A STUDY OF SOME CULTURAL CHARACTERISTICS AND BLOOD SERUM ANTIBODY TITRES OF ENTEROCOCCI ISOLATED FROM THE MOUTH AND FAECES

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I hereby declare that this dissertation is my own work, and has not been submitted or incorporated in another dissertation or thesis for any other degree.

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A C K N O W L E D G E M E N T S

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A B S T R A C T

Crevicular epithelium presents no effective barrier to the biologically active constituents of plaque. Moreover, it seems that the production of circulating antibodies, the development of a state of delayed hypersensitivity and the development of immunological tolerance all play a part in the pathogenesis of periodontal disease. An investigation was undertaken to study the cultural characteristics of enterococci isolated from the mouth and gut of a group of students, and to determinethe serum antibody titres to these organisms. Enterococci were isolated from faeces and the gingival crevice of 9 male dental students. Blood was obtained from each subject and allowed to clot. The serum was removed and stored at -20°C until required. Blood serum antibody titres to the bacteria were obtained by the indirect fluorescent antibody technique. In order to investigate for the presence of antibodies to homologous and heterologous enterococcal strains, each subject's serum was tested against all the strains of enterococci isolated. Higher antibody titres were obtained to oral enterococci than to faecal microorganisms. This investigation suggests that there are either differences in the immunogenic potential of oral and faecal streptococci or micro-environmental conditions in the host which favour antibody production to oral streptococci as opposed to streptococci in the gut. There are also differences in the cultural characteristics and bio=chemical reactions of the gut and mouth organisms. The

definition of enterococci is not entirely satisfactory and it is very likely that various strains of enterococci have very little in common.

C O N T E N T S

C H A P T E R III

C H A P T E R IV

C H A P T E R V

F I G U R E S

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T A B L E S

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CHAPTER I

1 ANTIGEN AND ANTIBODY FORMATION

HISTORY AND SIGNIFICANCE

The ability to resist infection is a property common to all living creatures and has been known for a long time. In his description of the plague in Athens some 2500 years ago, Thucydides reported that those who had recovered would not contract the disease a second time, even though they were tending to the sick and the dying. Little was known about the mechanisms by which immunity develops until fairly recently. It was in fact Pasteur who, in the last century, placed immunology on a scientific basis through his studies on microbes and the development of methods to induce immunity.

Immunology is concerned with the general field of resistance to infectious diseases, both naturally existing or artificially induced by the introduction of antigens. An antigen (immunogen) is a substance which, when introduced

into an organism, will elicit an immunological response. The nature of this response varies from one subject to the next but generally includes the production of circulating antibodies. The antigen has the ability to react specifically and in a demonstrable way with the antibody that is produced.

Some investigators make a distinction between the terms antigenicity and immunogenicity. They use the term antigenicity to refer to those characteristics of a molecule which are concerned with its capacity to interact with antibodies. On the other hand, the term immunogenicity is used to refer to those characteristics of molecules (immunogens) which elicit the production of specific antibodies (Abramoff, 1970). Thus, some molecules are capable of reacting with antibodies but are not able to elicit an antibody response. These molecules generally are relatively small in size and are called haptens. A distinction, therefore is made between molecules such as haptens which demonstrate properties of antigenicity but not immunogenicity (Davis et al, 1969).

The antigenicity of an antigen is determined by a number of factors including its molecular weight, the nature of the determinant groups and the degree of foreignness to the host. Generally the larger the molecular weight, the more antigenic the molecule. As far as the determinant groups are concerned Haurowitz (1952) considers that they should have a rigid structure and that this

property is a prerequisite for antigenicity. For example, it appears that the enhancement of the anti= genicity of gelatin by the addition of aromatic groups is due to the rigidity of the benzene rings of the aromatic groups. Lastly, in order to be immunogenic, molecules must be foreign to an organism; the more foreign they are, the more immunogenic the molecule will be for the organism.

In 1885 Ehrlich propounded the principle of "horror autotoxicus". This principle states that an animal will not produce antibodies to any substance normally found in his own circulation. There have been many theories put forward to explain this phenomenon. One of these is the clonal selection theory of Burnet (1959; 1961). This theory attempts to explain antibody formation and the ability of the antibody-producing mechanism to distinguish "self" from "not self". According to this theory somatic mutation of immunologically competent cells during early embryonic life gives rise to a popu= lation of genetically different cells. The range of cells thus formed is capable of mounting an immune response specific for any antigen which the body may subsequently encounter. Simple division of each mutated cell gives rise to a small group of cells called a clone. The cells in a clone are all identical and they are all capable of making one or at most a few specific antibodies. This theory further postulates that during early embryonic development "forbidden" clones that match "self"-antigens

are eliminated as they arise. Foreign antigens introduced at this stage are accepted as "self". If an antigen is introduced into an adult, the antigen selects and stimulates proliferation of that particular clone of cells which can react with it. Increase in size of the clone results in a concommitant increase in the amount of antibody produced. This theory is consistent with the evidence for genetic determination of antibody specificity; such specificity is mediated by a replicable antibody-synthesizing unit inheritable on a cellular level. The clonal selection theory could also account for other features of the immune response such as the anamnestic response, immunological tolerance and the phenomenon called the "doctrine of original antigenic sin".

An immunocompetent organism can respond to an antigen in a number of ways. It can stimulate the production of circulating antibodies, the development of immunological tolerance, or the development of a state of delayed hypersensitivity. These responses will be described below.

1.1 CIRCULATING ANTIBODIES

Circulating antibodies are called immunoglobulins and consist of two pairs of polypeptide chains, one heavy and the other light, held together by disulphide bridges. Both heavy and light chains contain variable and constant parts whose structure is genetically determined. The variable areas are situated at the end of the chains and

are primarily involved in specific interaction with the antigen.

The immunoglobulins form a family which may be divided into five main classes, namely, IgA, IgM, IgG, IgE and IgD. Immunoglobulin A (IgA) is the dominant immuno= globulin class in secretions such as saliva. These immunoglobulins are selectively secreted by the parotid gland and they are the only class of γ-globulins which binds to the skin, smooth muscle and mucous membranes (Campbell and Garvey, 1963). "Bound" IgA antibody, after interaction with antigen can cause the release of histamine and other pharmacologic agents at the local site of interaction. The significance of histamine release in unknown, but it is possible that IgA antibody may play a role in defending exposed body cavities to surface penetration by infective agents.

IgM has a high molecular weight and constitutes about 5 - 10 per cent of the total γ-globulin. In most primary immune responses, regardless of immunogen or species, IgM is the antibody that is first produced. However, this initial formation of IgM is transient and is followed within a few days by the synthesis of IgG which may continue for many months. A secondary antigenic stimulation results in a rapid increase of IgG production with antibody titres much higher than in the primary response. In humans IgG comprises about 85 per cent of total immunoglobulins.

IgE appears to be associated with reagens which are antibodies that can cause immediate hypersensitivity reactions. In such reactions the reagen becomes fixed to the surface of mast cells and basophils, ready to trigger cellular degranulation on combination with antigen.

Little is known of the functional properties of IgD, the last member of this family.

Despite their diverse characteristics the family of immunoglobulins also has common properties. They all cross-react antigenically which indicates that common structures exist in the molecules which appear to be associated with the antibody activity. All immunoglobulins probably are derived from plasma cells.

In oral infections cellular immune mechanisms and antibodies may take part in the pathogenesis of the diseases. Stimulation of the immune mechanism to produce antibody involves a series of events and two groups of lymphoid cells. The one group consists of the thymus-derived (T)cells and the bone marrow-derived, or bursa equivalent (B) cells; the other group consists of the macrophages. Responses to most antigens require a synergism and interaction between T cells and B cells which leads to the differentiation of B cells into antibody-producing cells. There are a number of hypotheses to explain the mechanisms of T

and B cell cooperation in the humoral antibody response. It has been suggested that synergism between the T and B cells is through the medium of two different determinants on the same antigen (Mitchison, 1969; Rajewski et al, 1969; Katz et al, 1970). Another explanation for the mechanism of T and B cell cooperation in the humoral antibody response is that put forward by Dutton et al (1971). They suggest that specifically activated T helper cells may release a nonspecific factor which triggers B cells that already have bound antigen through receptors on their surfaces. This hypothesis is supported by recent findings from several laboratories (Kishimoto and Ishizaka, 1974). Apparently the recognition structure for antigen on the surface of B cells is an immunoglobulin molecule with the same specificity as the secreted antibodies. There has been much controversy as to whether T cells have immunoglobulin receptors on their surface or not. In a recent study Hämmerling and McDevitt (1974) demonstrated that T cells are able to recognise antigen. They also observed profound differences between B and T cell antigen receptors.

The handling of many antigens by macrophages is an important step toward immune induction (Calderon and Unanue, 1974). On a molar basis, antigens taken up by macrophages are more effective than soluble antigen molecules. The experiments of Calderon and Unanue (1974) show a small and persistent release of antigen

by the macrophage. They conclude that the cell conserves a small pool of intracellular antigen in a form which is slowly catabolised; from this pool a small amount is slowly released by the cell. Mosier (1969) has shown that an intimate association of lymphocytes with macrophages is essential for primary antibody production *in vitro*. However, other observations indicate that intracellular digestion of antigen is not an essential feature of the antibody response (Talmage, 1970). It has been suggested by Pearson and Raffel (1971) that processing and breakdown of antigen into smaller molecules may favour the stimulation of delayed hypersensitivity in preference to humoral antibody production.

1.2 IMMEDIATE AND DELAYED HYPERSENSITIVITY

In the case of immediate hypersensitivity, a rapid response occurs when an animal makes contact with an antigen. On the other hand, when the response is slow, the reaction is called delayed hypersensitivity. It is a cell-mediated response in which the specific or immune reaction is the development of a population of T lymphocytes, sensitized to an antigen. The activated lymphocytes release at least two factors. One, the macrophage inhibition factor, inhibits the migration of macrophages and may provoke a local non-specific macrophage infiltration. The other factor exerts a non-specific cytotoxic effect on unrelated cells. Lehner

(1972) suggests that cell-mediated immunity against a number of oral microorganisms may be involved in the pathogenesis of periodontal disease.

1.3 IMMUNOLOGICAL TOLERANCE

According to Neter (1971) the main feature of immunological tolerance or unresponsiveness is the inability to mount a specific immune response to a given immunogen. This is thought to be due to prior contact with the antigenic determinant of the immunogen in a host otherwise capable of specific reaction to antigens of the same chemical or biologic groups. When an unresponsive state is induced, usually production of both circulating antibody and delayed hypersensitivity is affected (Dvorak et al, 1965; Weigle, 1966). However, in some cases, delayed hypersensitivity is preferentially affected (Borel, Fauconnet and Miescher, 1966). The state of tolerance may either precede or follow a phase of unimpaired immunologic capacity. It may occur naturally or it may be induced experimentally.

Under conditions of natural tolerance, animals do not make antibodies against their own tissue antigens. The ability to make antibodies is slowly developed in the embryo and all clones of immunologically competent lymphoid cells which contact self-antigens in embryonic life are destroyed. However, any self-antigens which are isolated from lymphoid cells (e.g. optic lens and thyroglobulin) will have corresponding intact lymphoid

clones able to make antibodies against these cells. When, as a result of trauma or infection, these self-antigens become accessible to the lymphoid cells, "auto-antibodies" could be directed against them (Claman, 1970). Similarly antibodies could be formed against endogenous gingival tissue components which have changed and developed new antigenic determinants during the course of chronic inflammation such as is found in periodontal disease (Willoughby and Ryan, 1970).

Experimental induction of immunological tolerance has been achieved with numerous antigens in both neonates and adults of many different species. Although the immunocompetence of mature animals is higher than that of immature animals, it is possible under certain cir= cumstances, to induce immunological tolerance in both normal adults and adults previously sensitized to antigen. The nature, dose and route of administration of the antigen also have a bearing on the induction of immunologic tolerance (Weigle, 1973).

It is difficult to induce a completely unresponsive state to many bacterial and viral antigens. These antigens are highly antigenic, are rapidly removed from the circulation and do not contact all the antigen-reactive cells in effective concentrations with the result that, at best, only a hyporesponsive state is obtained. Moreover, maintenance of this hyporesponsive state depends on repeated injections of antigens.

However, there are certain bacterial antigens which are capable of inducing a state of immunological tolerance. An example of such an antigen is purified pneumococcal polysaccharides, which, when injected into mice in moderate amounts, will induce immunological tolerance. The tolerogenic effects of pneumococcal polysaccharides possibly could be ascribed to the fact that mice lack specific depolymerases for the polysaccharides with the result that they persist for many months in vivo. Injections of minute amounts of the polysaccharide induce an apparent unresponsive state in which circulating antibody is absent but antibody-producing cells are present. The apparent unresponsive state probably results from a "treadmill" neutralization of the antibody by the persisting antigen. Administration of higher doses results in central inhibition characterised by absence of antibody-producing cells. A cardinal feature of maintenance of immunological tolerance appears to be the persistence of antigen.

The degree of immunological tolerance that is attained and its duration is also determined by the dose of antigen injected. Generally the larger the dose the greater the degree of immunological tolerance attained and the longer its duration. However, serum protein antigens are an exception in that multiple small doses injected into neonatal rabbits are more effective than one large injection. The phenomenon of "high-low zone tolerance"

was first described by Mitchison (1964). It appears that with certain antigen and species combinations, two doses of antigen, one high and one low, could produce an unresponsive or hyporesponsive state; doses between these two extremes would give rise to immunity. Weigle (1973) suggests that when it does occur, there are probably two requirements. Firstly, the antigen preparations must contain both an immunogenic and a tolerogenic form of the antigen; secondly, the animal must be able to respond to an immunogenic form of the antigen. Competition between the two different forms of the antigen could take place, the result depending on the absolute amount of each form.

Although some immunogens, when injected together, actually will enhance the production of antibodies against all the components of the mixture, there are situations when the response to one immunogen may be impaired when seemingly unrelated antigens are injected at the same time (Abramoff and Wolfe, 1956). Such interference with the immune response to one antigen is termed competition of antigens and may manifest itself in varying degrees up to complete suppression of the immune response.

Induction of immunological tolerance in immunized adults is more complex than in normal adults. In the case of sensitized adults, both virgin and memory cells are involved. It seems possible that induction of immunological tolerance to bacterial antigens involves the

elimination of memory cells which have arisen as a result of prior contact with the antigen or related antigens.

1.4 INDUCTION OF IMMUNOLOGICAL TOLERANCE - CELLULAR INTERACTIONS

Thymus-derived (T) and bone marrow-derived (B) lympho= cytes are involved in both humoral antibody production and acquired immunological tolerance. Moreover, an unresponsive state can be induced in both T and B cells; this is compatible with the presence of antigen receptors on both cell types. It also appears that only the T cells or B cells, but not both, need to be unresponsive for immunological tolerance to develop. Reaction of immunogen and tolerogen with receptor sites is a feature of both the immune response and the induction of immuno= logical tolerance; the manner in which this interaction will occur probably determines which of the two phenomena will take place (Weigle, 1973). Unresponsiveness to a number of antigens can be ended by immunization of the unresponsive animals with certain antigens that cross= react with the tolerated antigen. The work of Ruben, Chiller and Weigle (1973) suggests that antigen recogni= tion by the T cells manifests a wider range of cross= reactivity than that evidenced by the B cells.

CHAPTER II

1 DETECTION OF ANTIBODIES

The previous chapter was mainly devoted to a description of the mechanisms of antigen and antibody formation. In this chapter some of the methods used to detect antibodies are surveyed briefly.

Union between antibody and antigen to form an "antigen-antibody complex" results in a number of distinct reactions. These reactions are of practical value in that they form the basis for certain tests which are used to detect antibodies. Examples of such reactions are precipitation, agglutination and complement fixation in the presence of antigen. The indirect fluorescent antibody test is another method of detecting the reaction between antigen and antibody.

In the agglutination test antibody reacts with the surface antigens of particulate antigens such as bacteria or red blood corpuscles to produce visible clumps.

The visibility of the reaction is due to the relatively large size of the antigen (bacteria).

The precipitation test is based on the ability of some populations of antibodies to form precipitates in the presence of soluble antigen. The particles bearing the antigen are smaller in size than those participating in the agglutination test. Some antibodies will not give a positive precipitation test irrespective of whether the test is carried out in solution or whether it is carried out in a gel or agar medium. Lancefield used the pre= cipitation test to divide β-haemolytic streptococci into various groups.

Complement is a thermolabile complex of eleven different chemical substances. It is present in all normal sera and is not increased in quantity by antigenic stimuli such as infection. Whenever antigen and antibody react specifically together the resulting complex takes up complement. Complement fixation tests are designed to measure the capacity of certain antigen-antibody reactions to combine with complement.

Of particular interest to the present study is the indirect immunofluorescent technique of detecting anti= bodies. This technique depends on the property of fluorochrome dyes to emit fluorescence when exposed to ultraviolet light. Examples of such dyes are fluorescein which emits a yellow green fluorescence and rhodamine which emits a reddish orange fluorescence when excited

by ultraviolet light. These dyes are readily coupled to protein by means of coupling agents such as isothiocyanates. When fluorescein isothiocyanate (FITC) is conjugated to gamma globulin, the conjugate retains its specific immunologic reactivity. According to Nairn (1969) FITC is the reagent of choice for routine use. It appears that precipitating and non-precipitating sera can both be used successfully as fluorescent tracers. It is also advantageous to have ample samples from the same batch of antiserum as this allows valid and prolonged comparisons between different observations and projects (Nairn, 1969).

The sandwich technique can be used to detect the presence of antibodies to a known antigen. It is performed by successive layering of antigen, specific immune human antiserum and conjugated anti-human globulin serum. Antiglobulin serum is usually prepared from goat antiserum. When the first two layers of the sandwich are apposed, the known antigen combines with the antibody in the specific immune human serum. The antibody in the immune human serum adheres to the surface of the layer of antigen. Therefore, application of the third layer results in combination of the fluorescent antiglobulin (anti-antibody) in the conjugated serum with the antibody in the middle layer. It is possible to observe the labelled antibody by fluorescence microscopy. The immune human serum is serially diluted in order to ascertain the highest titre at which staining

still occurs. Figure 1 (Page 48) illustrates the principles underlying the sandwich method of detecting antibodies (Nairn, 1969).

Since antiglobulin serum can be obtained in high titre the small amount of antibody lost in conjugation and purification procedures is insignificant. Therefore, antiglobulin conjugates are of particular value when the serum used in the middle layer has a low antibody titre. If the weak serum were to be conjugated, its antibody titre would be reduced still further.

The sandwich technique is a much more sensitive test than single layer tracing. According to Nairn (1969) this extra sensitivity is due mainly to the additional combining sites provided by the molecules of the middle layer acting as antigen for the labelled antiglobulin (Figure 2) (Page 49).

CHAPTER III

1 THE PRESENT STUDY

According to Wilson and Miles (1957), Thiercelin in 1899 was the first to describe the enterococci. They are usually found in the gut (Shattock, 1962; Deibel, 1964) and most of the literature on the enterococci refers to microorganisms isolated from this location. However, they are also present in the oral cavity especially in the gingival crevice and in saliva (Socransky and Manganiello, 1971).

The definition of enterococci has never been satisfactorily agreed upon (Wilson and Miles, 1957). The criteria Breed, Murray and Smith (1957) use for differentiating the enterococci from other streptococci are their ability to grow in 6,5 per cent sodium chloride broth, at pH 9,6 and in 0,1 per cent methylene blue milk; they are able to decarboxylate tyrosine and possess the Lancefield group D antigen. These authorities include only two species in this group, namely, Streptococcus faecalis

(and its variants) and Streptococcus durans. Wilson and Miles (1957) have added another species, Streptococcus faecium, to this group, but state that the recognition of this species is rather contentious.

The species are identified by means of various tests, biochemical and other. However, these tests are not completely reliable. For instance, White (1963) reports that a test such as tolerance to 60°C for 30 min is closely related to the pH value of the medium. Moreover, recently isolated strains sometimes failed to grow under one or more of the tolerance conditions. On subculture, however, these strains may grow under the test conditions that previously gave negative results (Mannweiler, 1955). Therefore, in identifying these strains, dependence is best placed on a spectrum of characteristics possessed by the strain in question. Failure of a strain to comply in a few specific tests does not constitute sufficient grounds to negate speciation if it conforms with the overall species description; the occurrence of some transitional types is to be expected (Deibel, 1964).

Chapman (1944) cultured enterococci on tellurite strep= tococcus medium and found that they could be distinguished from the other streptococci because they produce dark brown or black, smooth, slightly raised colonies from 0,5 - 1,5 mm in diameter on this medium. Mitis salivarius agar and aesculin bile agar are two selective media which facilitate the isolation of the enterococci as a group.

Although enterococci constitute part of the normal flora of the alimentary canal, they are potential pathogens outside the digestive system. For instance, enterococci from the gut can cause urinary tract infections (Deibel, 1964) and are also occasionally associated with subacute bacterial endocarditis, peritonitis and meningitis (Wheeler and Foley, 1943). Oral enterococci may participate in infections such as gingivitis or periodontitis but it is difficult to assess their importance in the pathogenesis of these mixed infections. If they elicit a serum antibody response it may be taken as evidence of their active participation in the infection. Alternatively, bacteraemia resulting from the entry of oral streptococci into the blood stream may give rise to an antibody response. In patients who already have serum antibody titres to enterococci, the possibility always exists that the infection could have been produced by organisms harboured in the oral cavity or the gastro-intestinal tract. It seems possible that the antigenic constitution of oral and gut organisms and the intensity of their immune response may differ. The object of this investigation, therefore, was to determine whether there are, indeed, differences in the antigenic constitution of oral and faecal enterococci and their immune response, as well as differences in the cultural characteristics and biochemical reactions of the gut and mouth organisms.

In order to determine whether the antigenic constitution of the oral and faecal organisms and their quantitative immune response differed, it was decided to test each microbial strain isolated against homologous and heterologous sera. The serum antibody titres, therefore, were determined for the microbes isolated from the gingival sulcus and faeces of the subject (homologous antibodies) as well as for all the enterococci isolated from the other subjects used in the study (heterologous antibodies).

1.1 MATERIALS AND METHODS

The subjects for this examination were 9 male dental students. Their ages ranged from 22 - 44 yr (mean 24,6 yr). The periodontal index (Russell, 1956) as well as the oral hygiene index (Greene and Vermillion, 1960) was determined for each student. Samples for culturing the microbes were taken from the faeces and gingival crevice of each student by means of a wire loop. For primary isolation of the organisms crevicular samples were spread on mitis salivarius agar (to which tellurite had been added), as well as on blood agar and faecal samples were spread on aesculin bile agar. The inoculated media were incubated aerobically at 37°C for 24 hr. Enterococci formed small dark colonies on mitis salivarius agar and caused blackening around bacterial growth on aesculin bile agar. When Gram staining confirmed that they were streptococci, the organisms

were plated on 40 per cent bile agar and blood agar. After repeated subculture on blood agar pure cultures were obtained.

Identification of pure strains was based on some of the criteria enumerated by Breed et al (1957). These are shown in Table I (Page 53).

Two methods were employed to determine the Lancefield grouping of the organisms, namely, the micro precipitin technique and the indirect fluorescent antibody technique.

The micro precipitin technique evolved by Lancefield (1938), was closely followed in this study. Extracts of the various bacteria were obtained by first preparing 50 cc of broth culture from each organism. After centri= fugation of the broth cultures, the remaining bacterial sediment was removed and suspended in separate solutions of 2 cc of N/20 HCl in 0,85 per cent NaCl. The tubes containing the suspensions were placed in a boiling water bath for 10 min, cooled under running water and then centrifuged. The supernatant fluid was removed and neutralised. The resulting inactive precipitate was eliminated by further centrifugation. The final super= natant was used in the precipitin reaction.

The precipitin was reacted with a 1:16 dilution of Wellcome streptococcal grouping sera A, B, C and D (prepared in rabbits) in conical glass tubes prepared from lengths of 7 mm diameter glass tubing. The extract

was first introduced into the tubes, followed by the heavier serum which sank below the saline extract.

The results yielded by the micro precipitin technique were disappointing. This method was, therefore, discarded in favour of the indirect fluorescent antibody technique as described below. The reagents used were a 1:16 dilu= tion of Wellcome streptococcal grouping sera A, B, C and D as well as a 1:16 dilution of fluorescent anti- rabbit immunoglobulin (Wellcome).

Blood was obtained from each student and allowed to clot. The serum was removed and stored at -20°C until re= quired. Blood serum antibody titres to the bacteria were obtained by the indirect fluorescent antibody technique. The technique used was an adaptation of the revised (1967) Treponemal antibody-absorption (FTA - ABS) test first published by Hunter, Deacon and Meyer (1964). In order to investigate the cross-reaction of antibodies to the bacteria, each subject's serum was tested against all the strains of enterococci isolated.

Two circles, 1 cm in diameter, were cut on clean glass slides with a diamond pencil. Pure cultures were har= vested and the microorganisms suspended in phosphate buffered saline (PBS). The circles on the glass slides were covered with a suspension of antigen and allowed to dry. Following fixation in acetone for 10 min slides were stored at -20°C until required. The slides were

then washed in PBS for 10 min and gently dried with blotting paper. The sera of the subjects were thawed and serial dilutions (1 in 8, 1 in 16, 1 in 32, etc.) were prepared using buffered saline (Nairn, 1962). A sufficient number of slides was used to enable each organism isolated to be tested against homologous and heterologous sera. Bacteria on the slides were covered with dilutions of the sera, placed in a humid chamber and incubated at 37°C for 30 min. The following rinsing procedure was used to remove excess sera:-

1. Slides were rinsed with running PBS for approximately 5 s.
2. They were packed in slide carriers and placed in a dish containing PBS for 5 min.
3. Then agitated by dipping them in and out of PBS.
4. Using fresh PBS, steps 2 and 3 were repeated.
5. Slides were rinsed in running distilled water for approximately 5 s and gently dried with blotting paper.

Fluorescein anti-human globulin conjugate (Wellcome) was diluted to its working titre ($\frac{1}{40}$), in PBS and then applied over the smears. After incubation in a humid chamber for 30 min, the slides were rinsed as previously described, blotted dry and mounted for observation. The mounting medium consisted of phosphate buffer and glycerine in the ratio phosphate buffer:glycerine = 1:9.

The preparations were viewed using a Reichart Fluorpan microscope and a 'Fluorpan' widefield immersion

darkfield condensor with toric lens, and a 12 V 100 W halogen lamp. The microscope was also fitted with FITC interference filter and a red barrier filter. A bright ring of fluorescence surrounding the microorganisms indicated a positive reaction (Figure 3) (Page 50).

1.2 STATISTICAL ANALYSIS

The sample data used in the statistical analysis of this study are presented in a row by column arrangement in Tables II and III (Pages 51 and 52). These tables each contain nine rows and nine columns and include 9 x 9 = 81 cells. Each cell represents an individual item of data.

The following statistical analyses were carried out:-

1. The two-way (cross classification) analysis of variance. This test is used to test whether there are any significant differences between the row means (factor 1) or between the column means (factor 2) (Fatti, 1975).

   In the present study a two-way analysis of variance described by Johnson and Leone (1964) was used to test for row (organisms) and column (subject) effects, in the case of one observation per cell (or square of table). The data from Table II (Page 51) were used in conjunction with the

following formulae:-

xij = observation in i^{th} row and j^{th} cell

$xi.$ = mean of the i^{th} row

$x.j$ = mean of the j^{th} column

$x..$ = grand mean

Source	Sum of Squares	Mean Square	F Ratio
Between rows	$SS_1 = \sum_{i=1}^{9} 9(xi. - x..)^2$	$SS_1/8 = S_1^2$	S_1^2/S_3^2
Between columns	$SS_2 = \sum_{j=1}^{9} 9(x.j - x.)^2$	$SS_2/8 = S_2^2$	S_2^2/S_3^3
Residual	$SS_3 = SS_T - SS_1 - SS_2$	$SS_3/64 = S_3^2$	
TOTAL	$SST = \sum_{i=1}^{9} \sum_{j=1}^{9} (xij - x..)^2$		

2. The studentized range test was used to determine which columns or rows were significantly different (Johnson and Leone, 1964).

3. A student's t test was employed to determine whether a difference existed between the two tables representing the results for salivary organisms on the one hand, and gut organisms on the other hand (Johnson and Leone, 1964).

C H A P T E R IV

R E S U L T S

The serum antibody titres as well as Lancefield groupings obtained are shown in Tables II and III (Pages 54 and 55). They indicate variation in specificity and intensity of the immune response and that homologous sera did not necessarily show greater affinity for homologous microorganisms. Thus, for example, the titre obtained for serum 5 against its homologous microorganisms was lower than the titres obtained for microorganisms 2, 3, 4, 6, 7 and 8 (Table II) (Page 54). This may indicate that the differences in the serum antibody titres to the microorganisms (row differences) fall within the range of variability normally encountered when the immunogen (antigen) is a bacterium (Davis et al, 1969). It may also indicate that the antibodies show an imperfect specificity for the antigens and that the differences in titres observed (column differences) fall within the range of variability of antibodies

normally found (Boyd, 1966).

In order to test these hypotheses the two-way analysis of variance was undertaken:-

ANALYSIS OF VARIANCE TABLE : (DATA TABLE I)

Source	Sum of squares	df	Mean square	F ratio
Between rows	98618,47	8	12327,31	4,6475
Between columns	70913,58	8	8864,20	3,3419
Residual	169754,86	64	2652,42	
TOTAL	339286,91			

Conclusions:

Since $F(8,64)_{.95} = 2,10$

$F(8,64)_{.99} = 2,82$

$F(8,64)_{.999} = 3,86$

this analysis showed that row and column differences existed and were significant at the 0,1 per cent and 1 per cent levels (row differences, $P < 0,0001$; column differences, $P < 0,01$).

In other words, it indicated that the immune response

mounted by the different individuals (as evidenced by their respective antibody titres) differed significantly; this analysis also showed that the antigenic structure of Streptococcus faecalis is complex and varies from strain to strain.

Having established that row and column effects do exist, the next step was to determine which columns or rows in Table II (Page 54) were significantly different. A method based on the studentized range was used:-

xi.	(i,j)	x.j
2,67	1	18,67
13,33	2	16,89
18,67	3	32,00
120,89	4	112,89
22,22	5	31,11
37,33	6	27,56
27,56	7	10,67
64,89	8	16,89
8,89	9	49,78

At 95% : $q(64,9) = 4,55$

99% : $q(64,9) = 5,35$

where $q(64,9)$ denotes the studentized range statistic based on 64 degrees of freedom and 9 groups.

If the absolute difference was less than

$$d_1 = \sqrt{\frac{2652,42}{3}} \times 4,55 = 78,11$$

then the hypothesis that the two rows or the two columns have the same effect, was accepted at the 5 per cent

level. The test showed that at the 5 per cent level row pairs which were different were (4,2) (4,1) (4,3) (4,5) (4,6) (4,7) (4,9), whereas column pairs different were (4,1) (4,2) (4,3) (4,5) (4,6) and (4,8). At the 1 per cent level

$$D_2 = \sqrt{\frac{2652.42}{3}} \times 5.35 = 91,85$$

At the 1 per cent level row pairs which were different were (4,1) (4,2) (4,3) (4,5) (4,7) and (4,9); column pairs different were (4,1) (4,2) (4,7) and (4,8). These results indicated that row 4 as well as column 4 was significantly different from other rows or columns. It seems, therefore, that the strain of Streptococcus faecalis isolated from the gingival crevice of subject 4 differed significantly from most other strains of the same microorganisms isolated from the other subjects by having a more reactive antigenic constitution. Furthermore, the blood serum antibodies of subject 4 also differed significantly from those of the other subjects, indicating a quantitatively superior immune response to different antigens in this individual.

Generally, the antibody titres obtained for the gut microorganisms were lower than those obtained for the gingival sulcus. In order to determine whether these differences were significant a student's t test was undertaken. In this test $di = x_1 i - x_2 i$ was defined as the difference between the column means in Table I and Table II

(Pages 53 and 54) respectively, and then

$$\bar{d} = 18,87$$

and $$s_d^2 = 1386,82$$

Using a t test, the test statistic was : $\frac{9 \times 18,87}{\sqrt{1386,82}} = 4,5591$

Since $t(8, .95) = 2,306$

$t(8, .99) = 3,355$

$t(8, .998) = 4,501$

the hypothesis that the two tables do not differ was rejected at the 1 per cent level ($P < 0,01$). In other words, there was strong evidence that a difference existed between the titres of the two tables. Tables II and III (Pages 54 and 55) show that one of the faecal microorganisms (6FA) did not elicit an antibody response; salivary enterococci, however, all elicited an antibody response.

Table IV (Page 56) compares the highest titre obtained against crevicular enterococci of each subject with the Periodontal Index (Russell, 1956) and the Oral Hygiene Index (Greene and Vermillion, 1960). These results indicated that subject 4 had a Russell Periodontal Index of 1,5 denoting the presence of periodontitis with incipient tissue destructive effects. This subject also had a high serum antibody titre to a homologous strain of Streptococcus faecalis ($\frac{1}{512}$) and high titres to heterologous strains of enterococci. In this particular instance there was no positive correlation between the Oral Hygiene Index and Russell's Periodontal Index.

Differences were also observed in the cultural characteristics and biochemical reactions of gut and crevicular organisms. Faecal enterococci were considerably larger when viewed under the microscope than their crevicular counterparts (Figures 4 a and b) (Page 51); they also formed larger colonies (Figures 5 a and b) (Page 52), and displayed much more vigorous growth on artificial media. Oral strains were more susceptible to the inhibitory effects of 40 per cent bile in blood agar, 6,5 per cent sodium chloride, and 0,1 per cent methylene blue in milk, than faecal enterococci. When the latter were cultured on aesculin bile agar they grew prolifically. They also reduced the aesculin as evidenced by black pigmentation around the colonies. However, enterococci isolated from the gingival crevice grew sparsely on aesculin bile agar and most of them were unable to reduce the aesculin.

When crevicular organisms were inoculated on aesculin agar (without 40 per cent bile) there was still not, or only very slight, evidence of aesculin reduction. This finding is unexpected as some authorities regard aesculin hydrolysis as an important criterion for the identification of Streptococcus faecalis and Group D streptococci.

Grouping of microorganisms showed that 6 of the 8 crevicular, and 8 of the 10 faecal organisms belonged to the Lancefield group D. In the crevicular group 2

organisms were positive with B but one of these also with group A serum. One organism in the faecal group reacted with B, C and D sera, while 2 organisms did not group at all.

CHAPTER V

DISCUSSION

There seems to be general agreement that crevicular epithelium presents no effective barrier to the biologically active constituents of plaque.

MacPhee (1972) describes a conceptual model of the events which possibly occur in the gingivae in the course of gingivitis. Penetration of the crevicular epithelium by an antigen results in the accumulation of polymorphonuclear leucocytes. This event is followed shortly by the appearance of B lymphocytes and T lymphocytes. On contact with plaque antigens the B lymphocytes differentiate into plasma cells which synthesize humoral antibody. This explains the presence of immunoglobulins (IgG, IgA and IgM) in crevicular fluid which, according to Brandtzaeg and Kraus (1965), contains these immunoglobulins in proportions and concentrations comparable to those of plasma.

The locally-formed antigen antibody complexes activate the complement system which in turn causes the release of a number of biologically active proteins. These substances cause oedema and increased permeability of gingival tissue.

Processing and breakdown of antigen by macrophages will favour its combination with T cells. These cells are not only a source of primed antigen sensitive memory cells but they are also involved in the phenomenon of cellular immunity and delayed hypersensitivity.

Nissengard, Beutner and Gauto (1971) detected small numbers of IgE-immunocytes in sections of inflamed gingival tissues. It seems possible, therefore, that immediate hypersensitivity reactions are involved in the initial stages of periodontitis and that the lesion is perpetuated by such mechanisms as the action of complement and the effects of delayed hypersensitivity on the tissues. An Arthus-type reaction could be mediated in several ways. According to Brandtzaeg (1972) immunocytes may synthesize autoantibodies to altered IgG present in locally-formed immune complexes. Robinson and Schuffman (1971) report the production of such anti-antibodies in rabbits following prolonged immunization. Similarly a type of autohypersensitivity could be developed towards endogenous tissue components which have undergone change as a result of the inflam= matory process (Willoughby and Ryan, 1970).

Although it is generally concluded that cell-mediated immunity and the humoral response are jointly implicated in the pathogenesis of periodontal disease, the phenomenon of immunological tolerance probably also plays a role in determining the course of the disease.

Persistence of antigen is one of the main requirements for maintenance of immunological tolerance. Plaque does not only provide for a continuous source of antigens but it most probably also releases factors which promote local antigen penetration. Therefore it seems reasonable to suppose that tolerance could develop to some of these antigens.

It is also feasible that, in the presence of plaque, the sera of affected individuals will contain antibodies to a number of indigenous plaque bacteria. In such a situation competition of antigens might take place so that the immunogenicity of one or more antigens may be impaired by any of the other simultaneously invading antigens. The work of Radovich and Talmage (1967) suggests that a humoral factor produced in response to one antigen could inhibit response to another antigen.

As a rule an antiserum against a given antigen contains a diversity of antibodies not all alike (Boyd, 1966). Besides reacting with its antigen, an antiserum will usually also react with other antigens (called heterologous antigens) if sufficiently similar to the

antigen. The reactions with heterologous antigens are called cross-reactions (Davis et al, 1969). Boyd (1966) points out that the specificity of antibodies often becomes less as immunization continues and that the strength and extent of cross-reactions, therefore, increases with the duration of infection. According to Weigle (1973) immunological tolerance can be termi= nated by cross-reacting antigens. The microbes isolated in this study are regarded as normal inhabitants of the gingival sulcus or gut and it is likely that they fre= quently invade the underlying tissues (van Reenen, 1973). During phases of active invasion of the tissues the antibody titres would rise and drop again after the infection clears up. This could partly explain the degree of cross-reaction.

Table II (Page 54) indicates that in one instance only, namely, that of subject 4, did the serum antibody titre to homologous antigen exceed the serum antibody titre to heterologous antigen; the same observation applies to Table III (Page 55) in which subject 8 was the only exception to the rule. This anomaly could possibly be explained by the phenomenon called the "doctrine of ori= ginal antigenic sin" (Davis et al, 1969; Stainer et al, 1971). According to this principle a secondary (anamnes= tic) response can sometimes be elicited with an immunogen that resembles the immunogen involved in primary immuniza= tion. Most of the antibodies thus produced will then

react more strongly with the first than with the second antigen. Epidemiological studies of cross-reacting strains of influenza virus first drew attention to the "doctrine of original antigenic sin". This principle has proven useful in "serological archaeology" which is the testing of human sera with different strains of influenza virus during an epidemic. These investigations show that a particular patient's serum has a tendency to react less avidly with the strain causing his present illness than with a strain that caused his primary attack of influenza in a previous epidemic. Thus the study of sera from elderly patients has yielded results that led to identification of strains that probably caused major epidemics in the past (Davis et al, 1969). This phenomenon is compatible with Burnett's clonal selection theory (Stainer et al, 1971).

The findings recorded in Tables II and III (Pages 54 and 55) could also be explained on the assumption that various streptococcal strains do not necessarily cross-react antigenically but that most individuals were exposed to multiple streptococcal strains with the same or simi= lar antigenic structures to those isolated from the indi= viduals of this study, and that antibodies were produced against them. It is well known that different individuals may give quantitatively different immune responses to the same antigenic determinant depending on both host factors and antigenic composition, e.g. the nature of the "carrier molecule" of the antigen.

In the gingival crevice a mixed population exists consisting of different strains of antigenically dis= similar enterococci, together with other species of microorganisms to be found in this site. Taking sub= ject 7 in Table II (Page 54) as an example, it can be postulated that this subject was infected in succession, with two different strains of Streptococcus faecalis which bore antigenic resemblance to each other. The first strain produced a primary response and subse= quently succumbed to the defence mechanisms of the host (subject 7). The first strain of Streptococcus faecalis was then supplanted by another strain of the same species, namely, the strain isolated in the course of this study. Antibodies produced in the secondary response following infection with the second strain were directed at the original organism, thereby causing a relatively low titre ($\frac{1}{8}$) against the strain isolated. Subject 9, on the other hand, could conceivably have been subjected to repeated past infections by an anti= genically similar strain of Streptococcus faecalis to that isolated from subject 7 and hence elicited a much higher blood serum antibody titre ($\frac{1}{128}$) to this micro= organism. Likewise, the gingival crevice of subject 4 could possibly have been repeatedly invaded by the same strain of Streptococcus faecalis, thereby not only causing a high blood serum antibody titre ($\frac{1}{512}$), but

also producing clinical evidence of gingivitis.

The enterococci from both areas shared many similarities. Thus all strains tolerated 40 per cent bile, tellurite and 6,5 per cent sodium chloride and did not hydrolyze starch. However, this investigation confirms the view expressed by Wilson and Miles (1957) that the definition of enterococci is not entirely satisfactory. Thus the strains isolated from the gut produced larger colonies on agar than those from the gingival crevice. When viewed under the microscope the cells showed a similar size discrepancy. Moreover, one strain isolated from the gut and another from the gingival crevice possessed more than one Lancefield group antigen. Three other strains could not be grouped and presumably did not possess the antigens of groups A, B, C, or D. Yet all these organisms tolerated 40 per cent bile, tellurite and 6,5 per cent sodium chloride which are considered to be characteristic features of enterococci. This suggests that the Lancefield D antigen may not be an absolute diagnostic criterion for enterococci. However, at the time when this investigation was undertaken, the enzyme methods for preparing grouping extracts were not yet published. According to Lancefield (1972) these methods yield excellent results. Furthermore, it appears that the grouping sera used by some laboratories may not be of the same quality as those sera that Lancefield has at her disposal. It

is clear, therefore, that the technical difficulties experienced by many workers in demonstrating the Lancefield D antigen, may be due to either the quality of the commercial preparation used or the techniques used for the preparation of the antigen extract. This does not, however, detract from the statement made above that the detection of the antigen at present is not a useful diagnostic criterion for enterococci.

Three strains, one from the gut and two from the gingival crevice, possessed the Lancefield group B antigen. These strains may be classified as Streptococcus agalactiae yet they tolerated 0,1 per cent methylene blue, 6,5 per cent sodium chloride, reduced litmus milk and fermented mannitol, which is atypical for this organism. It seems that these strains have more in common with Streptococcus faecalis than Streptococcus agalactiae and it is for this reason that they were classified as enterococci.

One strain from the gingival crevice possessed the Lancefield group A antigen. This strain could be classified as Streptococcus pyogenes, yet it tolerated 0,1 per cent methylene blue, 40 per cent bile, 6,5 per cent sodium chloride, reduced litmus milk and fermented mannitol. It seems more feasible to classify this strain as Streptococcus faecalis rather than Streptococcus pyogenes.

Brock, Peacher and Pierson (1963) investigated a bacteriocin production of enterococci and concluded that it is very likely that each coccus is really a mixture of subtypes. Bacteriophage typing (Brock, unpublished data) and serology (Sharpe and Shattock, 1952) have not solved the taxonomic problems. They conclude that the various strains of enterococci have very little in common, and represent a group of or= ganisms so diverse that no grand design exists. The findings of the present study support their views, namely, that the enterococci are a diverse group of organisms. They possess characteristics, however, which justify their separation from other strepto= cocci.

It is clear, therefore, that the criteria used for differentiating the enterococci from other strepto= cocci require further investigation. In this respect it is suggested that the ability to grow in 6,5 per cent sodium chloride and tolerance of 40 per cent bile are more important criteria than those listed in Table I (Page 53) which include the Lancefield group D antigen.

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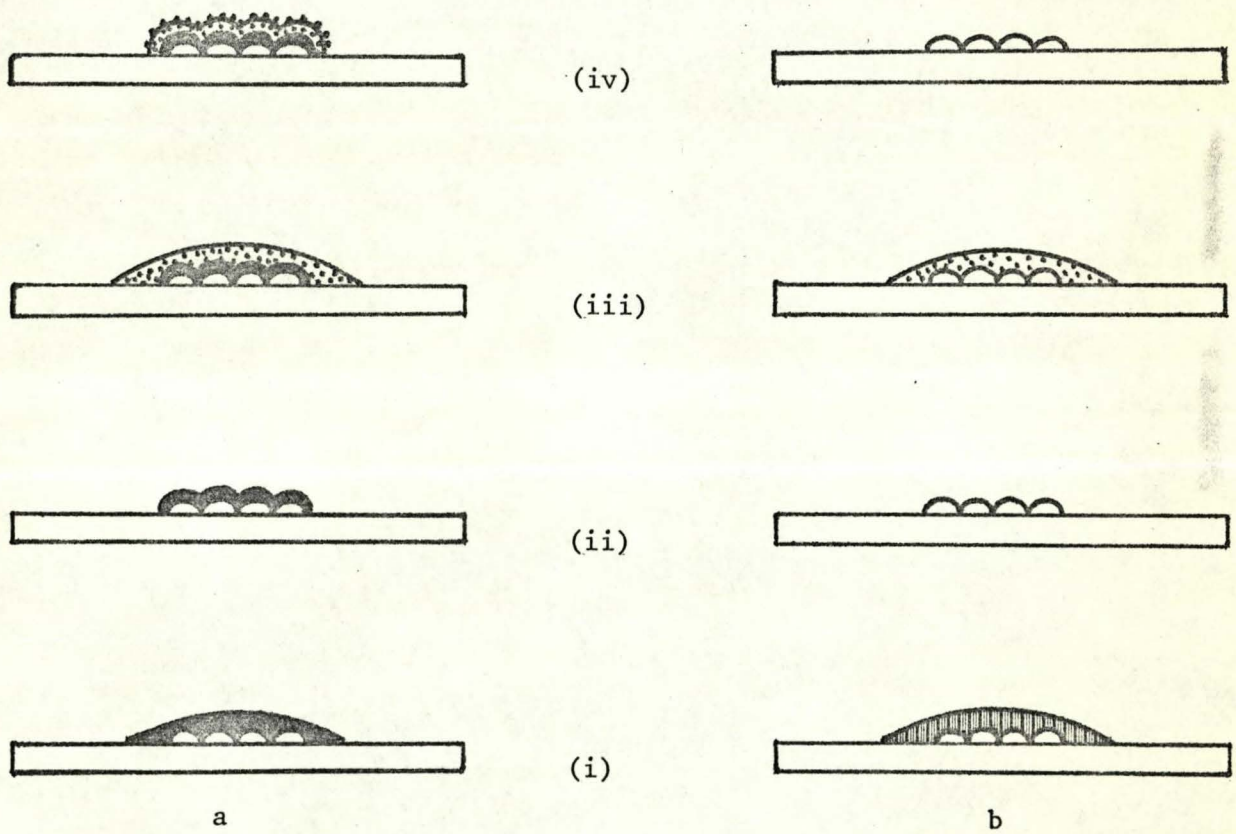


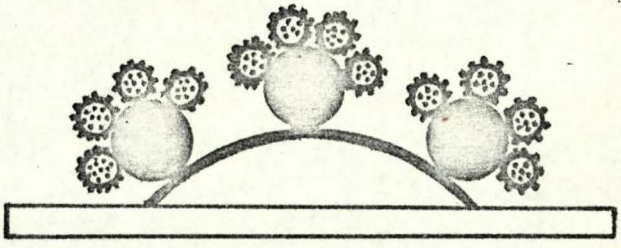
FIGURE 1 SANDWICH METHOD OF STAINING ANTIGEN

(i) The antigen (white semi-circles) is placed on 2 separate slides a and b. These preparations are treated respectively with specific antiserum (black) and non-immune serum (hatched).

(ii) As the non-immune serum does not react with the antigen it is removed from b by washing; the antibody in the immune serum has, however, combined with the antigen on a.

(iii) The conjugated antiglobulin serum (stippled) is applied.

(iv) The fluorescent antiglobulin has combined with the antibody attached to the antigen in a but is washed away from b.



F I G U R E 2 HYPOTHETICAL EXPLANATION OF EXTRA SENSITIVITY OF SANDWICH STAINING

Only 3 sites on the antigen (white semi-circle) are available for the unconjugated antibody (black), which acts as an antigen for the conjugated antiglobulin (stippled). In this representation the reactive sites for fluorescent staining are thus increased fourfold.

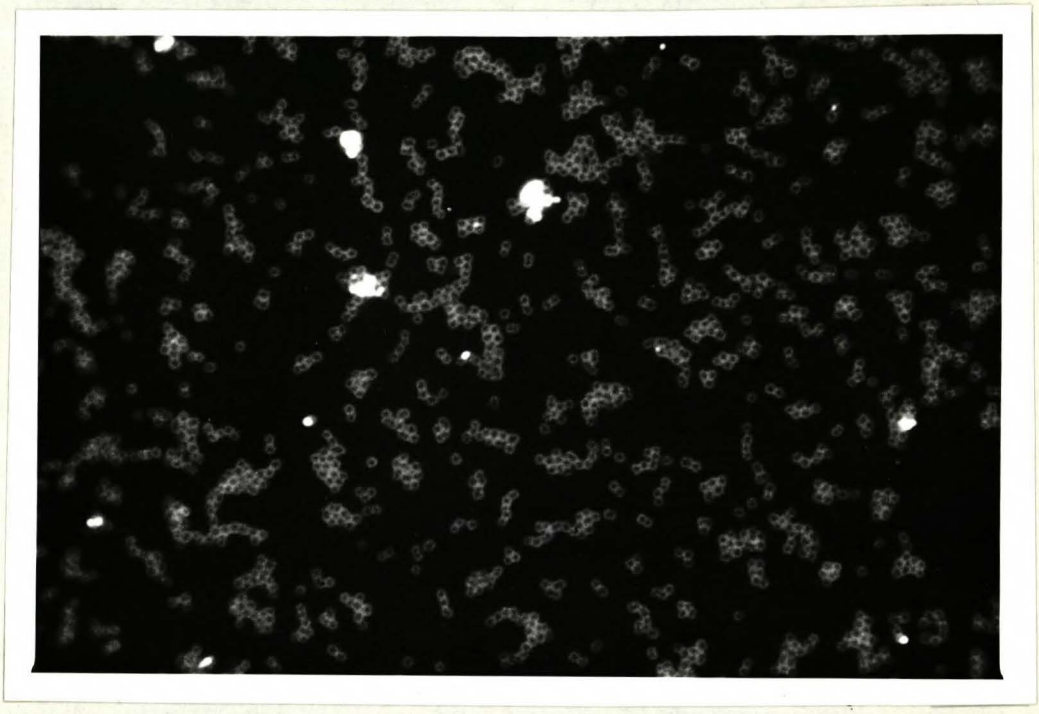
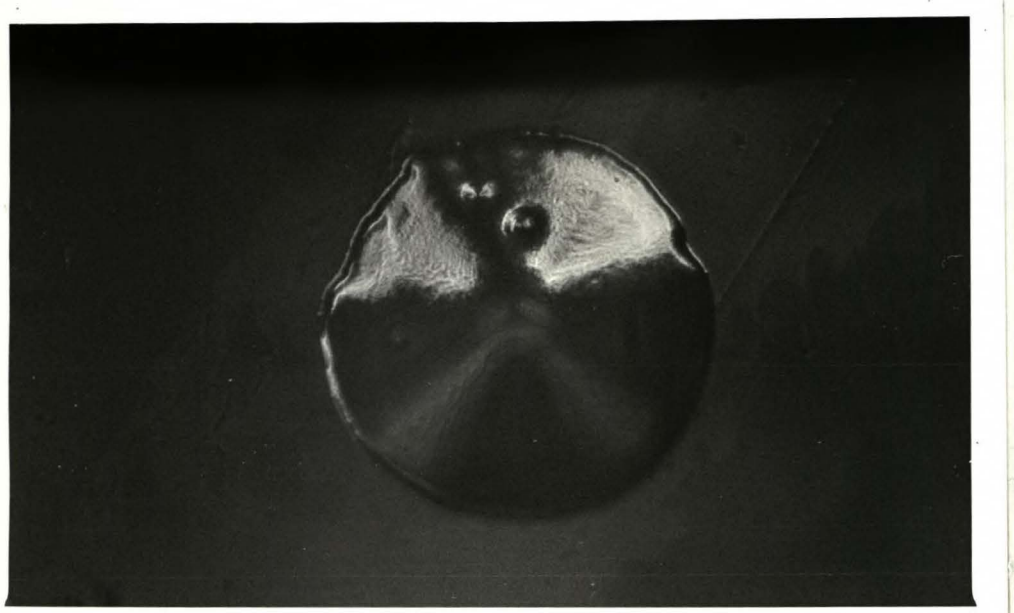


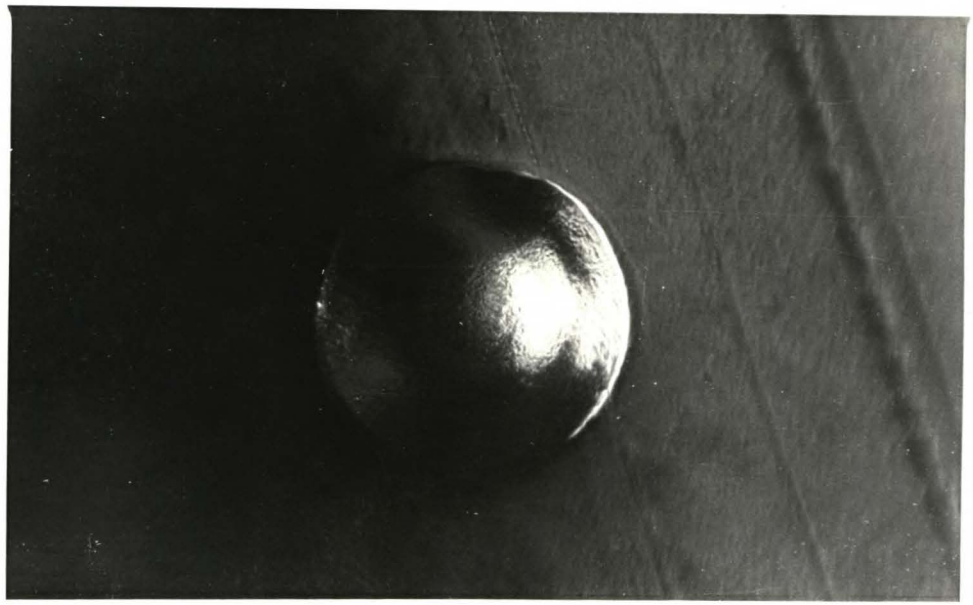
FIGURE 3

Photograph of streptococci showing positive fluorescent ring X 3000

(a)



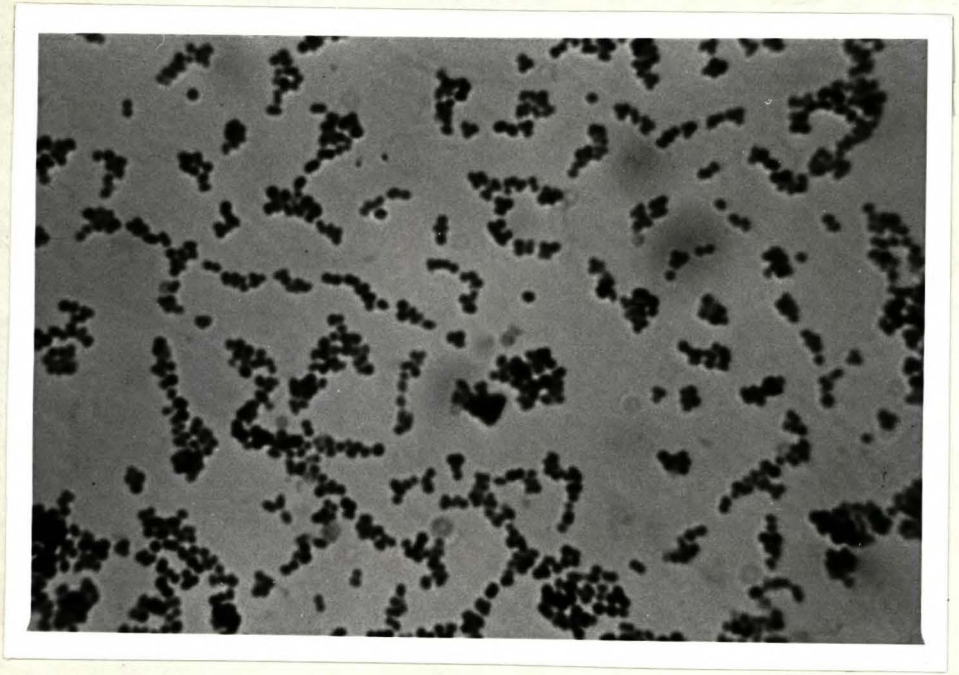
(b)



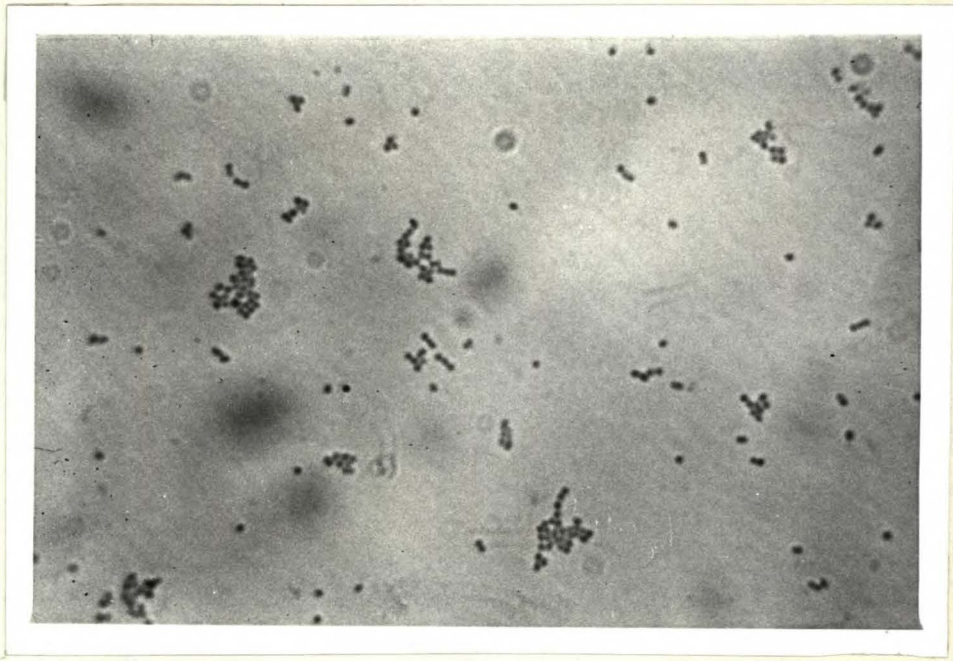
F I G U R E S 4 (a) and (b)

Photomicrographs illustrating the relative difference in size between colonies of enterococci from the gut (a) and from the gingival crevice (b) X 150

(a)



(b)



F I G U R E S 5 (a) and (b)

Photomicrographs illustrating relative difference in size of enterococci isolated from the gut (a) and enterococci isolated from the gingival crevice (b) X 2000

TABLE I CRITERIA USED FOR IDENTIFICATION OF ENTEROCOCCI

	Haemolysis	Tolerance of				Hydrolysis of		Reduction of litmus in litmus milk	Acid from Mannitol	Lancefield D Grouping
		40% bile in blood agar	0,1% Methylene blue in milk	6,5% Na Cl	Tellurite	Starch	Gelatin			
Streptococcus faecalis	α or ɣ	+	+	+	+	-	-	+	most +	D
Streptococcus faecalis variant liquifaciens	α or ɣ	+	+	+	+	-	+	+	most +	D
Streptococcus faecalis variant zymogenes	β	+	+	+	+	-	+/-	+	+	D
Streptococcus durans	β	+	-	+	+	-	-	-	-	D

TABLE II SERUM ANTIBODY (COLUMNS) AGAINST ENTEROCOCCI (ROWS) ISOLATED FROM GINGIVAL CREVICE OF 9 SUBJECTS

		S E R U M										
ENTEROCOCCUS	Subject	1	2	3	4	5	6	7	8	9	Total	Lancefield Group
S. faecalis	1	0	0	8	16	0	0	0	0	0	24	D
S. durans	2	16	16	16	32	16	8	0	16	0	120	D
S. faecalis	3	8	32	16	32	32	8	8	16	16	168	B
S. faecalis	4	64	32	128	512	64	64	32	64	128	1088	-
S. faecalis	5	16	0	32	128	8	16	0	0	0	200	D
S. faecalis	6	16	16	16	128	16	64	32	16	32	336	D
S. faecalis	7	16	16	8	32	16	16	8	8	128	248	D
S. faecalis	8	32	32	32	128	128	64	8	32	128	584	D
S. faecalis	9	0	8	32	8	0	8	8	0	16	80	A, B
T O T A L		168	152	288	1016	280	248	96	152	448	2848	

TABLE III SERUM ANTIBODY TITRES (COLUMNS) AGAINST ENTEROCOCCI (ROWS) ISOLATED FROM FAECES OF 9 SUBJECTS

		S E R U M										
ENTEROCOCCUS	Sub-ject	1	2	3	4	5	6	7	8	9	Total	Lancefield Group
S. faecalis var. zymogenes	1	32	0	8	32	64	0	64	8	16	224	D
S. faecalis var. liquifaciens	1	128	8	32	32	16	16	32	16	256	536	-
S. faecalis var. liquifaciens	2	32	8	8	8	8	0	16	0	64	144	B, C, D
S. faecalis	3	0	8	0	0	0	0	0	0	0	8	D
S. faecalis	4	8	8	8	8	8	0	0	0	0	40	D
S. faecalis	6	0	0	0	0	0	0	0	0	0	0	D
S. faecalis	7	8	0	0	0	16	16	8	16	0	64	-
S. faecalis var. zymogenes	7	8	16	8	64	8	0	64	0	32	200	D
S. faecalis	8	0	0	8	0	0	16	8	32	8	72	D
S. faecalis var. liquifaciens	9	0	0	8	0	0	16	8	0	0	32	D
T O T A L		216	48	80	144	120	64	200	72	376	1320	

TABLE IV PERIODONTAL INDEX, ORAL HYGIENE INDEX AND HIGHEST HOMOLOGOUS SERUM ANTIBODY TITRES FOR SUBJECTS

Subject	Periodontal Index	Oral Hygiene Index	Highest homologous serum antibody titres
1	0,66	1,93	0
2	0,33	0,33	$\frac{1}{16}$
3	0,5	1,49	$\frac{1}{16}$
4	1,5	1,00	$\frac{1}{512}$
5	0,2	0,33	$\frac{1}{8}$
6	0,07	0,66	$\frac{1}{64}$
7	0,2	2,26	$\frac{1}{8}$
8	0,28	0,8	$\frac{1}{32}$
9	0,06	0,82	$\frac{1}{16}$

Datum geleen Date borrowed	Handtekening van lener Borrower's signature	Datum terug Date returned	Voorletters leenklerk Loan clerk's initials

G.P.-S.1981-82 (B-B) 81/5233 (Z 218)

Datum geleen Date borrowed	Handtekening van lener Borrower's signature	Datum terug Date returned	Voorletters leenklerk Loan clerk's initials

G.P.-S.1981-82 (B-B) 81/5233 (Z 218)

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Some Cultural Characte-
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Datum geleen Date borrowed	Handtekening van lener Borrower's signature	Datum terug Date returned	Voorletters leenklerk Loan clerk's initials

G.P.-S.1981-82 (B-B) 81/5233 (Z 218)

Datum geleen Date borrowed	Handtekening van lener Borrower's signature	Datum terug Date returned	Voorletters leenklerk Loan clerk's initials

G.P.-S.1981-82 (B-B)

81/5233 (Z 218)

Datum | Handtekening | Tyd

MAANDAG

DINSDAG

WOENSDAG

TANDHEELKUNDE BIBLIOTEEK U.W.K.

BESPREKING VAN 'N GERESERVEERDE BOEK

Datum	Handtekening	Tyd
	DONDERDAG	
	VRYDAG	
	SATERDAG	