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Immunological Relationship Among Oral Anaerobic Spirochetes as Detected by Indirect Microhemagglutination

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A sodium deoxycholate-ethanol-extractable antigen was found to be common to three strains of oral anaerobic spirochetes by indirect microhemagglutination.

There is little doubt that anaerobic spirochetes are indigenous members of the normal flora of the human dentulous oral cavity. Light-microscopic studies have shown that the microbial population of subgingival dental plaque is normally composed of 0.6% spirochetes; however, with the onset of gingivitis there is an approximate 30-fold increase in the spirochetal population (7). Longitudinal studies on experimentally induced human gingivitis indicate that spirochetes can be detected within dental plaque immediately before the onset of gingival inflammation (9). Whether the spirochetes initiate the periodontal disease state or are secondary invaders who proliferate as a result of the additional living space created by the apical migration of the junctional epithelium has not yet been established. Based upon microscopic evidence, as well as cultural studies, it is safe to assume that the pathological periodontal pocket creates a suitable econiche for spirochetal multiplication. The purpose of the present investigation was to determine the immunological relationship among several strains of oral spirochetes by indirect microhemagglutination.

Our laboratory has isolated several strains of oral spirochetes from the subgingival plaque of patients with severe, chronic periodontal disease. Three of these strains were designated W, 11, and 14. The initial isolation procedure has been described previously (5). Stock cultures of each isolate were maintained on semisolid media consisting of PPLO broth (BBL Microbiology Systems, Cockeysville, Md.) plus 5 μg of cocarboxylase per ml, 10% sterile pooled rabbit serum, and 0.2% ion agar. The same medium lacking ion agar was employed for liquid cultivation. Electron microscopic studies revealed that all the isolates were identical morphologically and possessed the "2-4-2" axial filament arrangement. Specific immune serum was prepared against each spirochetal isolate in New Zealand white rabbits. The immunization schedule has also been previously described (5). Quantitation of specific spirochetal antibody was carried out by the microscopic agglutination test as ordinarily used for the quantitation of leptospiral antibody (3). Homologous microscopic agglutination titers were typically 1:10,000 or greater. Reciprocal adsorption microscopic agglutination studies were carried out according to the method of Kmety et al. (6) to determine the degree of antigenic cross-reactivity, if any, among the isolates with respect to surface antigens. The results (Fig. 1) indicate that although strains W and 14 share common surface antigens, all of the isolates may be considered as belonging to different serotypes. The definition of serotype is based upon the criteria established by the World Health Organization for the classification of *Leptospira*, which states: "two strains are considered to belong to different serotypes if, after cross-absorption with adequate amounts of heterologous antigen, 10% or more of the homologous titer regularly remains in each of the two antisera in repeated tests" (10). We propose to follow these criteria for the future classification of additional oral spirochetal isolates as they become available.

A sodium deoxycholate-ethanol extraction procedure similar to that described by Chang and McComb (2) was utilized to obtain antigens from each isolate. Twenty milliliters of an actively growing broth culture of each isolate was harvested at 10,000 × *g* for 30 min in a Sorvall RC2B refrigerated centrifuge. The sedimented spirochetes were suspended in sufficient 0.85% NaCl solution to give an optical density of 0.25 at a wavelength of 650 nm in a Spectronic 20 spectrophotometer. This procedure served to standardize the number of spirochetes per milliliter of diluent. A 0.5-g quantity of sodium deoxycholate (Sigma Chemical Co., St. Louis, Mo.) was dissolved per 10 ml of cell suspension and kept at 37°C for 2 h. Dark-field microscopy revealed that no intact spirochetes remained at the end of the incubation period. Cold absolute ethanol was added to a final concentration of 90%, and the mixture was placed at 4°C for 24 h. The alcohol precipitation yielded a flocculant,

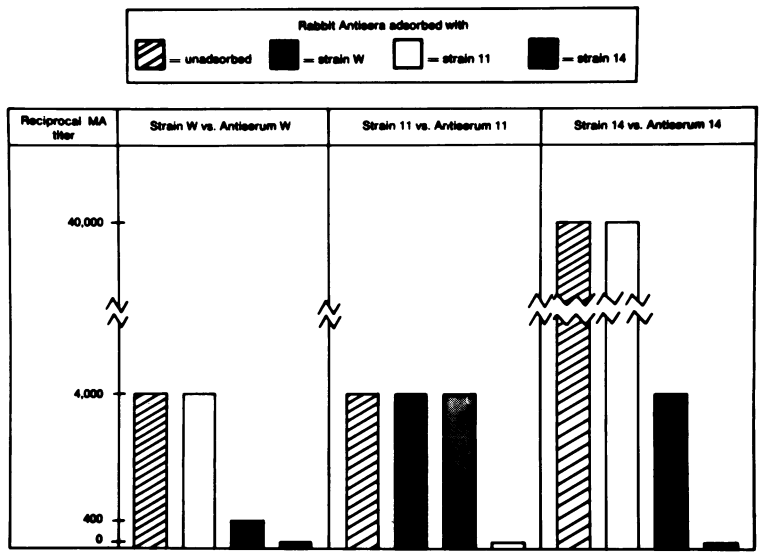

FIG. 1. *Results of reciprocal adsorption microscopic agglutination studies of three oral spirochetal isolates.*

whitish precipitate which was collected by centrifugation at 12,000 × *g* for 30 min. The precipitate was suspended in 10 ml of phosphate-buffered saline (pH 7.2) and was designated as the deoxycholate-ethanol spirochetal antigen (DES-Ag).

The indirect microhemagglutination test was performed according to the method of Boyden (1). Sheep erythrocytes were washed three times and suspended to a concentration of 2.5% in phosphate-buffered saline (pH 7.2). Equal volumes of 2.5% sheep erythrocytes and 1:20,000 tannic acid solution were mixed and incubated at 37°C for 10 min. The tanned erythrocytes were sedimented by centrifugation at 800 × *g* for 5 min, washed once, and suspended to 2.5% with phosphate-buffered saline (pH 7.2). The optimal concentration of antigen used to sensitize the erythrocytes was determined by preparing serial twofold dilutions of each DES-Ag in phosphate-buffered saline (pH 6.4) and adding equal volumes of antigen to 2.5% tanned sheep erythrocytes. After incubation at 37°C for 30 min, the erythrocytes were sedimented at 800 × *g* for 5 min and washed three times with 0.3% gelatin in phosphate-buffered saline (pH 7.2). The sensitized erythrocytes were suspended in the 0.3% gelatin solution to a final concentration of 1.25%. Tanned nonsensitized erythrocytes and nonimmune (prebleed) rabbit serum were utilized as controls. The indirect microhemagglutination assay was carried out in microtiter plates with U-shaped wells (Flow Laboratories, Inc., Rockville, Md.). Fifty microliters of homologous rabbit antiserum was added to 25 μl of sensitized erythrocytes in dilutions ranging from 1:10 to 1:2,560. All sera were heat inactivated at 56°C for 30 min and adsorbed with 10% packed sheep erythrocytes to eliminate heterophile antibodies. The plates were agitated for 30 s on a mechanical vibrator and allowed to stand at room temperature for 2 h. Agglutination patterns were read on a +4, +3, +2, +1, and negative basis, with +4 indicating a mat or carpet of cells displaying the greatest degree of agglutination. The highest dilution of antigen that gave a +3 agglutination with the homologous antiserum (1:10 dilution) was considered as 1 hemagglutination unit of DES-Ag. Utilizing this criterion, the optimal concentration of each antigen used to sensitize tanned erythrocytes in all subsequent studies was 16 hemagglutination units (Fig. 2).

To determine whether the DES-Ag was common to all of our isolates, the indirect microhemagglutination test was performed by sensitizing tanned erythrocytes with the optimal dilution of each antigen preparation and reacting it against both the homologous and heterologous rabbit antisera as well as against nonimmune rabbit serum. The results (Fig. 3) indicate that the titer of the homologous antisera was 1:1,280 in all cases, whereas with the heterologous antisera titers ranged from 1:80 to 1:320. The nonimmune rabbit serum did not react with any of the DES-Ag preparations. Thus, it appears that although the isolates represent different serotypes, the DES-Ag is common to all strains tested.

Imamura et al. (4) reported on a similar deoxycholate-extractable antigen, which was found to be common to six strains of *Leptospira*, and

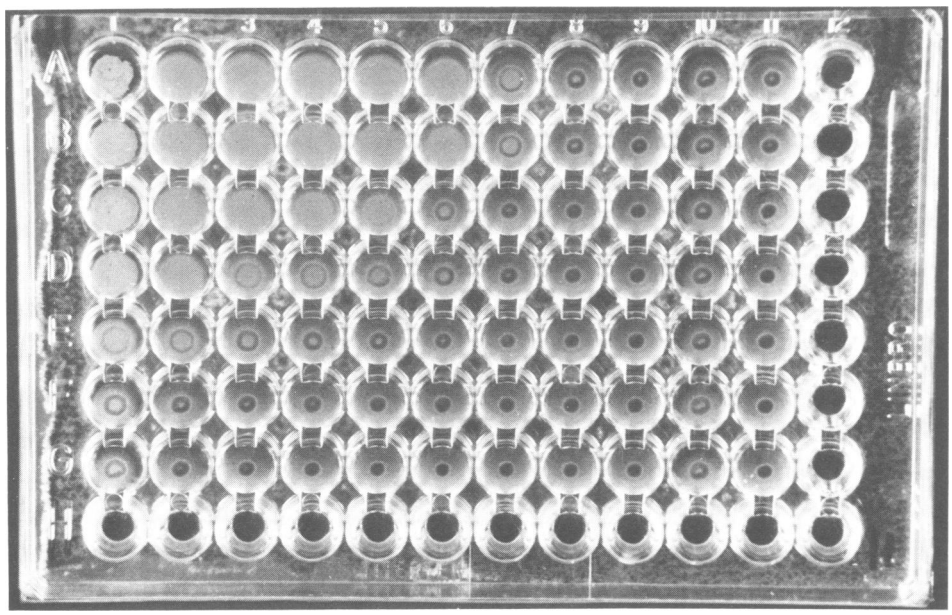

FIG. 2. *Determination of the optimal antigen dilution by block titration. DES-Ag was extracted from spirochete strain 11, and serial twofold dilutions of antigen (rows A-G) were reacted against 10-fold dilutions of the homologous rabbit antiserum. The highest dilution of antigen showing a +3 agglutination with a 1:10 dilution of the homologous antiserum is equal to 1 hemagglutination unit of DES-Ag.*

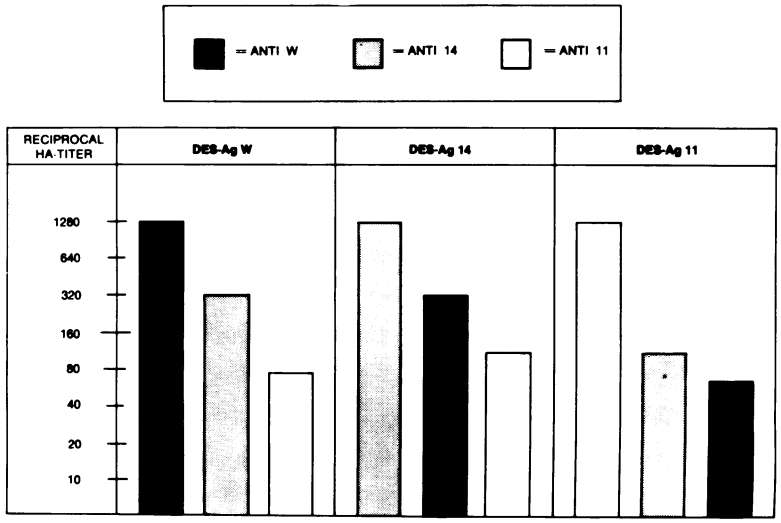

FIG. 3. *Antibody titers of homologous and heterologous rabbit antisera to DES-Ag of oral anaerobic spirochetes.*

on the usefulness of this antigen in detecting antibodies in the sera of patients with leptospirosis by indirect hemagglutination. Preliminary experiments in our laboratory indicate that DES-Ag may be useful in detecting humoral antibodies to oral spirochetes in the sera of patients with periodontal disease. Six patients with periodontal disease displayed titers ranging from 1:80 to 1:320 by indirect microhemagglutination, whereas three control patients without periodontal disease had titers of 1:20 or less. These findings are contradictory to those of Steinberg (8), who was unable to detect antibodies in the sera of patients with advanced

periodontitis by using intact cells of *Treponema microdentium* to sensitize tanned erythrocytes in the indirect hemagglutination assay. The possibility arises that by using intact cells of a single serotype as the sensitizing antigen, only those antibodies directed against the surface antigenic determinants of that specific serotype (or closely related serotypes) can be detected. DES-Ag, on the other hand, has been shown to be common to several serotypes and may represent a more universal antigen for the detection of spirochetal antibodies in the sera of patients with periodontal disease. The biochemical composition of DES-Ag is currently under investigation in our laboratory.

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