INFECTION AND IMMUNITY, Nov. 1978, p. 382-386
0019-9567/78/0022-0382$02.00/0

Vol. 22, No. 2
Printed in U.S.A.

Role of Bacterial Products in Periodontitis: Humoral Immune Response to *Eikenella corrodens*

D. A. JOHNSON, U. H. BEHLING, M. LISTGARTEN, AND A. NOWOTNY*

Center for Oral Health Research, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received for publication 18 April 1978

Eikenella corrodens can induce periodontitis-like disease in gnotobiotic rats. Some components of this bacterial cell elicit measurable humoral immune response during the development of the disease, but in this system endotoxin is not among the efficient immunogens. Because no humoral immune response could be seen to the endotoxin of *Eikenella corrodens* it is assumed that this endotoxin can act uncontrolled in monoinfected rats. Accordingly, the lack of protective humoral immune response to pathogenic components of *Eikenella corrodens* may be the major factor permitting the development of the disease described here. The possibility that both cell-mediated immunity and uncontrolled endotoxic action are parts of the pathomechanism of the disease is supported by our observations.

Eikenella corrodens, a microaerophyllic, gram-negative microorganism, has been isolated from the deep gingival pocket of a periodontosis patient by Newman et al. (7). Monoinfection of germfree rats at the age of 3 weeks with *E. corrodens* causes severe alveolar bone loss in 10 to 12 weeks (3; A. C. R. Crawford, S. S. Socransky, E. Smith, and R. Phillips, J. Dent. Res., Abstr. no. 275, 1977). The histopathological changes of the rat gingiva during the development of the disease are described by us in a recent publication (5).

Earlier longitudinal studies on the immune response in rats to *E. corrodens* during the development of the disease involved the measurement of cell-mediated immune (CMI) response. It was found that the development of the CMI reaches maximum levels in 4 to 6 weeks before subsiding rapidly to background levels. The disease developed only after the CMI response diminished. This finding has been interpreted by us as an indication of inefficient immune response to bacterial products, which permits the development of the periodontal disease (3). The results presented here were obtained by measuring the humoral immune response in monoinfected germfree rats during the development of the disease. In addition, we also present data from a pilot study in which we measured the humoral immune response to *E. corrodens* in monoinfected rats which were immune suppressed by whole-body irradiation throughout the course of disease development.

MATERIALS AND METHODS

E. corrodens cultivation. *E. corrodens* isolate no. 1073 has two morphologically distinct types. One, designated as CS-10-A, grows on blood agar as small, shiny, raised colonies. The other, designated as CS-10-B, shows under identical conditions spreading, dull growth, which also corrodes the blood agar surface. Electron microscopic sections of the two strains revealed minor differences between the two strains. The cell wall structure of CS-10-B is more convoluted than of CS-10-A. Also, CS-10-B cells are longer than CS-10-A cells, and their cytoplasm is more electron dense.

Accordingly, we cultivated the two strains separately but used the same medium, Todd-Hewitt broth, with 2 g of KNO_3 per liter, 5 mg of hemine per liter dissolved in 1 N NaOH (pH adjusted to 7.8), and 50 mg of L-cystein-hydrochloride. The bacteria were grown in a 5% CO_2 atmosphere under constant agitation by a slow magnetic stirrer.

Preparation of bacterial sonic extracts. A suspension of killed (0.5% phenol, final concentration) bacteria containing 10^8 to 10^9 cells in distilled water was sonically disrupted with a Branson Sonifier on ice for 15 min at 100 W.

Isolation of endotoxic lipopolysaccharides. A 1-g amount of harvested, washed, and lyophilized bacteria was extracted with 50 ml of 45% phenol at 70°C by the method of Westphal and Lüderitz (12). The water phase was dialyzed at 4°C for 3 days against distilled water, filtered, and precipitated by 2 volumes of methanol containing 0.1% $MgCl_2$. The precipitate was redissolved in 10 ml of distilled water and sedimented by ultracentrifugation at 100,000 × *g* for 2 h. The sediment was lyophilized. The same procedure was applied to both CS-10-A and -B strains.

Passive hemagglutination. The method of Neter

(6) was used to determine the humoral immune response to *E. corrodens* sonic extracts and to the phenol-water-extracted endotoxins. The endotoxins or the CS-10-A or -B sonic extracts were heated in a boiling-water bath for 2 h at neutral pH and used to coat sheep erythrocytes. The coating was carried out at 37°C, for 30 min, by shaking the cell suspensions gently at approximately 5-min intervals. The cell suspensions were centrifuged and washed three times with physiological saline. The final suspensions of coated sheep erythrocytes were prepared by dispersing 0.2 ml of packed-cell sediment in 10 ml of physiological saline.

The efficiency of the coating procedure was demonstrated by titrating coated cells with CS-10-A or -B antisera produced in rabbits immunized with whole *E. corrodens* bacteria. The titration was carried out in microtiter plates by the routine procedure of 1:2 serial dilutions of antiserum with saline before adding antigen-coated erythrocytes.

Infection and bleeding of germfree rats. Three-week-old germfree rats were infected by two different procedures. In procedure 1, a single, fully grown germfree rat, monoinfected with a mixture of *E. corrodens* (CS-10-A and CS-10-B), was placed in the isolator housing 32 3-week-old germfree rats. In procedure 2 all germfree rats were infected intraorally with the mixture of CS-10-A and -B strains by using a swab dipped into a broth culture. Simultaneously the sterile drinking water and sterile Keys 2000 diet of the rats were contaminated with the same mixture of the two strains. In procedure 3, the animals were first immunosuppressed by irradiation as described in detail below. The infection was induced in the same manner as in procedure 2. Pairs of animals were sacrificed every week starting 8 days after infection. Whole blood was collected and allowed to coagulate for 24 h at 4°C. The sera were separated by centrifugation and stored at −20°C until the end of the experiment, at which time all sera were tested.

Measurement of the degree of disease development. The decapitated heads were preserved in a mixture of 5% glutaraldehyde and 4% paraformaldehyde, buffered with sodium cacodylate to pH 7.2. The spaces between the three molars in all four quadrants were investigated with a dissecting microscope. Scoring of the disease was done as follows: no disease, 0; mild disease (teeth firmly in place, in line, and in contact with each other, but evidence of hair and bedding impaction visible), 1; moderate disease (teeth firm and in line but open contact points with hair and bedding impaction in the gingival sulcus), 2; advanced disease (teeth loose, out of line or lost, extensive hair and bedding impactions, alveolar ridge deformities), 3.

The periodontal status was assessed for each animal by adding the scores for all eight interdental spaces and dividing the total by 8. If n number animals was used per group, the sum of the scores was divided by $n \times 8$.

Immunosuppression of rats by whole-body irradiation. Monoinfected rats were transferred from the isolator into a sterile portable chamber by means of a sterile transfer sleeve. The portable cylindrical chamber was custom-made by our machine shop from 0.25-inch (ca. 0.64-cm) Lucite. The dimensions of the chamber measured 12 inches (ca. 30.48 cm) in diameter and 4.5 inches (ca. 11.43 cm) in height. The bottom of the chamber was outfitted with an air-tight "window" held in place by six wing nuts. The top side of the chamber held a passive antimicrobial air filter. The interior of the chamber was sterilized by means of ethylene oxide gas.

Whole-body irradiation of rats was achieved by means of a Gamma cell 40 irradiator (Atomic Energy of Canada Ltd.), which is a self-shielded unit containing two double-encapsulated 1,550-Ci cesium-137 sources. The above described portable chamber was placed into the irradiation compartment of the Gamma cell 40. The air exposure dose rate over the 12.5-inch target plane was determined at 97.5 R. Animals were exposed to a primary dose of 350 R which was followed by a maintenance dose of 150 R at 14-day intervals for the duration of the experiment. Although this irradiation dose schedule was sublethal, it effectively reduced the number of viable spleen cells to between 5 and 20% of nonirradiated monoinfected controls. This range of residual spleen cell survival reflects stages of radiation recovery between successive irradiation exposures.

RESULTS

Histopathology and the development of the disease. A detailed description of the histopathology of these monoinfected rats was recently reported by us (5). Of special interest was our observation that the connective tissue remained relatively free of inflammatory cells with the exception of isolated neutrophils. With time, the impaction of hair and bedding continued to affect primarily the junctional epithelium, although isolated hair or fragments of bedding material were also observed in the underlying connective tissue. Despite the encroachment of foreign bodies, no increase in the concentration of inflammatory cells in the connective tissue was detectable. In close proximity to the foreign materials, macrophages and giant cells could occasionally be detected. Even at a time when frequent interruptions in the continuity of the epithelial lining and the onset of marked osteoclastic activity could be observed, the gingival connective tissue continued to remain relatively free of typical lymphocytes and plasma cells.

In procedure 1 the animals showed the first sign of disease after 8 weeks. From then on, the disease developed rather rapidly and reached severe levels with great bone losses in 12 to 14 weeks. The animals which were infected by procedure 2 showed somewhat earlier the beginning of bone losses (week 5), but the progressing disease did not reach the extent shown in procedure 1 even after 14 weeks. The disease levels are indicated by bars in Fig. 1 for procedure 1 and in Fig. 2 for procedure 2.

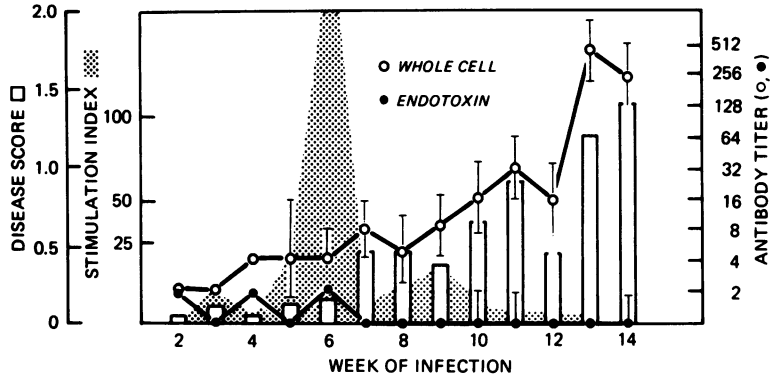

FIG. 1. *Correlation between disease development and rise of immune response in E. corrodens-monoinfected rats. Infection has been carried out by procedure 1. Vertical lines represent range in the titer values.*

The most rapid development of periodontal lesions was observed in rats which were strongly immune-suppressed by repeated whole-body irradiation before and after infection with *E. corrodens* (procedure 3). Table 1 shows the site and severity of the disease state for each of five individual rats used in this pilot study.

Passive hemagglutination assay. The coating of sheep erythrocytes with bacterial sonic extracts as well as with endotoxin was efficient, because the rabbit antisera to CS-10-A or C-10-B bacteria showed high titers to the sonic extract as well as to the isolated endotoxin.

Antibody titers. By using the sonic extract-coated sheep erythrocytes, the results of titration indicated that both procedures 1 and 2 produced increasing antibody levels to the bacterial sonic extract. The antibody titer increased from week 4 on and reached relatively high levels (titer 512) on week 14. The rate of increase of the antibody to the whole-cell sonic extract was parallel to the development of the disease.

In contrast, antiendotoxin titers remained undetectable in the rats infected by procedure 1 (Fig. 1) and were low but significant if procedure 2 had been used (Fig. 2). Figure 2 shows the antibody titer changes in procedure 2 to whole-cell sonic extracts of CS-10-A and CS-10-B bacteria, as well as the antiendotoxin titers. The humoral immune response to whole-cell sonic extracts was comparable in procedure 1 and procedure 2, but no detectable level of antibodies was produced to endotoxins in procedure 1, whereas procedure 2 gave definite antiendotoxin antibody levels.

In procedure 3, in which radiation immune-suppressed animals were infected with *E. corrodens*, no detectable agglutination titer could be detected for erythrocytes coated with either whole bacterial sonic extracts or their respective endotoxins of both strains. The severe suppression of a humoral response in these animals was to be expected; however, the more rapid and severe development of periodontal lesions in these animals weakens the popular notion that CMI hypersensitive state serves as a contributing mechanism leading to periodontal disease. Future studies will include a series of experiments in which monoinfected and irradiated monoinfected rats will be passively immunized with high-titer antiserum to *E. corrodens*. Similarly, parallel groups of animals will be hyperimmunized by repeated vaccination with nonviable bacterial sonic extracts or extracts of *E. corrodens*. The purpose of these experiments will be to test the contribution of an immune hypersensitive state to the development of periodontitis.

TABLE 1. *Progression of periodontal disease in radiation immune-suppressed gnotobiotic rats as a function of time*

Time (weeks) after initial infection	Location and severity of disease			
	Upper right jaw	Upper left jaw	Bottom right jaw	Bottom left jaw
3	M_1-M_2 (2)[a]	M_1-M_2 (3)	0	M_1-M_2 (2)
4.5	0	0	0	0
6	0	M_1-M_2 (3) M_2-M_3 (1)	0	0
7.5	M_1-M_2 (3)	M_2-M_3 (2)	0	0
9	M_1-M_2 (3) M_2-M_3 (2)	M_1-M_2 (1) M_1-M_3 (2)	0	0

[a] M_1, first molar; M_2, second molar; etc. Numerical score in parentheses indicates severity of disease (see text).

DISCUSSION

The results obtained indicate that although good humoral immune response is induced in rats to bacterial cell components, *E. corrodens*

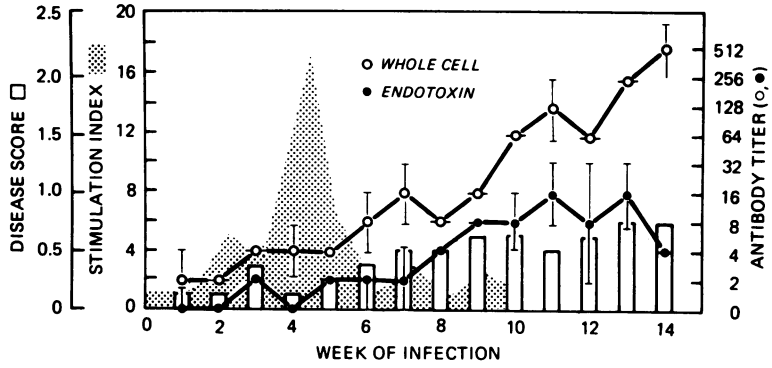

FIG. 2. *Correlation between disease development and rise of immune response in E. corrodens-monoinfected rats. Infection has been carried out by procedure 2. Vertical lines represent range in the titer values.*

endotoxin is not one of these immunogens. During the development of the disease, antibodies are produced to bacterial cell components other than endotoxin. The endotoxins of the CS-10-A and -B strains could not express their immunogenicity under the conditions of infection described here.

Lack of good anti-O titers (anti-endotoxic lipopolysaccharide) if one uses encapsulated bacterial cells is not an uncommon observation because the K (capsular) antigens can suppress the immunogenicity of the underlying O-antigen layers of bacterial cell envelopes. This explains why heat-killed gram-negative bacteria are used for good anti-O serum production, inasmuch as the heating destroys the sensitive K antigens.

It has been shown earlier (8, 9) that antiendotoxin antibodies are highly efficient in detoxifying isolated endotoxins. The lack of good humoral immune response to the endotoxin content of *E. corrodens* may play a significant role in the pathogenic mechanism of this model system. The constantly present endotoxin-containing cells and cell debris in the plaque will not be encountered by immunoglobulins which could neutralize the endotoxins.

The possibility that humoral antiendotoxin immune response may be an essential part of the rat defense mechanism becomes an even more acceptable assumption by comparing the result of procedures 1, 2, and 3. In procedure 1 the antiendotoxin response was zero, and the disease developed rapidly, leading to severe bone losses. In procedure 2, where the disease could not reach the same extent of bone destruction, a low but definite antiendotoxin titer could be uncovered. Although serum antibody levels do not wholly describe the local immune responses of the local periodontal lesion, they do represent a quantitative approximation of the host immune response. In the pilot study described as procedure 3, where the immune response capacity of the rats has been severely reduced by γ-ray irradiation, no antibody response could be manifested. Neither endotoxin nor other components of *E. corodens* elicited measurable antibody levels. In this group of rats, the most rapid and the most extensive destruction was observed. Although whole-body irradiation affects every mammalian cell in varying degrees, probably the most radiosensitive cells which are essential to life are the hemopoietic cells representing the immune system. This differentially destructive effect of sublethal radiation exposure on the immune system can be readily observed by measuring the immune response to an antigenic challenge and/or the histological examination of tissues constituting the immune system. In this preliminary study, we measured the antibody response as well as determined the surviving number of spleen cells at the time of sacrifice. The experiments by procedure 3 will be repeated with larger groups of rats in a more elaborate experimental design by varying several parameters. One conclusion which we feel to be sufficiently safe to draw at this point is that if the disease in the *E. corrodens*-monoinfected rats were accountable for destructive consequences of an immune response to *E. corrodens*, these immunosuppressed rats should not have developed any disease. On the contrary, we saw the most severe lesions manifested in this group of rats.

One should also entertain the possibility that both CMI and endotoxin action are parts of the pathomechanism in the gnotobiotic rat model system. Endotoxin can augment the manifestations of some CMI reactions; for example, as it was shown by Bordet in 1931 (1), guinea pigs sensitized by mycobacteria will show extensive hemorrhages if injected intravenously with endotoxin-containing culture filtrate. There are numerous references indicating similar interactions between CMI and endotoxic action (2, 4,

10, 11). It is quite likely that endotoxin or other possible toxic components of plaque microorganisms additively or synergistically aggravate the pathological effects of CMI-causing bacterial components, leading to a fully developed destructive periodontitis. As Fig. 1 shows, a short-lived but very intensive CMI to *E. corrodens* precedes the development of the bone loss. One should not dismiss the possibility that this transient CMI (as measured by lymphoblast as well as by skin assays) makes tissues more accessible to endotoxin at the same time when no neutralizing antiendotoxin antibodies were produced. The damage may be caused by the endotoxin, but it is the transient CMI which may facilitate the way for the endotoxin to reach its targets.

LITERATURE CITED

1. **Bordet, J.** 1931. Contribution a l'étude de l'allergie non specifique. C. R. Soc. Biol. **106**:1251-1254.
2. **Freund, J.** 1934. Hemorrhages in tuberculous guinea pigs at the site of infection of irritants following intravascular injections of injurious substances (Shwartzman phenomenon). J. Exp. Med. **60**:669-685.
3. **Johnson, D. A., U. H. Behling, C.-H. Lai, M. Listgarten, S. Socransky, and A. Nowotny.** 1978. Role of bacterial products in periodontitis: immune response in gnotobiotic rats monoinfected with *Eikenella corrodens*. Infect. Immun. **19**:246-253.
4. **LaGrange, P. H., and G. B. Mackaness.** 1975. Effects of bacterial lipopolysaccharides on the induction and expression of cell-mediated immunity. II. Stimulation of the efferent arc. J. Immunol. **114**:447-451.
5. **Listgarten, M. A., D. Johnson, A. Nowotny, A. Crawford, and S. S. Socransky.** 1978. Histopathology of periodontal disease in gnotobiotic rats monoinfected with *Eikenella corrodens*. J. Periotol. Res. **13**:134-148.
6. **Neter, E., L. F. Bertram, and C. F. Arbesman.** 1952. Demonstration of *Escherichia coli* O55 and O11 antigens by means of hemagglutination test. Proc. Soc. Exp. Biol. Med. **79**:255-257.
7. **Newman, M. G., S. S. Socransky, E. D. Savitt, D. A. Propas, and A. Crawford.** 1976. Studies of the microbiology of periodontosis. J. Periodontol. **47**:373-379.
8. **Nowotny, A., R. Radvany, and N. Neale.** 1965. Neutralization of toxic bacterial O-antigens with O-antibodies while maintaining their stimulus of non-specific resistance. Life Sci. **4**:1107.
9. **Radvany, R., N. Neale, and A. Nowotny.** 1966. Relation of structure to function in bacterial O-antigens. VI. Neutralization of endotoxic O-antigens by homologous O-antibody. Ann. N. Y. Acad. Sci. **133**:763.
10. **Suter, E., G. E. Ullman, and R. G. Hoffman.** 1958. Sensitivity of mice to endotoxin after vaccination with BCG (Bacillus Calmette-Guerin) (24282). Proc. Soc. Exp. Biol. Med. **99**:167-169.
11. **Trakatelis, A., W. R. Stinebring, and A. E. Axelrod.** 1963. Hyperreactivity to endotoxin in BCG treated guinea pigs and its relationship to delayed hypersensitivity. Proc. Soc. Exp. Biol. Med. **112**:349-352.
12. **Westphal, O., O. Lüderitz.** 1954. Chemische Erforschung von Lipopolysacchariden gram negativen Bakterien. Angew. Chem. **66**:407-417.