Role of Bacterial Products in Periodontitis: Immune Response in Gnotobiotic Rats Monoinfected with *Eikenella corrodens*

D. A. JOHNSON,1 U. H. BEHLING,1 C.-H. LAI,1 M. LISTGARTEN,1 S. SOCRANSKY,2 AND A. NOWOTNY*

Department of Periodontics, Center for Oral Health Research, University of Pennsylvania, Philadelphia, Pennsylvania 19104,1 and Forsyth Dental Center, Boston, Massachusetts 021152

Received for publication 22 June 1977

The development of humoral and cell-mediated immune responses to *Eikenella corrodens* (a bacterium that causes periodontal lesions in gnotobiotic rats) was measured and compared with the rate of appearance of macroscopic lesions. A possible inverse relationship was found. A strong cell-mediated immune response, as measured by skin reactivity and lymphocyte mitogenesis, occurred between 4 and 6 weeks after infection and subsided soon thereafter to a low response level. Humoral antibodies to endotoxin from *E. corrodens* could not be detected at any time. The disease developed only after the cell-mediated immune response diminished, thus suggesting that lack of an efficient immune response may permit the development of the disease. This is seemingly in contradiction to the assumption that tissue destruction in such cases is caused by the immune response and its products. We are inclined to believe, based on our findings reported here, that the lack of immune responsiveness to the bacterium and/or its products is the major causative factor in the development of periodontitis. At the same time, we wish to emphasize that occurrence of both phenomena during the long development of periodontal disease is possible.

Regarding the etiology of periodontal disease in man, one possibility is that products of bacteria present in dental plaque diffuse across the gingival epithelium and directly induce inflammation and tissue damage, including bone resorption (26–28). Endotoxin could be such a product of growing bacteria. Another possibility is that an immune response to bacteria or to bacterial products results in the release of mediators responsible for the observed inflammation and tissue lesions. This latter possibility was elaborated by Ivanyi and co-workers (12, 13, 15) and supported by further studies conducted in the laboratories of Horton and associates (6, 7). These authors reported that there is a strong correlation between the presence of moderate periodontal disease and an increased cellular immune response to autologous plaque antigens, as measured by lymphocyte transformation in vitro. They also observed a decreased lymphoblast response in patients who had severe periodontal disease. It was suggested that this latter effect may be due to the presence of blocking factors in the patients’ sera (14).

Further evaluation of these relationships has been greatly hindered for a long time by the lack of an animal model for periodontal disease.

The pathogenesis of periodontal disease in monoinfected rats has been described by a number of investigators who used a variety of infecting microorganisms (2, 3, 7, 11, 17, 18, 29). In brief, periodontal disease in this animal model first appears in the maxillary interdental molar areas but eventually involves the mandible as well. The lesion is associated with hair impaction and, in the case of gram-positive dental plaque-forming microorganisms, with massive accumulation of microorganisms on tooth surfaces. Gram-negative infections are devoid of such adherent microbial masses. In fact, gram-negative bacteria are few and appear infrequently in proximity of lesions. In no case is there frank invasion of the tissues by bacteria, a feature of the disease also common to the conventional disease seen in humans. The disease in the gnotobiotic rat is characterized by a progressive destruction of the interdental tissues, including gingiva and alveolar bone, in the presence of an inflammatory response that is relatively mild or absent (10). With progress of the disease, crater-like defects develop between the affected molars, which gradually lessen as a result of the loss of the supporting structures. This loosening is eventually accompanied by tooth migration, which results in teeth moving out of their normal alignment and, eventually, possible exfoliation.

It is feasible to grade the severity of periodontal disease on a semiquantitative basis by means...
of the scoring system outlined in Materials and Methods.

Although scoring of defleshed jaws for actual bone loss has been used frequently as a method to evaluate the severity of periodontal disease in rats (4), the method precludes histological studies on the same material.


In the present study, we determined quantitatively the humoral and cellular immune responses of such rats monoinfected with *E. corrodens*. Using this system, we wished to determine whether there is an interrelationship between the development of disease and the immune response in the rats.

**MATERIALS AND METHODS**

*E. corrodens*. The strain of *E. corrodens* used was designated CS10. It has been found to give rise to two morphological variants, one being shiny, smooth, and raised on agar surfaces, designated CS10A, and the other being a spreading and corroding type, designated CS10B.

For the cultivation of the bacteria, the following components were dissolved in 10 liters of distilled water: Trypticase (Baltimore Biological Laboratory [BBL], Cockeysville, Md.), 170 g; Phytone (BBL), 30 g; sodium chloride, 40 g; potassium nitrate, 10 g; and sodium formate, 10 g. After complete dissolution, the medium was autoclaved in 12-liter fermentation jars. Hemin was dissolved separately as follows. A 50-mg amount of hemin was mixed with 1 ml of 1 N NaOH and, after dissolution, diluted to 100 ml with distilled water. It was autoclaved, and 60 ml of this solution was added to the above autoclaved 10-liter mixture of nutrients. The jars were inoculated with 100 ml of a 24-h culture of the bacteria grown in the above medium and sealed. The bacteria were grown as still cultures at 37°C for 35 h. The cells were killed with 0.5% phenol, harvested by centrifugation at 1,000 × g for 30 min, and washed once with distilled water.

**Crude antigen preparation.** Lyophilized bacterial cells were suspended in saline in the required concentrations and subjected to sonic treatment on ice for 15 min at 100 W with a Branson Sonicator, using a 70% pulse.

**Endotoxin preparation.** Endotoxin was extracted from CS10A or CS10B by homogenizing 1 g of lyophilized cells in 35 ml of distilled water at 68°C for 15 min, after which 35 ml of 90% phenol (also warmed to 68°C) was added, and homogenization was continued for 10 min more. The mixture was then cooled to 4°C and centrifuged at 3,000 × g for 30 min in glass tubes. The phenol phase was extracted twice more with 35 ml of water at 68°C, as described above. The water phases were pooled and dialyzed against distilled water at 4°C for 3 days, changing the water twice daily. The dialyzed preparation was lyophilized. To prepare solutions for injection from lyophilized material, aliquots were suspended in saline and subjected to sonic treatment for 5 s to ensure even dispersion and facilitate dissolution.

**Induction of periodontal disease.** Twenty-four weanling germfree, Sprague-Dawley rats were placed in sterile isolators. In the first two series of experiments, the animals were infected by placing a fully developed rat, monoinfected with the CS10 strain, in the isolator. Fecal cultures were taken every 2 weeks to ascertain that the animals were infected with CS10 only.

Three large series of experiments were carried out. In the first series, the first rats were sacrificed 7 weeks after infection. Every week thereafter, up to 14 weeks, additional animals were removed from the isolator and their immune response was measured. In the second series, two rats were sacrificed every week between 2 and 14 weeks after infection. In the third series of experiments, Sprague-Dawley rats obtained from a commercial supplier in Boston as germfree animals were used. We found that the animals were infected with *Bacillus subtilis*. In spite of this, we started the experiment as described above, superinfecting these rats with *E. corrodens*. This time, the *Eikenella* infection was carried out by applying culture broth to each rat’s oral cavity with a swab. The first animals were sacrificed 1 week after the superinfection, and their spleens were used for lymphoblast assay in a fashion identical to that described below.

The animals were skin-tested under sterile conditions 24 h before sacrifice, as described below. At sacrifice, the spleens and sera of the animals were taken. The sera were frozen for later anti-CS10 determination by passive hemagglutination. The spleens were used immediately in the lymphocyte transformation assay.

Clinical severity score. At the time of sacrifice by decapitation, the heads were immediately fixed in a mixture of 5% glutaraldehyde and 4% paraformaldehyde, buffered to pH 7.2 with sodium cacodylate. The interdental spaces in the molar region were examined with a dissecting microscope, and the periodontal status was scored as follows: 0, no disease (teeth firm, in line, and in contact with one another, without any impacted hair or bedding in the gingival sulcus); 1, mild disease (teeth firm, in line, and in contact with one another, with evidence of some impacted hair or
bedding in the sulcus); 2, moderate disease (teeth firm and in line, open contact points, and definite impaction of hair and bedding); 3, severe disease (teeth out of line, possibly loose, with extensive impaction of hair and bedding, open contacts, and alveolar ridge deformities). For each time interval, the periodontal status was assessed by a mean severity score, obtained by adding the scores of all available interdental spaces in the molar regions (8 per animal, giving a total of 16 scores) and dividing by the number of spaces scored.

Endotoxicity assays. The local Shwartzman skin reactivity test was carried out as described earlier (24). The Limulus lysate clotting assay was carried out according to the procedure of Levin et al. (20).

Skin sensitivity tests. The skin tests were carried out by injecting 100, 10, or 1 μg of CS10A, CS10B, crude antigens, or similarly treated sonic extract of Escherichia coli O8 bacteria in 0.2 ml of saline. Similarly, endotoxin preparations were also injected in 20-μg quantities. All injections were given intradermally in the back skin of the rats under sterile conditions. The results were read after 24 h by shaving and skinning the animals. The diameter of maximum erythema and induration was measured in two directions at right angles.

Passive hemagglutination. Blood was collected at the site of decapitation and allowed to clot at room temperature. The serum was prepared by centrifugation and frozen at −20°C until used. The passive hemagglutination assays were carried out according to Neter et al. (21), using the endotoxin preparations from CS10A and CS10B strains. The successful coating of erythrocytes with CS10 endotoxins was checked by agglutinating these cells with CS10 antiserum, produced in rabbits.

Lymphocyte transformation. Spleen cells were obtained by tearing the organs in a small volume of RPMI 1640 medium enriched with 10% fetal calf serum (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.). 100 U of penicillin-streptomycin (GIBCO) was added. The cells were filtered through sterile gauze and adjusted to a concentration of 2 × 10^8 viable cells per ml. One milliliter of this suspension was added to triplicate tubes that contained one of the following preparations: 1, 10, or 100 μg of sonically treated CS10A or CS10B and 20 μg of endotoxin from CS10A or CS10B; 10 μg of concanavalin A (Sigma Chemical Co., St. Louis, Mo.); or saline. The culture tubes were maintained at 37°C for 68 h in a 5% CO2 incubator after which they received 1 μCi of tritiated thymidine (New England Nuclear Corp., Boston, Mass.) in 10 ml of saline. The tubes were then incubated for an additional 4 h and harvested as described previously (16). Radioactivity incorporation into the cells was determined with a Mark I scintillation counter (Searle Radiographics Inc., Cherry Hill, N.J.). The results were expressed as counts per minute of the sample divided into the counts per minute of the saline controls, giving a stimulation index.

Microviscosity of living cell membranes. The microviscosity of cell membranes can be measured by evaluating the fluorescence of an incorporated probe (1,6-diphenyl-1,3,5-hexatriene). The viscosity is determined by exciting the probe with polarized light and measuring the depolarization of the fluorescence caused by the rotation of the probe in the membrane. Highly viscous membranes hinder the rotation of the probe, and, consequently, the emitted light maintains a high degree of polarization. Alternatively, in a membrane of low viscosity, only a low degree of polarization is obtained.

The effect of endotoxin from CS10A, CS10B, and Serratia marcescens on the microviscosity of WI-38 cell membranes was measured with an Elscint Microviscosimeter MV-1 (Elscint, Ltd., Rehovot, Israel). The cells were used by a modification of the procedure described by Inbar and Shinitzky (8) with a fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene. Accordingly, a 2 × 10−6 M 1,6-diphenyl-1,3,5-hexatriene dispersion was made by adding 0.1 ml of 2 × 10−3 M 1,6-diphenyl-1,3,5-hexatriene in tetrahydrofuran to 100 ml of phosphate-buffered saline, which was stirred vigorously for 15 min at 25°C, resulting in a clear dispersion. N2 was bubbled through the liquid for an additional 15 min to drive off the tetrahydrofuran. A total of 109 WI-38 cells in 50 ml of phosphate-buffered saline was then labeled by adding them to an equal volume of the 1,6-diphenyl-1,3,5-hexatriene dispersion. This mixture was incubated with frequent mixing at 25°C for 30 min, after which the cells were washed three times with phosphate-buffered saline. The cells were then diluted to give consistent incident light and readings after two successive 1:10 dilutions.

The cells were exposed to endotoxin preparations at 100 μg/ml of phosphate-buffered saline and the microviscosity was measured at 10 min and 1 h. Buffer without endotoxin was used as a negative control. The results are reported as poise (P) units, which relate directly to microviscosity (9).

RESULTS

Endotoxicity assays. In the Shwartzman and Limulus lysate assays, the CS10A phenol-water extracts were found to have activities comparable to similar quantities of S. marcescens endotoxins extracted by the same procedures. The CS10B endotoxin was much less active in the assays described above than the S. marcescens standard preparation.

Microviscosity changes due to exposure to endotoxin. Microviscosity changes due to endotoxin exposure are given in Table 1. As can be seen, there was a marked decrease in the microviscosity of the cell membranes exposed to CS10B endotoxin. CS10A endotoxin had little effect, whereas S. marcescens endotoxin resulted in a marked increase in microviscosity. The buffer control did not change.

Clinical signs. In the second experiment, the first clinical signs of periodontal disease were observed in week 6. With one exception (at week 3, when one rat showed a mild lesion), there were no signs of disease until week 6. Mild to moderate disease was present during weeks 7, 8, and 9 in all rats. Severe disease first appeared at week 10. All the experimental animals in both the first and the second groups developed mod-
erate to severe disease during these last weeks.

Passive hemagglutination. The sera from all rats in these two experiments showed no antibody titer against the CS10A or CS10B endotoxin-coated cells. The rabbit sera, which were obtained by immunizing animals with CS10 organisms, gave a titer of 256, thus indicating that the sheep erythrocytes were sufficiently coated with the endotoxins.

Lymphocyte transformation. In the first series of experiments, in which the first rats were sacrificed 7 weeks after infection, low to moderate stimulation could be detected. During the following weeks, periodontal disease developed in the animals to its full severity. All concanavalin A stimulation indexes were in excess of 10-fold stimulation during the same period. The results are shown in Fig. 1.

The results of the second series of experiments, which included the period of time before the appearance of clinical symptoms (2 weeks after infection), showed a sharp peak of lymphoblast reactivity to CS10 antigens during weeks 5 and 6, as indicated in Fig. 2 through 4. Figure 2 shows the stimulation indexes obtained when CS10A crude antigen was used to stimulate the spleen cells. Figure 3 was obtained by using CS10A endotoxin. Figure 4 shows the results where CS10B crude antigen was used for the lymphocyte transformation assay. Figure 5 was obtained when CS10B endotoxin was used in the same assay. In Fig. 5, a reduced peak can be seen in weeks 5 and 6. The stimulation indexes obtained by using S. marcescens endo-

![FIG. 1. Lymphoblast transformation, group 1. Symbols: O, stimulation obtained by whole bacterial sonic extract of CS10B; , stimulation by whole bacterial sonic extract of CS10A; , stimulation by S. marcescens endotoxin. All indexes are compared with saline controls.](image1)

![FIG. 2. Lymphoblast transformation: CS10A crude antigen, group 2. Each point represents the geometric mean of the values obtained from two animals.](image2)

![FIG. 3. Lymphoblast transformation: CS10A endotoxin, group 2. Each point represents the geometric mean of the values obtained from two animals.](image3)

![FIG. 4. Lymphoblast transformation: CS10B crude antigen, group 2. Each point represents the geometric mean of the values obtained from two animals.](image4)

**TABLE 1. Measurement of WI-38 cell membrane microviscosity**

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Microviscosity (P) at:</th>
<th>0</th>
<th>10 min</th>
<th>1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS10A endotoxin</td>
<td>0.196</td>
<td>0.194</td>
<td>0.193</td>
<td></td>
</tr>
<tr>
<td>CS10B endotoxin</td>
<td>0.196</td>
<td>0.180</td>
<td>0.182</td>
<td></td>
</tr>
<tr>
<td>S. marcescens endotoxin</td>
<td>0.196</td>
<td>0.210</td>
<td>0.214</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>0.196</td>
<td>0.195</td>
<td>0.196</td>
<td></td>
</tr>
</tbody>
</table>

*All endotoxin preparations were at 100 μg/ml of phosphate-buffered saline.

Poise values of viscosity (P) represent units of force per unit of area required to maintain a unit of difference in flow velocity between two parallel layers that are a unit of length apart. P is expressed as dynes per second per square centimeter. Therefore, P relates directly to viscosity.
toxin peaked in week 6. The stimulation index went down after this to low levels, which is in agreement with the results obtained during the first experiment.

The lymphoblast assay results in the *B. subtilis* - and *E. corrodens*-infected animals showed a quite different pattern. Positive stimulation could be seen 1 week after the superinfection with *Eikenella*, which lasted for about 3 weeks, subsiding from that time to insignificant levels. Only CS10B endotoxin caused a slight increase in the stimulation index; CS10A was without effect. The lesions developed again at the time when the lymphoblast assay declined. In this system, neither the lymphoblast assay nor the degree of periodontal lesions was as clearly expressed as in animals monoinfected with *E. corrodens* (see Fig. 7).

**Skin tests.** The results of the skin tests are summarized in Table 2. Figure 6 represents the skin reactions to CS10A endotoxin. The results obtained by using CS10B endotoxin were quite similar; therefore, they are not presented here. The reactions to the injections of crude CS10A and CS10B antigens, using whole sonic extracts of cells, were weaker and less reproducible. Most of the reactions with these preparations occurred during the first half of the infection period, during weeks 4 through 7.

**DISCUSSION**

Various compounds, including many drugs, have been found to have effects on the microviscosity of cell membranes in similar systems. The results obtained in this study indicate that endotoxins also affect the microviscosity of the cell membrane. The drop in microviscosity observed with the CS10B endotoxin was unexpected in view of other experiments in our laboratories that indicate that endotoxin preparations tend to cause an increase in microviscosity. This intriguing result is most probably due to unique structural characteristics of the CS10B endo-

---

**Fig. 5. Lymphoblast transformation: CS10B endotoxin, group 2.** Each point represents the geometric mean of the values obtained from two animals.
ROLE OF BACTERIAL PRODUCTS IN PERIODONTITIS

The results of the lymphocyte transformation assay clearly show that there is a strong response early in the period of infection, peaking at weeks 5 and 6. This peak occurred before the time when clinical signs appeared. As these symptoms progressed, the stimulation index dropped to a greatly reduced level. It is even more important that these results were paralleled by the reactions observed in the skin test. The greatest skin reactivity was seen between weeks 4 and 7 of infection. Little or no reaction could be detected before or after this period. The greatest reactions both in the lymphoblast assay and in the skin test were observed when purified endotoxin from CS10A or CS10B was used instead of crude antigens obtained by sonic treatment of whole bacteria. These findings indicate that endotoxins are important antigenic components of these organisms.

The findings with the B. subtilis- and E. corrodens-infected rats showed both development of disease and the lymphoblast reactivity at a much earlier time than in monoinfected rats. This may be due to the fact that in these animals the superinfection was carried out directly, by a mouth swab with virulent Eikenella bacteria, instead of exposing the germfree animals to a monoinfected animal in the same isolator, which was the procedure used in the first two series of experiments.

The fact that the degree of lymphoblast stimulation as well as the degree of periodontal lesion development was much reduced in these animals may be attributed to two possible factors. One is that the source of the animals was different than the source in the first two experiments. According to unpublished observations from S. S. Socransky's laboratory, the germfree colony at Forsyth Dental Center is much more susceptible to Eikenella infection-caused periodontal lesions than are other germfree rat lines. An other conclusion is that infection with other bacteria in addition to Eikenella (B. subtilis, in this case) may render the animals less susceptible to the effects of Eikenella. Figure 7 summarizes the findings.

A relatively low level of reactivity was seen to S. marcescens endotoxin in the lymphocyte transformation assay or to E. coli O8 cell homogenate crude antigen in the skin assay. We assume, based on the fact that gram-negative endotoxins contain serologically cross-reactive components (23), that these reactions are caused by such components of the preparations used.

The inverse relationship between the state of disease in the animals and their cellular immune response to CS10A and CS10B antigens, demonstrated in these studies, appears to be similar to the human situation, in which the response to peripheral lymphocytes drops off as symptoms of the disease increase to severe levels (14). Further similarities are seen when comparing our findings with the study of Lehner et al. (19), in which the immune response to specific bacterial antigens was measured during experimental gingivitis in human volunteers. This response peaked after 5 weeks and then dropped back to a baseline level.

The finding that the disease increases in severity at the time when the intensity of immune reactions drops markedly may be explained by assuming the development of a high zone of tolerance. This phenomenon results in a decreased efficiency of the immune response, which may allow the accumulation of bacteria and their products. Endotoxin may very well be one such product, and it has already been shown to stimulate bone resorption and collagenase release, in addition to the induction of inflammation and the release of a number of mediators (5, 6).

Furthermore, we wish to emphasize here again that not all endotoxins are identical, either bio-

FIG. 6. Skin test response to CS10A endotoxin. Results are expressed as products of two diameters.

FIG. 7. Summary of findings. Symbols: 0, stimulation by CS10B endotoxin as compared with saline controls; 0, stimulation by CS10A endotoxin compared with controls. Bars indicate the clinical severity score.
logically or chemically. There are endotoxins that are lipid soluble, such as those produced by heptoseless rough mutants of some gram-negative bacteria; some others are relatively low in lipid content and still demonstrate some endotoxic properties (1, 25, 30). We still think it is quite reasonable to assume that some of these newly isolated gram-negative bacteria growing in the gingival pockets of some patients may produce unusual endotoxins or other bacterial products that will lead to the development of periodontal disease as soon as the immune response against them fails. Indeed, our findings indicate that E. corrodens endotoxin is significantly different from S. marcescens and other common endotoxins with respect to its effect on the microviscosity of cell membranes, as discussed above. The importance of this finding may lie in the possible enhanced transport of materials across the membranes of cells directly affected by the locally produced E. corrodens endotoxins. Such an event may lead to accelerated cell destruction in the gingiva and may also lead to enhanced activation of osteoclasts. Experiments testing the above possibilities are in progress in our laboratories.

It may be necessary to emphasize at this point that we do not conclude from these studies that endotoxic lipopolysaccharide from E. corrodens is the sole bacterial product involved in the pathogenesis of periodontitis. The highly unusual effect of E. corrodens on membranes may make the affected cells more accessible to harmful bacterial products other than endotoxins. The reason we used isolated endotoxin as well as whole cell homogenates in some of the assays was to include in the reactivity measurements endotoxins as well as other components of the bacterial cell. In a comparison of the findings in Fig. 4 and 5, it is clearly visible that whole cell homogenate was much more active than isolated endotoxin in the lymphoblast assay, which points to the correctness of our assumption.

Finally, we wish to point out that the two possibilities, namely, either destructive immune response or lack of protective immune response to plaque products, may both occur during the development of periodontitis. It is possible that in the phase of high immune responsiveness, harmful mediators were produced, and their effects will be manifested only some time later, which time may coincide with the energy to plaque products. It is also possible that direct harmful effects of plaque products on gingival tissue and on bone structure cause the destruction, and, while a good immune response is mounted against them, they will be kept under control efficiently. It may be even more reason-
able to assume that some of the extremely heterogeneous plaque constituents will induce the production of harmful mediators of cellular immune responses, whereas others will induce damage to the periodontium via nonimmune mechanisms. The existences and involvements of all the above mechanisms are not mutually exclusive.

ACKNOWLEDGMENTS

We acknowledge the excellent technical help of Robert Kalwinski, Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pa. We also thank R. Christopher Miller for his help in microviscosity measurements. Our most sincere thanks are extended to Elscint, Ltd. (Rehovot, Israel), who loaned the microviscosimeter to us for this study.

This research was supported by Public Health Service grants 5 RO1 DE-03685 and DE-02623 from the National Institute of Dental Research.

LITERATURE CITED

13. Ivanyi, L., and T. Lehner. 1971. Lymphocyte transfor-