plaque and gingivitis scores

Dental plaque accumulated heavily throughout the experiment, no differences being seen between the three groups of rats.

All animals had clinically healthy gingiva on day 0. None of the rats in group A developed clinical signs of gingivitis during the experiment. In group B2 some of the animals had gingival bleeding on days 3, 7, and 14. In group B1 all animals had gingival bleeding on days 7 and 14 (Fig. 1).

Antibody titers

Serum antibody titers against the four strains of bacteria that were used to provoke gingival inflammation are shown in Fig. 2.

In general, titers were low in group A, moderate in group B2, and high in group B1. Peak responses were usually seen on day 7, but even on day 0 high titers were found in some animals, particularly in group B1.

Numeric density

A considerable number of lymphocytes were seen in all groups on day 0 (Table 1). In group A this number remained fairly constant until day 14, when a slight increase was seen. In group B1 the number increased throughout the experiment and had almost tripled by day 14. In group B2 the number doubled between days 3 and 7 and remained at this level on day 14.

Plasma cells were very rare on day 0, but their number increased substantially throughout the experiment in all groups, except for a slight decrease on day 14 in group B1.

Macrophages were absent or scanty in all groups. No systematic changes in the number of macrophages were seen during the experiment.

Immunocytochemistry

The immunocytochemistry studies showed few gingival cells that were positive for the markers OX6 (B lymphocytes) and W3/25 (T helper lymphocytes). No systematic staining pattern was seen, and no differences were found between the experimental groups.

Discussion

The heavily increased numbers of gingival lymphocytes and plasma cells in all animal groups during the experiment clearly indicate that an inflammatory reaction took place in relation to the introduction of the four microorganisms. This is in agreement with previous studies, which show that all four bacterial species can colonize the oral cavity and cause periodontal disease in rats (17–20).

In group A the lymphocytes appeared later and in smaller numbers than in the older rats. On day 14, when the experiment was terminated, the histologic pictures in

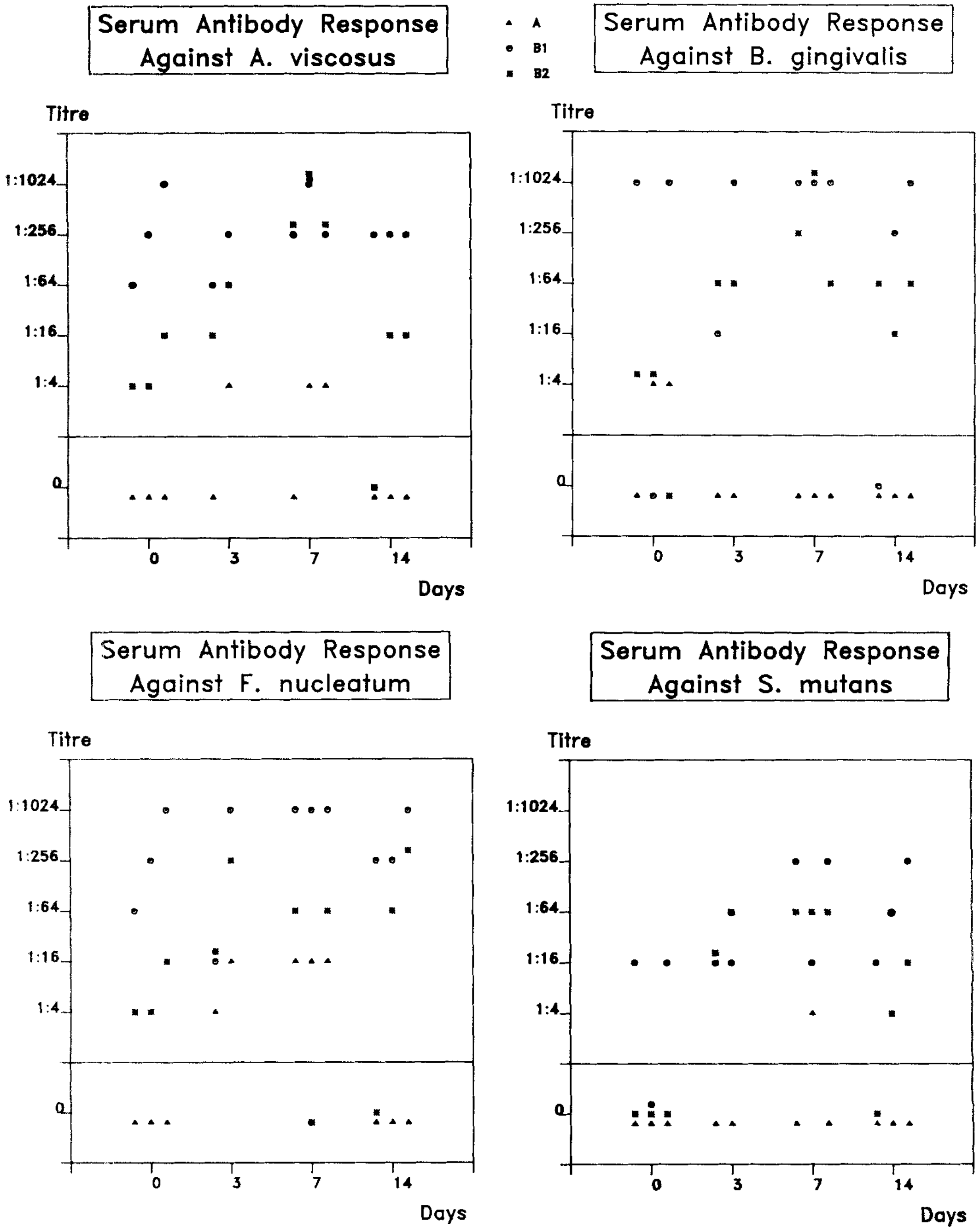

Fig. 2. Serum antibody titers against the four bacterial strains in each group of rats during the experiment. Each symbol represents one animal.

groups A and B2 were similar, with almost equal numbers of lymphocytes and plasma cells, but in group B1 the ratio was 3:1 in favor of lymphocytes. These results are quite contrary to the findings in man, in whom lymphocyte numbers are equal in adults and children, whereas plasma cells are much more numerous in adults (7).

Table 1. Numeric density of gingival inflammatory cells in the three experimental groups during the experiment

Cells, no./mm³	Day			
	0	3	7	14
Lymphocytes				
Group A	11.4 ± 0.6	10.8 ± 0.5	10.8 ± 1.0	14.2 ± 1.4
Group B1	11.3 ± 0.6	15.9 ± 1.3	16.8 ± 1.4	31.8 ± 2.7
Group B2	11.6 ± 0.9	9.5 ± 0.6	19.2 ± 2.1	18.1 ± 1.6
Plasma cells				
Group A	0	9.3 ± 0.5	10.3 ± 0.9	15.5 ± 1.5
Group B1	0	5.9 ± 0.4	16.7 ± 1.3	10.1 ± 0.9
Group B2	3.9 ± 0.3	8.7 ± 0.6	14.2 ± 1.4	14.7 ± 1.3

The serum antibody measurements showed high titers against all bacteria in group B1 already on day 0, indicating that the immunization procedure was successful. In group A the amounts of specific antibody were low on day 0, in some cases undetectable, which suggests that previous contact with the four microorganisms was negligible. In group B2 the titers on day 0 were slightly higher than in group A; this could be due either to previous contact with the bacteria or to a greater amount of cross-reacting antibody in the older animals.

During the experiment the titers remained high in group B1 and low in group A. In group B2 the titers increased markedly from day 0 to day 7, also against *B. gingivalis* and *S. mutans*, for which the starting titers were just as low as in group A. This strongly suggests that the older animals were more capable of raising an antibody response than the younger ones.

In general, the histologic and serologic findings all indicate a level of immunologic responsiveness that was higher in adult than in young rats. The level was particularly high in rats that had been injected with dead bacteria of the four strains involved in the experiment. This difference between the groups was also reflected by the clinical state of their gingiva, since all animals in group B1, none in group A, and 50% in group B2 bled on probing during the experiment. These results are similar to the findings in man, in whom gingival inflammatory reactions to dental plaque are slower and less pronounced in younger individuals (2–6, 9).

In summary, the reactions seen in adult immunized rats suggest that sensitization to plaque antigens could be a major factor contributing to the increased tendency to develop gingivitis seen in adults. However, the serum antibody findings seem to indicate that the immune system of older individuals has a capacity to react against plaque antigens which cannot solely be explained by specific priming to the antigens.

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