HUMORAL IMMUNE RESPONSE IN MICE FOLLOWING IMMUNIZATION WITH *Prevotella intermedia* and CROSS-REACTIVITY WITH SPECIES OF *Prevotella, Bacteroides* and *Porphyromonas*

BY

MARTHA ROMA SHEPERTYCKY

A Thesis submitted to the Faculty of Graduate Sudies in Partial Fulfillment of the Requirements for the Degree of

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For my parents

Who built a new life for themselves in Canada,

instilling in their children the belief that

hard work, honesty, and knowledge are integral to success.

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ABSTRACT

Members of the genus *Bacteroides* (*Prevotella, Porphyromonas*) are resident in the supragingival and subgingival plaque environments in humans and animals (Slots, 1979; White and Mayrand, 1981; Moore, 1987). Recovery of *Bacteroides* (*Prevotella, Porphyromonas*) from oral cavities of children is rare, since children often exhibit little periodontal disease, despite large accumulations of plaque (Zambon et al., 1981). In contrast, in adults with periodontal disease, these organisms constitute a significant proportion of the cultivable flora.

Serological studies of the genus *Bacteroides* (*Prevotella, Porphyromonas*) by Ebersole et al. (1988) demonstrated that both black-pigmented and non-pigmented *Bacteroides* possess distinct surface moieties, to which rabbits and humans mount an immune response. Ebersole et al. also demonstrated that systemic antibody levels are modified by colonization and invasion of the gingival sulcus by periodontal microorganisms (Ebersole et al., 1987).

Elevated humoral responses to periodontopathic organisms in humans have been demonstrated (Ebersole, et al.,1982(b), Ebersole et al., 1987). Wide cross-reactivity among the black-pigmented *Bacteroides* has been demonstrated using hyperimmune rabbit serum (Bowden and Nolette, 1990; Ebersole, et al., 1988). The elevated immunoglobulin levels to organisms isolated from periodontally diseased patients may be specifically induced in response to colonization by these organisms, due to cross-reactive stimulation by similar organisms, or both.

This study was initiated to study the degree of stimulation of the humoral immune system required to generate cross-reactive antibody to related organisms, following immunization of mice with a commensal oral microorganism - *Prevotella intermedia* BH18/23 (genotype II). C57BL/6 and Balb/c mice were immunized with live, unaltered cells of *P. intermedia* using various routes, quantities of cells, booster

injections and addition of Freund's compete adjuvant. Pooled serum obtained from control non-immunized mouse groups was used to determine the natural antibody repertoire present in these animals. Germ-free mouse serum (NIH strain) was probed to ascertain their level of natural antibody.

Non-detergent treated outer membranes were prepared from various species of the genera *Prevotella*, *Bacteroides* and *Porphyromonas*. Crude outer membranes were selected for this study, to reflect the wider range of antigens, which could be recognized *in vivo* by an animal's immune system. SDS-polyacrylamide gel electrophoresis was used to separate the constituent antigens, and immunoblotting was used to probe the mouse sera for response to antigens in the protein profiles of homologous and heterologous organisms. Human child and adult sera were used to compare the antigen recognition profiles with that seen in mouse antibody profiles. ELISA assays provided a relative quantitative assessment of antibody levels following immunization, and aided in the determination of the degree of specificity of the generated response.

Anti-mouse goat IgG and IgM conjugate was capable of recognizing several antigens in the outer membrane profiles of most species of *Prevotella*, *Bacteroides* and *Porphyromonas* tested. Control mice recognized significantly more antigens, or developed the antigens with greater intensity compared to goat conjugate alone, signifying that the mouse carries natural antibodies to the species of *Prevotella, Bacteroides* and *Porphyromonas* tested. Germ-free (NIH strain) mice also carried a low level of antibody capable of recognizing antigens in *P. denticola*, *P. asaccharolyticus, P. gingivalis* , *P. oralis* and *P. buccae*. Antigen development by germ-free mouse sera was very weak, although levels were higher than that developed by the goat anti-mouse conjugate in the absence of mouse serum.

A comparison of antigens recognized by mouse sera (immunized with *P. intermedia*), hyperimmune rabbit sera (immunized with *P. intermedia*) and human child and adult sera, showed striking similarity in major antigens recognized by

antibody from all hosts. Mouse serum (M3) recognized only 7 antigens in the IgG profile, while hyperimmune rabbit sera produced antibody recognizing 15 antigens in the outer membrane of the homologous organism. Both mouse and rabbit sera showed similar recognition of antigens to both human child and adult profiles. The children's sera recognized most of the antigens present in the adult serum, although the intensity of antigen development on immunoblots was lower with the childrens' sera. These results show that the mouse model provides a simplified picture of immune response, similar to that seen in other animals.

Low dose immunization of C57BL/6 mice (10^3-10^4 viable cells of *P. intermedia* BH18/23)(2-3 weeks old) produced antibody, not previously present prior to immunization, which was capable of recognizing unique antigens in the outer membrane of the homologous organism (IgG - 26, 34, >100 kDa, IgM - >100 kDa). Only one cross-reactive antibody response was generated to *P. asaccharolyticus* (IgG - 65 kDa). Low dose immunization of 4-5 weeks old C57BL/6 and Balb/c mice did not produce antibody capable of recognizing unique antigens. Intra-species differences in the natural antibody repertoire of the two mouse strains could be detected, with the most pronounced difference being the recognition of a 50 kDa antigen of *P. loescheii* by C57BL/6 mouse IgM.

High dose immunization of C57BL/6 mice (10^6-10^7 viable cells *P. intermedia* BH18/23) produced not only antibody that recognized antigens not seen prior to the immunization only to the homologous organism (IgG - 26, 34, >100 kDa; IgM - >100 kDa), but also higher levels of antibody to antigens which were recognized by control mice (IgG - 31, 44 kDa). A similar high dose immunization of Balb/c mice produced similar response to the homologous organism, as well as produced cross-reactive IgG and IgM antibody capable of recognizing antigens in the outer membranes of *P. intermedia* BH20/30 (genotype I), *P. melaninogenica* and *P. denticola.*

The addition of Freund's complete adjuvant with a single dose of *P. intermedia* intramuscularly in C57BL/6 and Balb/c mice resulted in the development of more cross-reactive responses. Most of these cross-reactive responses to heterologous organism were of the IgG isotype (C57BL/6 IgG - *P. denticola, B. fragilis, P. loescheii),* while IgM cross-reactive antibody was produced only in response to *B. fragilis* by both C57BL/6 and Balb/c mouse sera.

Chronic low dose immunizations for 6 weeks of C57BL/6 mice (10^6-10^7 viable cells *P. intermedia* BH18/23) produced sera which recognized primarily two antigens in the homologous profile (IgG - 31 and 44 kDa) with little significant changes seen in antigen pattern recognition to heterologous organism outer membranes.

In contrast, the production of a hyperimmune serum, by high dose immunization of C57BL/6 mice together with Freund's complete adjuvant and intravenous booster injections, produced serum which showed extensive cross-reactivity to most outer membranes, primarily in the IgG isotype, and only IgM cross-reactivity with *P. intermedia* BH20/30.

A study of individual mouse responses to chronic low dose immunizations with *P. intermedia* BH18/23 showed that individual mice produced similar antibody profiles, although the quantity of immunoglobulin produced to each antigen recognized in the outer membrane, by each individual mouse, varied somewhat. This could be attributed to the variation in the actual number of viable cells injected into each animal due to interstitial loss of sample during injection, or death of viable cells during the immunization time period.

ELISA assays of sera from various immunization experiments was tested against whole cells and outer membranes of *P. intermedia* BH18/23, *P. melaninogenica, P. asaccharolyticus, P. gingivalis* and *B. fragilis.* Results showed that the highest optical density was obtained from the hyperimmune mouse sera to the homologous organism, followed by a slightly lower optical density value from mice immunized with low chronic

doses of viable cells to the homologous organism. ELISA assays of sera from individual mice immunized with low chronic doses of *P. intermedia*, showed similar values of antibody within the immunized group, which were much higher than the optical density values obtained from control mice.

Ebersole and Holt in 1988 suggested that antibody generated to periodontal microorganisms isolated from the periodontal microflora of diseased patients is a specific inductive process. These studies of controlled immunizations of mice with a single commensal oral microorganism, further strengthens this specific induction theory, showing that immunization with *P intermedia* BH18/23 (genotype II) produces antibody primarily directed against antigens in the homologous organism as evidenced by immunoblot analysis and ELISA. Low dose chronic immunization of mice with viable cells of *P. intermedia*, a situation which may be encountered in periodontal disease, enhanced the response to selected antigens in the outer membrane of the homologous organism, showing a selectivity of reaction to certain antigens in the outer membrane, and not a generalized increase in response to all antigens. Significant cross-reactive responses to heterologous outer membranes were detected by production of a hyper-immune mouse serum (high dose immunization together with Freund's Complete Adjuvant and booster doses), primarily by the IgG isotype. As it is unlikely that a host would receive such stimulation of the immune system by its resident flora, or from an organism in the flora of the periodontal pocket, with the possible exception of cases of severe periodontal breakdown, the significance, if any, of the production of weak cross-reactive responses generated to heterologous organisms in the periodontal disease process remains unknown.

CHAPTER 1

LITERATURE REVIEW

1:1) INTRODUCTION

The complex ecological environment of the oral cavity harbors a diverse population of microbes. Although the oral flora is similar in all humans, components of the indigenous oral flora are unique to each individual, and the oral flora differs from those found elsewhere in the body. Microorganisms from the surrounding environment are constantly being introduced into the oral cavity, and while some of the invaders are integrated into the resident normal flora, others reside only temporarily in the mouth, i.e. transient flora. Infections caused by a single species of microorganism create an interaction between the host's defense system and the specific microorganism. However, in mixed infections, a much more complicated pathologic picture arises. Each species in a mixed infection has an effect on other species present, thus inter-species ecological interactions occur, creating predominant and less dominant organisms. The ecological distribution of organisms is affected by the host defenses, and conversely the host defences are influenced by the various microbial species.

The activity of the host immune system in relation to the severity of disease, measured by antibody levels or response of lymphoid cells from peripheral blood has been studied extensively. Various factors such as: the nature and amount of the antigen, its route of introduction and the genetic constitution of the host, affect the production of antibodies by an animal. Some of the mechanisms stimulating the emergence of an antibody repertoire are known, but many areas of the immune response are still unclear. The study of immune responses in mono-infections or after immunization with bacterial products is relatively straight forward, whereas responses in mixed infections are more difficult to assess. The interplay and relative roles of humoral, mucosal, and

cell-mediated immunity makes the understanding of responses by the immune system even more difficult.

Microbiological investigations of periodontal disease have suggested an association of distinct subgingival microorganisms with different types of periodontal disease (Moore, 1987; Moore et al., 1985; Moore et al., 1987). Ebersole et al. demonstrated that systemic antibody levels are modified in periodontal disease as a result of colonization and invasion of the gingival sulcus by microorganisms (Ebersole et al., 1982(a); Ebersole et al., 1982(b); Ebersole et al., 1983; Ebersole et al., 1987). Generation of higher antibody titres in the host serum to a specific periodontal pathogen may therefore be utilized in detecting subgingival colonization by the microorganism and identifying active sites of periodontal destruction.

Members of the genus *Bacteroides*, *Prevotella* and *Porphyromonas* are associated with the Gram-negative anaerobic microflora of human periodontal pockets (Slots, 1979; Spiegel et al., 1979; White and Mayrand, 1981). An elevated antibody level to oral *Bacteroides* (*Porphyromonas* and *Prevotella*) is demonstrable in periodontally diseased patients (Ebersole et al., 1986). Serological studies of the genus *Bacteroides* (*Prevotella and Porphyromonas*) by Ebersole et al. in 1988, showed that both black-pigmented and non-pigmented species possess distinct surface antigenic moieties, to which rabbits and humans can mount an immune response. Immunization of an animal with one species of *Bacteroides* (*Prevotella, Porphyromonas*) generates antibody, which cross-reacts with other strains of black-pigmented *Bacteroides*, *Prevotella* and *Porphyromonas*. The antibody response elicited may be specific, while some of the response may be due to previous exposure of the host to various antigens on similar microorganisms among the resident flora (e.g. gut-associated organisms).

The current study was initiated to further explore the development of specific and cross-reactive humoral immune responses in mice following immunization with a

relatively non-virulent strain of black-pigmented *Bacteroides* (*Bacteroides intermedius=Prevotella intermedia* BH18/23 - a genotype II, serogroup B isolate from a clinical periodontal pocket). This species is commonly recoverable from the human resident oral flora. Immunization regimens varied in the quantity of viable cells injected, the route of injection, age and genetic strain of mice and the use of adjuvant for augmentation of the immune response. Serum obtained from control mice was used to determine the natural repertoire of antibodies present in non-immunized animals, while serum obtained from immunized animals was used to study the most immunogenic antigens in outer membrane preparations of a variety *Bacteroides*, *Prevotella* and *Porphyromonas* strains and cross-reactivity among these species. Serum obtained from humans - adults and children, was also tested against outer membrane preparations of various *Bacteroides*, *Prevotella* and *Porphyromonas* to ascertain the antigenic profile recognized by humans, and comparisons were drawn between the profiles recognized by human sera and sera from mice immunized with *P. intermedia*.

1:2) GENERAL IMMUNOLOGY

2:1) INTRODUCTION

The immune system is responsible for the protection of a host from infections. Immune responses are specifically directed against an invading pathogen, i.e. bacteria, viruses, fungi or parasites, and/or any toxic materials produced by these agents. Although the immune system defends the host against infections by microorganisms and larger parasites, a response can be elicited by almost any macromolecule, termed an antigen, which is foreign to the host. The specificity and ability of the host to identify self-antigens as non-foreign, ensures that immune responses will only be mounted to

eliminate foreign antigens. Failure of a host's immune system to recognize self-antigens can result in a self-destructive process.

Two main types of immune responses have been recognized:

(1) humoral or antibody responses and

(2) cell-mediated responses.

Antibody responses involve the generation of protein molecules called immunoglobulins. Immunoglobulins circulate throughout the blood stream and other body tissues, where they bind to antigens, which stimulated their production. Cell-mediated responses require the production of specialized cells, which react directly with foreign antigen.

Specialized white blood cells termed lymphocytes, are vital components in both types of immune responses. T lymphocyte cells are responsible for recognition in cell-mediated immunity, but they also play a role in the generation of antibody by B lymphocytes. T lymphocytes are produced in the thymus from pleuripotent hemopoeitic stem cells, which migrate to the thymus via blood.

Antigen-stimulated B lymphocytes are the precursor cells that mature into plasma cells, and subsequently produce and secrete antibody. In mammals, B cells are produced by hemopoeitic tissues, such as adult bone marrow or fetal liver. In birds, precursor cells from hemopoeitic tissues migrate to the Bursa of Fabricius - a gut-associated lymphoid tissue, where the B cells are subsequently generated.

The immune system is capable of responding to numbers of foreign antigens in a highly specific manner. Antigenic structures varying only slightly in composition (e.g. amino acid sequence of a protein or optical isomers) can be differentiated by the immune system. The clonal selection theory developed in the 1950s by Burnet (Burnet, 1959; Ada and Nossal, 1987) suggested that each lymphocyte was committed to respond to a single foreign antigen, which had been determined prior to the lymphocyte's exposure to that specific antigen. This specific commitment is manifested in the form of a cell-

surface receptor protein (present on IgM molecules), which can specifically recognize and bind the antigen. Interaction between macrophages, T cells and the subsequent specific binding of a foreign antigen to its corresponding lymphocyte causes proliferation and maturation of the specific lymphocyte clone.

The part of an antigen that binds to the antigen-binding site on an antibody molecule or on a lymphocyte is called the antigenic determinant or epitope. Thus a single antigen molecule can activate many lymphocyte clones, which bear complementary binding receptors. In order to ensure that specific lymphocytic clones within the entire clonal population of lymphocytes encounter an introduced antigen, antigens are trapped in secondary lymphoid organs, such as lymph nodes, through which T cells continuously recirculate. Most lymphocytes possess surface receptors (glycoproteins), which are responsible for the homing of these cells to specific tissues within the body. For example, a glycoprotein found on some lymphocytes will selectively recirculate them through the Peyer's patches, while different surface glycoproteins activate the lymphocytes to travel through the lymph nodes. Upon stimulation of a specific lymphocyte by an antigen, the lymphocyte loses its original homing receptors and acquires new receptors which direct the activated cells to sites of inflammation.

The host develops a state of immunological tolerance to self-antigens, so that these antigens are recognized as non-foreign. The immune system is genetically capable of responding to self, but eliminates self-reactive lymphocytes. This elimination occurs in the primary lymphoid organs, upon recognition of their respective antigens. The production of reactive lymphocytes to self-antigens is continuous through life, therefore maintenance of self-tolerance requires the presence of self-antigens and elimination of reactive lymphocytes.

2:II) HUMORAL IMMUNE RESPONSE

The generation of a humoral immune response involves the formation of specific antigen-triggered proteins called immunoglobulins. Immunoglobulins are formed in the lymphoid tissues of the body, in response to the presentation of a foreign antigen. Antigens possess a specific structure, called antigenic determinants. The resulting immunoglobulin produced in response to a given antigen is designed to specifically fit the unique structure of the antigenic determinant.

Synthesis of antibodies occurs primarily in the spleen, gut-associated lymphoid tissue, lymph nodes, and bone marrow, but antibody synthesis may be demonstrated in any area of the body containing lymphoid tissues, with the exception of the thymus.

The principal cells involved in generation of antibody are B lymphocytes, although participation by T cells, plasma cells and macrophages is required in the initiation, activation or secretion of antibody. T lymphocytes provide regulatory mechanisms within the immune system, i.e. T helper cells assist in antibody production, while T suppressor cells inhibit antibody production.

a) Structure of Antibodies

The basic structural unit of an antibody molecule consists of four polypeptide chains: 2 identical light (L) chains and two identical heavy (H) chains. These four chains are held together by a combination of non-covalent and covalent disulfide bonds. The molecule is composed of two identical halves; each half containing the same antigen binding site.

Antigen binding sites of immunoglobulins are present at the tips of the Y-shaped bivalent molecules (the Fab portion) and are composed of portions of both the heavy and light chains. The bivalent Y-configuration of an immunoglobulin allows for the cross-

linking of antigen molecules into large lattices, provided the antigen has three or more antigenic determinants.

The amino acid sequences of the Fab portion vary from one monomer to another, and the resulting variations in chemical structure account for the ability of different antibodies to recognize and bind many different antigens. Any single antibody molecule has only one type of variable region in its protein chains, hence it will only bind to one unique antigenic determinant. Antibodies with the same antigen-binding sites can have different tail regions (Fc portions), which allow the antibody to possess different properties, such as activation of the complement cascade or the ability to bind to phagocytic cells.

b) Classes of Immunoglobulins

Immunoglobulins (Ig) have been divided into five classes: IgA, IgD, IgE, IgG and IgM, each with its own H chain - α, δ, ε, γ and μ respectively. In addition, four subclasses of IgG have been recognized, each with its own particular heavy chain ($\gamma 1$, $\gamma 2$, $\gamma 3$, $\gamma 4$). The specific γ heavy chain imparts a unique property to the IgG molecule.

IgM is the first class of antibody to be produced by an activated B cell (plasma cell) in primary response to the initial exposure to an antigen. Antibodies of the IgM class comprise about 5-10% of total antibody present in human serum. This immunoglobulin generally does not leave blood vessels and enter surrounding tissues, or cross the placenta to provide immunity to the fetus. The rapidity of IgM production and it's high effectiveness as a protective antibody, plays a very important role in the primary humoral response. IgM is a pentamer, consisting of ten potential antigen-binding sites, therefore it is very effective in the crosslinking of particulate antigens. Subsequent binding of an antigen to the Fab (antigen-binding) region of a secreted pentameric IgM induces the Fc region (tail) to bind to and activate the first component of

the complement cascade. The resulting activation of the complement cascade unleashes a biochemical attack on the invading microorganism (antigen) recognized by the antibody.

IgG is the major immunoglobulin found in human serum, accounting for about 80-85% of antibody. During the secondary immune response (pg. 11), IgG is produced in large quantities upon encountering the antigen to which the host had been previously sensitized. IgG antibody readily crosses walls of blood vessels and enters tissue fluids, i.e. maternal IgG antibodies can cross the placenta and confer passive immunity to the fetus. The activation of the complement cascade, and the ability of IgG to bind to certain receptors present on macrophages and neutrophils, allows simultaneous activation of both humoral and cell-mediated responses by this immunoglobulin. Opsonization - the attachment of IgG molecules to bacterial antigens, enhances cytotoxic killing of the bacterial cell by phagocytes.

IgA comprises about 15% of all serum antibodies, and is the principal class of immunoglobulin found in secretions, such as milk, saliva and tears. It consists in one of two forms, i.e. as a four-chain monomer or a dimer of two units, carrying a single J (joining) chain and a secretory chain (secretory IgA). The presence of the secretory chain renders the immunoglobulin more resistant to proteolytic breakdown, which is especially important in the transport of IgA through the blood to the surface of secretory tissues. The main function of secretory IgA is to prevent the attachment of pathogens, particularly viruses, to mucosal surfaces.

IgD antibodies comprise about 0.2% of the total serum concentration of immunoglobulins. A high concentration of IgD is present on the surface of B-cells, especially in newborns, and therefore it plays a role in the initiation of the immune response. Upon initial antigen presentation, some virgin B lymphocytes produce cell-surface IgD molecules, with the identical antigen-binding sites as the corresponding IgM molecule produced. IgD is rarely synthesized by B cells, but is present in large quantities on the membrane of circulating B lymphocytes. Although the precise role of

IgD is not known, it may be involved as a receptor for antigen, or possibly play a role in antigen-triggered lymphocyte differentiation.

IgE is present on the surfaces of basophils and mast cells, where the IgE molecule acts as a receptor for antigen. IgE constitutes only about 0.002% of the total serum antibody concentration. The binding of antigens to IgE molecules located on basophils or mast cells triggers these cells to secrete a variety of amines, such as histamine and serotonin. These amines cause peripheral vasodilation and increased permeability of blood vessels, and are thereby largely responsible for the clinical manifestations of such allergic reactions as hay fever, asthma, and hives.

c) Antigen Presentation

Upon the entry of an antigen into an appropriate lymphoid organ, a sequence of events develops, i.e. processing of the antigen, recognition and binding of the antigen to an antigen-sensitive lymphocyte. Macrophages (antigen-presenting cells) are involved in the processing of antigen, and the formation of an interaction between appropriate T and B cells. The processing procedure results in alteration of the antigen, with no change to the portion containing the antigenic determinant.

Some antigens, called T-independent antigens, do not require the collaboration of T cells in the stimulation of B cells. T-independent antigens are usually polymeric structures with repeating antigenic determinants, which can either crosslink the cell surface of a B cell or bind to the B cell together with a mitogen (compounds capable of causing proliferation of B cells). T cell independent antigens activate B cells to produce plasma cells (which almost exclusively produce IgM) with the production of very few or no memory cells.

T-dependent antigens require the interaction of T helper cells for the activation of an appropriate B cell. Upon recognition of a T-dependent antigen by a T cell, the T cell

releases a specific T helper factor which activates the B cell. T cell dependent antigens induce B cell production of IgM, IgG and IgA, as well as memory cells.

d) Primary Immune Response

The clonal selection theory of Burnet (1959) states that clones of lymphocytes are present in the human body since birth, and that they are predetermined to respond to a particular antigen. On exposure to an antigen, the antigen finds or selects the specific clone which has been programmed to identify it. Each B cell and its clone synthesizes only one combination of variable region on the heavy and light chains of a given immunoglobulin molecule, to form an antibody-combining site. The exact identity of this site in the Fab fragment is determined during fetal development.

The first IgM antibodies produced by a B cell are not secreted, but inserted into the plasma membrane, where they serve as receptors for the antigen. The binding of antigen to a B cell receptor causes its proliferation and maturation into either memory cells or active B cells (plasma cells) capable of producing large amounts of soluble, non-membrane bound antibody possessing the same antigen-binding sites as the surface receptor.

The active plasma cells synthesize and secrete IgM and this initial response is called the primary immune response. Five to eight divisions of the B cell are required for transformation into a plasma cell. Due to the necessary time required for the differentiation of a B cell into a plasma cell, the primary immune response is characterized by a lag in immunoglobulin production after exposure to antigen, and a relatively short duration of antibody production.

Plasma cells are found in the germinal centers of lymph nodes, the spleen and diffuse lymphoid tissues of the alimentary and respiratory tracts. Each plasma cell lives for only about 2 days, but is capable of producing about 2,000 antibody molecules

per second. The remaining progeny produced by a stimulated B cell become memory cells, i.e. cells which are capable of recognition of the antigenic determinant to which the parent cell was exposed. Memory cells are disseminated throughout the blood, lymph and tissues for extended periods and become a reservoir of antigen-sensitive cells that are primed with a memory factor for future immunoglobulin production.

e) Secondary Immune Response

The secondary response to the same antigen produces a shorter lag period prior to generation of antibody, and a greater response of longer duration than that of the primary response. Upon reintroduction of the same immunogen, memory cells proliferate rapidly and synthesize a large quantity of antibody (IgG). This immunological memory can be explained by the clonal selection theory. In a mature host, T and B cells in secondary lymphoid tissues are present in at least three stages of maturity: virgin cells, active cells and memory cells. Virgin cells, upon presentation of antigen, can mature to become active cells that generate a humoral response, i.e. secrete antibody or initiate cell-mediated responses. Some virgin cells are stimulated to produce memory cells, which do not respond immediately to the antigen, but are readily inducible upon the next presentation of the same antigen. Immunological memory is thus increased by clonal expansion, i.e. by the proliferation of antigen-triggered virgin cells, creating memory cells. Memory cells have a longer life-span than the virgin cells, and can respond much quicker upon the re-introduction of the same antigen.

2:III) CELL MEDIATED IMMUNITY

Cell mediated immunity describes localized reactions by lymphocytes and phagocytes to intracellular pathogens and foreign cells. Although, T lymphocytes are the

primary effector cells in cell-mediated defense of a host infected by viruses, bacteria, or fungi, antibody can play a role by coating infected target cells, which aids in their recognition by the cellular components of the immune system.

a) Types of T cells

T cells are the key component involved in the generation of a cell-mediated response. Based on their function, T cells can be subdivided into two main categories, i.e. effector cells and regulatory cells. Cytotoxic T cells, together with B cells are the main effector cells of the immune system. Helper T cells (T_h) and suppressor T cells (T_S) function in the regulation of antibody responses, and are collectively termed regulatory T cells. Helper and cytotoxic T cells do not bind free antigen.

The cell-mediated response occurs upon the presentation and stimulation of T helper cells by antigen. Foreign antigens are recognized by specialized antigen-presenting cells located in most tissues. Antigen-presenting cells are derived from bone marrow, and comprise an assortment of cells, including dendritic cells in lymphoid tissues, Langerhans cells in skin, and certain macrophages. Their function is to present the processed antigen to a T helper cell.

b) Antigen Recognition by T cells

Antigen recognition by T cells occurs in combination with the major histocompatability complex (MHC) glycoproteins. The major histocompatability complex is composed of cell-surface glycoproteins which are genetically identical to host tissues. Thus, T cells are able to determine whether an antigen is foreign or self.

The recognition of antigen together with the MHC complex on a target cell surface signals the T lymphocyte that it is making contact with another cell. Cytotoxic T cells

recognize foreign antigen in association with class 1 MHC products, and since most nucleated host cells possess class 1 MHC glycoproteins, the cytotoxic T cell has the potential to recognize any host cell, which has become infected by a virus. T helper cells and most of the T cells that proliferate in response to antigen, recognize antigen in association with class 2 MHC glycoproteins, which are expressed mostly on antigen-presenting cells and B cells. T cells possess a surface receptor (Tr) capable of recognition of the MHC proteins and the antigen. A T4 peptide is present in association with the T receptor for the recognition of MHC class 2 antigens, while a T8 peptide is present in association with MHC class 1 antigen receptors. The T4 and T8 peptides are thought to send activation signals to T cell.

Antigen processing by the antigen-presenting cell occurs by endocytosis of the foreign protein. The antigen-presenting cell does not synthesize the antigen to which it has been exposed, but after partial degradation of the antigen, returns it to the cell surface. Antigen-presenting cells can process and present virtually any antigen they encounter to an appropriate T helper cell. This lack of specificity implies that many of the proteins ingested and degraded will be host (self) proteins. Together with B cells, which can also present antigen to T helper cells, these specialized antigen presenting cells are the main types of cells that normally express class 2 MHC glycoproteins. Degraded fragments of foreign protein bound to class 2 MHC glycoproteins are presented to T helper cells, in a similar manner to the presentation of virus-particles bound with class 1 MHC glycoproteins to a cytotoxic T cell.

c) T cell Proliferation

The initiation of T cell proliferation involves not only the recognition of antigen and MHC determinants, but also depends on the passing of signals between antigen presenting cells and lymphocytes. Local chemical mediators, called interleukins are

secreted by the T cell-stimulated antigen-presenting cells. Antigen binding and the secretion of interleukin-1 (IL-1) by an antigen presenting cell does not directly stimulate the proliferation of helper T cells. IL-1 causes the T cell to stimulate its own proliferation by inducing the T cell to secrete a growth factor called interleukin-2 (IL-2), and produce cell surface IL-2 receptors. The binding of IL-2 to the T cell's receptors stimulates the T cell to grow and divide. When the antigen is eliminated, the T cell eventually stops production of the IL-2 receptors and proliferation ceases. Helper T cells aid in the proliferation of other T cells, including cytotoxic T cells, which can be induced to express IL-2 receptors. The expression of IL-2 receptors is strictly dependent on antigen stimulation, therefore only those T cells, which have encountered antigens can proliferate. Helper T cells, which secrete IL-2 when stimulated by antigen, also secrete other interleukins such as γ-interferon. γ-interferon attracts macrophages and activates them to become more efficient at phagocytozing and destroying invading microorganisms. Another important effect of γ-interferon is to induce the expression of class 2 MHC glycoproteins on the surface of some cells, that normally do not express them, enabling these cells to present antigen to helper T cells. T helper cells can therefore recruit extra antigen-presenting cells, when the need arises.

d) T Regulatory Cells

In addition to T helper cells, another T regulatory cell is the T suppressor cell. Helper T cells and suppressor T cells work as a unit, and are thought to control the activity of B cells and cytotoxic cells, i.e. the major effector cells of the immune system. Helper T cells act directly on these effector cells, while suppressor T cells are thought to act indirectly by inhibiting the helper T cells, required for activation of the immune response. Suppressor T cells often interact with the helper T cell, by recognizing

antigenic determinants associated with antigen-binding sites of the helper T cell receptor, called idiotypes.

e) Cytotoxic Killer Cells

Four general cell-mediated cytotoxic effector mechanisms have been identified: (1) specifically sensitized effector cytotoxic T cells reacting directly with target cells; (2) specifically sensitized effector lymphocytes (TD) reacting with antigen and releasing mediators that kill target cells (cytotoxins) or activate other effector cells (macrophages); (3) nonsensitized lymphocytes (natural killer cells) activated by immunoglobulin antibody-Fc binding (antibody-dependent cell-mediated cytotoxicity or ADCC) and (4) activated macrophages.

Cytotoxic T cells (Tc) have the ability to bind to their target cell upon recognition of the antigen in association with MHC determinants.

Natural killer cells (NK cells) recognize determinants expressed on neoplastic or virally infected cells. These lymphocytes which lack T and B cell markers are able to cause death in certain target cells without specific antibody mediated killing.

Viruses have the ability to proliferate inside host cells, where they are protected from recognition by antibodies, therefore eluding a humoral immune system. Cytotoxic T cells or natural killer cells (NK cells) function primarily by killing virus-infected cells, prior to the assembly of newly replicated virus particles within the host cell. Since cytotoxic T cells have destructive potential, it is vital that the host be able to respond very specifically. Natural killer cells (NK cells) are large granular lymphocytes, which have the capability of recognizing the fragments of viral molecules bound to class 1 MHC glycoproteins, which appear on the surface of virally infected cells. This recognition allows the natural killer cell to differentiate an infected cell from a normal host cell. The attachment of cytotoxic T cells to such target cells,

stimulates the cytotoxic cells to release pore-forming proteins called perforins, which polymerize in the target cell plasma membrane to form transmembrane channels. These transmembrane channels create holes in the cell membrane, and lead to the eventual destruction of the target cell. Perforins, are homologous to the complement component C9, and are stored in secretory vesicles. By methods of local exocytosis, the secretory vesicles containing perforins are released at the point of contact with the target cell.

T-Killer cells possess receptors for the Fc portion of antibody, therefore, much like a phagocytic cell, the killer cell can recognize antibody. The presence of antibody attached to the virally infected target cell surface, allows the natural killer cell to form a bridge with the target cell, thus activating the NK cell and triggering mechanisms of destruction of the target cell. This system of extracellular killing is termed antibody dependant cell-mediated cytotoxicity (ADCC).

Macrophages play a central role in cell-mediated responses, both in the initiation of the response by presentation of antigen (antigen-presenting cells) and as effectors in inflammation as microbicidal cells. Antigen presentation to a T lymphocyte, stimulates the T cell to release factors called lymphokines. Lymphokines released by antigen-stimulated T cells act primarily on macrophages, causing their subsequent activation. The T cell activation is a specific event, while the subsequent macrophage activation is non-specific. Lymphokines which are released in response to the antigens of one microorganism, can therefore bring about the destruction of a different microorganism residing in the activated macrophage.

2:IV) MUCOSAL IMMUNITY

Mucosal surfaces represent the largest area of contact between the immune system and the environment. Microorganisms and their associated products, as well as substances found in ingested food and inhaled air, act as constant sources of foreign

antigen, capable of stimulating the systemic and/or secretory immune responses. Lymphoid tissue accumulation in various sites throughout the body function in the processing and handling of such antigens (Brostoff and Challacombe, 1987b).

The most studied area of mucosal associated lymphoid tissue (MALT) are the Peyers Patches, found in the gut. Each Peyers patch area can be divided into three major regions: (a) the dome, (b) the lymphoid follicle (B-cell zone) and (c) parafollicular area (T-cell zone). A unique epithelium called lymphoepithelium, which consists of cuboidal epithelial cells, a reduced number of goblet cells, and specialized antigen-sampling cells called either follicular-associated epithelial cells (FAE cell) or microfold cells (M cells) covers each Peyers patch. M cells function as antigen-presenting cells, capable of phagocytozing particulate antigens such as viruses and whole bacteria. The uptake of proteins by the M cells, does not result in their lysosomal enzyme degradation, and such immunogenic intact proteins can be transmitted to the underlying lymphoreticular cells in the dome region. The follicles, which lie beneath the dome region, are comprised of one or two germinal centres, which contain B cells committed to IgA production. Unlike other germinal centres located elsewhere in the body, B cell differentiation into plasma cells rarely occurs in this area.

Antigenic stimulation of mucosal associated lymphoid tissue induces a secretory response by synergistic central and local intervention. Gut associated lymphoid tissue (GALT) and bronchial associated lymphoid tissue (BALT), as well as small areas of lymphoid tissues found in association with the oral mucosa are responsible for the release of IgA precursor cells. The primary signal leading to the release of IgA precursors destined for secretory tissues arises in the GALT or BALT area, while a secondary signal arises in the local tissues, leading to the subsequent proliferation and differentiation of these cells into plasma cells. The local response is specific, such that antigen presented in one particular area of the body will lead to the generation of secretory IgA (sIgA) at that particular site only: e.g. antigen placed on one part of the

intestine, will result in a specific sIgA response only in that area of the intestine, without concomitant response in non-immunized areas of the intestine.

Secretory-IgA has been recognized as the main immunoglobulin responsible for mucosal immunity. Its ability to neutralize antigens such as viruses, toxins and enzymes, prevent the uptake of antigens from the intestinal tract and inhibit the adherence of bacteria to epithelial surfaces is extremely beneficial in the prevention of infectious diseases contracted through mucosal surfaces (Brostoff and Challacombe, 1987a).

Secretory IgA differs from serum IgA in structure. Serum IgA is a typical four-polypeptide chain structure consisting of two alpha- and two light chains, while secretory IgA consists contain dimeric, disulphide linked molecules with additional polypeptides: J (joining) chain and a secretory component (SC). The secretory component is produced by epithelial cells found in secretory glandular tissues of mucosal surfaces of the gastrointestinal and respiratory tracts. Stabilization of the quaternary structure of sIgA and increased resistance to various proteolytic enzymes is evident after the binding of the secretory component to one of the monomeric subunits of the dimeric IgA.

Antigen-stimulated B cells derived from the GALT and BALT areas, enter the regional lymph nodes and the general circulation via the thoracic duct. These precursor cells then home to the lamina propria of intestinal and respiratory tracts, salivary, lacrimal, mammary and cervical uterine glands, where they differentiate into IgA plasma producing cells, producing secretory antibodies specific for the ingested or inhaled antigen.

A significant number of mature T cells, most of which are T helper (Th) cells or T suppressor (Ts) cells, are found in the MALT regions. The oral administration of antigen, processed by MALT, results in the simultaneous induction of (a) Ts cells, which migrate to the periphery and mediate oral tolerance, i.e. suppressing systemic immune

responses (IgG and IgM), and (b) IgA-specific Th cells, which direct IgA-specific B cell responses for sIgA synthesis at distant mucosal sites. Three types of T contrasuppressor cells have been identified: (a) Tc inducer cell, (b) Tc transducer cell and (c) Tc effector cell. Their presence in the MALT region may be required for the potentiation of immune responses to orally encountered antigens, since T contrasuppressor cells can abolish oral tolerance by enhancing Th cell activity and preventing suppression of mucosal sIgA function.

Factors such as antigen dose, frequency of immunization, and nature of antigen influence the secretory response elicited. Large doses of ingested antigen are usually necessary to stimulate secretory responses (Michalek et al., 1977). Low doses of antigen create a state of unresponsiveness (oral tolerance) (Michalek et al., 1977; Challacombe, 1983). Single immunizations with antigen produce small detectable sIgA responses, whereas more consistent antibody titres are evident after repeated immunizations (Taubman and Smith, 1977). Live organisms,used as oral vaccines, appear to be more immunogenic than inactivated bacteria in the generation of sIgA (Fubara and Freter, 1973). The requirement for larger doses in induction of secretory immune responses, may imply that there is a need for the organism to adhere to the mucosa in order to become an effective immunogen. This phenomena may also be a reflection of antigen dilution in the environment of the gut. Particulate antigens, whether viable or inactivated, are more effective than soluble antigens in inducing secretory antibodies (Challacombe, 1983). Soluble antigens are more likely to give rise to tolerance.

Secretory immune responses after gut immunization are short lived. Secondary immunization gives antibody production of similar duration, but in slightly higher titres. This suggests that after the migration of antigen-sensitive cells from Peyers patches to secretory tissues, the cells do not continue to secrete antibody in the absence

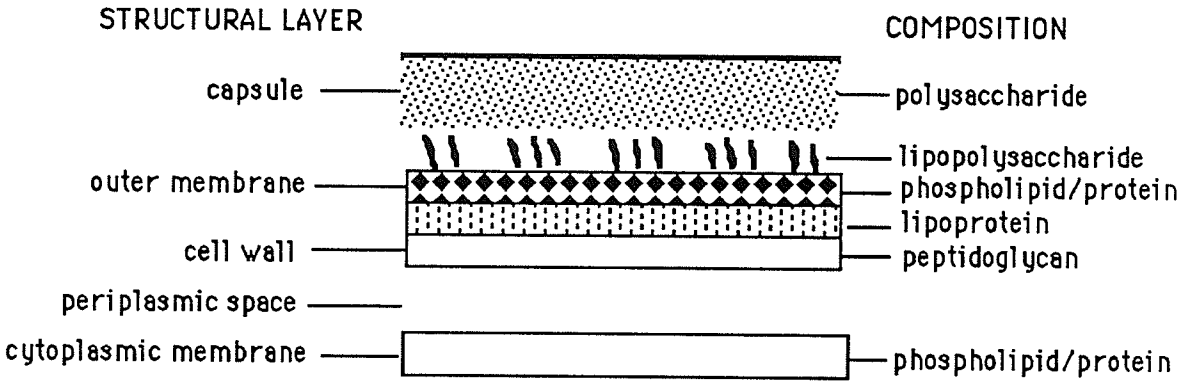
of local or continued antigenic challenge. Amplification of the local antibody response can only be induced if the antigen is once again presented at the site.

1:3) ANTIGENIC STRUCTURE OF GRAM-NEGATIVE MICROORGANISMS

3:1) ANTIGENIC STRUCTURE OF CELL WALL

The ultrastructure of the Gram-negative cell wall is composed of several distinct layers and is more complex than that of Gram-positive organisms. Two distinct layers can be identified outside the cytoplasmic membranes: i.e. a relatively thin peptidoglycan layer similar in chemical composition and physical structure to that present in Gram-positive organisms and a lipopolysaccharide/phospholipid/protein outer membrane, lying external to the peptidoglycan layer. The outer membrane is composed of a typical lipid bilayer, similar to that seen in most biologic membranes. Lipoprotein and protein bridges tightly bind the lipid bilayer to the underlying peptidoglycan backbone (Figure 1.1). The lipopolysaccharide component of the outer membrane is of particular importance, as it is capable of stimulating an antibody response, when injected into animals. Enterobacterial lipopolysaccharide includes a polysaccharide region and a lipid region (termed Lipid A) that are covalently linked. The polysaccharide region component consists of a core, with a basic structure common to all Gram-negative organisms and a variable terminal segment (O antigen) that is exposed on the outer surface of the bacteria, and is a major surface antigen. The O antigen usually determines the immunochemical specificity of the bacterium, while the core polysaccharide consists of a number of hexoses, in addition to 2-keto-3-deoxyoctaonate (KDO) and heptose. Lipid A is a toxic component capable of inducing inflammation in the host.

Figure 1.1 - Diagram of the Gram-Negative Bacterial Cell Envelope



modified from Taussig, 1984

3:II) OUTER MEMBRANE PROTEINS

The protein components of outer membrane of Gram-negative bacteria have been extensively studied by using SDS-polyacrylamide gel electrophoresis (PAGE) to determine their molecular weights by comparison with known molecular weight standard proteins. The outer membranes of Gram-negative bacteria account for approximately 9-12% of the cellular protein, and contain few enzymes, in contrast to the inner cell membrane. Early studies of the outer membranes of *Escherichia coli* demonstrated major outer membrane proteins - proteins which were very prominent in SDS-PAGE profiles. Subsequent studies revealed that some 10 outer membrane proteins comprise approximately 80% of the total outer membrane protein, of which 100,000 copies of each protein per cell could be found (Osborn and Wu, 1980). In addition, minor protein components have been described, which are implicated in such functions as vitamin B12 uptake, iron transport, nucleoside transport, maltose uptake, phage receptors, and blocking and stabilization of mating aggregates, and some to which no function has been ascribed.

Various modifications to the technique of outer membrane preparation have been developed, including the use of ionic and non-ionic detergents. Although these techniques have allowed for better characterization of structural and functional relationships of the outer membrane constituents within the membrane, it is still unknown to what extent these preparations differ from the *in situ* outer membrane, especially in the generation of immune response by the host. Antibodies generated to purified outer membrane proteins contain antibodies reactive against minor protein contaminants, lipopolysaccharides and denatured antigens, indicating that antigenic determinants may be exposed, which are normally not recognized *in vivo* because of their strong association with phospholipids or lipopolysaccharide.

A study by Sears et al. (1984) of human sera in patients experimentally challenged with *Vibrio cholera* showed that they generated antibodies which reacted mainly to protein antigens, not lipopolysaccharides. Antibodies from these subjects recognized outer membrane proteins from heterologous serotypes in addition to those of the homologous (immunizing) strain. This suggests that a part of the membrane protein may represent a common antigenic determinant among serotypes of a species.

The Gram-negative outer membrane also contains porins, which are trimeric proteins arranged in pores extending across the outer membrane. Porins allow the nonspecific diffusion of molecules <800 m.w, in contrast to the cytoplasmic membrane, which excludes all hydrophilic molecules unless there is a specific transport mechanism for the specific substance. The outer membrane of Gram-negative organisms, being much less permeable to hydrophobic or amphipathic molecules, yet permeable to low molecular weight substances, partially accounts for the relatively high antibiotic resistance seen in Gram-negative compared to Gram-positive organisms.

3:III) LIPOPOLYSACCHARIDES

Lipopolysaccharides are a major component of the outer-membrane of Gram-negative cells responsible for the integrity and functional conformation of the intact cell. Electron microscopic studies by Okuda et al. (1985), showed that LPS from *P. gingivalis*, for example exhibited trilaminar and cord-like structures consistent with the outer membrane, and was of a width equal to that of the outer membrane.

Mansheim et al. in 1979 studied the major surface antigens of *Bacteroides melaninogenica*, which were present in the outer membrane and discovered that the lipopolysaccharide present in this organism was chemically distinct from lipopolysaccharide present in other facultative Gram-negative organisms in that it lacked two distinct core sugars: 2-keto-3-deoxyooctonate (KDO) and heptose (almost

universally present in facultative bacteria), as well as β-hydroxymyristic acid - the predominant fatty acid present in the lipid A moiety. Lipid A is responsible for the biologic potency of lipopolysaccharide. The lipopolysaccharide of *P. asaccharolyticus* showed little biologic activity, contrasting with the properties of LPS in other facultative bacteria. The lipopolysaccharides from *Actinobacillus* and *Fusobacterium* species contain small amounts of KDO, and large quantities of heptose and β-hydroxymyristic acid (Hamada et al., 1988).

The biologic activity of lipopolysaccharide from *B. gingivalis* is less than that of enterobacterial LPS, but it can stimulate bone resorption (Sveen and Skaug, 1980). Koga et al. (1985) demonstrated that *P. gingivalis* has the ability to act as a mitogen, a polyclonal B cell activator and also stimulate interleukin 1 production, properties comparable to *E. coli* LPS.

3:IV) CAPSULE

Capsules located on the external surface of the Gram-negative cell have been associated with conferring virulence on the organism, by protecting the cell from phagocytosis by polymorphonuclear neutrophils. Gram-negative capsules are composed primarily of polysaccharide heteropolymers and a small percentage of protein (Luft, 1964; Okuda et al., 1987). The capsule of an organism may be also capable of masking lipopolysaccharide, thus shielding it from activating the complement system. Studies by Okuda et al. (1987) of the structure of capsules of black-pigmented *Bacteroides* from humans showed the presence of capsules, of varying widths in all strains of black-pigmented *Bacteroides*.

3:V) PERIPLASMIC SPACE

Gram-negative bacteria secrete proteins-enzymes into the periplasmic space between the cell wall and the outer membrane. These enzymes are capable of degradation and detoxification, and are similar to those secreted by Gram-positive bacteria into the environment. Therefore the Gram-negative periplasmic space could be considered the bacterial equivalent of eukaryotic lysosomes. The outer membrane functions in preventing leakage of secreted periplasmic proteins and in the case of Gram-negative enteric bacteria - protection from bile salts and hydrolytic enzymes of the host environment.

1:4) MICROBIAL FLORA OF THE ORAL CAVITY

The human body supports a diverse oral microflora. The wide variety of environments in the oral cavity: teeth, mucous membranes, gingival crevice, tongue, and saliva, as well as factors such as: salivary flow and composition, crevicular fluid, inter-species relationships, as well as microbial adherence and metabolism, influence the development of the resident oral flora (Hardie and Bowden, 1974; Gibbons and van Houte, 1975; Socransky, 1977).

Although yeast, protozoa and occasionally viruses have been isolated from the oral cavity, bacteria are by far the most predominant microorganism present in the human mouth. The environment of the oral cavity is so diverse and modified by outside factors, such as diet, hormones, etc. that description of "normal flora" becomes difficult. Sampling of a specific site in the mouth may detect certain organisms, which are unique to that particular site, and not present in other areas of the oral cavity. Similarly,

repeat sampling of a particular site in an individual over a period of time, can produce both qualitative and quantitative differences in the composition of microflora.

Cellular adherence and growth are the two major characteristics necessary for oral colonization by bacteria. In an open system, such as the oral cavity, the organisms must attach to the tissues in order to withstand the cleansing forces of saliva. Oral bacteria vary in their abilities to attach to different oral surfaces, and their adherence properties have been related to their natural intraoral distribution (Gibbons and van Houte, 1973; Gibbons and van Houte, 1975). Discussion of the development of oral flora will be limited to the colonization of the mouth from birth to development of a climax community (a relatively stable state)(Alexander, 1971), and the development of dental plaque with special emphasis on on the oral flora associated with periodontal disease.

4:1) DEVELOPMENT OF THE ORAL FLORA

At birth, the oral cavity of a newborn is sterile, but acquisition of an oral microbiota commences within hours (Socransky and Manganiello, 1971). The first pioneer species colonizing oral tissues are streptococci, in particular *Streptococcus salivarius* (McCarthy et al., 1965). The metabolic processes of this organism alter the environment of the oral cavity, allowing colonization by other microbial species. Among the great variety of microorganisms entering the infant mouth, only selected species become established as part of the resident flora. Contaminating organisms such as fecal lactobacilli from the vagina and rectum of the mother, which are transmitted to the newborn during delivery do not become established in the infant's oral cavity (Carlsson and Gothefors, 1975; Carlsson et al., 1975). In monkeys it has been shown that the oral flora of mothers reflects that of their progeny, indicating that direct contact may also play a role in the colonization of the oral cavity in human children (Delaney, et al.,

1988), while other studies have suggested that individuals harbor clones of species of black-pigmented *Bacteroides* which have DNA fingerprint patterns unique to the individual-host (Loos et al., 1988).

Frisken et al. (1990) examined the oral cavities of children from birth to 2 1/2 years of age for the presence of periodontopathogenic bacteria. Although no such organisms were isolated in the first week after birth, *P. intermedia,* and *P. melaninogenica* were detected within the first month of life. Sixty two percent of the children under 6 months harbored *Eikenella corrodens.* No evidence of colonization by *A. actinomycetemcomitans* was obtained in any of the children studied. Although these organisms were detected in the mouths of children, it is unclear whether they were present as transient or permanent members of the oral flora.

By the first year of life most children possess an oral flora composed of *Streptococcus*, *Staphylococcus*, *Neisseria*, *Veillonella*, and possibly *Actinomyces*, *Lactobacillus* and *Fusobacterium.* The eruption of teeth also brings a change in the environment and available habitats, i.e. anaerobic gingival crevices, and subsequently increases in a number of strictly anaerobic species, such as anaerobic streptococci and motile Gram-negative rods. Organisms, which adhere to dental hard tissues, e.g. *Streptococcus sanguis, Streptococcus mutans* and *Actinomyces viscosus* are detected as teeth erupt. *S. sanguis* attaches to teeth and some mucosal surfaces more effectively than does *S. mutans.* Therefore colonization by *S. sanguis* precedes that of *S. mutans* (Gibbons and van Houte, 1975).

Some microorganisms increase in numbers at the onset of puberty: e.g. black-pigmented *Bacteroides* and spirochaetes. The presence of low numbers of *B. melaninogenicus* and spirochaetes in the gingival crevices of adults suggest that these organisms may be dependent on the presence of such factors as hemin or vitamin K, and the hormones-estradiol and progesterone (Wojcicki et al., 1987). As teeth are lost throughout adult life, the microflora eventually resembles that of the mouth of an infant.

As the microbial community develops in the oral cavity, it becomes more complex, until such time as a stable "climax" community is established (Alexander, 1971) (Table 1.1). A climax community is one which remains relatively stable in its populations and therefore is most commonly seen in such areas as approximal plaque, where the bacterial population is somewhat screened from many of the dramatic physical and physiological changes that can occur in the oral environment.

4:II) DEVELOPMENT OF DENTAL PLAQUE

Plaque can be defined as a tenacious microbial deposit which forms on hard surfaces within the mouth and consists of microbial cells and their products, together with molecules, mainly derived from saliva (MacFarlane and Samaranayake, 1989). Within minutes of mechanical cleaning, the tooth develops an acquired pellicle, composed by the selective adsorption of different salivary glycoproteins, immunoglobulins enzymes, blood-group reactive substances and/or bacterial extracellular polysaccharides (van Houte, 1979). The binding of pellicle to the tooth surface is dependent on electrostatic ionic interaction between calcium ions and phosphate groups in the enamel surface and oppositely charged groups in salivary molecules. The presence of pellicle on teeth is not a pre-requisite to plaque formation, but its rapid rate of formation on a cleaned tooth surface, usually precedes the attachment of bacteria. Studies of the initial phase of plaque formation on cleaned tooth surfaces have shown that bacteria adhere initially in areas sheltered from oral cleansing forces, such as fissures, areas of imperfection-cracks, roughness and near the gingival margin (Lie, 1978).

Table 1.1 - Bacteria Detected at Various Stages of Plaque Development on Cleaned Solid Surfaces in the Mouth of Adults

TIME AFTER CLEANING	SPECIES PRESENT
0-15 min.	*S. sanguis, S. salivarius, A. viscosus,* "corynebacterium"
1-18 hr.	*S. sanguis, S. mitis, S. epidermidis, A.viscosus, Peptococcus sp.*
24-48 hr.	*S. sanguis, S. salivarius, A. viscosus, R. dentocariosa, L. casei, Veillonella sp., Fusobacterium sp., Neisseria sp.*
3-5 days	*S. sanguis, S. salivarius, A. viscosus, A. naeslundii, A. odontolyticus, R. dentocariosa, L. buccalis, E. saburreum, A. israelii, P. melaninogenicus, Neisseria sp., Veillonella sp., Lactobacillus sp.*
6-14 days	At this time, plaque reaches its most complex community

(Bowden et al., 1979)

The adherence of bacteria to the pellicle may be initiated by single cells of bacteria or aggregates of limited size, usually via mucinous glycoproteins or immunoglobulin A (Liljemark et al., 1981). Subsequent accumulation of bacteria occurs by proliferation of the attached cells, as well as by adherence of unattached cells to bacteria already attached to the tooth surface (coaggregation). Bacteria found in plaque, such as *S. mutans, S. salivarius* and *A. viscosus* are known to synthesize many extracellular polysaccharides, which aid in retention and increase plaque mass. An increase in bacterial numbers not only cause plaque to spread laterally over the tooth surface, but also to increase its thickness.

Ultrastructurally, the innermost layer of plaque consists of densely packed predominantly coccoid and rod-shaped bacteria. The body of plaque is composed of many filamentous bacteria, oriented perpendicular to the tooth surface, in addition to coccoid and rod-shaped cells. In subgingival plaque, the surface layer in contact with the gingival tissues contains many motile, Gram-negative rods and spirochaetes (Listgarten et al., 1975).

The composition of plaque is dependent on the conditions under which it develops. Subgingival plaque develops in the presence of gingival crevicular fluid and little or no saliva. The presence of a low oxidation-reduction potential, lack of direct effects of diet, special nutrients in the gingival fluid and different tooth surface, i.e. cementum, is responsible in part for the variation seen between the composition of subgingival and supragingival plaque. A transitional flora consisting of Gram-positive organisms, characteristic of supragingival plaque and Gram-negative organisms predominantly found in subgingival plaque separates the two environmentally diverse populations. In general, a healthy gingival crevice is populated by nonmotile rods and cocci, predominantly Gram-positive species of *Streptococcus* and *Actinomyces* (Slots, 1977).

4:III) ORAL FLORA IN PERIODONTAL DISEASE

The accumulation of microbial plaque on or near the cervical region of teeth and its extension apically along the root surface has been linked to the development of periodontal disease. The flora involved in periodontal disease is exceedingly complex, with more than 325 different taxa identified (Moore, 1987). Although correlations can be drawn between the isolation of certain microorganisms and disease states, all correlations must be considered carefully, and not taken as proof of causal relationships. Organisms isolated may only be indicators of a change in local environmental conditions, which subsequently favor the etiologic agents of the disease.

Due to the complexity of the process leading to periodontal disease, classification of these diseases has changed over the years. The separation into gingival diseases (no associated loss of attachment) and periodontal diseases (loss of attachment and bone resorption) have remained unaltered. The redefinition of subdivisions of periodontitis presently in use is presented in Table 1.2.

4:IV) GINGIVITIS

Supragingival plaque associated with healthy gingival tissues appears to be relatively thin, and consists predominantly of Gram-positive, coccal-shaped microorganisms. Organisms commonly cultured from a "normal" disease-free supragingival flora includes: *Streptococcus sanguis, Streptococcus mitis, Actinomyces viscosus, Actinomyces naeslundii, Rothia dentocariosa* and *Veillonella* species (Listgarten, 1976; Moore LHV, et al., 1987).

Gingivitis is an inflammatory process of the gingiva, in which the junctional epithelium, although altered by the disease, remains attached to the tooth at the original level (Genco, 1990). Gingivitis is associated predominantly with a change from Gram-

Table 1.2 Periodontal Disease Classification

A. Periodontitis in Adults
   1) Acute Adult Periodontitis, Classification I, II, III, IV
   2) Epidemiologic: moderately and rapidly progressing periodontitis
   3) Clinical based on treatment: refractory and recurrent
   4) Clinical based on history: recurrent acute necrotizing ulcerative periodontitis and postlocalized juvenile periodontitis

B. Periodontitis in Juveniles
   1) Localized juvenile periodontitis
   2) Generalized juvenile periodontitis

C. Periodontitis with systemic involvement

D. Miscellaneous conditions affecting the periodontium

(Genco, 1990)

positive streptococcal flora to a more complex flora including Gram-negative and spiral bacteria (Moore LHV et al., 1987). The supragingival flora associated with gingivitis appears to represent an overgrowth of some of the bacteria found in plaques of healthy sites (Listgarten, 1976). A study by L.H.V. Moore et al. in 1987 concluded that no major differences in composition of flora foun in naturally-occurring and experimental gingivitis could be seen from a bacteriologic standpoint. The subgingival flora studied represented a transition between flora associated with health, and that associated with periodontal disease.

In chronic gingivitis, the microflora is comprised of about 45% Gram-negative bacteria and approximately 45% of the recoverable organisms are anaerobic (Socransky et al., 1982). Predominant isolates includes various species of *Actinomyces, Streptococcus, Fusobacterium* and *Bacteroides*, as well as *Eikenella corrodens* and *Capnocytophaga gingivalis* (Loesche et al., 1985; Tanner et al., 1984). On the other hand, acute necrotizing gingivitis has been associated with high proportions of *Prevotella intermedia* and *Treponema* (Loesche et al., 1982).

4:V) PERIODONTITIS

Periodontitis is a condition which affects the periodontal attachment apparatus, composed of the alveolar process, connective tissues and cementum. The disease may occur in young adolescents or otherwise healthy adults. Some forms of periodontitis are very rapidly progressing and require rapid diagnosis for treatment purposes. The disease is often characterized by bursts of activity, occurring randomly throughout the dentition. The presence of bacterial invasion of the subepithelial periodontal tissues in severe forms of periodontitis has been documented (Slots and Genco, 1984).

The microbiota isolated from the gingival sulcus has been related to various periodontal disease states. Many of the organisms can be isolated in health, but in varying proportions of the recoverable flora. Some bacteria have been associated with certain periodontal diseases, and these are summarized in Table 1.3.

Advanced adult periodontitis lesions are characterized by a cultivable subgingival flora comprised of approximately 75% Gram-negative and 90% anaerobic organisms (Slots, 1977). The disease process is characterized by development of periodontal pockets and attachment loss apical to the cemento-enamel junction. This loss of attachment and pocketing may be present on any surface of single-rooted or multi-rooted teeth. As the disease progresses, advanced stages may exhibit tooth mobility. The types of Gram-negative organisms isolated appear to vary from individual to individual, and site to site. Isolates such as *Porphyromonas gingivalis, Prevotella intermedia, Actinobacillus actinomycetemcomitans* and various species of *Fusobacterium, Wolinella* and non-pigmenting *Bacteroides* have been associated with adult periodontitis.

Localized juvenile periodontitis occurs primarily in teenagers and young adults. The disease is clinically characterized by pocket formation, loss of connective tissue attachment and alveolar bone loss affecting mainly first molars and incisors, in the presence of minimal plaque accumulation. A strong correlation has been made between the presence of *Actinobacillus actinomycetemcomitans* - a large Gram-negative coccobacillus, and the lesions of localized juvenile periodontitis (Moore et al., 1985). The organism has been recovered with greater frequency and in higher numbers from lesions of juvenile periodontitis than from healthy sites in the same individual or from lesions of gingivitis or periodontitis (Slots et al., 1980). An association of high levels of *P. gingivalis* together with *A. actinomycetemcomitans* may be required for generalized advanced periodontal destruction in young adults (Zambon et al., 1984).

Table 1.3 Subgingival Microorganisms Associated with Periodontal Disease

DISEASE	MICROORGANISM
Acute necrotizing ulcerative gingivitis	*Prevotella intermedia*
Adult periodontitis	Intermediate-sized spirochetes *Actinobacillus actinomycetemcomitans* *Prevotella intermedia* *Porphyromonas gingivalis* *Bacteroides forsythus* *Capnocytophaga gingivalis* *Eikenella corrodens* *Eubacterium* species *Fusobacterium nucleatum* *Propionibacterium acnes* *Streptococcus intermedius* *Wolinella recta*
Localized juvenile periodontitis	*Actinobacillus actinomycetemcomitans* *Bacteroides* species
Generalized juvenile periodontitis	*Actinobacillus actinomycetemcomitans* *Porphyromonas gingivalis* *Prevotella intermedia* *Capnocytophaga* *Eikenella corrodens* *Neisseria*
Periodontal abscess	Gram-negative anaerobic rods *Porphyromonas gingivalis* *Fusobacterium* species *Capnocytophaga* *Vibrio* species
Periodontitis associated with insulin-dependent diabetes mellitus	*Actinobacillus actinomycetemcomitans* Anaerobic *vibrios* *Campylobacter* *Capnocytophaga*
Periodontitis associated with non-insulin-dependent diabetes mellitus	*Porphyromonas gingivalis* *Fusobacterium* species *Wolinella recta*
Pregnancy gingivitis	*Prevotella intermedia*
Refractory or recurrent periodontitis	*Actinobacillus actinomycetemcomitans* *Bacteroides forsythus* *Porphyromonas gingivalis* *Prevotella intermedia* *Wolinella recta*

(Zambon, 1990)

The lesions of treated and untreated progressively destructive periodontal lesions have shown a significant correlation with the presence of *A. actinomycetemcomitans, P. gingivalis and P. intermedia*, either alone or in combination (Slots, 1982).

1:5) CLASSIFICATION OF *BACTEROIDES*

The 1st edition of Bergey's Manual of Systematic Bacteriology (1984) places the genus *Bacteroides* in the family of *Bacteroidaceae.* This family is described as a group of "obligately, anaerobic, Gram-negative, nonsporeforming, straight, curved or helical rods, that are either motile or non-motile; chemoorganotrophic; metabolizing carbohydrates, peptones or metabolic intermediates" (Holdeman et al., 1984), which in addition to *Bacteroides* includes *Fusobacterium, Leptotrichia, Butyrivibrio, Wolinella, Selenomonas* and other genera.

Castellani and Chalmers in 1919 first described *Bacteroides* as Gram-negative, obligately anaerobic nonsporeforming rods, which were either motile with peritrichous flagella or nonmotile. Since 1919, many more species of *Bacteroides* have been isolated, and in the 1st edition of Bergey's Manual of Systematic Bacteriology (Holdeman et al., 1984), 39 species are included in this genus (Table 1.4). The type species for the genus *Bacteroides* is *Bacteroides fragilis* (Veillon and Zuber, 1898; Castellani and Chalmers, 1919). The genus *Bacteroides* as listed in the 1st edition of Bergey's Manual of Systematic Bacteriology is characterized as having chemoorganotrophic metabolism, with the ability to metabolize carbohydrates, peptones or metabolic intermediates, and produce succinate, acetate, lactate, formate and propionate, as end products of metabolism.

As will be described later in this section, the genus *Bacteroides* is phenotypically a very diverse group which possesses a broad range of DNA base ratios mol%G+C from

TABLE 1.4 Species of the Genus *Bacteroides* Listed in the 1st Edition of Bergey's Manual of Systematic Bacteriology (Holdeman et al., 1984)

Name of organism	Isolators of organism	Date of first description
B. amylophilus	Hungate and Hungate	1956
B. asaccharolyticus	Finegold and Barnes	1977
B. bivius	Holdeman and Johnson	1977
B. buccae	Holdeman et al.	1982
B. capillosus	Kelly	1957
B. coagulans	Eggerth and Gagnon	1933
B. corporis	Johnson and Holdeman	1983
B. denticola	Shah and Collins	1982
B. diastonis	Eggerth and Gagnon	1933
B. disiens	Holdeman and Johnson	1977
B. eggerthii	Holdeman and Moore	1974
B. fragilis	Castellani and Chalmers	1919
B. furcosus	Hauduroy et al.	1937
B. gingivalis	Coykendall et al.	1980
B. gracilis	Tanner et al.	1981
B. hypermegas	Harrison and Hansen	1963
B. intermedius	Johnson and Holdeman	1983
B. levii	Johnson and Holdeman	1983
B. loeschii	Holdeman and Johnson	1982
B. macacae	Coykendall et al.	1980
B. melaninogenicus	Roy and Kelly	1939
B. microfusus	Kaneuchi and Mitsuoka	1978
B. multiacidus	Mitsuoka et al.	1974
B. nodosus	Mraz	1963
B. oralis	Loesche et al.	1964
B. oris	Holdeman et al.	1982
B. ovatus	Eggerth and Gagnon	1933
B. praeacutus	Holdeman and Moore	1970
B. pneumosintes	Holdeman and Moore	1970
B. putredinis	Kelly	1957
B. ruminocola	Bryant et al.	1958
B.splanchnicus	Werner et al.	1975
B.succinogenes	Hungate	1950
B. thetaiotaomicron	Castellani and Chalmers	1919
B. termiditis	Holdeman and Moore	1970
B. uniformis	Eggerth and Gagnon	1933
B. ureolyticus	Jackson and Goodman	1978
B. vulgatus	Eggerth and Gagnon	1933
B. zoogleformans	Cato et al.	1982

28 to 61. It is now generally accepted among taxonomists, that a difference of >10% indicates species, which are unrelated at the generic level.

Although Bergey's Manual subdivides *Bacteroides* into species on the basis of production of acid from lactose, many common classification methods divide *Bacteroides* species on the basis of: (a) the presence or absence of pigment production by colonies grown on medium containing blood and by (b) the ability of the organisms to ferment glucose. The subdivision into black-pigmented *Bacteroides* and non-pigmented *Bacteroides* is not an approved designation, but this terminology is commonly used among bacteriologists, when referring to this genus.

The natural habitat of most *Bacteroides* species appears to be mucous membranes of humans, other animals and insects. Diseases of the colon, oral cavity, upper respiratory tract and female genital tract have been associated with various species of this genus. Most of the discussion of taxonomy of this genus will be limited to *Bacteroides* strains of human origin (Table 1.5), although occasional reference will be made to non-human species of *Bacteroides,* when this will aid in explanation of the subdivision of the genus/species.

5:1) BLACK-PIGMENTED *BACTEROIDES*

In 1921, Oliver and Wherry (Oliver and Wherry, 1921) first identified a small, pigmented, anaerobic Gram-negative rod from a variety of sites including the oral cavity, urine, human feces, respiratory tract, as well as post-surgical infections. Since this organism produced black colonies when grown on blood agar plates, and the pigment was considered to be melanin, they named the rod *Bacterium melaninogenicum.*

The bacterium was subsequently described in the third edition of Bergey's Manual of Determinative Bacteriology (Bergey et al., 1930) as *Haemophilus melaninogenicus,*

TABLE 1.5 *Bacteroides* Species Isolated from Human Sources

BLACK PIGMENTED *BACTEROIDES*		NONPIGMENTED *BACTEROIDES*	
Fermentative	Nonfermentative	Fermentative	Nonfermentative
P. denticola [a]	*P asaccharolyticus* [b]	*P. bivius* [a]	*B. coagulans*
P. intermedia [a]	*P. endodontalis* [b]	*P. buccae* [a]	*B. forsythus*
P. melaninogenica [a]	*P. gingivalis* [b]	*B. disiens*	*W. gracilis* [e]
P loescheii [a]		*B.capillosus*	*B. pneumosintes*
P. corporis [a]		*B. diastonis*	*T. praeacutus* [f]
		B. eggerthii	*B. ureolyticus*
		B. fragilis	
		M. hypermegas [c]	
		M. multiacidus [d]	
		P oralis	
		P. oris	
		B. ovatus	
		B. splanchnicus	
		B. thetaiotaomicron	
		B. uniformis	
		B. vulgatus	
		B. zoogleoformans	

(Kornman, 1988)

[a] - Previously *Bacteroides*, reclassified as *Prevotella*
[b] - Previously *Bacteroides*, reclassified as *Porphyromonas*
[c] - Previously *Bacteroides*, reclassified as *Megamonas*
[d] - Previously *Bacteroides*, reclassified as *Mitsuokella*
[e] - Previously *Bacteroides*, reclassified as *Wolinella*
[f] - Previously *Bacteroides*, reclassified as *Tissierella*

Current reclassification, see section 4:III

because better growth was obtained on a solid medium containing X and V growth factors, characteristic of the members of the genus *Haemophilus.* In 1938, Prevot concluded that the genus *Bacteroides* was invalid and proposed that *Haemophilus melaninogenicus* be changed to *Ristella melaninogenica.* This proposal was however, not accepted in the fifth edition of Bergey's Manual, and the name *Bacteroides melaninogenicus* was retained (Roy and Kelly, 1939).

In 1947, Schwabacher et al. proposed yet another name for *B. melaninogenicum,* based on its ability to produce pigment. Since the organism was classified by Wilson and Miles (1945) in the group *Fusiformis*, Schwabacher et al. suggested the name *Fusiformis nigrescens.* In spite of the proposals for a name change, the 7th edition of Bergey's Manual of Determinative Bacteriology retained the name *Bacteroides melaninogenicus* as the proper description of the organism (Kelly, 1957).

In spite of the work of Sawyer et al. in 1962, and Courant and Gibbons in 1967 which demonstrated biochemical and immunological heterogeneity among strains of *Bacteroides melaninogenicus,* this name was retained till 1970.

In 1970, Holdeman and Moore, recognized that this group of black-pigmenting organisms was quite diverse in regard to their ability to ferment glucose. A proposal was made to further subdivide *B. melaninogenicus* into subspecies, i.e. *B. melaninogenicus,* subspecies *asaccharolyticus* - organisms which were non-fermentors, *B. melaninogenicus,* subspecies *intermedius* - weak or intermediate fermenting organisms, and *B. melaninogenicus,* subspecies *melaninogenicus* - organisms that were strong fermentors (Holdeman and Moore, 1970). In view of the significant differences in DNA G+C% content between these subspecies, they were then raised to the species level as *B. melaninogenicus, B. intermedius*, and *B. asaccharolyticus* (Finegold and Barnes, 1977).

The heterogeneity demonstrated between the oral and non-oral asaccharolytic strains (Shah et al., 1976; van Steenbergen et al.,1979) led Coykendall (Coykendall et al., 1980) to propose the name *B. gingivalis* to include asaccharolytic species isolated from the oral cavity. The non-oral asaccharolytic *Bacteroides* species retained the name *Bacteroides asaccharolyticus.* Further studies of the electrophoretic mobility of malate dehydrogenase (Shah and Williams,1982), protein (Strom, et al., 1976; Swindelhurst et al., 1977), cellular fatty acid profiles (Miyagawa, et al., 1979; Shah and Collins, 1980) composition of mucopeptide (Shah, et al.,1976; Williams, et al., 1975), and DNA hybridization studies (Coykendall et al., 1980) have substantiated this division between oral and non-oral asaccharolytic *Bacteroides.*

Studies by Reed et al. in 1980, showed that oral strains of asaccharolytic black-pigmented *Bacteroides* do not share major antigens with the nonoral *B. asaccharolyticus* or with either *B. melaninogenicus,* subsp. *intermedius,* or *B. melaninogenicus* subsp. *melaninogenicus*, suggesting that the oral asaccharolytic strains represented an antigenically distinct group of microorganisms. In 1984, van Steenbergen et al. re-examined some original isolates of asaccharolytic black-pigmented *Bacteroides* from infected tooth pulps by Sundqvist (Sundqvist, 1976). DNA homology revealed little in common, with either the authentic *B. asaccharolyticus* or *B. gingivalis,* therefore a new species: *Bacteroides endodontalis* was proposed (van Steenbergen et al., 1984). *B. endodontalis* could be differentiated from the non-oral strain of *B. asaccharolyticus,* by a lower DNA G+C content and the absence of common cell wall proteins, as differentiated by polyacrylamide gel electrophoresis.

Asaccharolytic black-pigmented *Bacteroides* have been isolated from other mammals, such as beagle dogs (Syed, 1980), and cats (Love et al., 1984). Laliberte and Mayrand (1983) found that all the oral asaccharolytic strains of black-pigmented *Bacteroides* isolated from dogs, differed from their human counterpart *B. gingivalis* in being catalase positive. At least 4 genotypes of asaccharolytic black-pigmented

Bacteroides from the mouths of dogs (Kaczmarek and Coykendall, 1980) and 5 phenotypes from soft-tissue infections of cats (Love et al., 1984) have been recognized.

Parent et al. in 1986 examined human and animal biotypes of *B. gingivalis* and compared antigens from three human strains of *B. gingivalis* with those of four animal strains isolated by Laliberte and Mayrand (1983) using crossed immunoelectrophoresis revealing:

(a) antigenic relatedness between the two groups, i.e. 4 cross-reacting antigens shared by both human and animal biotypes and

(b) serological specificity, i.e. two serotypes were evident among the human and animal strains

Two antigens specific to the human strains and two antigens specific to the animal strains were identified, indicating that serotype-specific antigens distinguished each biotype. Serotype I of *B. gingivalis* included the catalase-negative human biotype, while serotype 2 included the catalase-positive animal biotype.

The saccharolytic black-pigmenting *Bacteroides* spp. now include the following: *B. melaninogenicus*; *Bacteroides loescheii*, formerly grouped with *B. melaninogenicus* (Holdeman and Johnson, 1982); *Bacteroides denticola*, formerly a DNA homology group within *B. melaninogenicus* (Shah and Collins, 1981), (Holdeman and Johnson, 1982); *Bacteroides corporis*, formerly a DNA homology group and serogroup within *B. intermedius* (Johnson and Holdeman, 1983) and *B. intermedius* which contains two DNA homology subgroups (Johnson and Holdeman, 1983).

5:II) NON-PIGMENTED *BACTEROIDES*

Presently, there are over 30 species of non-pigmenting *Bacteroides*, which are commonly differentiated by the production of indole from tryptophan, acid production from sugars, and growth in the presence of 20 percent bile. Human isolates of non-

pigmented fermentative *Bacteroides* can be divided into species commonly isolated from the intestinal tract, oral cavity and urogenital tract (Table 1.6).

Intestinal, oral and urogenital fermentative *Bacteroides* species can be readily differentiated from each other by their ability to grow in the presence of 20% bile, hydrolyze esculin and produce indole from tryptophan (Table 1.7).

Non-pigmented, non-fermentative *Bacteroides* are composed of a small number of organisms: *B. coagulans, B. forsythus, B. gracilis, B. pneumosintes, B. praeacutus* and *B. ureolyticus.* This group is difficult to identify, because they are difficult to grow. Some of the organisms, such as *B. ureolyticus* and *B. gracilis* require hydrogen and fumarate for growth. These organisms are often difficult to distinguish from *Campylobacter concisus* and *Wolinella recta.* Table 1.8 lists commonly used chemical tests used in the identification of these organisms.

5:III) RECENT CHANGES IN THE TAXONOMY OF *BACTEROIDES*

Since the publication of the 1st edition of Bergey's Manual of Systematic Bacteriology (1984), several changes in the taxonomy of the genus of *Bacteroides* have occurred. Many of the initial classifications were based on phenotypic characteristics. With the development of techniques to study the chemical characteristics and genetics of these organisms, better delineation of this genus has now been achieved.

As mentioned previously, the genus *Bacteroides* as described in the Ist edition of Bergey's manual of Systematic Bacteriology includes a phenotypically diverse group of obligately anaerobic Gram-negative non-sporeforming rods, which possess a DNA base composition range of 28-61 G+C%, although it is generally accepted that a difference of more than 5% G+C indicates that two organisms belong to separate species, while a difference of >10% indicates species that are unrelated at the genus level (Austin and Priest, 1986). Due to this wide range in DNA homology, the genus exhibits a variety of

TABLE 1.6 Non-Pigmented Fermentative *Bacteroides* Species from Humans

INTESTINAL	ORAL	UROGENITAL
B. fragilis	*P. oris*	*P. bivius*
B. vulgatus	*P. buccae*	*B. disiens*
B. diastonis	*P. oralis*	
B. multiacidus	*B. zoogleoformans*	
B. ovatus	*M. hypermegas*	
B. thetaiotaomicron		
B. uniformis		
B. eggerthii		
B. splanchincus		
B. capillosus		
M. hypergmegas		

(Kornman, 1988)

TABLE 1.7 Differentiation of Non-Pigmented Fermentative Human Bacteroides Species

	Intestinal	Oral	Urogenital
Growth in 20% bile	+	-	-
Esculin hydrolysis	+	+	-
Indole production	variable	-	-

(adapted from Kornman, 1988)

TABLE 1.8 Differentiation of Non-Pigmented Non-Fermentative *Bacteroides* Species

	Indole production	Nitrate reduction	Urease production	Fumarate and H_2O_2 requirement
B. coagulans	+	-	-	
B. forsythus	-		-	
B. gracilis	-	+	-	+
B. pneumosintes	-	-	-	
B. praeacutus	-	+	-	
B. ureolyticus	-	+	+	+

(Kornman, 1988)

cellular morphologies and is biochemically and physiologically heterogenous (Shah and Collins, 1985). The type species of *Bacteroides*: *Bacteroides fragilis* has a mol%G+C content of 41-44. Other members of the *Bacteroides fragilis* group (*B. diastonis, B. eggerthii, B. ovatus, B. thetaiotaomicron, B. uniformis and B. vulgatus*) form a well defined group with a mol%G+C range of 40-48. Three species: *Bacteroides capillosus*, *Bacteroides microfusus* and *Bacteroides multiacidus* possess relatively high DNA G+C ratios (56-61), while others: *B. praeacutus, B. hypermegas, B. termiditis, B. coagulans, B. furcosus and B. ureolyticus* have very low G+C ratios (28-37%). *Bacteroides multiacidus* and *Bacteroides microfusus* have now been reclassified into two new genera as *Mitsuokella multiacidus* (Shah and Collins, 1982a; Williams and Shah, 1980) and *Rikenella microfusus* (Collins et al., 1985). Enzymatic studies (Shah and Williams, 1982) revealed that *B. multiciadus and B. microfusus* contain only malate dehydrogenase, while *B. hypermegas* contained only traces of NAD-dependent glutamate dehydrogenase. *Bacteroides furcosus* and *B. praeacutus* are particularly distinctive, lacking both malate dehydrogenase and glutamate dehydrogenase. Based on the diversity of G+C ranges and variations in enzyme activity, the following changes to genus have been made: *B. furcosus* to *Anerorhabdus furcosus* (Shah and Collins, 1986), *B. hypermegas* to *Megamonas hypermegas* (Shah and Collins, 1982b), *B. praeacutus* to *Tissierella praeacutus* (Collins and Shah, 1986a) and *B. termiditis* to *Sebaldella* (Collins and Shah, 1986b).

Studies of the fatty acids of *Bacteroides* species have demonstrated that the genus can be divided into two groups: (a) taxa which contain major amounts of *iso*- and *anteiso*-methyl branched chain acids in addition to straight chain saturated acids and (b) taxa which contain predominantly straight-chain saturated and monosaturated fatty acids (Migayawa et al., 1979). Most of the *Bacteroides* species contain branched fatty acid chains, while *B. succinogenes* almost exclusively contains straight-chain fatty acids.

Based on this absence of branched fatty acids, *B. succinogenes* has now been classified in a new genus *Fibrobacter* (Montgomery et al., 1988).

B. amylophilus has been reclassified into a new genus *Ruminobacter* (Stackenbrandt and Hippe, 1986), in spite of its similar DNA base composition (mol%G+C 40-42) to the *B. fragilis* type strain. This separation was based on the absence of sphingolipids and a low level of *iso/anteiso*-methyl branched fatty acids. *B. amylophilus*, a rumen species, is unrelated to *B. fragilis* and 16S-rRNA oligonucleotide sequencing (Paster et al., 1985) has shown that this species belongs to the purple photosynthetic bacteria.

The *B. oralis-B. melaninogenicus* group (Table 1.9) contains many of the species of *Bacteroides* most commonly isolated from the oral cavity. As their G+C ratio is 40 to 50 mol per cent, they differ from the newly defined Bacteroides group, and have been reclassified into the genus *Prevotella* (Shah and Collins, 1990). The pigmented, asaccharolytic species of *B. asaccharolyticus*, *B. endodontalis* and *B. gingivalis* are reclassified as genus *Porphyromonas* (Shah and Collins, 1988) based on their production of large quantities of protohaem on blood agar plates and predominance of isomethyl branched long chain fatty acids.

Based on the availability of data of the phenotypic, genetic and chemical composition of *Bacteroides* species, Hardie supported the proposal by Shah and Collins (Shah and Collins, 1989), that suggested that the genus *Bacteroides* be limited to species which possess the following features (Hardie, 1989):

(a) obligate anaerobic, Gram-negative, non-sporeforming rods,

(b) production of major amounts of acetic and succinic acids from glucose, plus smaller amounts of other acids,

(c) contain malate dehydrogenase, glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase,

(d) Have a DNA base composition of within the approximate range of 40-48% G+C,

TABLE 1.9 PROPOSED TAXONOMIC REVISION TO THE GENUS *Bacteroides*

SPECIES	TAXONOMIC STATUS	REFERENCE
B. fragilis group: B. fragilis, B. caccae, B. diastonis, B. eggerthii, B. merdae, B. ovatus, B. stercoris, B. thetaiotaomicron, B. uniformis, B. vulgatus,	genus *Bacteroides*	Shah and Collins, 1989
B. oralis/ melaninogenicus group: *B. bivius, B. buccae, B. buccalis, B. corporis, B. denticola, B. intermedius, B. disiens, B. loescheii, B. oralis, B. oulorum, B. oris, B. ruminicola, B. veroralis, B. heparinolyticus, B zoogleoformans, B. melaninogenicus,*	*Prevotella* *P. intermedia* *P. melaninogenica*	Shah and Collins, 1990
Pigmented asaccharolytic group: *B. gingivalis, B. endodontalis, and B. asaccharolyticus*	*Porphyromonas*	Shah and Collins,1988
B. amylophilus	*Ruminobacter*	Stackenbrandt and Hippe,1986
B. capillosus	new genus	Collins and Shah, 1987
B. coagulans.	new genus	Collins and Shah, 1987
B. furcosus	*Anaerorhabdus*	Shah and Collins, 1986
B. gracilis	*Wolinella*	
B. hypermegas	*Megamonas*	Shah and Collins,1982
B. levii	uncertain(possibly related to BPB-asacch group)	
B. macacae	uncertain (possibly related to BPB-assach group)	
B. microfusus	*Rikenella*	Collins et al., 1985
B. multiacidus	*Mitsuokella*	Shah and Collins, 1982
B. nodosus	uncertain (not *Bacteroides)*	
B. praeacutus	*Tissierella*	Collins and Shah, 1986
B. putredinis	uncertain (possibly related to *B. macacae* and BPB-asacch group)	
B. polypragmatus	uncertain (not *Bacteroides*)	
B. pneumosintes	uncertain (not *Bacteroides*)	
B. splanchnicus	uncertain (not *Bacteroides*)	
B. succinogenes	*Fibrobacter*	Montgomery et al., 1988
B. termiditis	*Sebaldella*	Collins and Shah, 1986
B. ureolyticus	*Campylobacter* or *Wolinella*	

(Shah and Collins, 1989; Collins and Shah, 1987)

(e) possess sphingolipids,

(f) possess predominantly straight-chain saturated, *anteiso*-methyl branched and *iso*-methyl branched long-chain fatty acids, and

(g) contain menaquinones (major components MK-10, MK-11) as sole respiratory quinones.

As illustrated in Table 1.9, the only members which would be included into the genus *Bacteroides* using the above criteria, are the *Bacteroides fragilis* group. Members of the *Bacteroides melaninogenicus-Bacteroides oralis* groups and many other *Bacteroides species* are of uncertain generic position and will require further taxonomic studies (Shah and Collins, 1989; Collins and Shah, 1987).

5:IV) SEROLOGICAL ANTIGENIC STUDIES

Antigenic subgroups within *Bacteroides intermedius* (*P. intermedia*) have been recognized by Mouton et al. in immunofluorescence studies, and by Gmur et al. in enzyme-linked immunosorbent assays (Mouton et al., 1981; Gmur and Guggenheim, 1983; Gmur and Wyss, 1985). Although two DNA homology groups I and II have been identified in the *P.intermedia* group, they have not yet been raised to species level due to the lack of readily available distinguishing phenotypic traits. In 1988, Nakazawa et al. demonstrated the presence of three serogroups within *P. intermedia*: A, B, and C (Nakazawa et al., 1988). Strains categorized as belonging to DNA homology group I (*P. int.* I) in the above study, were members of either serogroup A or C, while those categorized as being DNA homology II (*P. int.* II) were members of serogroup B. Data from Nakazawa et al. (1988) and previous studies support the division of *P. intermedia* by DNA homology.

Enzyme-linked immunosorbent assays have also been used to study the antigenic differences among *Bacteroides.* A study by Ebersole et al. in 1988 using formalinized whole cells, both as antigen in the ELISA test and for antiserum production demonstrated 30-40% cross-reactivity between the 2 homology groups of *P. intermedia,* and a 20-25% similarity of surface antigens between *P melaninogenica* and *P. loescheii. P. denticola* was antigenically similar to *P. intermedia* and *P. melaninogenica* by 10-15%. The remaining *Bacteroides* tested (*P. gingivalis, P. oralis, B. levii, W. gracilis, P buccae, P. corporis)* showed less than 5% similarity to other *Bacteroides* species.

Bowden and Nolette in 1990 compared the profiles of antigens from whole cells developed by Western blotting of saccharolytic oral-pigmented strains of *Bacteroides.* Blot profiles of the various species studied were developed with rabbit antisera, each prepared against one of the strains used. Visual comparison, and densitometric analysis of these blots showed extensive cross-reactivity among *P. intermedia, P. melaninogenica*, *P. denticola*, and *P. corporis*, with less cross-reactivity to *P. gingivalis*, *P. asaccharolyticus* and *B. buccae.* Similar to the findings of Ebersole et al. in 1988, this study found approximately 50% similarity between the two homology groups of *P. intermedia*, and 70% similarity between the two different strains of *P. melaninogenica.* A higher degree of similarity was, however, noted by Bowden and Nolette between *P. intermedia* and *P. melaninogenica* (62%). Antisera absorbed with one of the cross-reacting species was then used for development of further immunoblot profiles. Absorption of the antisera with one cross-reacting strain removed some of the reactions to the homologous strain, and in many cases totally removed or significantly reduced the reactions to extracts of other cross-reacting species. This suggests that a range of antigens exist which are common to these pigmented bacteria. This phenomenon was seen even with strains which showed low antigenic similarity from analysis of whole cell profiles, where relatively few antigens would be expected to be common to both species. The explanation provided by the authors for development of few bands after

absorption despite low similarities between strains on pattern analysis, was that antigens of different molecular weights may share common epitopes, and therefore absorption of the antiserum may remove antibodies which recognize antigens that occur at different points in the profiles of different species.

1:6) BACTEROIDES VIRULENCE FACTORS AND PATHOGENICTY

6:1) PATHOGENICITY OF *BACTEROIDES* SPECIES

A pathogenic organism is defined as one capable of producing disease in a susceptible host. With very few exceptions, species of the genus *Bacteroides* in pure culture, do not produce significant disease in animals or humans. However, the presence of *Bacteroides* in mixed bacterial infections, seems to augment the disease process. Experiments by MacDonald and Gibbons, showed that subcutaneous infections in rabbits could be produced by a combination of a black-pigmented *Bacteroides* together with one or more other organisms (MacDonald and Gibbons, 1962). In 1965, Socransky and Gibbons showed that mixtures of either oral or intestinal bacteria, which did not include *B. melaninogenicus* (*P. melaninogenica*) were unable to produce transmissible disease upon subcutaneous inoculation of groins in guinea pigs. The subsequent addition of a pure culture of *P. melaninogenica* to the mixture of organisms restored infectivity.

Brook et al. in 1984, confirmed that a possible synergistic relationship existed between *Bacteroides* species and most anaerobic Gram-positive cocci, as well as between most anaerobic Gram-positive cocci, *P. aeruginosa* and *S. aureus*. Injection of mice with aerobic or anaerobic Gram-positive cocci together with *Bacteroides*, significantly increased the mortality among the animals. The accompanying microbiota in these mixed infections was therefore thought essential to the produce growth factors for *Bacteroides* species.

Although the synergistic mechanisms of infectivity with *Bacteroides*-associated mixed infections are not fully understood, some relationships have been identified. Naphthoquinone, a vitamin K-related compound enhances the growth of *Bacteroides* (MacDonald et al., 1963). Mayrand and McBride showed that non-virulent *P. gingivalis* strains became infective when injected into guinea pigs together with a hemin-agar mixture. The agar appeared to retard the rapid diffusion of hemin from the injected area (Mayrand and McBride, 1980). Substitution of hemin by succinate, an end-product of glucose metabolism by such organisms as: *Klebsiella pneumoniae*, *Actinomyces viscosus* and *Escherichia coli*, fulfilled the hemin requirement of *B. asaccharolyticus* (*P. gingivalis*)(Mayrand and McBride, 1980). Pathogenic *B. gingivalis* (*P. gingivalis)* strains grown on a hemin-free medium produced progeny, which were avirulent when injected subcutaneously in mice (McKee et al., 1986).

Bacteria capable of causing disease are considered virulent, that is, they have the capacity of overcoming host defence mechanisms. Virulence is actually a product of many interacting variables, involving both the microorganism and host. A virulence factor is one property or characteristic of a pathogenic microorganism that permits disease production by the organism. *Bacteroides* species, although considered relatively non-virulent, when in pure culture, possess several characteristics capable of producing varying degrees of host destruction, when present in mixed infections. Some of the characteristics which impart virulence to the microorganism may be antigenic, and initiate a host response. With the increased technology available for studying antibody and cell-mediated responses, a better understanding of the pathogenic properties of these microorganisms will result.

6:II) VIRULENCE ASSOCIATED CHARACTERISTICS

a) Adherence

The production of disease in a host, depends on the colonization/adherence and subsequent survival of a microorganism within its host. Surface structures of most Gram-negative bacteria (including *Bacteroides* species) such as: external appendages, capsular material and endotoxic lipopolysaccharide mediate such attachment. External appendages identified in *Bacteroides* species include fimbriae or pili, and adhesins

Fimbriae or pili, the proteinaceous, polymerized, nonflagellar appendages that project from the surface of microorganisms, have been recognized in various species of *Bacteroides*. *Bacteroides* species possess a range of pili, which differ morphologically, functionally, and chemically, as evidenced by electron microscopy (Yamamoto et al., 1982), hemagglutination studies (Mayrand et al., 1980), epithelial attachment studies (Okuda et al., 1981) and immunological studies (Okuda and Takazoe, 1978).

Agglutination of erythrocytes has been associated with the presence of fimbriae-like structures on bacterial surfaces. Okuda and Takazoe demonstrated the ability of oral strains of *Bacteroides* to agglutinate human erythrocytes as well as erythrocytes from some animals (Okuda et al., 1974). In 1978, Slots and Gibbons demonstrated the presence of fimbriae on *Bacteroides asaccharolyticus* (*P. gingivalis*), and associated their presence with a possible role in the colonization of periodontal pockets. It is now believed that fimbriae may play a role in the attachment of *Bacteroides* to the crevicular epithelium of the periodontal pocket, saliva-coated hydroxyapatite, (Okuda et al., 1981), certain Gram-positive bacteria (Slots and Gibbons, 1978), and red blood cells (Okuda and Takazoe, 1974). The binding to saliva-coated hydroxyapatite and epithelium is inhibited by serum and crevicular fluid, therefore the incorporation of certain

Bacteroides species, namely *P. gingivalis* into dental plaque may be dependent on its binding ability to Gram-positive organisms (Slots and Gibbons, 1978).

Okuda et al. reported that although all strains of *B. gingivalis, B. asaccharolyticus* and *B. melaninogenicus* possessed fimbriae-like structures, these species differed in their haemagglutinating capabilities (Okuda et al., 1981). Different kinds of pili or a pilus with several functional attachment sites may be present on cells of a given *Bacteroides* species, as the attachment of *P. gingivalis* to some surfaces was inhibited by serum or crevicular fluid, while attachment remained intact to other surfaces (Slots and Gibbons, 1978). Findings by Yoshimura et al. (1984) showed that purified fimbriae did not exhibit either hemagglutination activity or hemagglutination inhibition. In a study by Suzuki et al. (1988) both fimbriate and afimbriate strains of *P. gingivalis* had comparable hemagglutinating activity, suggesting that possibly more than one type of fimbriae may exist on the *P. gingivalis* bacterial surface (some being involved in hemagglutination, and others not), or that hemagglutination is not associated with fimbriae altogether.

b) Adhesins

Although some data has suggested that fimbriae/pili are responsible for the hemagglutination of *Bacteroides*, there is also evidence that a non-fimbrial haemagglutinating adhesin may be present on the cell surface of the bacterium (Boyd and McBride, 1984; Okuda et al., 1986; Yoshimura et al., 1984). The fractionation of *P. gingivalis* outer membranes by Boyd and McBride into two components revealed different binding activities of the two fractions (Boyd and McBride, 1984). The first membrane fraction comprised mainly of protein and carbohydrate material (possibly a capsular-polysaccharide-protein complex) possessed bacterial aggregating activity associated with the aggregation of Gram-positive microorganisms. A charged component group on

the cell wall of the Gram-positive organisms, may serve as the receptor for the adhesin. The second membrane fraction, consisting of low-molecular weight lipopolysaccharide, protein and loosely bound lipid, contained hemagglutinating activity. It was postulated that interference with binding to one of these receptor fractions, would not necessarily preclude the binding to another. This ability of the organism to bind both to host tissue and other bacteria, may be of ecological advantage. Similarly, the shedding of "blebs"- outer membrane fragments containing these agglutinins, by *Bacteroides* may serve a protective function, by binding antibody which could potentially interfere with adherence and colonization by the organism.

In 1989, Mouton et al. characterized and identified the bacterial cell surface component of *P. gingivalis* responsible for adherence of the organism to erythrocytes, (hemagglutination). Cross immunoaffinity electrophoresis studies identified two significant polypeptides of about 33 and 38 kDa mass which were not linked by disulphide bridges, yet probably shared at least one epitope. These polypeptides were shown to be distinct from polypeptides typical of fimbrial preparations (41 to 43 kDa).

6:III) AVOIDANCE OF THE HOST DEFENCES

Upon adhering to a suitable surface, the organism must be able to survive in this environment. Survival entails avoiding the host immune response. *Bacteroides* species possess several protective mechanisms to avoid elimination by the host such as: the ability to form "blebs" - outer membrane fragments, encapsulation, hydrolysis of complement and antibody, and the production of toxins and enzymes. The recognition by the host of some of these components, which are also antigens probably plays a significant role in diseases involving *Bacteroides*.

a) Outer Membrane Vesicles (Blebs)

The production of membranous extensions or outgrowths of the outer membrane (blebs) of several Gram-negative bacteria including *Bacteroides* has been recognized (Grenier and Mayrand, 1987; Gamazo and Maryon, 1987). The release of outer membrane vesicles occurs during normal growth, but can be amplified by the manipulation of culture conditions. Vesicle production of *P. gingivalis* is closely related to the growth condition (McKee et al., 1986) Under hemin limitation *P. gingivalis* is avirulent and produces large numbers of blebs, which are both cell bound, and free. In conditions of excess hemin, few extracellular vesicles are present. The gingival crevicular hemin concentration is usually quite low, therefore the production of vesicles would most likely be stimulated.

SDS-polyacrylamide gel profiles reveal that the polypeptide pattern of vesicles is similar, but not identical to that of outer membranes of the corresponding cells. Grenier and Mayrand (1987) found that generally, fewer polypeptide bands were seen in the outer membrane preparations, than in vesicles. They believed that the additional bands present in the PAGE profiles of vesicles may be the result of either concentration of the proteins during the process of bleb formation, or the result of proteases degrading the surface molecules of vesicles. On the otherhand, Deslauriers et al. 1990, found that the extracellular vesicle preparations showed a noticeably larger number of proteins, than the 3-4 weak bands reported by Grenier and Mayrand, suggesting that extracellular vesicles retain most of the complement of outer membrane components.

Extracellular vesicles produced by *Actinobacillus actinomycetemcomitans* and various *Bacteroides* species have been identified in dental plaque. Vesicles produced by *Actinobacillus actinomycetemcomitans* exhibit leukotoxic activity, endotoxic activity and contain a bone-resorption-inducing factor (Nowotny et al., 1982). Grenier and

Mayrand (1987) showed that vesicles produced by *P. gingivalis* exhibited proteolytic activities similar to that of whole cells.

Extracellular vesicles may possibly promote the adherence between homologous cells, as well as mediate the attachment between two non-coaggregating bacterial species. Vesicles-blebs may play a significant role in periodontal disease by serving as a vehicle for the delivery of toxins and proteolytic enzymes. Since vesicles are small in size, it is believed that they would be able to cross anatomic mucosal barriers, which would otherwise be impermeable to bacterial cells. These structures may have been responsible for the immunofluorescence seen by Pekovic and Fillery (1984) at the surface, as well as in the interstitial space between epithelial cells. In addition to the antigenic diversity of these vesicles, which may be able to induce a complex host immune response, it is postulated that *in vivo*, extracellular vesicles may protect the infecting bacteria by competing for antibodies directed to the whole cell, thus impeding specific antibacterial defense mechanisms.

b) Capsule

Bacterial capsules are external electron-dense layers often secreted by the cell, which have various functions: physiochemical barriers, protection against dessication of the cell by binding of water molecules, and antiphagocytic properties, which aid the cells in avoiding engulfment by polymorphonuclear leukocytes. Capsules may also aid in the attachment of bacteria to other bacteria, and thus play a role in synergisitic infections.

The presence of a capsule, or an outer slime layer in *B. melaninogenicus* (*P. melaninogenica*) was first identified by Takazoe et al. (1971). The capsule consisted of a thick layer composed of a fine fibrillous network external to the triple-layered outer cell wall membrane. Isolation of the capsular material was achieved by treating whole cells with acetate buffer at low pH. This purified capsular material was termed K-

antigen. Staining of the capsular material indicated that the capsule was composed of a polysaccharide heteropolymer (Luft, 1964).

Not all *Bacteroides* species or serogroups of a species possess capsules. Encapsulated species of *Bacteroides* demonstrate greater virulence, producing enduring abscesses. Strains lacking a capsule produce either no lesions, or lesions which resolved within a week (Takazoe et al., 1971; Takazoe, et al., 1975).

In addition to increasing bacterial virulence, the capsular layer imparts antiphagocytic properties to the *Bacteroides* cells, making them resistant to killing by polymorphonuclear leukocytes (Okuda and Takazoe, 1973). The capsular K-antigen of *Bacteroides* is capable of masking and preventing lipopolysaccharide from activating the complement system. Capsular material associated with the cells of black-pigmented *Bacteroides* not only inhibits their killing by phagocytes, but also the killing of concomitant facultative anaerobes and aerobic bacteria, by a mechanism which is dependent on the presence of serum (Ingham et al., 1981). In 1982, Sundqvist and Johansson showed that capsules played a role in providing resistance to the bactericidal activity of human serum.

c) Lysis by Complement

Polymorphonuclear leukocytes (PMN) play a crucial role in the cell-mediated host response to disease. Any pathogen with the ability to suppress the PMN response, could significantly increase its virulence. An organism can induce PMN chemotaxis by complement activation, generation of chemotactic products (C3a) or by the release of compounds, which are directly chemotactic (e.g. N-formyl methionyl peptides). Cells of black-pigmented *Bacteroides* are capable of activating both the alternative (Okuda et al., 1978) and classical complement pathways (Okuda and Takazoe, 1980). *Bacteroides* lipopolysaccharide activates the complement system and produces chemotactic factors

for PMNs. However, capsular material and soluble extracellular products from the organisms can interfere with chemotaxis (Okuda et al., 1978). By masking the lipopolysaccharide, the *Bacteroides* capsule may serve to decrease the chemotactic stimulus. *Bacteroides* species are also capable of elaborating non-chemotactic products, which can compete for and block chemotactic receptors on PMNs, thus further reducing their chemotactic ability (Van Dyke et al., 1982). Due to these various evasive characteristics of *Bacteroides*, a strong PMN response is not mounted to this genus, although PMNs do migrate toward accumulations of infecting black-pigmenting *Bacteroides* cells, as evidenced by the pyogenic nature of these infections.

Complement serves various functions in host defence against bacterial invasion. The activation of complement may result in the release of peptides with biological activities affecting: the vasculature (histamine, serotonin), leukocyte function and cellular immune response. Deposition of C3-derived opsonins, such as C3b and iC3b on the bacterial surface results in more efficient recognition, ingestion and killing of such bacteria by phagocytes.

Fragments of complement proteins: C3, C4 and factor B have been identified in gingival crevicular fluid from patients with periodontal disease (Schenkein and Genco, 1977). An increase in the severity of gingivitis and periodontitis was evidenced in the presence of C3 - cleavage fragments (Niekrash and Patters, 1986). The inactivation of complement factors by bacterial pathogens present in the gingival crevice, enhances the virulence of the periodontal organisms.

Schenkein (1988) studied the ability of *P. gingivalis* to degrade the complement components C3, C4 and factor B, which have been demonstrated in gingival crevicular fluid. It was shown that at serum protein concentrations approaching that of gingival crevicular fluid, and under conditions of time and temperature permissive of complement activation, *P. gingivalis* proteases were not likely to destroy the complement proteins. However, the author suggests that this may not reflect the

situation with complement proteins bound on the bacterial cell surface, which would be involved in complement-mediated opsonization and complement-mediated killing of the cell.

d) Degradation of Immunoglobulins

Antibodies against bacteria may protect the host from infection by inhibiting attachment, cell killing, opsonization, and neutralization of toxins, enzymes and other noxious bacterial products. Saliva contains predominantly secretory IgA antibodies, while the gingival crevicular fluid and gingival tissues contains IgG (Brandztaeg and Tolo, 1977). Secreted and cell-bound immunoglobulin proteases from bacteria can interfere with the protective action of host antibodies. Proteolytic destruction of immunoglobulins can result in an increase in bacterial adherence, a decrease in bacterial lysis due to complement, a reduction of phagocytosis, as well as a decrease in the antibody-neutralization of toxins and enzymes. Thus, degradation of immunoglobulins could paralyze the host humoral defense mechanisms and permit bacterial invasion of tissues.

Black-pigmented *P. gingivalis, P. intermedia, P. melaninogenica* and *P. endodontalis* can completely degrade immunoglobulin G molecules and use the resulting fragments as nutrients to increase their own growth (Kilian, 1981; Grenier et al., 1989). *P. gingivalis, P. asaccharolyticus* and *P. melaninogenica* are also capable of splitting serum and secretory IgA. Kilian demonstrated that a majority of strains of these three bacterial species degrade IgA1; IgA2 is cleaved by *P. gingivalis* and *P. intermedia* (Kilian, 1981). The degradation of IgG and IgA by black-pigmented *Bacteroides* occurs in two stages. The initial stage of degradation results in the breakage of the immunoglobulin molecule into larger fragments, followed by a subsequent degradation into smaller peptides (Grenier et al., 1989).

e) Production of Toxic Substances

Bacteroides species produce various toxic substances, capable of interfering with the replication and function of other cells. Butyrate and propionate, the characteristic end products of metabolism, are potent inhibitors of various human and animal tissue culture cell lines (van Steenbergen et al., 1982b). Black-pigmented *Bacteroides* elaborate potentially cytotoxic products such as indole, ammonia, fatty acids, and such volatile sulfur compounds as: hydrogen sulfide, dimethyl sulfide and methylmercaptan (Tonzetich and McBride, 1981), which may possibly interrupt the metabolism of various organisms or augment destruction of periodontal tissues (Socransky, 1970). *P. gingivalis* releases a substance, which induces collagen breakdown in cultures of rat mucosal keratinocytes. Two separate substances possessing proteolytic capabilities, promote sequential reactions, i.e. induction of the secretion of procollagenase and subsequent activation of this enzyme (Birkedal-Hansen, 1987). These results suggest that *P. gingivalis* has the potential to destroy periodontal connective tissues directly with its own collagenase, or indirectly by inducing host collagenase, in the absence of local immune responses.

Despite the ability of black-pigmented *Bacteroides* to induce breakdown of tissues, it has been postulated that several prerequisites must be fulfilled in order for the initiation of a destructive phase by these organisms (Socransky et al., 1982). Unless these multiple factors are present, for example: adherence and various enzymatic activities necessary for the production of small peptides, tissue destruction will not proceed.

f) Enzyme Production

Bacteroides species produce an array of lytic enzymes, which may contribute to their pathogenicity (Table 1.10).

Collagen, the major constituent of gingival tissues, is destroyed by proteolytic enzymes in periodontal disease. Studies of strains of *Porphyromonas gingivalis* have shown their ability to produce enzymes which degrade collagen. Collagenase is produced by exponentially growing cell cultures of *P. gingivalis*, especially in a peptide-deficient medium; it can also occur in cultures of *P. melaninogenica, B. fragilis, B. thetaiotaomicron, B. capillus* and *P. oris* (Gibbons and MacDonald, 1980; Mayrand et al., 1980). The presence of free thiol groups and metal ions facilitates the activity of this bacterial collagenase (Sunqvist et al., 1987). In 1988, Birkedal-Hansen et al. suggested that *P. gingivalis* collagenase be defined as a true collagenase, based on its ability to dissolve reconstituted collagen (type I) fibrils and cleave the helical domains of types I, II, and III collagen.

Heparinase production by *Bacteroides* has been shown to be associated with the development of thrombophlebitis, since the heparinase can inactivate the anticoagulant effect of heparin, which is normally present in the serum of man (Steffen and Hentges, 1981). Specific proteases produced by *P. gingivalis* are also capable of degradation of human plasma proteinase inhibitors (Nilsson et al., 1985). Since proteinase inhibitors modulate the activity of proteinases released by polymorphonuclear leukocytes, the proteolytic activity of *P. gingivalis* may permit a more rapid progression of periodontal disease by efficiently paralyzing the host's various defences (clotting, fibrinolysis and contact activation systems) against invading microorganisms.

P. gingivalis also elaborates superoxide dismutase and peroxidase, which may help the organism resist the deleterious effects of oxygen and hydrogen peroxide,

Table 1.10 Enzymes Elaborated by *Bacteroides* species Capable of Dissolving and Digesting Tissue or Cellular Material

ENZYME	ORGANISM
Collagenase	*Porphyromonas gingivalis* *Porphyromonas asaccharolyticus* *Prevotella melaninogenica* *Bacteroides fragilis* group *Bacteroides capillus* *Prevotella oris*
IgA protease	*Porphyromonas gingivalis* *Porphyromonas asaccharolyticus* *Prevotella melaninogenica*
IgG protease	*Porphyromonas gingivalis* *Porphyromonas asaccharolyticus* *Prevotella melaninogenica*
IgM protease	*Porphyromonas asaccharolyticus*
Hyaluronidase	*Bacteroides fragilis* group
Chondroitin sulfatase	*Bacteroides fragilis* group
Fibrinolysin	*Bacteroides fragilis* Black pigmented *Bacteroides*
Neuraminidase	*Bacteroides fragilis* group *Prevotella melaninogenica* *Prevotella oralis* *Prevotella bivius*
Heparinase	*Bacteroides fragilis* group
DNase	*Porphyromonas asaccharolyticus* *Prevotella melaninogenica* *Bacteroides fragilis* group *Prevotella ruminicola*

(Hoftstad, 1984)

allowing colonization of periodontal pockets and invasion of gingival tissues (Amano et al., 1986).

1:7) HUMORAL IMMUNE RESPONSE TO PERIODONTOPATHIC MICROFLORA

The number of different microbial taxa that reside in the periodontal pocket has been estimated at over 300 (Moore et al., 1982b). Gram-negative bacteria have been reported to play a significant role in the etiology and pathogenesis of different forms of human periodontal disease (Slots and Listgarten, 1988; Socransky, 1977). In periodontal diseases, as in other diseases, the host's immune response is crucial in mediating inflammation and concomitant periodontal destruction. In many infectious diseases caused by a single microorganism, the host immune response is quite efficient in eliminating the offending agent and restoring health. The question that arises in studying immune responses to the microorganisms involved in periodontal disease is whether these organisms are considered as exogenous pathogens (transient) or endogenous opportunistic pathogens (resident flora). In periodontal disease, this picture is further complicated by the multiplicity of bacterial species that can be isolated from the periodontal pocket, and some qualitative similarities of the bacterial species isolated from both diseased and healthy sites. Demonstration of antibody to a specific microorganism responsible for an infectious disease, where isolation of the organism is difficult, has been used to confirm disease/infection. The difficulty in understanding the significance of antibody detected in response to oral bacteria found in subgingival plaque, lies in the fact that no clear description and definition of the inductive principals of antibody formation in response to these organisms has been made.

Most studies of the humoral response in periodontal disease compare the antibody response in the diseased periodontal patient to that of "normal" healthy-controls, although little is known about the "normal antibody response" of humans to bacteria

colonizing the oral cavity in health. Knowledge of which bacterial antigens are relevant in the pathogenesis of periodontal disease, as well as the origin of these antigens is limited, but assumptions are made that the immune response is mounted against significant antigens. Although studies have demonstrated increased/high levels of antibody to components of the oral microflora isolated from periodontal lesions, it is still unclear what functions these antibodies play in the disease process: i.e. protective or destructive. As little is known of the role of antibody as a defense mechanism in health or in early disease, the value of antibody as an indicator of disease cannot easily be ascertained.

The response by humans to infection with periodontopathic bacteria involves both the cellular and humoral arms of the immune system. In this review, I will try to concentrate mainly on studies of the humoral response to oral microbial flora, with special emphasis placed on antibody generation to Gram-negative bacteria, particularly the black-pigmented *Bacteroides* (*Prevotella, Bacteroides* and *Porphyromonas*).

The healthy gingival crevice is usually colonized by Gram-positive nonmotile rods and cocci, such as *Streptococcus* and *Actinomyces* (Slots, 1977). With the development of periodontal disease, the subgingival flora changes to include many more Gram-negative and anaerobic species (Socransky et al., 1982, Moore, 1987). *P. gingivalis*, for example is rarely found in children, and antibody levels to the organism are also low in children (Mouton et al., 1981b). However, recent findings have shown that other black-pigmented *Bacteroides* (*P. intermedia* and *P. melaninogenica*) can be isolated from mouths of children (Frisken et al., 1990) and newborns, and also that enzyme-linked immunosorbent assays have demonstrated the presence of serum antibody reactive to dental pathogens in young children (mean age 9.4 years, age range: 6-14 yrs.)(Cole, 1991).

Mashimo et al. (1976) reported that natural antibodies reactive with organisms from dental plaque are present in most adults, although few data have been based on sera of subjects with a healthy periodontium. Hofstad found that most children older than 1 year of age and blood donors have circulating antibodies to the lipopolysaccharide of oral *B. melaninogenicus* (presumably *P. intermedia*)(Hofstad, 1974). Mouton et al. (1981b) found that the development of serum antibodies to *P. gingivalis* followed the general immune response pattern; in the first months of life there was a rapid increase of IgM activity followed by IgG and after 6 months IgA. Specific IgG activity to *P. gingivalis* was found in 44% of cord blood sera, but after a few weeks post-partum the levels decreased, indicating catabolism of the IgG molecules transferred across the placenta to the fetus. Few children from 6 months to 6 years of age demonstrated IgG antibodies reactive with oral *P. gingivalis*, but between ages 6 to 12, the proportion of reactive children doubled, and a threefold increase in the level of specific IgG antibody was detected. Low levels of antibodies reactive with oral *P. gingivalis* were present in up to 84% of normal adults, indicating that these antibodies are natural antibodies, and may play a protective role. Patients with adult periodontitis had levels of IgG antibodies to *P. gingivalis* that were on average, five times higher than those found in normal adult subjects. Patients with localized juvenile periodontitis, acute necrotizing ulcerative gingivitis and edentulous subjects had low levels of antibodies to *P. gingivalis*, comparable to those found in normal subjects. Mouton et al. (1981b) and Taubman et al. (1982), found that serum antibody levels to *P. intermedia* showed no differences among the subjects with or without periodontal disease.

Elevated systemic antibody levels to subgingival bacteria appear to correlate with colonization of the pocket (Ebersole et al., 1984; Ebersole, et al., 1987), since plaque samples from individuals with high serum antibody levels to periodontopathic organisms harbored the respective organisms. In renal transplant patients with depressed immune systems, a reduced immunological reactivity against antigens from

subgingival plaque bacteria is noted, accompanied by a milder and less destructive form of periodontitis (Tollefsen et al., 1986). The decreased production of specific antibody to periodontopathic organisms demonstrated in immunosuppressed renal patients may be dependent on the general immune status of the host and could provide some degree of protection from antibody mediated tissue destruction in the host.

Naito et al. (1984) studied serum and gingival crevicular fluid from normal healthy adults and peridontitis patients for IgG antibodies to antigens from various bacteria commonly isolated from subgingival plaque. The antibody levels to *P. gingivalis* in serum and gingival crevicular fluid were significantly higher in patients with periodontitis. Some patients with periodontitis had levels of antibody to *P. gingivalis* which were somewhat lower than the others, possibly showing that their periodontitis was related to infection with organisms other than *P. gingivalis*. No remarkable differences between the diseased and control groups in antibody titres to *P. intermedia*, *P. loescheii* and *Capnocytophaga* were observed.

Microbiological data suggests that *A. actinomycetemcomitans* can be found in 36% of the normal population in dental plaque, periodontal pockets and buccal mucosa, however the levels of colonization are always low (Zambon, 1985). Studies by Ebersole et al. in 1982(a) showed a significantly increased level of anti-*A. actinomycetemcomitans* serotype *b* IgG antibody in nearly 90 % of the localized juvenile periodontitis patients compared to only 40% in the generalized juvenile periodontitis, 25% in adult periodontitis and 10% or less in the control groups.

Most patients with adult periodontitis and severe progressive periodontitis exhibit higher serum levels against *P. gingivalis* than those in subjects with healthy gingiva (Mouton et al., 1981(b); Ebersole et al., 1982(b); Ebersole et al., 1986; Taubman et al., 1982). Mansheim et al. (1980) suggested that serum IgG levels were not significantly elevated with the degree of periodontitis, but rather with the age of the subject. Measurement of antibody to *P. gingivalis* by ELISA showed significantly higher

levels in about 60% of patients with advanced destructive periodontitis (Ishikawa et al., 1988) compared to healthy subjects. Taubman et al. (1982) noted that virtually all normal and edentulous subjects failed to manifest antibody to *P. gingivalis*, whereas all but 26% of the adult periodontitis patients had antibody to this organism. Various antigenic components present in *P. gingivalis*, such as lipopolysaccharide in the outer membrane, fimbriae, proteolytic enzymes, collagenase and trypsin-like enzyme may play stimulatory roles in the development of an antibody response in disease.

Loasrisin et al. (1990) studied the major antigenic proteins of *P. gingivalis* recognized by serum antibodies of patients with rapidly progressing periodontitis and adult periodontitis using SDS-polyacrylamide gel electrophoresis and Western blotting. The healthy group of patients-controls, contained bands reactive to proteins, but at low levels in comparison to the rapidly progressing periodontitis and adult periodontitis patients. The serum from adult periodontitis patients frequently reacted with high molecular weight proteins (82, 57, 44 kDa), while rapidly progressive periodontitis patient serum reacted with lower molecular weight components (44, 27, 25, 22, 18 kDa), suggesting a specific antibody response pattern upon invasion of the host periodontium with *P. gingivalis*. The authors suggested that low molecular weight proteins may be the major antigens in early onset periodontitis, either because of their rapid penetration or greater immunogenicity. In addition, adsorption of the serum with the homologous microorganisms was far more efficient in removing antibody activity than with heterologous bacteria, suggesting a specificity of the antibody produced in response to *P. gingivalis* (Table 1.11).

Watanabe et al. (1989) similarly demonstrated by Western blotting that sera from adult and young adult patients with severe periodontitis reacted strongly with bands of sonicates of *P. gingivalis* cells (46, 27 and 14 kDa), and outer membrane antigens (76, 57, 51, 46, 35 and 19 kDa) compared to that of sera from controls and mild periodontitis patients (Table 1.11).

Table 1.11 Detection of Major Antigenic Bands of *P. gingivalis* Cell Sonicates and Outer Membranes by Immunoblotting Using Human Sera from Patients with Rapidly Progressing, Adult and Severe Peridontitis

Antigens (kDa)	LAOSRISIN et al., 1990		WATANABE et al., 1989	
	Adult Periodontitis	Rapidly Progressing Periodontitis	Adult Severe Periodontitis	
	Cell Sonicates	Cell Sonicates	Cell Sonicates	Outer Membrane
82	x			
76				x
57	x			x
51				x
46			x	x
44	x	x		
35				x
27		x	x	
25		x		
22		x		
19				x
18		x		
14			x	

ELISA analysis of the reaction of human serum IgG antibodies to sonicate preparations, leukotoxin, group carbohydrates and lipopolysaccharide moieties of *A. actinomycetemcomitans* revealed that antibody reacted to specific determinants on the microorganism (Ebersole et al., 1983). Patients with localized juvenile periodontitis reacted most strongly with sonicates, leukotoxin and group carbohydrate antigens, compared to normal and other periodontally diseased groups. Significantly increased responses to the leukotoxic group carbohydrate antigen of *A. actinomycetemcomitans* in sera from localized juvenile type periodontitis compared to other disease was found. Serum IgM antibodies to the lipopolysaccharide of *A. actinomycetemcomitans* were increased in localized juvenile periodontitis, generalized periodontitis and adult periodontitis patients, such that no differentiation among the types of disease on the basis of the antibody frequency and/or level to lipopolysaccharide could be shown. This similarity of response to lipopolysaccharides may represent a more generalized cross-reaction among other Gram-negative organisms inhabiting the gingival crevice.

Higher serum levels of IgG antibody were detected in serum and gingival crevicular fluid of untreated adult periodontitis patients, compared to treated adult periodontitis patients or gingivitis patients (Murray et al., 1989). The ratios of gingival crevicular fluid antibody to serum antibody in the untreated adult periodontitis groups were not significantly higher than ratios of the treated groups, suggesting that possibly the local immune response is a result of leakage of serum into the gingival crevice, and not a site-specific response to infection.

The assumption that the increased antibody production is in response to colonization by periodontopathic microorganisms, would suggest that institution of treatment modalities by scaling, root planing and periodontal surgery would diminish the level of antibody found in both gingival crevicular fluid and serum. The levels of specific antibody in crevicular fluid may be dependent on a number of factors including serum levels, local antibody synthesis, the rate of fluid flow and passage of antibody, the

degree of degradation by bacterial enzymes, together with specific and non-specific binding of antibody in the pocket. Baranowska et al., (1989) compared the levels of IgG antibody to *P. gingivalis* in sequential-controlled volume samples of crevicular fluid from healthy and diseased sites of patients with untreated periodontitis, and drew comparisons with levels present in serum. Generally, although a wide range of levels of antibody were detected in crevicular fluid samples, specific IgG antibody levels to *P. gingivalis* were lower than those in serum. It is suggested that the crevicular fluid is derived from serum and that levels of specific antibody are reduced during passage through tissues and the healthy sulcus or periodontal pocket. Ebersole et al. (1985) demonstrated that in 9% of his subjects, there was a higher concentration of antibody in the crevicular fluid than in serum, suggesting a possible local synthesis of antibody in these tissues. Tew et al. (1985) found higher levels of antibody in the majority of crevicular fluid samples compared to serum in young patients with severe periodontitis. The concept that local synthesis is related to the level of inflammatory response, would imply that an increase in antibody levels would be seen between diseased and healthy conditions, and this relationship could not be demonstrated by Baranowska et al. (1989).

Study of a large family with a high prevalence of early-onset periodontitis showed that sera from family members contained antibodies for putative periodontal pathogens not found in their pocket flora, and conversely, putative periodontal pathogens were isolated from subjects in which no serum antibodies to these bacteria were present (Williams et al., 1985). One explanation of these findings could be that some potential pathogens may not elicit a humoral immune response, because they can only colonize the periodontal pocket, and not infect. Infection may result in the production of protective levels of antibodies and such infections may occur sequentially. Infection (not simply colonization) by a given species may result in the production of protective levels of antibodies and result in microbial clearance, conferring protective immunity to

reinfection by the same or a closely related species. Reinfection by an unrelated species could occur, with repetition of the whole process. Moore et al. in 1985 concluded that the bacterial composition at individual sites appears to be dynamic, with the flora at affected sites differing significantly in some people at any one time, or differing in others from week to week. This fluctuation in microbial populations could result in an episodic progression of periodontal disease, and explain fluctuations seen in antibody levels and bacterial colonization.

In spite of the reported general increases in antibody to periodontopathic organisms seen in serum and gingival crevicular fluid in diseased individuals, reports of lower levels in patients relative to that in healthy control individuals have been documented (Doty et al., 1982; Ishikawa et al., 1988). Ishikawa found that high anti-*P. gingivalis* antibody levels were associated with all three types of periodontitis patients (advanced destructive periodontitis, juvenile periodontitis and adult periodontitis). The presence of high anti-*P. gingivalis* titres was almost always associated with elevated antibody levels to other periodontopathic organisms. However, elevated antibody levels to *A. actinomycetemcomitans* were not always found in the juvenile periodontitis patients, but patients with high-IgG titre to this organism showed a more destructive form of bone loss. Doty et al. (1982) reported that serum IgG antibody to oral organisms was significantly lower in diseased patients than in control subjects. The IgG and IgA antibody titres to certain periodontal disease-associated microorganisms were depressed in the diseased individuals, when compared to those of healthy controls. No difference however, was noted in the IgM titres. Self-immunization of the gut-associated lymphoid tissue via swallowing or local sensitization with periodontally associated antigens may cause a depression of the antigen-specific humoral response, which was not demonstrated by Doty et al. in the IgM response of diseased patients. Ebersole et al., (1982a) also found a small number of low-titre responders among the juvenile periodontitis group. In these patients it is believed that

the depressed host response may be related to decreased chemotactic ability of polymorphonuclear leukocytes.

Many of the Gram-negative species in the oral cavity are known to be capable of polyclonal B cell activation through nonspecific signals, which elicit the formation of antibody in the absence of specific induction (Bick et al., 1981). Studies by Ebersole and Holt (1988) using absorption suggest that specific antibody induction by periodontopathogens in the subgingival microbiota occurs, although little information available demonstrates unequivocally the presence of the organism or antigen eliciting these antibodies. Studies of these sera adsorbed with bacteria showed that absorption with the homologous organism was most efficient in removing antibody activity. Distribution of IgG and IgM antibody isotypes in response to periodontopathic organisms also varied among individual patients in the disease categories. If antibody generation was primarily due to polyclonal B cell activation, similar levels of antibody of all isotypes would be expected. An initial response of increases of 60-200% in IgG activity to intact bacteria is against outer envelope antigens, and this is consistent with classic host responses to bacterial infections. Periodontitis patients had antibody of significantly higher avidity to *P. gingivalis* than normal subjects, a result similar to those seen in non-human primates immunized with *P. gingivalis* (Ebersole and Holt, 1988). Antibodies that are polyclonally activated are generally of low avidity.

Gmur (1985) studied antibody levels against four different isolates of *Prevotella intermedia* representing the two DNA homology groups (van Steenbergen et al., 1982(a); Johnson and Holdeman, 1983) and three monoclonal antibody serogroups (Gmur and Guggenheim, 1983; Gmur and Wyss, 1984). Individual human sera showed very different antibody titres against the four bacterial strains. As it is known that *P. intermedia* colonizes the host at sites outside of the oral cavity, response to this "non-oral" bacteria may impair the interpretation of studies on the generation of humoral immune responses in periodontal disease.

Although there is a general consensus that antibody production to oral bacterial antigens is a very specific process, Mallison et al. (1989) showed that antibodies specific for non-oral antigens can be produced in large quantities in the inflamed gingiva of humans. Injection of humans with tetanus toxoid, followed by booster injections demonstrated a 5- to 10- fold increase in serum anti-tetanus antibody. Gingival antibody synthesis specific for these non-oral antigens also occurred, suggesting that B cells arrive at inflamed sites, and receive signals necessary for differentiation into plasma cells.

Mallison et al. (1989) studying rabbits, immunized with non-oral antigens found that antigen-specific plasma cells responsive to non-oral antigens could be found in the inflamed gingiva for months after the animal was immunized. Rabbit peripheral blood taken 7 days after a booster immunization with a non-oral antigen was also capable of some specific antibody production in culture. The addition of periodontitis-associated bacteria, which are known to be polyclonal B-cell activators, markedly increased the amount of transient antibody produced in the culture. Introduction of antigens at sites other than the mouth may, therefore, produce transient local oral responses to periodontal disease organisms, and these may occur whenever an immune response is stimulated. In the case of a persistent oral antigen, such as that seen in periodontal disease, high levels of specific gingival antibody synthesis may become persistent.

In classical infections, antibody titres to the infecting microorganism increase, and subsequently decrease upon control of the disease. A longitudinal study by Mouton et al. (1987) studied antibody to *P. gingivalis* in adult patients with chronic periodontitis and periodontally healthy individuals using ELISA of serum samples. One group of patients with chronic periodontitis had no IgA antibody, but carried levels of IgG and IgM similar to those of the healthy population, suggesting low levels of *P. gingivalis* together with higher levels of other bacteria in the periodontal lesions (low-reactive group). The second group of patients had detectable levels of IgA and significantly higher levels of

IgG antibody to *P. gingivalis*, reflecting colonization by high numbers of *P. gingivalis* (high-reactive group). No cyclic pattern of humoral immune response was observed over the period monitored, which one would expect if the host responded to exacerbations and remissions in periodontal disease (Socransky et al., 1984). Also in the periodontitis patients examined by Mouton et al. (1987), no peak level of antibody immediately post-treatment (scaling and rootplaning) was detected, contrary to some studies which believe that treatment provoked active immunization with the microflora present in the gingival crevice (Ebersole et al., 1985). In many instances microbial species have been detected in the subgingival plaque, which do not apparently elicit increased antibody responses after treatment (Ebersole et al., 1985). The increased antibody level seen in serum post-scaling by Ebersole et al. occurred approximately 2 to 4 months post-treatment; with antibody returning to pretreatment levels by 8-12 months. Although Mouton et al. (1987) did not see a peak of antibody in their patients immediately post-treatment, antibody titres of IgG to *P. gingivalis* decreased to approximately 50% one year period post-treatment, suggesting that the treatment effectively reduced the immune challenge, although the antibody levels did not reach those of periodontally healthy subjects.

An increase in the clinical indices of periodontal disease (plaque index, gingival index, periodontal pocket depth and alveolar bone loss) was positively correlated with increased antibody to *P. gingivalis* (Naito et al., 1985). Treatment of the individuals showed a general decrease in antibody titre to *P. gingivalis*; similar decreases in antibody to other periodontal organisms did not occur.

The studies of levels of serum antibodies in patients with varying forms of periodontal disease show a correlation, although not perfect between the predominant organisms found in the lesions and antibody titres to these organisms. Future studies of the significant antigens to which antibodies are directed, and on the functional

capabilities of the antibodies recovered from diseased lesions (inhibition of toxic products, bactericidal capabilites, opsonization) are required.

1:8) IMMUNE RESPONSE STUDIES IN EXPERIMENTAL ANIMALS

Elevated humoral immune responses to periodontal pathogens have been demonstrated in various human and animal studies, although the precise function of antibody formation in the disease process is still poorly understood. Increases in antibody levels may play a protective role in periodontal disease by inhibiting the initial colonization by bacteria of the gingival sulcus, the antibody generated also may play a role in the progression of the disease process or indicate a state of disease remission or healing. In early periodontal disease, the low levels of antibody to periodontopathogens in the gingival crevice may be insufficient to prevent and inhibit colonization of the pocket by the invading organisms, while the subsequent increases in antibody titres detected in advanced periodontal disease, may be insufficient to influence disease progression. Experimental models of periodontal disease using animals including mice, rats, cats, dogs and monkeys have been designed to help clarify the processes involved in generating immune responses to periodontal pathogens in humans.

8:1) MODULATION OF COLONIZATION BY IMMUNIZATION

Several studies have explored the role that immunization of an animal with periodontal pathogens plays in the modification of colonization of the host and the ecology of the subgingival flora in periodontal disease.

Nisengard et al. in 1989, studied the effect of immunization with *B. macacae* - the monkey equivalent to the human species of *P. gingivalis*, on ligature induced periodontitis in cynomolgus monkeys. During a 12 week period, monkeys were

immunized weekly either with washed, heat-killed *B. macacae* cells in an emulsion of Freund's complete adjuvant, or sham-immunized with saline. Gingival health was maintained in the animals by scaling and weekly pumice prophylaxis of the dentition. Following the 12 week period of immunization and prophylaxis, ligatures were introduced into the gingival sulci of mandibular molars to mechanically induce periodontitis, and subsequently the gingival condition was followed for 6 months. Immunization led to increased antibody levels to *B. macacae*, while ligation increased the amount of plaque, gingival inflammation and bone loss. Peak antibody levels occurred either during the immunization phase or during the first three months following ligation, and declined during the second three months of the ligation phase. Following 6 months of ligation *B. macacae* constituted 1.7% of the cultivable flora in the immunized non-ligated monkeys, 2.1% in the immunized ligated monkeys, and 5.6% in the sham-immunized ligated monkeys. In contrast, the percent of *P. intermedia* present in both the immunized, ligated and sham-immunized, ligated groups remained similar. These results suggest that systemic immunization with *B. macacae* reduces the recolonization of the subgingival crevice by this organism, reflected by the reduction in the percentage of black-pigmented *Bacteroides* and anaerobes isolated and therefore may also modulate the course of ligature induced periodontitis.

In 1989, McArthur et al. orally immunized squirrel monkeys with viable cells of *Porphyromonas gingivalis*, while simultaneously ligating five teeth in one quadrant of the animal with bacterium-soaked suture material to facilitate colonization by black-pigmented *Bacteroides*. Prior to the immunization of each animal, microbiologic cultures were obtained to confirm the absence of black-pigmented *Bacteroides* in the subgingival flora of the test animal. Microbiologic sampling confirmed that *P. intermedia* colonized both the immunized and sham-immunized animals to a similar extent and no cross-reactivity between the monkey-derived *P. intermedia* and *P. gingivalis* could be detected. Immunization of animals by *P. gingivalis* however, resulted

in an increased level of immunoglobulin G anti-*P. gingivalis* in the serum and this was associated with a significant reduction in the colonization of the gingival crevice by black-pigmented *Bacteroides.*

A similar study using squirrel monkeys, studying the effect of antibody formation on colonization of the gingival crevice by *P. intermedia,* in response to immunization with *P. intermedia* was performed by Clark et al. in 1991. Squirrel monkeys rendered free of black-pigmented *Bacteroides*, by a single scaling, 10 days of tetracycline therapy and toothbrushing three times a week, were immunized with *P. intermedia*, or sham-immunized with phosphate buffered-saline, followed by ligation of teeth to facilitate colonization by black-pigmented *Bacteroides*. Two weeks after tooth ligation, *P. intermedia* could be detected in 5 of the 6 sham-immunized animals, and only in 3 of the 6 immunized animals. Results demonstrated that immunization led to an increased level of anti-*P. intermedia* IgG antibodies in the serum of the animals, which was associated with a reduction in the frequency of re-colonization of the gingival sulcus by indigenous *P. intermedia.*

Okuda et al. (1988) similarly showed that colonization by *P. gingivalis* in ligated hamsters could also be significantly reduced by subcutaneous immunization with whole cells or with purified hemagglutinin. Hemagglutinin induced specific antibody, which may be effective against other strains of the *P. gingivalis*, while no cross-reactivity could be detected to other black-pigmented *Bacteroides.* Local passive immunization with rabbit IgG anti-whole *P. gingivalis* or anti-bacterial hemagglutinin antibodies also resulted in a reduction of colonization by *P. gingivalis.*

8:11) CHANGE IN DISEASE PATHOGENESIS BY IMMUNIZATION

In studies by Chen et al. (1987) mice were immunized intraperitoneally with either an invasive or non-invasive strain of *P. gingivalis*, *P. intermedia* or Ringer's

solution and subsequently challenged with the invasive *P. gingivalis strain* to study the effect on development of a subsequent septicemia or a spreading infection. Mice immunized with the invasive *P. gingivalis* strain localized the infection, while animals immunized with either the non-invasive *P. gingivalis* strain, *P. intermedia*, or Ringer's solution developed spreading infections. Results from this study demonstrated that prior immunization of an animal with an invasive *P. gingivalis* strain alters the subsequent pathogenesis of the disease. Immunization with *P. intermedia* had no effect on the invasiveness of *P. gingivalis*, suggesting that protection is species specific.

A study by Dahlen and Slots (1989) used tissue cages implanted in the backs of New Zealand rabbits to study the interaction between *P. gingivalis* and systemic antibody formation. Tissue cages directly expose the bacterial cells to complement, antibodies, phagocytic cells and other antimicrobial mechanisms of the host, and mimic the infectious process which may be expected in periodontal disease. Following successful implantation of 4 metal tissue cages in each animal, 0.1 ml. of an individual bacterial suspension (*P. gingivalis, P. intermedia, A. actinomycetemcomitans* or *B. fragilis*) was injected into each tissue cage. Clinical and microbiologic examinations were performed at 2 hours, 24 hours, 3, 7 and 14 days after inoculation. Infectivity was evaluated according to clinical signs, as well as leukocyte and bacterial counts in material aspirated from tissue cages. ELISA testing of pre- and post-immunization serum with sonicated cells as antigen was used to assess the amount of antibody produced by each animal. Rabbits which were immunized with *P. gingivalis,* and then challenged with a subsequent injection of pure culture of *P. gingivalis* into the tissue cage showed complete elimination or markedly lower post-inoculation bacterial counts and weaker tissue response reactions compared to non-immunized animals. The suppression of *P. gingivalis* could either be due to antibody or cellular-mediated mechanisms, or both. In contrast, *P. gingivalis* injected together with *A. actinomycetemcomitans* caused significantly more severe infections than observed in mono-infected animals and even

hyperimmune rabbits could not fully prevent the establishment of *P. gingivalis* and its associated pathology.

Chen et al., in 1990 studied the effect of intraperitoneal immunization of mice with either whole bacterial cells, a lithium diiodosalicylate (LIS) extract or lipopolysaccharide (LPS) of *P. gingivalis* on modulation of the pathogenic disease process in these animals. Two weeks after the final intraperitoneal immunization, the mice were challenged with a subcutaneous injection of the *P. gingivalis* used for the initial immunization. Mice immunized with *P. gingivalis* whole cells did not develop septicemia or secondary lesions, whereas mice immunized with LIS bacterial extract had few secondary lesions and no septicemia. Mice immunized with LPS, and non-immunized mice demonstrated secondary abdominal lesions and septicemia following subcutaneous challenge.

8:III) EFFECT OF IMMUNIZATION ON HUMORAL IMMUNE RESPONSE

Exposure of an animal to an antigen initiates the development of an antibody repertoire. Several animal studies have been designed to explore the effects of immunization on the development of antibody, and mechanisms involved in modification of antibody generation.

Studies of the nature of the humoral responses of animals in the previously described experimental infections have been made. In the study by Chen et al. (1990), it was found that serum antibody was induced by immunization with whole bacterial cells and LIS extract. The LIS extract contained both proteins and lipopolysaccharide, however the LPS content was less than that in the LPS preparation. As no elevation in the antibody titres (IgG and IgM) was found in response to immunization with LPS extract, it was felt that the *P. gingivalis* LPS used in this study required an association with protein in order to be immunogenic.

A study by Ebersole et al. in 1991 further studied the role of production of antibody post-immunization on progression of the periodontal disease process. Nonhuman primates (*Macaca fascicularis*) were parenterally immunized with 10^9 cells of *Porphyromonas gingivalis, B. fragilis* or *Prevotella intermedia*, together with an equal volume of incomplete Freund's adjuvant. Each test animal received a total of three injections over a period of 6 weeks. Control animals were immunized in a similar fashion, using phosphate buffered saline. Baseline analysis of each animal included microbiologic sampling of the gingival crevice, ELISA testing for presence of antibody and clinical observations involving plaque indices, bleeding indices, radiographic analysis of bone density, attachment levels and probing depths. After obtaining the baseline data, three adjacent teeth in one quadrant of each animal (immunized and non-immunized) were ligated, with the contralateral teeth serving as controls.

Results showed that all animals in the study carried baseline levels of antibody to *P. gingivalis, P. intermedia*, and *B. fragilis*. The predominant type of immunoglobulin detected to *P. gingivalis* and *P. intermedia* was IgG, with the levels to *P. intermedia* being approximately fourfold greater than to *P. gingivalis*. In contrast, levels of IgM to *B. fragilis* were 30 to 100 times greater than to IgG or IgA. Immunization of the animals produced an approximately two-fold increase in serum immunoglobulins (IgG, IgM and IgA), which were highly specific to the homologous organism used for immunization. Little change in immunoglobulin levels were seen post-immunization and post-ligation. Levels of antibody to *P. intermedia* and *B. fragilis* declined during ligature-induced disease, while the *P. gingivalis* antibody remained elevated. The authors believe that the resident *P. gingivalis* population in the subgingival plaque may provide the host with a constant low level of antigen, capable of maintaining these elevated immunoglobulin levels.

On the otherhand, the numbers of *P. gingivalis* organisms colonizing the gingival pocket during periodontal disease were significantly reduced post-immunization.

Immunization of the animals with *P. gingivalis* not only reduced the number of *P. gingivalis* colonizing the gingival crevice, but also significantly reduced the numbers of *P. intermedia* colonizing the gingival crevice. Conversely, this effect was not demonstrated with immunization of animals with *P. intermedia* on the emergence of *P. gingivalis* in the periodontal pocket. Immunization with *P. gingivalis, P. intermedia* or *B. fragilis* had little or no effect on the emergence of such organisms as: *P. melaninogenica, P. denticola, P. loescheii, E. corrodens A. actinomycetemcomitans, W. recta* or *Capnocytophaga spp.* in the subgingival microflora of the periodontal pocket.

Clinical studies showed that ligated tooth sites in immunized animals demonstrated higher bleeding on probing, more loss of attachment and bone density loss compared to non-immunized animals. The authors concluded that inspite of elevated systemic antibody response by the host after immunization with *P. gingivalis, P. intermedia*, and *B. fragilis*, and its subsequent modifying influence on colonization by these periodontopathogens, other ecological parameters play a significant role in disease process.

8:IV) EFFECT OF ANIMAL AGE ON DEVELOPMENT OF IMMUNE RESPONSE TO ORAL BACTERIA

Children rarely develop periodontal disease compared to adults (Sweeney et al. 1987). Although periodontal pathogens are frequently present in the mouths of young children (Frisken et al., 1987; Van Oosten et al. 1988), many differences have been demonstrated between child and adult periodontal disease. These differences could be attributed to an increased immune reactivity by adults to microorganisms resulting from immunologic priming by prolonged contact with dental plaque antigens.

In 1989, Lekic et al. studied the role of age and immunization of the development of gingivitis in rats. After exposure of the healthy rats of varying ages (1 month, 3

month and 3 month-immunized) to *S. mutans*, *A. viscosus*, *F. nucleatum* and *P. gingivalis* given orally together with 5% sucrose, the gingival condition was assessed histologically and clinically, and serum antibody titres obtained. These microorganisms were selected on the basis that previous studies showed that they have the capability to colonize the oral cavity and cause periodontal disease in rats. No young rats developed gingivitis during the experiment, whereas half of the adult and all of the adult immunized rats bled on probing 7 and 14 days after immunization. Antibody levels to bacteria were low in the young rats, moderate in the older rats, and high in adult immunized rats. The authors concluded that prior exposure of the animal to plaque antigens may be responsible for the increased antibody levels and clinical signs of disease.

CHAPTER 2

RATIONALE AND APPROACH TO PRESENT STUDY

2:1) RATIONALE

Members of genera *Prevotella, Bacteroides* and *Porphyromonas* (previously included in *Bacteroides*) have been isolated in both health and disease in humans and animals (Slots, 1979; White and Mayrand, 1981; Moore, 1987). Although few mon-infections with species of these genera occur, their presence in mixed infections often augments the disease process (MacDonald and Gibbons, 1962; Socransky and Gibbons, 1965). Colonization of the periodontal pocket by *Bacteroides*, *Porphyromonas* and *Prevotella* occurs in various types of periodontal disease and is associated with an increase in antibody production (Ebersole et al., 1982(b); Ebersole et al., 1987). However, wide cross-reactivity among species of *Bacteroides, Prevotella and Porphyromonas*, and specifically among the black-pigmented *Bacteroides*, has been shown using hyperimmune rabbit sera (Bowden and Nolette, 1990, Ebersole et al., 1988). It could be proposed therefore, that elevated humoral immune responses in periodontally diseased patients to organisms isolated from their subgingival microbiota may be specifically induced in response to colonization by these organisms, or due to cross-reactive stimulation by similar organisms, or a combination of both. Studies using sera from periodontally diseased patients have suggested that a specific immune response is mounted to periodontopathic organisms, as adsorption of their serum with cross-reactive bacterial strains only partially removes the antibody response (Ebersole and Holt, 1988). In addition, antibody produced to periodontopathic microorganisms in diseased patients is of higher avidity compared to sera from normal subjects, suggesting that its formation is not simply the result of polyclonal B cell activation (Ebersole and Holt, 1988).

Recently studies by Cole et al. (1991) have shown that although sera from young children reacted with black-pigmented *Bacteroides*, a significant portion of the reactivity could be removed by adsorption of the serum with *B. fragilis* - a common member of gut flora. The studies described above suggest that immune responses to subgingival bacteria can arise from specific stimulation and also as a result of antibody to cross-reactive bacteria.

Antibody reactive with periodontopathic microorganisms could be induced by several mechanisms:

1) Polyclonal B cell activation producing antibody of low-avidity, cross-reactive with more distantly related bacteria. It would be expected that this reaction would occur locally via accumulated cells present in the periodontal pocket, directed at the pocket flora.

2) Cross-reactive responses to similar shared epitopes among microorganisms, e.g. Animals immunized with *Prevotella intermedia* produce sera, which shows cross-reactivity with *P. melaninogenica* and

3) Natural antibody formed in response to antigens shared with gut-associated flora (*B. fragilis*).

If antibody production in response to immunization with one particular organism (*P. intermedia*) from the subgingival flora is highly specific, it would be expected that little or no change in antigenic profile to heterologous organisms would be generated. In contrast, it could be expected that since the host is exposed to similar antigens from commensal microorganisms present in the gut flora (*B. fragilis*), that immunization with *P. intermedia* may also stimulate the host to activate memory cells already present in the antibody repertoire, and significant cross-reactivity among the species would be evident. This controlled immunization of mice with a single commensal oral microorganism was designed to further examine the development of immune response.

The purpose of the present studies was to gain some insight into the degree of stimulation of the humoral immune system that would generate antibodies cross-reactive with related organisms. Such cross-reactivity has been shown in human serum, but it is not clear whether a short exposure to bacteria, which could occur during acute phases of periodontitis, would stimulate specific or cross-reacting antibody. In addition, the studies which have shown extensive cross-reactivity between *P. intermedia* and other bacteria have used hyperimmune rabbit sera. A mouse model was selected using two inbred strains: Balb/c and C57BL/6. The test organism was a strain of *Prevotella intermedia*, genomic species 2, strain BH18/23.

2) APPROACH

Two genotypes of *Prevotella intermedia* have been described, i.e. genotype I which plays a factor in periodontal disease and may be limited in its habitat to the periodontal pocket, and genotype II, which is a constituent of the normal commensal flora with a wide habitat including supra- and sub-gingival plaque. Members of genotype II can produce an immunosuppressive factor, which could subsequently enhance pathogenicity of this organism and promote virulence in infections with other organisms (Shenker, 1991). A member of genotype II *Prevotella intermedia* BH18/23 (a laboratory isolate) was selected for immunization of mice in this study, as it is a common isolate of the oral cavity and has been shown to be cross-reactive with other black-pigmented *Bacteroides* (*Bacteroides*, and *Prevotella*) species.

In addition to immunization by various routes, quantities of cells, and booster injections, two genetically dissimilar strains of inbred mice (C57BL/6 and Balb/c) of varying ages were immunized with *P. intermedia* to ascertain the role played by genetic composition and age of mice in formation of a humoral response. Mice were primarily immunized intravenously with varying doses of viable cells. A contrasting situation was

created by injecting mice with high doses of the microorganism, and organisms together with Freund's Complete Adjuvant followed by booster injections to produce a hyperimmune serum. Pooled sera obtained from control non-immunized mouse groups was also used to determine the repertoire of natural antibodies present in these animals, as it was expected that the animals may carry some cross-reacting antibody, due to stimulation of the immune system by the gut flora.

Non-detergent treated outer membranes were prepared from various species of the genera *Prevotella, Porphyromonas* and *Bacteroides.* Crude outer membrane preparations were chosen, as opposed to detergent treated preparations to reflect a wider range of antigens, which would be recognized *in vivo* by the immune system. SDS - polyacrylamide gel electrophoresis was used to separate the constituent antigens, and immunoblotting used to probe the mouse sera for response to antigens in the protein profiles of homologous and heterologous cells. In addition to the immunized mouse sera, human adult and children's sera were used to compare to the antigen recognition profiles developed by the mouse sera. ELISA assays provided a quantitative assessment of differences in antibody after immunization, and also determined the degree of specificity of the immune response.

CHAPTER 3

MATERIALS AND METHODS

3:1) STRAINS OF BACTERIA USED FOR OUTER MEMBRANE PREPARATION

Representative species of the genera *Prevotella*, *Bacteroides* and *Porphyromonas* were selected and outer membranes prepared (Table 3.1). All strains were reconstituted in Basal Medium (Appendix A) from previously freeze-dried ampoules, and grown anaerobically (80% N_2,10%H_2,10% CO_2) on blood plates supplemented with hemin and menadione/Vitamin K (Amundrud et al., 1985) (Appendix A). Culture purity was determined by Gram-staining and examination of colony morphology on blood plates under magnification. The identity of the strains was confirmed by acid end product analysis and the results of test runs in the API system (API Laboratory Products Ltd., St. Laurent, Que.).

During the course of this study, bacteria were subcultured at 4-day intervals onto supplemented blood agar (Appendix A). Freeze-dried ampoules of each representative strain were kept for future reference and reconstituted as required.

In 1988, Jonsson et al. using polyacrylamide gel electrophoresis verified that the *Prevotella intermedia* strains isolated by Bowden and used in this study were representative of the species described by Johnson and Holdeman (1983): i.e. BH18/23 represented VPI 8944 group (genotype II), and BH20/30 - VPI 4197 group (genotype I).

3:2) OUTER MEMBRANE PREPARATION

2:I) GROWTH OF CELLS FOR OUTER MEMBRANE PREPARATION

Universal bottles containing approximately 20 ml. of sterile basal media (Appendix A) were inoculated with a single colony of bacteria, which had been grown for 4 days on supplemented blood agar under anaerobic conditions. The Universal bottles containing the microorganism were placed in an anaerobic chamber for 4-5 days, until sufficient growth was visible. A small amount (approximately 2-3 ml.) of the bacterial suspension was removed, centrifuged, and the cells Gram-stained and plated onto supplemented blood agar for growth under aerobic and anaerobic conditions to confirm purity of culture.

Upon verification of culture purity, 1-2 ml. of the universal bottle was used as inoculum for 6-8 1-liter bottles of basal (complex) medium. The individual 1 liter bottles were grown anaerobically for 4-7 days.

Growth in each 1 liter bottle was once again checked for purity, as described previously (Gram-staining and O_2/AnO_2 growth), labelled for identification purposes, and processed separately till such time as culture purity (from O_2/AnO_2 growth) was confirmed.

2:II) PREPARATION OF OUTER MEMBRANES

Bacterial cell suspensions were centrifuged for 20 minutes at 10,000 R.P.M on a RC5C-Sorvall Centrifuge (head SS34)(Dupont, Connecticut). The pellet was resuspended in approximately 150 ml. of 2x EDTA buffer (Appendix A), cells were dispersed by pipette and sonication, and the suspension was heated for 30 minutes in a shaking water bath (60^oC).

Table 3.1. Sources of *Prevotella*, *Bacteroides* and *Porphyromonas* used for the Preparation of Crude Outer Membranes

	SOURCE
Black Pigmented Oral Strains	
Prevotella intermedia BH 18/23	Dr. G. Bowden
Prevotella intermedia BH 20/30	Dr. G. Bowden
Prevotella melaninogenica 25845	ATCC
Prevotella denticola 33185	ATCC
Prevotella loescheii 15930	ATCC
Porphyromonas gingivalis 18/10	Dr. G. Bowden
Black Pigmented Non-oral Strains	
Prevotella corporis 33547	ATCC
Porphyromonas asaccharolyticus 25260	ATCC
Non-Pigmented Oral Strains	
Prevotella oralis 33269	ATCC
Prevotella buccae 33574	ATCC
Porphyromonas gingivalis 18/10	Dr. G. Bowden
Non-Pigmented Non-oral Strains	
Bacteroides fragilis 8560	NCTC

ATCC= American Type Culture Collection
NCTC= National Collection of Type Cultures

The heated suspension of cells was dispersed using a Tekmar cell disrupter/homogenizer (Cincinnati, Ohio) for 2 minutes. Cells were subsequently mechanically pressed through a 25 guage needle, followed by centrifugation of the suspension at 15,000 RPM for 30 minutes on a RC5C-Sorvall Centrifuge (head SS34 at 27,000 G)(Dupont, Connecticut). The supernatant containing the outer membranes was further centrifuged at 50,000 RPM on a L8-70M Ultracentrifuge (26,500 G) (Beckman, Ontario) for 90 minutes.

The pellet was resuspended in a small amount of Hepes buffer (10 mM, pH - 7.4) and sonicated (Kontes Ultrasonic Cell Disrupter) to obtain a uniform suspension. The suspension was ultracentrifuged at 50,000 RPM on a L8-70M Ultracentrifuge (Beckman, Ontario) (26,500 G) for 60 minutes and the supernatant discarded. The resulting pellet was washed twice more and the final pellet was resuspended in about 5 ml. of Hepes buffer.

2:III) STORAGE OF OUTER MEMBRANE PREPARATIONS

Outer membrane preparations of the individual species were aliquoted into several Eppendorf tubes and stored at -4^{o}C. Single Eppendorf tubes of the membranes from each species were opened for analysis, while the remaining tubes were left frozen to prevent possible deterioration of the preparations upon continued freezing and thawing.

3:3) MOUSE IMMUNIZATION

3:I) MOUSE STRAINS

Two inbred strains of mice: C57BL/6 and Balb/c were immunized. The strains were obtained from either the University of Manitoba, Central Animal Care breeding

facility or Charles River Supply House. Inbred strains of mice of identical genetic constitution were used to decrease the incidence of genetic variation in immune response produced between individual mice. The mice ranged in age from 2-3 weeks to 5-6 weeks old, at the initiation of the immunization regime. Mice were maintained on the regular diet used by the University of Manitoba, Central Animal Care facilities.

3:II) PREPARATION OF CELLS (ANTIGEN) FOR IMMUNIZATION

Cells of *P. intermedia* BH18/23 were grown anaerobically on supplemented blood agar plates for 4-5 days. One standard loopful (4 mm) of BH18/23 (*P. intermedia*) was scraped from the blood plate and suspended into 3.0 ml. of sterile saline. The cells were dispersed into solution using a sterile Pasteur pipette and vortex mixer. A small quantity of cell suspension was removed aseptically and placed in a Petroff Hauser Bacteria Counter (Arthur H. Thomas Co., Philadelphia, Pa. U.S.A.) to obtain an approximate quantity of cells present in the suspension. Cells were counted using phase contrast microscopy under magnification (1,280x). The average value of counts from 16 small squares, located in 4 arbitrary large squares (n) (each small square equals 1/400 mm^2 by 1/50 mm^2) was used to obtain an average count value and the number of cells per ml. calculated using the following formula:

(n) x 400 x 50 x 1,000 = organisms/ml. of cell suspension

An appropriate dilution in sterile saline was prepared based on the above calculation, bearing in mind that the inoculum volume to be used for each mouse immunization would be 0.1 ml/mouse. Prior to and post- immunization of mice, the cell suspension used for immunization was spiroplated using a Spiralplater (Spiral Systems Inc., Cincinnati, Ohio) onto supplemented blood agar and grown anaerobically

for 4 days. Counts of the plates were used to approximate the number of viable cells used for each immunization, with an average of 75% of cells viable during the immunization.

In cases where Freund's complete adjuvant was used, equal amounts of diluted cell suspension and Freund's adjuvant (Difco Laboratories, Detroit, Michigan) were emulsified using a double headed needle and a syringe. The dilution used was adjusted to allow for the addition of an equal volume of Freund's Adjuvant.

3:III) IMMUNIZATION TECHNIQUE

Mice were immunized according to the experimental regimes listed in Table 3.2. M denotes individual mouse groups. Throughout immunizations, a constant volume of 0.1 ml. antigen/mouse was maintained. Prior to immunization, mouse tails were immersed in warm water, causing peripheral vasodilation. Intravenous injection of mice was made by injecting viable cells into mouse tail veins using a 27-guage needle. Intramuscular injections were given into the flank region using a 27-guage needle, while subcutaneous injections were given in posterior back area.

Mice were anaesthetized prior to sublingual injections. The mouth was pried open with a 25-guage needle tip, to aid in localization of the floor of the mouth and a 30-guage needle was used to deliver the 0.1 ml. volume of antigen. Particular care had to be taken to ensure that the injection did not result in suffocation or drowning of the mouse, as the floor of the mouth in mice has a small surface area, lying in close proximity to the trachea.

3:IV) EXSANGUINATION OF MICE

Mice from each group were anaesthetized with ether, until a sufficient level of anaesthesia was achieved and the chest cavity was opened. This method allowed continued slow pumping of blood from the heart while blood was collected using a sterile Pasteur

Table 3.2 Mouse Immunization Regimes

Experiment 1

Injection of 10^6-10^7 viable cells of BH18/23 into tail veins of C57BL/6 mice (4-5 weeks old)

M1- one injection of 10^6-10^7 cells; sacrificed at day 10 (10 mice)
M2- one injection of 10^6-10^7 cells; sacrificed at day 20 (10 mice)
M3- two injections of 10^6-10^7 cells: one at day 0, and second at day 10; sacrificed at day 20 (10 mice)
M4- control - no injections; sacrificed at day 20 (10 mice)

Experiment 2

Injection of 10^3-10^4 viable cells of BH18/23 into tail veins of C57BL/6 mice (2-3 weeks old)

M5- one injection of 10^3-10^4 cells; sacrificed at day 10 (10 mice)
M6- one injection of 10^3-10^4 cells; sacrificed at day 20 (10 mice)
M7- two injections of 10^3-10^4 cells: one at day 0, and second at day 10; sacrificed at day 20 (10 mice)
M8- control - no injections; sacrificed at day 20 (10 mice)

Experiment 3

Injection of 10^3-10^4 viable cells of BH18/23 into tail veins of C57BL/6 mice (4-5 weeks old)

M9 - one injection of 10^3-10^4 cells; sacrificed at day 10 (10 mice)
M10- one injection of 10^3-10^4 cells; sacrificed at day 20 (10 mice)
M11- two injections of 10^3-10^4 cells: one at day 0, and second at day 10; sacrificed at day 20 (10 mice)
M12- control - no injections; sacrificed at day 20 (10 mice)

Experiment 4

Injection of 10^3-10^4 viable cells of BH18/23 into tail veins of Balb/c mice (4-5 weeks old)

M13- one injection of 10^3-10^4 cells; sacrificed at day 10 (10 mice)
M14- one injection of 10^3-10^4 cells; sacrificed at day 20 (10 mice)
M15- two injections of 10^3-10^4 cells: one at day 0, and second at day 10; sacrificed at day 20 (10 mice)
M16- control - no injections; sacrificed at day 20 (10 mice)

Table 3.2 Cont.

Experiment 5

Injection of 10^6-10^7 viable cells of BH18/23, together with Freund's Complete Adjuvant I.M. into C57BL/6 mice (4-5 weeks old)

M17- one injection of 10^6-10^7 cells with FCA; sacrificed at day 4 (10 mice)
M18- one injection of 10^6-10^7 cells with FCA; sacrificed at day 17 (10 mice)
M19- one injection of 10^6-10^7 cells with FCA; sacrificed at day 32 (10 mice)
M20- one injection of FCA and saline (control); sacrificed at day 32 (10 mice)
M21- control - no injections; sacrificed at day 32 (10 mice)

Experiment 6

Injection of 10^6-10^7 viable cells of BH18/23, together with Freund's Complete Adjuvant I.M. into Balb/c mice (4-5 weeks old)

M22- one injection of 10^6-10^7 cells with FCA; sacrificed at day 4 (10 mice)
M23- one injection of 10^6-10^7 cells with FCA; sacrificed at day 17 (10 mice)
M24- one injection of 10^6-10^7 cells with FCA; sacrificed at day 32 (10 mice)
M25- one injection of FCA and saline (control); sacrificed at day 32 (10 mice)
M26- control - no injections; sacrificed at day 32 (10 mice)

Experiment 7

Injection of 10^6-10^7 viable cells of BH18/23 into tail veins of Balb/c mice (4-5 weeks old)

M27- one injection of 10^6-10^7 cells; sacrificed at day 10 (10 mice)
M28- one injection of 10^6-10^7 cells; sacrificed at day 20 (10 mice)
M29- two injections of 10^6-10^7 cells: one at day 0, and second at day 10; sacrificed at day 20 (10 mice)
M30- control - no injections; sacrificed at day 20 (10 mice)

Experiment 8

Total of 6 injections of 10^3-10^4 viable cells of BH18/23 into tail veins of C57BL/6 mice, on a weekly basis (4-6 weeks old)

M31- 6 injections of 10^3-10^4 cells at one week intervals; sacrificed after 6 weeks (20 mice)
M32- control - no injections; sacrificed after 6 weeks (10 mice)

Table 3.2 Cont.

Experiment 9

Injection of 10^7-10^8 cells of BH 18/23 together with Freund's Complete Adjuvant I.M.(flank) and subcutaneously (back), with 2 booster injections of 10^7-10^8 cells into tail veins at the end of weeks 3 and 5 of C57BL/6 mice (4-6 weeks old)

M33- one injection subcutaneously and one injection I.M. of 10^7-10^8 cells of BH 18/23 together with Freund's Complete Adjuvant at day 0, followed by IV tail injections of 10^7-10^8 cells of BH 18/23 at the end of week 3 and 5; sacrificed after 7 weeks (20 mice)
M34- one injection subcutaneously and one injection I.M. of Freund's complete adjuvant in saline; sacrificed at week 7 (10 mice)
M35- control - no injections; sacrificed at week 7 (10 mice)

Experiment 10

Total of 6 injections of 10^3-10^4 viable cells of BH18/23 into tail veins of C57BL/6 mice, on a weekly basis (4-6 weeks old)

M36- 6 injections of 10^3-10^4 cells at one week intervals; sacrificed after 6 weeks (10 mice)
M37- control - no injections; sacrificed after 6 weeks (5 mice)

Collected serum from each mouse was kept separate.

Experiment 11

Total of 6 injections of 10^3-10^4 viable cells of BH18/23 into tail veins of C57BL/6 mice (4-6 weeks old), on a weekly basis, followed by one sublingual injection of 10^7-10^8 viable cells of BH18/23

M38- 6 injections of 10^3-10^4 cells at one week intervals, followed by one sublingual injection of 10^7-10^8 viable cells; sacrificed after 7 weeks (15 mice)
M39- control - no injections; sacrificed after 7 weeks (10 mice)

pipette. In most cases, with the exception of groups M36 and M37, the mouse sera obtained from each group of mice were pooled together. Mouse sera collected from each mouse in Groups 36 and 37 was stored individually.

After an adequate volume of blood was collected from each mouse, the mouse was killed by cervical dislocation.

3:V) GERM-FREE MOUSE SERA

Serum from germ-free mice (NIH strain) was obtained from Georgetown University, Faculty of Medicine and Dentistry (National Institute of Health breeding facility).

3:4) PREPARATION OF MOUSE SERUM

To facilitate the clotting of red blood cells in the whole blood collected, each tube containing pooled blood was stirred with a sterile Pasteur pipette. The mouse serum was placed in a refrigerator (4^{o}C) overnight for further clot separation. The following day, the blood was centrifuged in a Dynac Centrifuge (Clay Adams Division, Parsnippay, N.J.) for 30 minutes and the resulting serum drawn off by sterile pipette. To remove any particulate matter, the serum was filtered through a 0.45 mm Millex-HV filter (Millipore products Division, Bedford) and aliquoted into individual Eppendorf tubes, sealed with Parafilm and stored at -4^{o}C. On average, approximately 200-300 µl. of serum was obtained per mouse. As with the outer membrane preparations, serum was aliquoted in several Eppendorf tubes and single tubes of serum were used for development of immunoblots, avoiding deterioration of the serum on repeated freezing and thawing.

3:5) HUMAN SERA

Human adult sera collected from 6 periodontal patients, with varying periodontal disease states, was obtained through the Periodontal Department at the University of Manitoba, Faculty of Dentistry. The pooled serum was stored frozen at -4°C until required.

Serum from young children (mean age: 9.4 years, Range: 6-14 years) was donated by M. Cole (University of Georgetown, Washington, D.C.). The serum collected was part of a study of colonization/immune response patterns in children.

3:6) RABBIT SERA

New Zealand white male rabbits of approximately 2-3 kg. in weight were used for preparation of antisera to *P. intermedia* BH18/23. Prior to immunization of the rabbits, blood was obtained and sera tested for the presence of natural antibodies to the immunizing organism. Pre-immune serum was tested against whole cells of *P. intermedia* using the Ouchterlony double diffusion method (Roitt, 1988).

P. intermedia BH18/23 was grown anaerobically on supplemented blood agar plates for approximately 4 - 5 days. The cells were scraped from the plate and washed three times in sterile saline. An equal volume of cells and Freund's Complete Adjuvant (Difco, Detroit, MI.) was emulsified together and 0.2 ml. of the solution injected subcutaneously into the rabbit at 4 sites (one above either shoulder, and one above each flank) followed by a single 0.2 ml. injection I.M. into the flank. After two weeks, the rabbit was bled from an ear vein and the serum tested by double diffusion for presence of antibody to the homologous organism. If no precipitin lines developed, or a very weak reaction occurred, the animal was boosted with a 0.2 ml. injection of whole cells in saline, into an ear vein. Booster doses were repeated at 1 week intervals, until such

time as an adequate antibody response was generated. Serum was obtained by bleeding the animal from ear veins or a heart puncture under sedation.

3:7) POLYACRYLAMIDE GEL ELECTROPHORESIS

Separation of proteins in the outer membranes was accomplished using polyacrylamide gel electrophoresis as defined by Amundrud et al. (1985). A level of 5 µg. of protein (assayed by the Bio Rad/Bradford method) was chosen as optimal for resolution of outer membrane constituents by immunoblotting. Samples were run at 100 volts constant voltage at a constant temperature of 20°C through the stacking gel, and on a constant current of 40 mAMPS through a 12 % (1.5 mm thick) separating gel. Each gel contained a lane loaded with six low molecular weight standards (Pharmacia LKB, Piscataway, NJ), included to allow calculation of the molecular weights of the outer membrane antigens. Bacterial outer membrane samples were run in lanes 2-9 of a 10 lane gel, to prevent distortion of the profiles in the outer lanes.

3:8) WESTERN BLOTTING TECHNIQUE

The method of immunoblotting used was based on that described by Towbin and Gordon in 1985. Proteins were transferred to Immobilon PVDF Transfer membrane (Millipore Corporation, Bedford, MA) in a transfer buffer (Tris 15.15g; glycine 72.05g; methanol, 1000 ml; distilled water, 3,912.8ml; pH 8.3) using a Transfor system (Hoeffer, Technical Marketing Inc, Ont). Pyronin (1%)(Fisher Scientific Co, FairLawn, N.J.) was used to mark individual lanes of the gel prior to placement on the PVDF Transfer Membrane. The marked immunoblot blot could subsequently be cut into strips for development by the appropriate serum. Transfer of the outer membrane antigens from the gel was made by applying a 0.5 A current for 16 hours at 10°C. After

the transfer was completed, the immunoblot was cut into separate lanes, and the low molecular weight standards stained using fix (methanol-45%; acetic acid 10%; distilled water 45%) and Coomassie blue dye (BioRad, Ont.). The portion of the membrane which was to be cut into individual strips was stored in a plastic bag with Tris/Tween/Saline at -20°C, until it was developed.

3:9) DEVELOPMENT OF IMMUNOBLOTS

Immunoblots were developed using the methods described by Renart et al. (1979). Stored frozen immunoblots were thawed and rinsed in Tris/Tween/Saline solution (sodium chloride 17 g.; Tris, 2.42 g.; Tween 20 (Sigma), 4 g.; distilled water 2,000 ml.)(pH 8.2). The membrane was then blocked with a 4% solution of Bovine serum albumin (Sigma, Albumin, Bovine, Fraction V, No. A-2153) in Tris/Tween/Saline for 1 hour at 37°C. The blocked immunoblot strips were rinsed with a solution of Tris/Tween Saline 1 hour at 37°C consisting of 4x15 minute washes.

An appropriate dilution of mouse serum (1:100-250) was prepared using a 0.8% Bovine serum albumin solution (Sigma, Albumin, Bovine, globulin free, No. A-7638) in Tris/Tween/Saline and the membrane reacted for 1 hour at 37°C. Following incubation of the membrane with mouse serum, the immunoblots were rinsed 4 times with Tris/Tween/Saline for 15 minute periods. Detection of the antigen/antibody complexes were made by incubation of the immunoblots for 1 hour at 37°C with either a peroxidase conjugated Affinipure goat anti-mouse IgG (H&L) (Jackson Immunoresearch Laboratories, Inc.) or peroxidase conjugated Affinipure goat anti-mouse IgM, μ chain specific (Jackson Immunoresearch Laboratories, Inc., Avondale, PA.) at a dilution of 1:2,000 to 1:3,000. Peroxidase conjugated rabbit immunoglobulins to human IgG (γ chain) (DAKO Immunoglobulins, Denmark) and peroxidase conjugated rabbit immunoglobulins to human IgM (μ chain) (DAKO Immunoglobulins, Denmark) were used

at a concentration of 1:3,000 - 1:6,000 for the development of immunoblots exposed to human sera. Immunoblots reacted with rabbit sera were exposed to peroxidase-conjugated swine immunoglobulin to rabbit IgG or IgM (DAKO Immunoglobulins, Denmark). After three 15 minute rinses with Tris/Tween Saline, the membrane was developed using a solution of 100 ml. 0.1M phosphate buffered saline (OXOID)(pH 7.3), together with 50 mg. of Sigma 3, 3^1-diaminobenzidine diamine and 100 µl of 30% hydrogen peroxide (Fisher, NJ). The membrane was reacted for approximately 2 minutes and the reaction stopped by placing the blot into distilled water at 4°C overnight. Washed membranes were dried between paper towels and stored flat in the dark.

9:I) CONTROL OF IMMUNOBLOTTING REAGENTS

Antibody to the genera *Prevotella*, *Bacteroides* and *Porphyromonas* was detected in the commercial preparations of goat anti-mouse IgG and IgM. To control for the background antibody in the goat anti-mouse serum, most blots contained a lane which was only reacted with the goat anti-mouse sera. This allowed detection of natural antibody carried by goat for comparison to the profiles developed by the mouse sera. Molecular weights of antigens detected in the outer membrane profiled by the goat antibody are shown in Table 4.1.

3:10) IMMUNOBLOT ANALYSIS

10:I) DETERMINATION OF MOLECULAR WEIGHTS

Calculation of molecular weights of bands present on immunoblots was determined by measuring the migration distance of each protein of the Pharmacia low molecular weight standards on the stained immunoblot, in relation from the point of origin to the bottom of the immunoblot, i.e.

$$Rf = \frac{\text{distance protein has migrated from origin}}{\text{distance from origin to reference point}}$$

The Rf values of the standards were plotted against the log of their molecular weights using the Cricket Software program (Cricket Software Inc., Malvern, PA.) on a Macintosh SE computer. This program provided a formula for slope of this line, and by insertion of a unknown Rf value, a log value of the unknown molecular weight could be obtained. Conversion of the log M.W., using the antilog function (10^x) gave the molecular weight of any unknown band.

3:11) ELISA ASSAYS

11:I) WHOLE CELL SUSPENSION PREPARATION

Cultures of *P. asaccharolyticus* (BM4), *B. fragilis* NCTC 8560, *P. melaninogenica* ATCC 25845, *P. gingivalis* 18/10 and *P. intermedia* BH18/23 were grown anaerobically for 4 days on supplemented blood agar. The cells were harvested and suspended in 4 ml. of sterile saline. Several drops of formaldehyde 37%w/w (Fisher Scientific , Fair Lawn, N.J.) were added to each culture suspension to stabilize the cells.

11:II) DETERMINATION OF CELL SUSPENSION CONCENTRATION

Polycarbonate filters (Nucleopore - .22 microns) were placed in Petri plates and dried in a conventional hot air oven. The dried filters were quickly weighed, prior to

their rehydration upon exposure to room air. The weighed filter was then placed on top of a nitrocellulose 0.45 micron support filter and connected to a vacuum. The suspension of cells was vortexed, and 100 microliters of suspension was gently placed onto the polycarbonate filter. The filters were rinsed twice with distilled water; the membrane removed from the suction unit, and placed into the oven.

The weight of the dry cells plus membrane was determined, and from this a concentration of cells (mg/ml) of suspension was calculated. The original cell suspension was added to coating buffer (0.05M carbonate buffer, pH 9.6 + 0.02%NaN_3)(Appendix B) to obtain a concentration of 10 mg/ml,which was then used to coat plates for the ELISA assay.

11:III) COATING OF ELISA PLATES - WHOLE CELLS

Attachment of the cells to the flat bottomed polystyrene microtiter plates (Immulon-Dynatech Lab Inc. Chantilly VA) was achieved by pipetting 100 µl. of prepared cell suspension in coating buffer (100 µg/ml.) into the appropriate wells. This concentration had been previously found to give optimal coating of wells by whole cells of microorganisms (Cole and Ciardi, 1983). The plates were sealed with plastic wrap and placed in 4°C refrigerator overnight, or until required.

11:III) COATING OF ELISA PLATES - OUTER MEMBRANES

Attachment to the flat bottomed polystyrene microtiter plate (Immulon-Dynatech Lab Inc. Chantilly VA) of the outer membrane preparations was achieved by pipetting 100 µl. of outer membranes in coating buffer (10 ug/ml.) into the appropriate wells. This concentration had been previously found to give optimal coating of wells by outer membranes of microorganisms. The plates were sealed with plastic wrap and placed at 4°C overnight, or until required.

11:V) ELISA ASSAY

Once plates were sensitized by antigen, the coating/cell suspension solution was gently tipped off, and the plates rinsed three times with 299 µl. of blocking buffer (0.1% BSA + 0.02% NaN_3 in Phosphate buffer saline, pH 8.0)(Appendix B) to block nonspecific binding. Blocking of the plates was accomplished by leaving the final rinse of blocking buffer in the wells for 1 hour at room temperature.

The appropriate dilution of mouse first antibody was prepared with PBS-Tween, just prior to use (1:25-1:12,800). Dilutions of sera to be tested were prepared in PBS-Tween (0.1% Tween 20-polyoxyethylenesorbitan monolaurate + 0.02% NaN_3 in PBS, pH 8.0)(Appendix B) just prior to commencement of the assay. One hundred microliters of the serum dilutions were dispensed into the wells, sealed with plastic wrap and incubated on a shaker at room temperature for 1 hour. The serum dilutions were gently shaken out of the wells after incubation, and the wells rinsed three times with 299 µl. of PBS-Tween.

Horse-radish peroxidase (HRP) conjugate (Peroxidase conjugated affinipure goat anti-mouse IgM, µ chain specific-Jackson Immunoresearch Lab, West Grove, PA, or Peroxidase-conjugated affinipure goat anti-mouse IgG, Fc fragment specific, Jackson Immunoresearch Lab, Inc. West Grove, PA) was mixed with horse-radish peroxidase diluent (0.1% BSA in PBS, pH 8.0, no NaN_3)(Appendix B) just prior to assay. This solution was kept in the dark to prevent any reactivity to light. One hundred microliters of the HRP conjugate (1:500-1:10,000) was added to each well, sealed, covered to prevent exposure to light and incubated for 1 hour at room temperature. The plates were once again rinsed three times with 299 µl. of PBS-Tween.

One hundred microliters of substrate solution (citrate-phosphate buffer- 4 parts 0.1M citric acid with 6 parts of 0.2 Na_2HPO_4-pH4.5 + 1 mg/ml of ortho-phenylenediamine + 0.012% H_2O_2)(Appendix B) was pipetted into each well, and the optical density of the plates read at approximately two minute intervals, or until such

time as an adequate color developed. Incubation of the plates with substrate was performed at room temperature in the dark, on a gentle shaker. Optical density was read at 450 nm. in a Titertek Multiscan spectrophotometer (Flow Laboratories, Helsinki, Finland).

11:VI) CONTROL OF REAGENTS

Nonspecific background binding was measured by incubating cells with conjugate (anti-mouse) and substrate, but no serum. Each assay included a positive control, consisting of conjugate and substrate alone, and a negative control, consisting of cells, substrate and serum, but no conjugate. Duplicate determinations were made for each sample on the same plate. The optimal working dilution of the HRP conjugate was determined by serial dilutions in preliminary assays.

Background optical density readings to all organisms, with the exception of *P. gingivalis* were low, ranging from 0.0015 to 0.126. The horse-radish peroxidase (HRP) controls to *P. gingivalis* (with no mouse serum) showed substantial background staining with both IgG and IgM (optical densities ranging from 0.173 to 0.625 units). These control values were high, yet lower values (ranging from .022-.1295 optical density units) were recorded for wells containing mouse sera and the HRP anti-Ig. A possible explanation for this phenomenon of high control values, is that the mouse serum sterically inhibited the attachment of the HRP anti-mouse Ig to the antigen. In the wells, without mouse serum, the HRP anti-mouse Ig could bind directly to the cells/outer membranes, giving an O.D. of higher magnitude compared to that of wells containing mouse serum.

To minimize this phenomenon of a high background to *P. gingivalis*, the HRP anti-mouse IgG and IgM were absorbed with whole cells of *P. gingivalis*. The cells were removed by filtration (0.45 micron filter) and centrifugation (10,1000 RPM for 5

minutes). Results using the absorbed HRP-IgG and IgM showed background of negligible value, and somewhat decreased the O.D. values of wells containing mouse sera.

11:VII) CALCULATION OF OPTICAL DENSITY

ELISA plates were read using the Titerek Multiskan spectrophotometer, which produced a computer printout of all optical density values obtained during a plate readout at a given time interval. Optical density values ranged from a 0.00 value to 3.00.

The average value for each set of duplicate samples was calculated, subtracting the optical density average obtained by the control wells containing only HRP IgG or IgM conjugate.

11:VIII) STATISTICAL ANALYSIS

Statistical analysis of the data obtained from the ELISA assays of individual mouse sera from Groups 36 and 37 (individual animals) employed a 2 sample student t-test, capable of showing a statistically significant difference between the immunized and non-immunized individual animals.

CHAPTER 4

RESULTS

4:1) PRESENCE OF NATURAL ANTIBODY TO *PREVOTELLA, BACTEROIDES* AND *PORPHYROMONAS* IN MICE (C57BL/6 AND BALB/C)

1:1) CONTROL OF REAGENTS - NONSPECIFIC BINDING OF ANTI-MOUSE IgG and IgM

The extent of non-specific binding by horse-radish peroxidase affini-pure goat anti-mouse IgG and IgM to antigens present in outer membranes *Prevotella, Bacteroides* and *Porphyromonas* species was determined by reacting the anti-mouse IgG or IgM conjugate (2nd antibody) with the Western blots of the various species, in the absence of mouse first antibody. Analysis of affini-pure reagents showed a significant amount of non-specific cross-reactivity with antigens present in the outer membrane profiles (Table 4.1). Both anti-mouse IgG and IgM had non-specific binding ability to all outer membranes tested, with the exception of *P. intermedia* (BH20/30). For this reason, all Western blots from immunization experiments contained a single control lane, which was exposed to only the anti-mouse IgG or IgM conjugate.

Variations over time could be seen in the pattern of non-specific recognition by the anti-mouse IgG and IgM between Western blots of membranes of the same species. This difference in the intensity of band recognition could be explained by such factors as: a difference in amount of outer membrane preparation loaded into gel wells, length of transfer time to the PVDF membrane or deterioration of the anti-mouse conjugate over time.

Absorption of the goat anti-mouse IgG and IgM conjugate was not done, as absorption of the conjugate by whole cells of cross-reacting strains could remove antibody against mouse IgG and IgM and it was considered better to include controls onto each blot (see above).

Table 4.1 Antigens (kDa) of *Prevotella, Bacteroides*, and *Porphyromonas* Outer Membranes Recognized by Non-specific Binding of Anti-Mouse IgG or IgM Conjugates Detected by Western Blotting

Outer Membrane Antigens	IgG	IgM
P. intermedia (BH18/23)	16 31 44	16 31 44
P. intermedia (BH20/30)	- -	- -
P. melaninogenica (25845)	17 41	17 41
P. denticola (33185)	19	19
B. fragilis (8560)	16.5	16.5
P. loescheii (15930)	14 96 >100	14 96 >100
P. corporis (33547)	45 68	45 68
P. asaccharolyticus (25260)	28 68 93 >100	28 68 93 >100
P. gingivalis (18/10)	21 22 96 98	21 22 96 98
P. oralis (33269)	16.5 32	16.5 32
P. buccae (33547)	18 32	18 32

1:II) NATURAL ANTIBODY TO OUTER MEMBRANES PRESENT IN SERUM FROM CONTROL MICE (C57BL/6)

Non-immunized mice (control mice) possessed natural antibodies recognizing various antigens in the outer membrane profiles of *Prevotella*, *Bacteroides* and *Porphyromonas* species (Table 4.2). Several of these antigens recognized by control mice were identical to those detected by non-specific binding of goat anti-mouse IgG or IgM conjugate in the absence of mouse serum. However, when these were detected by sera from control mice, they were often of greater intensity compared to that seen by the non-specific binding of the conjugate alone, indicating that control mice carry antibody of higher titre or avidity to the respective antigen. Similarity in patterns of band recognition by non-specific binding of the goat anti-mouse serum and the control mouse serum would suggest that these antigens are part of the natural antibody repertoire of both animals and possibly are generated by each animal in response to similar microorganisms resident in the gut flora. It should be noted however, that Balb/c mice have higher levels of natural antibody (IgM) to outer membranes. This was shown by control mice (Groups M16 and M30, Tables 4.15, 4.16, 4.17).

1:III) NATURAL ANTIBODY TO OUTER MEMBRANES PRESENT IN SERUM FROM GERM-FREE MICE (NIH STRAIN)

Serum from germ-free mice (NIH strain) was reacted with outer membranes of *Prevotella*, *Bacteroides* and *Porphyromonas* species. Although these mice are germ-free, given sterilized foods and kept in special germ-free environments, weak reactions to some antigens in the outer membrane profiles were identified at a 1:100 mouse serum dilution (Table 4.3). Germ-free mouse sera detected similar antigens to those recognized by the goat anti-mouse IgG and IgM conjugate and the control mouse natural antibody (Table 4.4). The control immunoblots developed with only the goat anti-mouse serum (which was identical to that used in previous experiments) did not demonstrate

Table 4.2 Outer Membrane Antigens (kDa) Recognized by IgG and IgM in Control Mouse Sera (C57BL/6) Detected by Western Blotting.

Outer Membrane Antigens	IgG	IgM
P. intermedia (BH18/23)	16 31 44	16 31 44
P. intermedia (BH20/30)	67	67
P. melaninogenica (25845)	17 41	17 24 32 41
P. denticola (33185)	19 26 29 40	19 26 29 40 45 47
B. fragilis (8560)	16.5	16.5
P. loescheii (15930)	14 33 46 50 96 >100	14 96 >100
P. corporis (33547)	27 29 45 63 68	27 29 45 63 68
P. asaccharolyticus (25260)	28 86 93 >100	28 68 93 >100
P. gingivalis (18/10)	21 22 24 96 98	21 22 24 96 98
P. oralis (33269)	16.5 32 52	16.5 32 41 45 52 65
P. buccae (33547)	18 32 73	18 32 73

Table 4.3 Antigens (kDa) of *Prevotella, Bacteroides*, and *Porphyromonas* Outer Membranes Recognized by Antibodies from Germ-free NIH Mouse Serum (1:100) Detected by Western Blotting

Outer Membrane Antigens	IgG	IgM
P. intermedia (BH18/23)	- -	- -
P. intermedia (BH20/30)	- -	- -
P. melaninogenica (25845)	- -	- -
P. denticola (33185)	19*	19*
B. fragilis (8560)	- -	- -
P. loescheii (15930)	- -	- -
P. corporis (33547)	- -	- -
P. asaccharolyticus (25260)	28* 68* 93*	28* 68* 93*
P. gingivalis (18/10)	24 96* 98*	24 96* 98*
P. oralis (33269)	 32* 45 52	27 32* 45 52
P. buccae (33547)	18*	18*

* - Band intensity much greater than that seen by nonspecific binding of goat anti-mouse conjugate

Table 4.4 Comparison of Antigens Recognized by Goat Anti-Mouse IgG and IgM Conjugate, C57BL/6 Mouse Natural Antibodies (Control Mice) and Germ-free NIH Strain Mouse Sera, as Detected by Western Blotting

	Non-specific Binding by Goat Anti-Mouse Conjugate		Control Mice (C57BL/6)		Germ-free Mice (NIH strain)	
Outer Membrane Antigens	IgG	IgM	IgG	IgM	IgG	IgM
P. intermedia (BH18/23)	16 31 44	16 31 44	16 31 44	16 31 44	- -	- -
P. intermedia (BH20/30)	- -	- -	67	67	- -	- -
P. mel. (25845)	17 41	17 41	17* 41*	17* 24 32 41*	- -	- -
P. denticola (33185)	19	19	19* 26 29 40	 26 29 40 45 47	19*	19*
B. fragilis (8560)	16.5	16.5	16.5*	16.5*	- -	- -
P. loescheii (15930)	14 96 >100	14 96 >100	14 33 46 50 96* >100*	14 33 46 50 96* >100*	- -	- -
P. corporis (33547)	 45 68	 45 68	27 29 45* 63 68*	27 29 45* 63 68*	- -	- -
P. asacch. (25260)	28 68 93 >100	28 68 93 >100	28 68* 93* >100*	28 68* 93* >100*	28* 68* 93*	28* 68* 93*
P. gingivalis (18/10)	21 22 96 98	21 22 96 98	21 22 24 96* 98*	21 22 24 96* 98*	 24* 96* 98*	 24* 96* 98*

Table 4.4 Cont.

P. oralis (33269)	16.5 32	16.5 32	16.5 32 52	16.5 32 41 45* 52 65	32* 45 52	27 32* 45 52
P. buccae (33547)	18 32	18 32	18 32	18 32 73	18*	18*

* - Band Intensity greater than attributed by non-specific Binding by Goat Anti-mouse Conjugate alone

non-specific binding to antigens present in the outer membranes of *P. intermedia* BH18/23, *P. intermedia* 20/30, *P. melaninogenica*, *P. denticola*, *P. loescheii*, *P. gingivalis* and *B. fragilis*. The outer membrane profiles developed with germ-free mouse sera were negative to outer membranes of *P. intermedia* (BH18/23), *P. intermedia* (BH20/30), *P. melaninogenica*, *B. fragilis*, *P. loescheii*, *P. corporis*. In contrast, serum from germ-free mice detected antigens in the outer membranes of *P. denticola*, *P. asaccharolyticus*, *P. gingivalis*, *P. oralis* and *P. buccae*. In all cases, except for *P. oralis* and *P. gingivalis*, the bands recognized were identical to those resulting from non-specific binding of the goat anti-mouse IgG and IgM conjugates, but of greater intensity. One additional unique band (27 kDa) was recognized in the outer membrane profile of *P. oralis* by the germ-free mouse serum. The generation of some antibody by germ-free mice can possibly be attributed to a response to food antigens.

4:2) SIMILARITY OF OUTER MEMBRANE ANTIGEN RECOGNITION BY MOUSE, RABBIT, CHILD AND ADULT HUMAN SERA.

Immunoblots of outer membrane antigens of *Prevotella*, *Bacteroides* and *Porphyromonas* species developed by immune mouse sera (M3), hyperimmune rabbit sera, child and adult human sera were compared for similarity of antigen recognition. Mouse serum (M3) used in this analysis was produced by immunization of C57BL/6 mice with 10^6-10^7 viable cells of *P. intermedia* BH18/23, at day 0 followed by a similar booster immunization at day 10. To show the range of antigens present in outer membrane preparations, Western blots were developed with sera from rabbits hyperimmunized with *P. intermedia* BH18/23.

Immunoblots developed with C57BL/6 mouse sera (M3) showed the simplest pattern of antigen recognition, detecting 7 major bands in the *P. intermedia* BH18/23 outer membrane preparation. Human child serum developed 10 bands in the *P. intermedia* outer membrane immunoblot, six of which were common to the mouse serum

profile (Table 4.5)(Figure 4.1). Pooled human adult serum from periodontal patients with varying clinical diagnoses detected 12 antigens in the profile, including the 10 detected by children's serum. Two additional bands (36, 38 kDa), which were not developed by the children's serum, were recognized by the adult serum (Table 4.5). Although the bands developed by the pooled child serum were identical in molecular weight to those detected by human adult serum, children's sera often gave a weaker response to the antigen.

The M3 sera (produced by immunization of mice with *P. intermedia*) also developed antigenic bands in outer membrane immunoblots of *P. loescheii* (33, 84, >100 kDa) and *B. fragilis* (16.5 kDa), which were the same molecular weight as those detected in these species by human adult and child sera. In contrast to mouse and human sera, the hyperimmune rabbit serum developed a complicated pattern of antigenic bands in the *P. intermedia* profile, suggesting that the use of hyperimmune serum may not be valid for comparison in studies of the immune response in clinical disease situations. Mouse sera (M3) showed closely similar patterns of antigen recognition to those from child and adult, in *P. intermedia* BH18/23 profiles and other selected *Prevotella* and *Bacteroides* species (*P. loescheii*, *P. corporis*, *B. fragilis*, *P. buccae*), although the pattern in mice was more simple with fewer bands recognized. Table 4.5 shows the various antigen bands in the outer membrane profiles recognized by C57BL/6 mouse serum, human child and adult serum and hyperimmune rabbit serum. The close similarities between the molecular masses of the antigens detected by the mouse and human serum, suggest that the immune response in the mouse recognizes similar antigens of *Prevotella intermedia* to the response in young and adult humans.

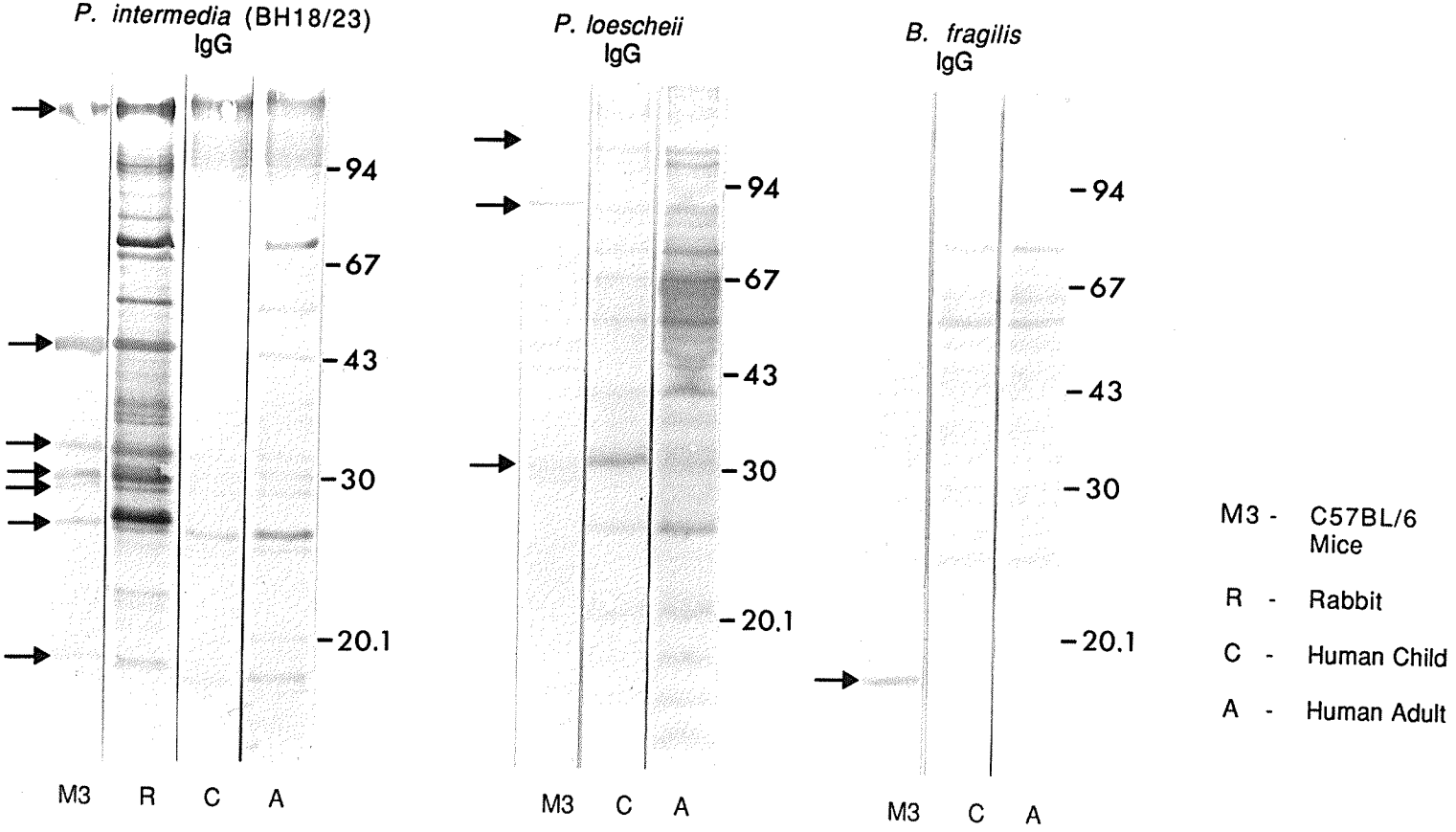
Table 4.5 Antigens (kDa) of Outer Membrane Recognized by Immune Mouse (M3), Hyperimmune Rabbit, Human Child and Adult IgG (Figure 4.1)

P. intermedia BH18/23				*P. loescheii* 15930			B. fragilis 8560		
M3	R	C	A	M3	C	A	M3	C	A
16	16	16	16		14	14	16.5	16.5	16.5
		20	20		16	16			19
	23				26	26		25	25
26	26	26	26	33	33	33		27	27
		28	28		39	39		28	28
29	29				55	55		29	29
31	31	31	31		67	67		53	53
34	34	34	34		74	74		64	64
	36		36	84	84	84		76	76
	38		38	>100	>100	>100			
44	44	44	44						
	56	56	56						
	69								
	73	73	73						
	80								
	85								
>100	>100	>100	>100						

M3 - Mouse serum
R - Hyperimmune Rabbit Serum
C - Human Child Serum
A - Human Adult Serum

FIGURE 4.1

SIMILARITY OF OUTER MEMBRANE PROTEINS OF *PREVOTELLA*, *BACTEROIDES*, AND *PORPHYROMONAS* RECOGNIZED BY C57BL/6 MOUSE, RABBIT, CHILD AND ADULT HUMAN SERA.



4:3) WESTERN BLOT ANALYSIS OF THE OUTER MEMBRANE ANTIGENS OF *PREVOTELLA, BACTEROIDES* AND *PORPHYROMONAS* RECOGNIZED BY SERA FROM MICE IMMUNIZED WITH *PREVOTELLA INTERMEDIA* BH18/23

3:1) Low Dose (10^3-10^4 viable cells) Immunization of 2-3 week old C57BL/6 mice (GROUPS M5-8)

Three groups of C57BL/6 mice were immunized with a low dose of viable *P. intermedia* BH18/23 cells: i.e.

M5: one injection of 10^3-10^4 viable cells I.V., sacrificed after 10 days,

M6: one injection of 10^3-10^4 viable cells I.V., sacrificed after 20 days,

M7: two injections of 10^3-10^4 viable cells at day 0 and day 10, sacrificed after 20 days,

M8: control group: no injections-sacrificed after 20 days.

Immunization of the C57BL/6 mice with one low dose of viable *P. intermedia* cells (Groups M5 and M6) over a period of 10 or 20 days, did not generate IgG or IgM antibodies capable of recognizing new antigens in the *Prevotella*, *Bacteroides*, or *Porphyromonas* outer membranes. However, immunization with a low dose of viable *P. intermedia* cells, followed by a booster injection, produced antibodies to antigens in both the homologous organism (26, 34 and >100 kDa) and *P. asaccharolyticus* (65 kDa antigen - IgG), which were not recognized prior to immunization (Table 4.6). No cross-reactivity to other strains of *Prevotella*, *Bacteroides*, or *Porphyromonas* resulted from immunization of the young mice with either a single low dose, or booster dose of viable cells.

Note: All tables showing results from immunization experiments will exclude any non-specific binding antigens or antigens present in control animals, unless the intensity of band recognition is significantly higher than that developed by the control sera

Table 4.6 Antigens (kDa) of *Prevotella*, *Bacteroides* and *Porphyromonas* Recognized by C57BL/6 Mouse Antibody Following Low Dose Immunization with *P. intermedia* BH18/23 (Groups M5-8)

Outer Membrane Preparations	IgG	IgM
P. intermedia (BH18/23)	26 34 >100	>100
P. intermedia (BH20/30)	- -	- -
P. melaninogenica (25845)	- -	- -
P. denticola (33185)	- -	- -
B. fragilis (8560)	- -	- -
P. loescheii (15930)	- -	- -
P. corporis (33547)	- -	- -
P. asaccharolyticus (25260)	65	- -
P. gingivalis (18/10)	- -	- -
P. oralis (33269)	- -	- -
P. buccae (33547)	- -	- -

NOTE: Antigens recognized by control mouse serum or by goat anti-mouse conjugate non-specific binding are not included in this table.

3:II) Antigens Detected by Sera from Two Genetically Different Mouse Strains (C57BL/6 and Balb/c) Immunized with a Low Dose of *P. intermedia* BH18/23 (Groups M9-16)

Two genetically different, inbred mouse strains - C57BL/6 and Balb/c (4-5 weeks old) were immunized with a low dose of viable *P. intermedia* cells (10^3-10^4 cells) to study the effect genetic makeup of an animal would present on formation of antibody.

The C57BL/6 mice immunization groups were designated as follows:

M9: one injection of 10^3-10^4 viable cells I.V., sacrificed after 10 days,

M10: one injection of 10^3-10^4 viable cells I.V., sacrificed after 20 days,

M11: two injections of 10^3-10^4 viable cells at day 0 and day 10, sacrificed after 20 days

M12: control group: no injections-sacrificed after 20 days,

The Balb/c mouse groups were labelled:

M13: one injection of 10^3-10^4 viable cells I.V., sacrificed after 10 days,

M14: one injection of 10^3-10^4 viable cells I.V., sacrificed after 20 days,

M15: two injections of 10^3-10^4 viable cells at day 0 and day 10, sacrificed after 20 days,

M16: control group: no injections-sacrificed after 20 days.

This low dose immunization of both mouse strains did not produce sera capable of recognizing new bands in the outer membrane profiles of the *Prevotella*, *Bacteroides* or *Porphyromonas* outer membranes, differing from antigens recognized by control mouse sera. In addition, no significant differences were seen in the intensity of antigen band development between the various immunization groups, i.e. M9-11, or M13-15, indicating that low dose immunization even with a booster dose did not produce a significant impact on development of new antibodies.

Although there was no generation of new antibody from the immunization experiment, differences in the general pattern of recognition outer membrane antigens between mouse strains (C57BL/6 and Balb/c) were evident in *P. corporis* (IgG), *P. intermedia* BH18/23 (IgM), *P. intermedia* 20/30 (IgM), *P. melaninogenica* (IgM), *P. denticola* (IgM), *B. fragilis* (IgM), *P. corporis* (IgM), *P. oralis* (IgM) and *P. buccae* (IgM)(Tables 4.7 and 4.8). The most pronounced difference seen was in the IgM profile of C57BL/6 mice to *P. loescheii*, where a 50 kDa pair of antigens showed a strong reaction, compared to the intensity seen in the immunoblot developed by Balb/c serum (Figure 4.2).

3:III) High dose (10^6-10^7 viable cells) Immunization of C57BL/6 Mice (Groups M1-4)

Three groups of C57BL/6 mice (4-5 weeks old) were immunized with a high dose of viable *P. intermedia* BH18/23 cells: i.e.

M1: one injection of 10^6-10^7 viable cells I.V., sacrificed after 10 days,

M2: one injection of 10^6-10^7 viable cells I.V., sacrificed after 20 days,

M3: two injections of 10^6-10^7 viable cells at day 0 and day 10, sacrificed after 20 days,

M4: control group: no injections-sacrificed after 20 days.

Western blotting of outer membranes of *Prevotella*, *Bacteroides* and *Porphyromonas* showed that immunization of C57BL/6 mice with a high dose (10^6-10^7 viable cells) of *P. intermedia* BH18/23 (M1-4 sera) stimulated the formation of antibody (IgG and IgM) recognizing specific antigen bands in the outer membrane of the homologous organism (Table 4.9)(Figure 4.3). Figure 4.3 shows that a single immunization of the M1 group, stimulated IgG antibody formation to the 26, 31, 34, 44 and 100 kDa and IgM antibody to antigens >100 kDa. A single immunization with 10^6-10^7 viable cells, followed by a rest period of 20 days (M2), showed stronger

Table 4.7 Differences in Intra-species (C57BL/6 and Balb/c) Mouse IgG Recognition of Antigens (kDa) in Outer Membrane Profiles of *Prevotella*, *Bacteroides* and *Porphyromonas*, Detected by Western Blotting. (Groups M9-16).

Outer Membrane Antigens	IgG C57BL/6 mice	IgG Balb/c mice
P. intermedia (BH18/23)	- -	- -
P. intermedia (BH20/30)	- -	- -
P. melaninogenica (25845)	- -	- -
P. denticola (33185)	- -	- -
B. fragilis (8560)	- -	- -
P. loescheii (15930)	- -	- -
P. corporis (33547)	65	53 55
P. asaccharolyticus (25260)	- -	- -
P. gingivalis (18/10)	- -	- -
P. oralis (33269)	- -	- -
P. buccae (33547)	- -	- -

NOTE: Antigens recognized by control mouse serum or by goat anti-mouse conjugate non-specific binding are not included in this table.

Table 4.8 Differences in Intra-species (C57BL/6 and Balb/c) Mouse IgM Recognition of Antigens (kDa) in Outer Membrane Profiles of *Prevotella*, *Bacteroides* and *Porphyromonas*, Detected by Western Blotting. (Groups M9-16).

Outer Membrane Antigens	IgM C57BL/6 mice	IgM Balb/c mice
P. intermedia (BH18/23)	- -	- -
P. intermedia (BH20/30)	- -	- -
P. melaninogenica (25845)	- -	15
P. denticola (33185)	- -	- -
B. fragilis (8560)	- -	28 75
P. loescheii (15930)	50[a]	- -
P. corporis (33547)	73	- -
P. asaccharolyticus (25260)	- -	- -
P. gingivalis (18/10)	- -	- -
P. oralis (33269)	- -	32[b] 52[b]
P. buccae (33547)	- -	45 60 68

[a] - Antigen recognized by both C57/BL6 and Balb/c mice, although intensity of band recognition was significantly greater in the C57BL/6 mouse sera

[b] - Antigen recognized by both C57/BL6 and Balb/c mice, although intensity of band recognition was significantly greater in the Balb/c mouse sera (antigen present in both Balb/c control and immunized mouse groups)

NOTE: Antigens recognized by control mouse serum or by goat anti-mouse conjugate non-specific binding are not included in this table.

FIGURE 4.2

RECOGNITION OF OUTER MEMBRANE ANTIGENS OF *PREVOTELLA* AND *BACTEROIDES* BY C57BL/6 and BALB/C MOUSE IgM ANTIBODY.

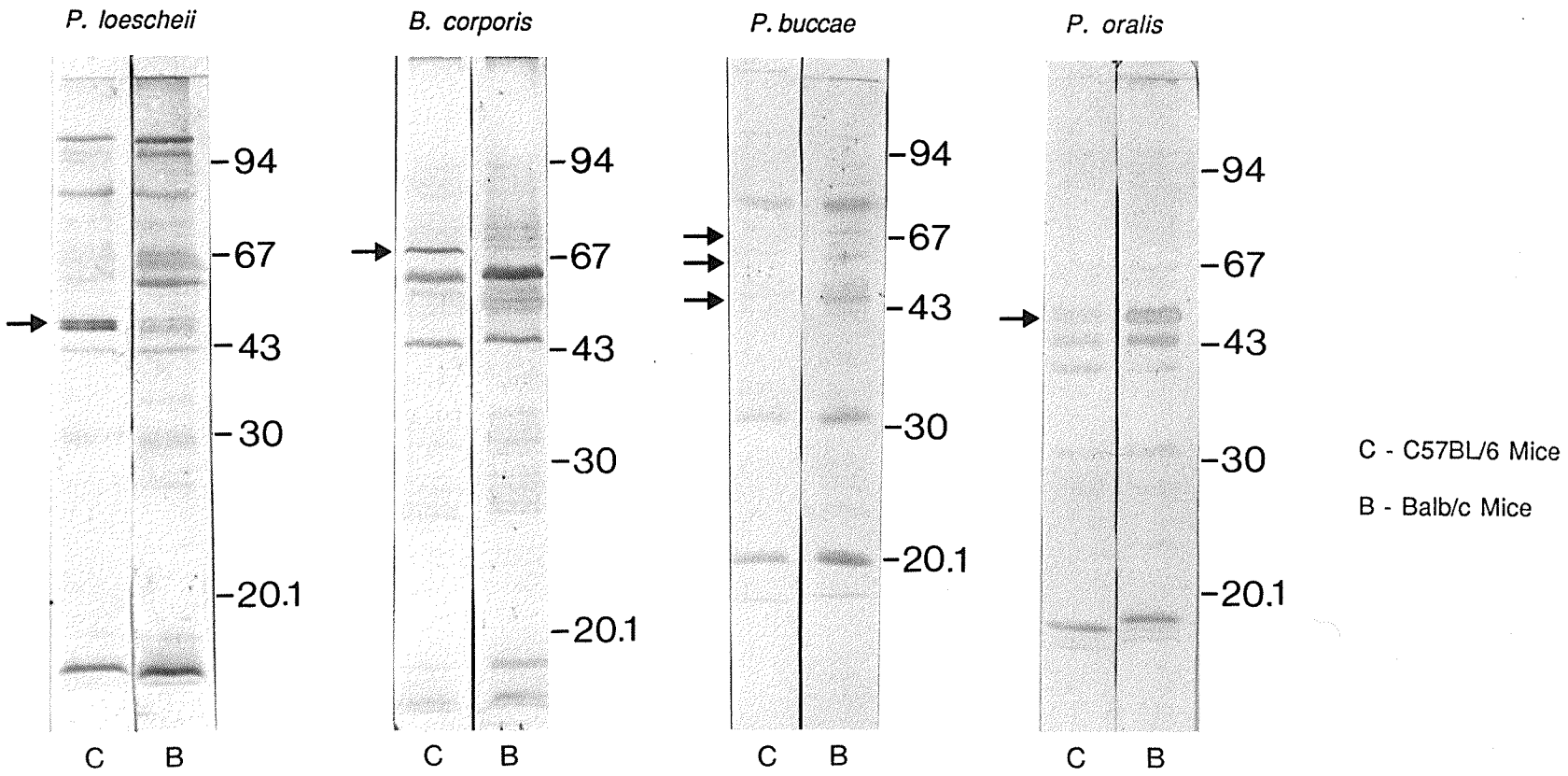


Table 4.9 Antigens (kDa) of *Prevotella*, *Bacteroides* and *Porphyromonas* Recognized by C57BL/6 Mouse Serum Following High Dose immunization with *P. intermedia* BH18/23 (Groups M1-4)

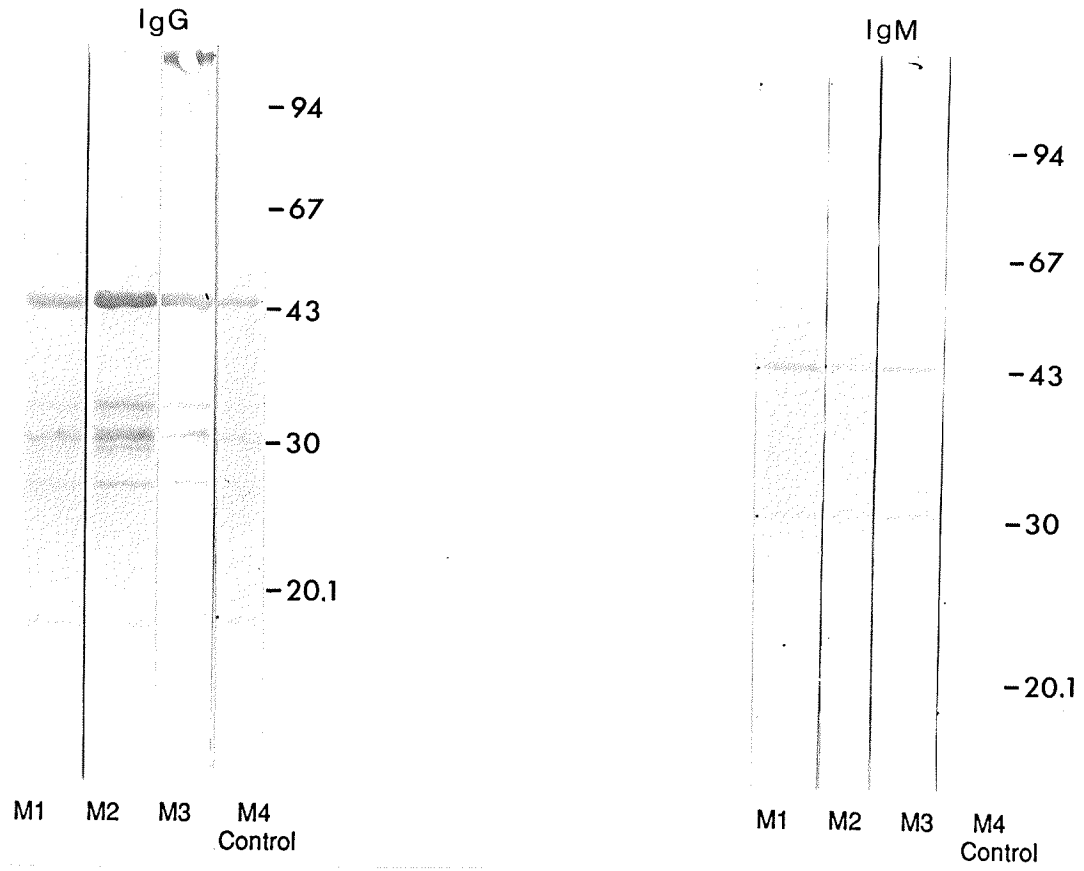
Outer Membrane Antigens	IgG	IgM
P. intermedia (BH18/23)	26 31* 34 44* >100	>100
P. intermedia (BH20/30)	- -	- -
P. melaninogenica (25845)	- -	- -
P. denticola (33185)	- -	- -
B. fragilis (8560)	- -	- -
P. loescheii (15930)	- -	- -
P. corporis (33547)	- -	- -
P. asaccharolyticus (25260)	- -	- -
P. gingivalis (18/10)	- -	- -
P. oralis (33269)	- -	- -
P. buccae (33547)	- -	- -

* -Intensity of antigen band recognition is significantly greater than seen in control mouse sera

NOTE: Antigens recognized by control mouse serum or by goat anti-mouse conjugate non-specific binding are not included in this table.

FIGURE 4.3

C57BL/6 MOUSE ANTIBODY AGAINST *PREVOTELLA INTERMEDIA* (BH18/23) OUTER MEMBRANE ANTIGENS.



responses by IgG to the previously mentioned antigens (Table 4.9). The administration of a booster dose of similar number of cells, 10 days following the initial immunization (M3) further intensified the recognition of antigens in both IgG and IgM profiles (Figure 4.3).

3:IV) High dose (10^6-10^7 viable cells) Immunization of Balb/c mice (Groups M27-30)

Three groups of Balb/c mice were immunized with a high dose of viable *P. intermedia* BH18/23 cells: i.e.

M27: one injection of 10^6-10^7 viable cells I.V., sacrificed after 10 days,

M28: one injection of 10^6-10^7 viable cells I.V., sacrificed after 20 days,

M29: two injections of 10^6-10^7 viable cells at day 0 and day 10, sacrificed after 20 days,

M30: control group: no injections-sacrificed after 20 days.

Immunization of Balb/c mice (4-5 weeks old) with one high dose of *P. intermedia* BH18/23 (Group M27 - 10 days) stimulated the formation of antibody (IgG and IgM) recognizing specific bands in outer membrane profiles, not seen prior to immunization. Immunization of mice with one high dose of viable cells followed by a period of 20 days (Group 28) produced a stronger response to these same antigens. A booster immunization at day 10 (group M29) further accentuated the recognition of the same antigens as those detected by sera from groups M27 and M28.

Table 4.10 shows the antigens bands recognized in the outer membrane of the homologous organism *P. intermedia* BH18/23 - (IgG - 26, 34, 44, 76, >100 and IgM - 36 and >100 kDa), as well as cross-reactive responses to the outer membranes of *P. intermedia* BH20/30, *P. melaninogenica*, and *P. denticol*a. Several of the cross-reactive

Table 4.10 Antigens (kDa) of Outer Membranes Recognized by Balb/c Mouse Sera Following High Dose Immunization with of *P. intermedia* BH18/23 (Groups M27-30)

Outer Membrane Antigens	IgG	IgM
P. intermedia (BH18/23)	26 31* 34 44* 76 >100	 34 36 >100
P. intermedia (BH20/30)	 43 >100	34
P. melaninogenica (25845)	32* 41*	32* 41*
P. denticola (33185)	29* 40*	 32 40*
B. fragilis (8560)	- -	- -
P. loescheii (15930)	- -	- -
P. corporis (33547)	- -	- -
P. asaccharolyticus (25260)	- -	- -
P. gingivalis (18/10)	- -	- -
P. oralis (33269)	- -	- -
P. buccae (33547)	- -	- -

* - Intensity of antigen band recognition is significantly greater than seen in control mouse sera

NOTE: Antigens recognized by control mouse serum or by goat anti-mouse conjugate non-specific binding are not included in this table.

antigens recognized in the outer membranes of *P. melaninogenica* (IgG - 32 and 41 kDa; IgM - 32 and 41 kDa) and *P. denticola* (IgG - 29 and 40 kDa; IgM - 32 and 40 kDa) were those detected by sera from the control mouse group (M30), but there was a significant increase in the reactivity of the antibody to these antigens after immunization (Figure 4.4).

3:V) Response to Immunization of C57BL/6 and Balb/c Mice with A Single Injection of *P. intermedia* BH18/23 together with Freund's Complete Adjuvant (Groups M17-26)

Four to five week old C57BL/6 mice and Balb/c mice were immunized with a single injection of 10^6-10^7 viable cells of *P. intermedia* BH18/23 together with Freund's complete adjuvant, and sacrificed at various time intervals. The C57BL/6 mouse groups consisted of:

M17: one injection of 10^6-10^7 viable cells together with FCA I.M., sacrificed after 4 days,

M18: one injection of 10^6-10^7 viable cells together with FCA I.M., sacrificed after 17 days,

M19: one injection of 10^6-10^7 viable cells together with FCA I.M., sacrificed after 32 days,

M20: control - one injection of FCA and saline I.M., sacrificed after 32 days, and

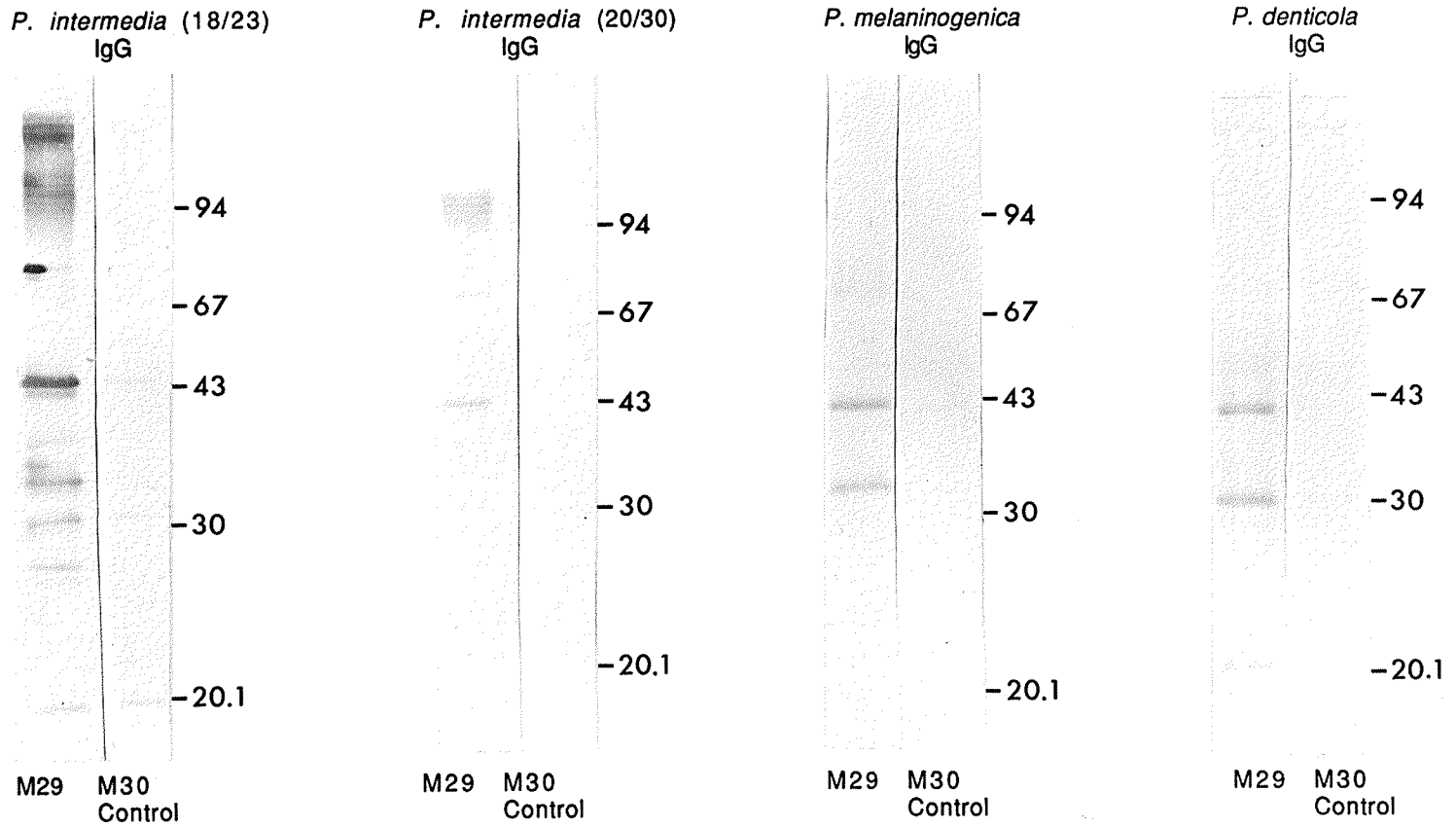
M21: control - no injections-sacrificed after 32 days.

Balb/c mice were immunized in the same manner as outlined above (group designations from M22-M26).

The intramuscular immunization of mice with 10^6-10^7 viable cells of *P. intermedia* BH18/23 together with Freund's Complete Adjuvant generated IgG and IgM antibody, which detected antigens in outer membranes of both homologous organism and

FIGURE 4.4

ANTIGENS RECOGNIZED BY ANTIBODY FROM BALB/C MICE FOLLOWING IMMUNIZATION WITH *PREVOTELLA INTERMEDIA* BH18/23.



cross-reactive organisms (*P. denticola* - IgG and IgM [C57BL/6], *B. fragilis* - IgG and IgM [C57BL/6] and IgM [Balb/c], and *P. loescheii* - IgG [C57BL/6]), which were not recognized by control mice (Table 4.11)(Figure (4.5). One antigen to *P. intermedia* BH18/23 was recognized by C57BL/6 IgM, while three antigens were developed by the IgG. In comparison, Balb/c IgM to *P. intermedia* BH18/23 recognized 1 additional antigen, with IgG developing 6 antigens. Figure 4.5 shows the unique antigens recognized by IgG and IgM (M19 and M24) in response to immunization with *P. intermedia* together with Freund's Complete Adjuvant. It was apparent that there were differences between the two mouse strains. C57BL/6 mouse serum recognized a range of antigens in *P. denticola* and *P. loescheii*, which were not detected by Balb/c mice. Immune serum from Balb/c mice detected more antigens in *P. intermedia* (18/23) and *B. fragilis* outer membranes.

Antibody production in response to a single immunization with *P. intermedia* and Freund's Complete Adjuvant, increased over the experimental period of time with the strongest recognition of antigens seen by the M19 (C57BL/6) and M24 (Balb/c) sera.

3:VI) Chronic Low Dose Immunization of C57BL/6 Mice with 10^3-10^4 viable cells of *P. intermedia* BH18/23 over a Period of 6 weeks.(Groups M31-32)

It was thought that components of the resident flora of a host might provide a consistent low dose stimulation of the immune system. An attempt to reproduce this situation in the mouse was made by giving a series of low dose injections.

C57BL/6 mice were immunized with 6 low dose injections of 10^3-10^4 viable cells of *P. intermedia* BH18/23 over a 6 week period (Group M31). Low chronic immunization stimulated IgG antibody which reacted strongly with two antigens (34 and 44 kDa) of the homologous organism. Few other antigens in the *P. intermedia* outer membrane profile were identified by the IgG present in the serum from M31 group. The

Table 4.11 Specific and Cross-reactive Antigens (kDa) of Outer Membranes Recognized by Antibodies from Mice Immunized with a Single Dose of *Prevotella intermedia* BH18/23 together with Freund's Complete Adjuvant I.M. (Groups M17-26)

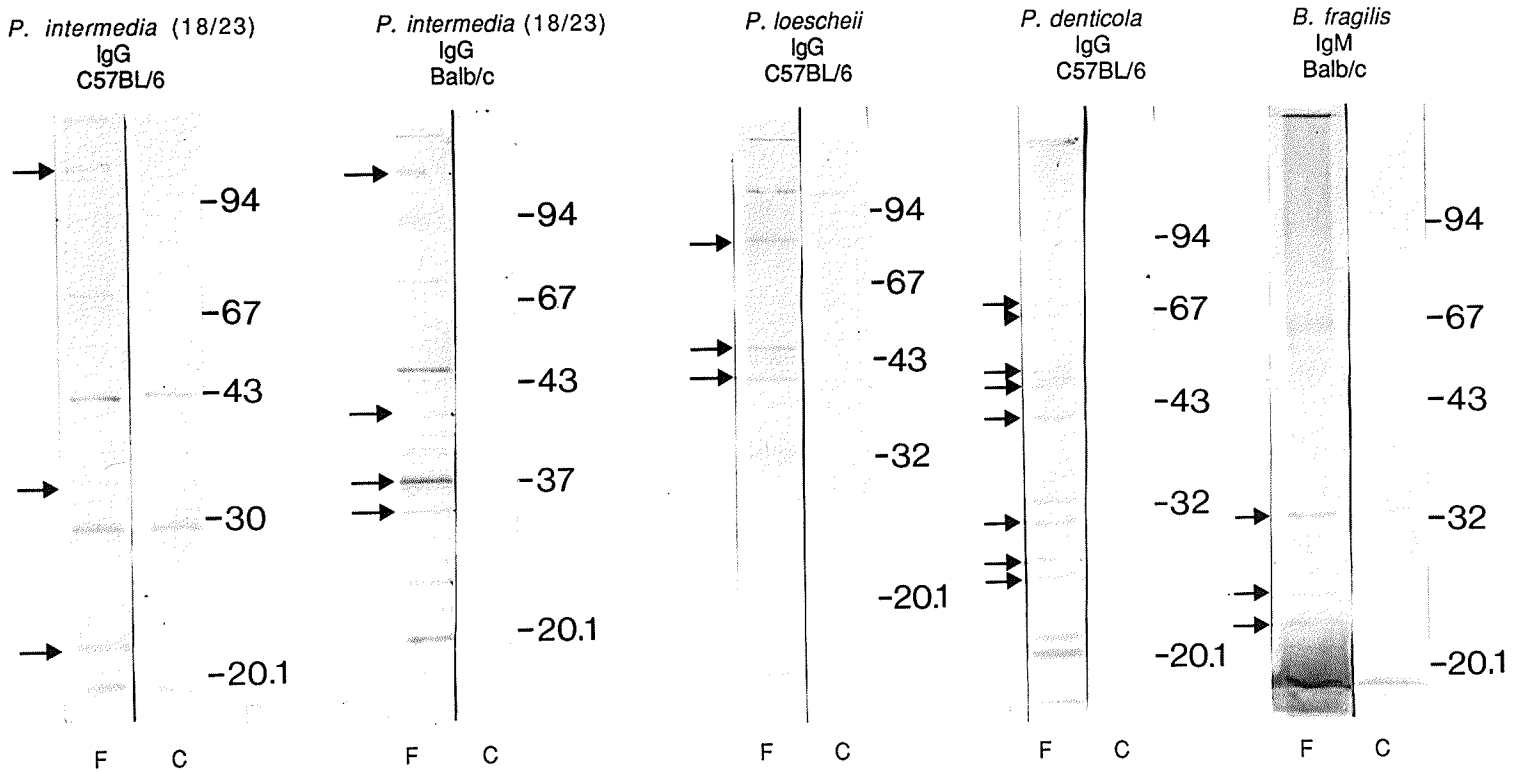
	C57BL/6 mice		Balb/c Mice	
	IgG	IgM	IgG	IgM
P. intermedia (BH 18/23)	23 34 >100	26	16 27 30 37 43 >100	27
P. intermedia (BH 20/30)	- -	- -	- -	- -
P. mel. (25845)	- -	- -	- -	- -
P. denticola (33185)	26* 27 29* 40 45* 47* 67 69	- -	- -	- -
B. fragilis (8560)	52	32	- -	22 25 32 65
P. loescheii (15930)	38 46 82	- -	- -	- -
P. corporis (33547)	- -	- -	- -	- -
P. asacch. (25260)	- -	- -	- -	- -
P. gingivalis (18/10)	- -	- -	- -	- -
P. oralis (33269)	- -	- -	- -	- -
P. buccae (33547)	- -	- -	- -	- -

* - Intensity of band recognition greater than that seen in control mouse sera

NOTE: Antigens recognized by control mouse serum or by goat anti-mouse conjugate non-specific binding are not included in this table.

FIGURE 4.5

ANTIGENS RECOGNIZED BY ANTIBODY FROM C57BL/6 AND BALB/C MICE FOLLOWING IMMUNIZATION WITH A SINGLE DOSE OF *P. INTERMEDIA* (BH18/23) AND FREUND'S COMPLETE ADJUVANT.



F - Freund's Complete Adjuvant
C - Control Mice

M31 serum produced by C57BL/6 mice in response to this low dose chronic immunization appeared specific for only *P. intermedia* BH18/23, showing no cross-reactivity with antigens of other organisms tested (Figure 4.6). Lane L in Figure 4.6 shows the outer membrane antigens recognized by M31 serum, compared to lane C (M32 - control mice). It was of interest that a series of bands of antigens (ladder) was developed in the 94 kDa region of the blot. Such ladder effects are seen with profiles of lipopolysaccharides (Deslauriers et al., 1990). Although the control lane also showed these bands developed by IgG, the response in the IgM lane L appeared to be specific and was absent from the control lane C (Figure 4.6). Negligible cross-reactive responses by M31 sera are demonstrated to *P. melaninogenica* - IgG, *P. denticola* - IgG, *P. loescheii* - IgG and *B. fragilis* - IgG.

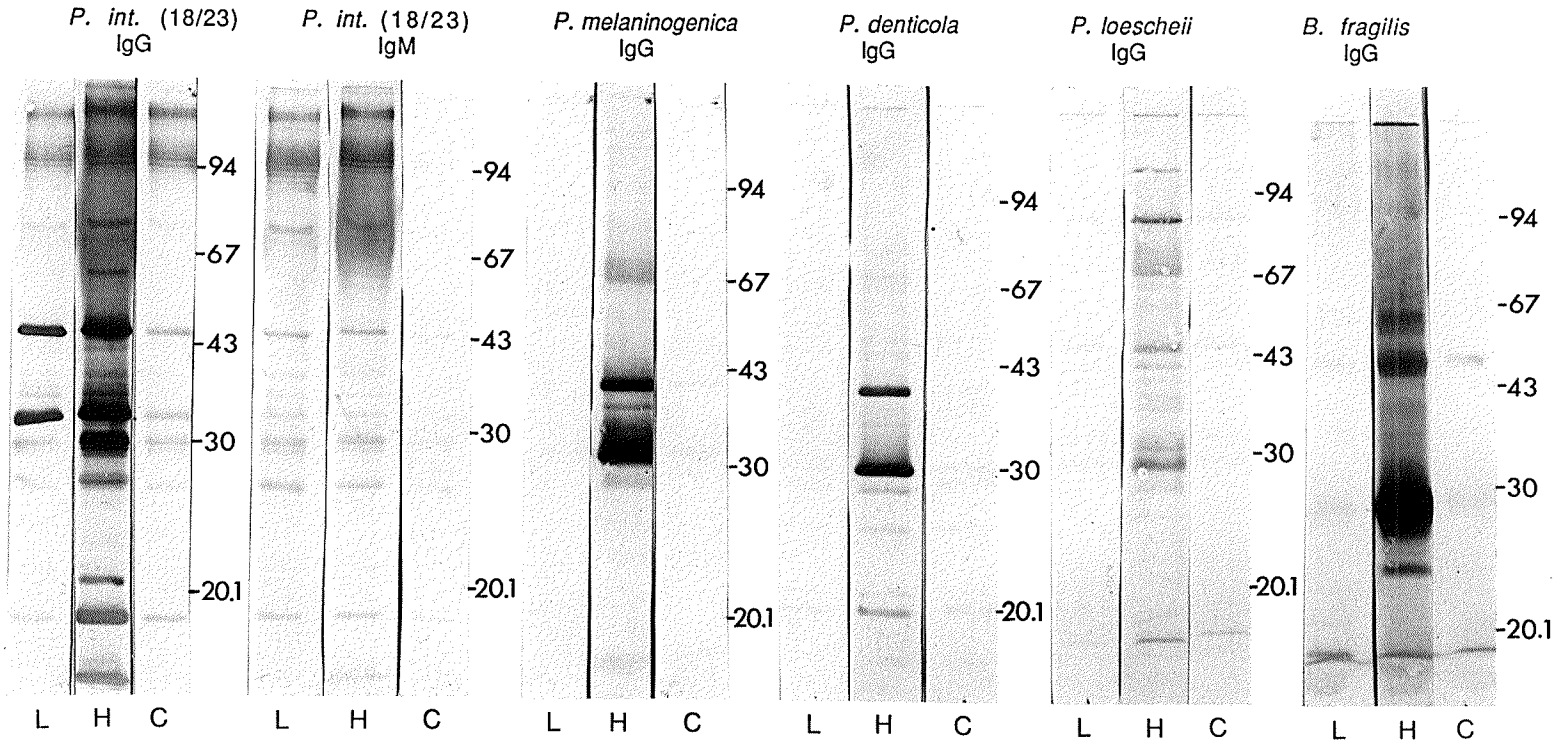
3:VII) Chronic Low Dose Immunization of C57BL/6 Mice with 10^3-10^4 viable cells of *P. intermedia* BH18/23 over a Period of 6 weeks, Followed by a High Dose Sublingual Injection (Groups M38-39)

The results of the chronic immunization indicated that a specific response to the immunizing strain developed. It was thought that such chronic low dose immunization, could mimic the situation in periodontal disease (where the resident flora could sensitize the patient). Consequently mice sensitized by several low doses of *P. intermedia* were subsequently boosted with a localized injection in the oral cavity to model an acute local increase of an oral organism.

C57BL/6 mice (4-6 weeks old) were immunized weekly with a low chronic dose (10^3-10^4 viable cells) of *P. intermedia* BH18/23 for a 6 week period, followed by a high dose (10^6-10^7 viable cells) sublingual injection. Serum obtained from this immunization recognized specific bands in the homologous outer membrane profile of *P. intermedia* BH18/23 (IgG and IgM), as well in the outer membranes of *P. intermedia* BH20/30 (IgG and IgM), *P. oralis* (IgG) and *P. corporis* (IgM) (Table 4.12).

FIGURE 4.6

ANTIGEN PROFILES OF SELECTED OUTER MEMBRANE REACTED AGAINST SERA FROM C57BL/6 MICE AFTER CHRONIC IMMUNIZATION WITH LOW DOSES OF *P. INTERMEDIA* BH18/23 AND IMMUNIZATION WITH FREUND'S COMPLETE ADJUVANT FOLLOWED BY BOOSTING.



L - Low Dose Chronic Immunization (M31)
H - Hyperimmune Immunization (M33)
C - Control (M34)

Table 4.12 Specific and Cross-reactive Antigens (kDa) of Outer Membranes Recognized by Antibodies from Mice Immunized with a Chronic Low Dose Immunization of *P. intermedia*, Followed by a High Dose Sublingual Injection (Groups M38-39)

Outer Membrane Antigens	IgG	IgM
P. intermedia (BH18/23)	34* 44* >100	16* 26 44 >100
P. intermedia (BH20/30)	>100	>100
P. melaninogenica (25845)	- -	- -
P. denticola (33185)	- -	- -
B. fragilis (8560)	- -	- -
P. loescheii (15930)	- -	- -
P. corporis (33547)	- -	20 33 37 63* 68*
P. asaccharolyticus (25260)	- -	- -
P. gingivalis (18/10)	- -	- -
P. oralis (33269)	32*	- -
P. buccae (33547)	- -	- -

* - Intensity of band recognition greater than that seen in control mouse sera

NOTE: Antigens recognized by control mouse serum or by goat anti-mouse conjugate non-specific binding are not included in this table.

3:VIII) Reactivity of Hyperimmune Serum from C57BL/6 Mice (Groups M33-34)

Hyperimmune rabbit serum has been used to show the potential for cross-reactivity within the *Prevotella*, *Bacteroides* and *Porphyromonas* species. The earlier results (Section 4.2) had shown a close similarity between mouse (M3) and human response to *P. intermedia*. It was not clear however, if the mouse, given sufficient stimulation of its immune system, could produce antibody showing the same range of cross-reactivity between these organisms, or if its immune response was limited.

Hyperimmune serum was produced by injecting C57BL/6 mice with a high dose (10^6-10^7 viable cells) of *P. intermedia* BH18/23 together with Freund's Complete Adjuvant followed by booster I.V. injections of the same magnitude (M33). The hyperimmune mouse serum showed wide cross-reactivity with the outer membranes of the majority of strains examined (Table 4.13)(Figure 4.6). Table 4.13 shows the relative strength of response noted to the various strains of *Prevotella, Bacteroides* and *Porphyromonas* tested. In Table 4.13, which shows cross-reactive response to outer membranes of *Prevotella*, *Bacteroides* and *Porphyromonas* generated by hyperimmune mouse serum (bands common to the control mice have not been included). The IgM response was generally similar to the control mice, but the IgG was increased substantially. Lane H in Figure 4.6 shows the extensive cross-reactivity of this hyperimmune serum to *P. melaninogenica*, *P. loescheii*, and *B. fragilis*. In Figure 4.6, both the low chronic dose mouse sera - M31 (Lane L) and the hyperimmune sera - M33 (Lane H) were used at the same dilution (1:100) when developing immunoblots, to illustrate the difference in antibody production in response to hyperimmunization of the mice.

Table 4.13 Presence of Cross-Reactive Responses to Outer Membranes of *Prevotella*, *Bacteroides* and *Porphyromonas* Generated by Hyperimmune Serum Produced in C57BL/6 Mice (Group M 33)

Outer Membrane Antigens	IgG	IgM
P. intermedia (BH18/23)	+ +	+ +
P. intermedia (BH20/30)	+	+
P. melaninogenica (25845)	+ +	- -
P. denticola (33185)	+	- -
B. fragilis (8560)	+ +	- -
P. loescheii (15930)	+	- -
P. corporis (33547)	+ +	- -
P. asaccharolyticus (25260)	- -	- -
P. gingivalis (18/10)	+	- -
P. oralis (33269)	+ +	- -
P. buccae (33547)	+	- -

++ - strong response
+ - medium response
- - - weak response

3:IX) Response of Individual C57BL/6 Mice to Chronic Low Dose Immunization (10^3-10^4 viable cells) with *P. intermedia* BH18/23 (Groups M36-37)

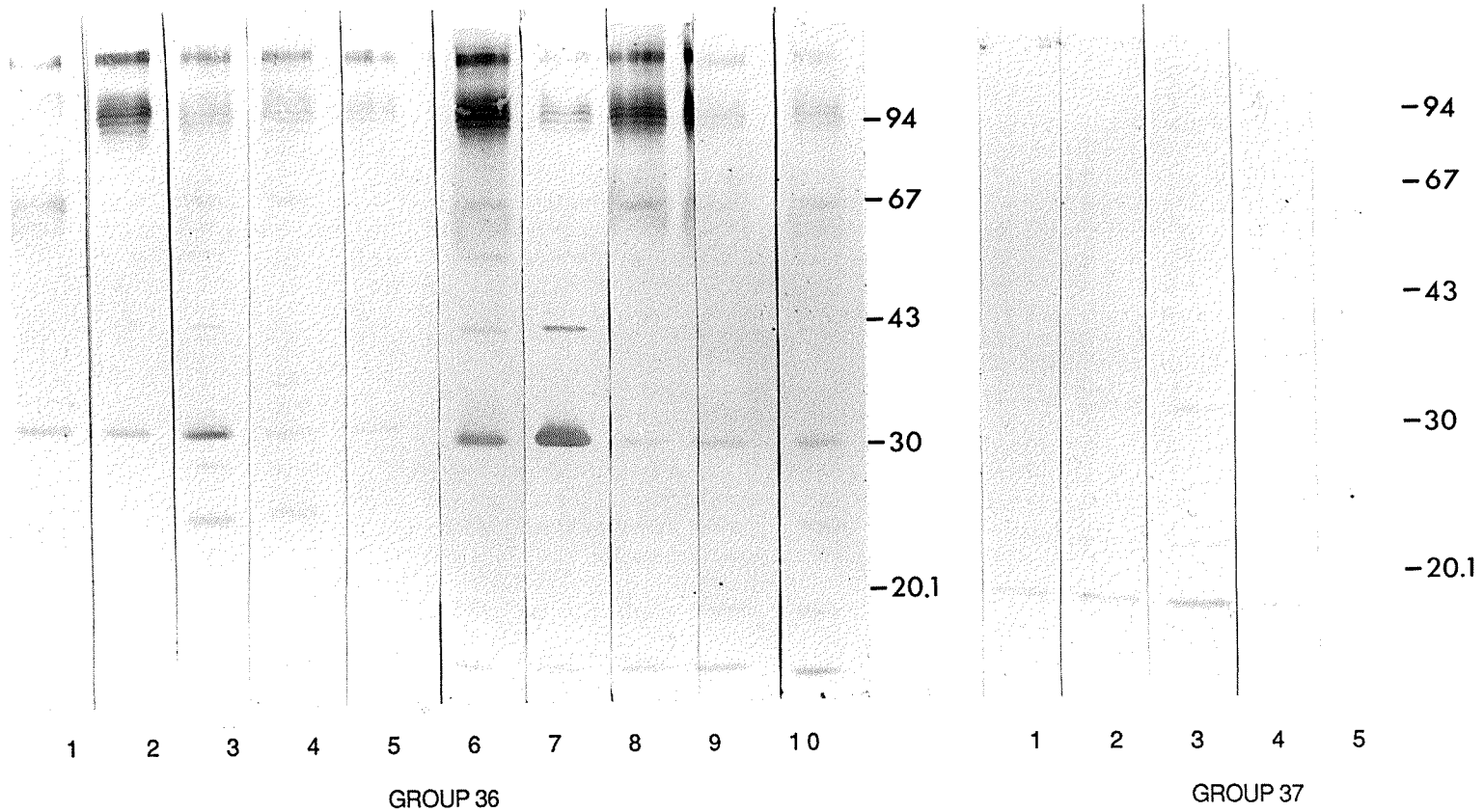
Up to this point, all of the sera used, both from experimental and control mice were pooled for each mouse group. The pooled sera provided a general picture of the response to immunization, but did not show the possibility for variation between mice within a group. Consequently, serum was collected for analysis from individual mice. Low chronic immunization was selected for these studies, as it was thought that this most closely resembled the situation of the response to organisms within the resident flora.

Ten C57BL/6 mice were immunized with 6 low dose injections of 10^3-10^4 viable cells of *P. intermedia* BH18/23 over a 6 week period of time (M36), together with a control group of 5 mice (M37) receiving no injections; both groups being sacrificed after 6 weeks. Sera obtained from individual animals in the M36 and 37 groups was stored separately, to allow comparison of the response of each mouse to immunization. Due to the small quantity of serum obtained (only 200-300 µl.), only the IgG antibody response to the homologous organism - *P. intermedia* BH18/23 outer membrane was studied.

Individual mice immunized with *P. intermedia* formed IgG antibody capable of recognizing several antigens in the outer membrane profiles (26, 31, 34, 44, 66 and >100 kDa), not seen in the control (M37) mice (16 kDa). Three mice (M36-2, M36-6, and M36-8) produced stronger IgG responses to the outer membrane antigens compared to the remaining seven animals in group M36 (Figure 4.7). Factors such as: difference in actual numbers of viable cells injected into each individual animal - quantity, interstitial loss of sample during injection or loss of cell viability due to time spent during immunization (1st animal vs. 10th animal), may account for the differing amounts of antibody produced. In spite of these quantitative differences, little qualitative difference was noted between the patterns of antigens in the

FIGURE 4.7

OUTER MEMBRANE ANTIGENS OF *P. INTERMEDIA* (BH18/23) DEVELOPED BY IgG ANTIBODY FROM INDIVIDUAL MICE (C57BL/6) IMMUNIZED WITH LOW CHRONIC DOSES OF *P. INTERMEDIA* (BH18/23) OVER A PERIOD OF 6 WEEKS.



profile detected by the mice in group M36. As in Figure 4.6, a ladder effect in the 94 kDa region is seen, particularly in mice #2, 6, and 8 of Groups M36.

ELISA assays of the individual mouse sera from groups M36 and M37 against outer membranes of the homologous organism were also performed. Results showed that little variance in the quantities of IgG and IgM produced by the mice in groups M36. Substantially higher values were detected in group M36, compared to the M37 - control group (Section 4.4).

4:4) COMPARISON OF ANTIBODY REACTIVITY OF MOUSE SERUM USING ELISA

Western blotting provides a clear indication of the antigens recognized by antibody in a given serum. However, although differences in the intensity of the reaction can be detected, it is not possible to easily provide an objective assessment of the reactivity of the serum. In order to gain some insight into the degree of reactivity of the sera, pooled sera and sera from individual mice were analyzed by ELISA.

Enzyme linked immunosorbent assays of pooled mouse sera (M9-M35) to both formalinized whole cells and outer membranes of *P. intermedia* BH18/23, *P. melaninogenica* 25845, *B. fragilis* 8560, *P. asaccharolyticus* 25260 and *P. gingivalis* BH18/10 were performed. The species of bacteria used as antigen for the ELISA assay were selected for specific reasons: *P. melaninogenica*: for its potential cross-reactivity with the homologous organism used for immunization, *B. fragilis* - a representative of the gut flora, *P. gingivalis* - an organism implicated in periodontal disease, showing little cross-reactivity to *P. intermedia*, and *P. asaccharolyticus*: an organism, which consistently produced a strong reaction with the mouse sera.

Preliminary ELISA runs showed that the optimum dilutions of mouse sera for assays were at levels of 1:100 for IgG, and 1:50 for IgM. No comparisons were drawn between the relative reactivity of mouse sera to whole cells compared to that of outer

membranes, as the ELISA assays were performed with a significant time interval separating experiments. Possible deterioration of either the outer membranes or HRP-anti mouse immunoglobulin could account for the weaker mouse immunoglobulin response to outer membranes. An arbitrary value of 0.20 O.D. units was used as the minimum value recorded for IgG response to whole cells and IgG and IgM response to outer membranes, while a value of 0.40 was selected as the minimum for the IgM response, taking into account that the overall values for responses to whole cells was much higher (Tables 4.14-4.18).

The serum which showed the most reactivity was from Group M33 (immunization of the animals with high dose of viable *P. intermedia* cells together with Freund's Complete Adjuvant, followed by two I.V. boosting immunizations). Antibody levels of both anti-mouse IgG and IgM were highest to both whole cells and outer membranes of the homologous organism used for immunization - *P. intermedia*. An elevated, but lower level of response was given by M31 serum (low chronic dose I.V. immunization) and M29 sera (2 I.V. injections of high dose *P. intermedia* over 20 days). A relatively low level of reactivity of the sera to the other organisms used in the ELISA assays would suggest that the various immunization regimes did not significantly affect the amount of antibody to these organisms.

It is of interest that the pooled mouse sera gave relatively high readings to IgM against whole cells to *P. melaninogenica, P. asaccharolyticus* and *B. fragilis*. In Table 4.15 mouse groups M13. 14. 15. 16. 27. 28. 29. 30. and 33 gave readings between 0.936 to 1.48. Low levels for IgM (0.426 to 0.619) were given by 11 of the 21 mouse groups, when *B. fragilis* whole cells were used as antigen. However, IgM against *P. asaccharolyticus* was as high or higher than that against *P. melaninogenica* with the same groups giving the highest values (M13-16, M27-30). These groups of mice were both Balb/c. Immunization did not have an impact on the readings, as equivalent levels were given by control mice. The other groups in table 4.15 and 4.17 were C57BL/6. These

Table 4.14 ELISA ASSAY OPTICAL DENSITY READINGS AT 450 NM. USING MOUSE SERA AGAINST *PREVOTELLA INTERMEDIA* BH18/23 WITH *P. INTERMEDIA* AS ANTIGEN.

Mouse strain	Mouse serum	IgG - 1:100		IgM - 1:50	
		Whole Cells[a]	Outer Membranes[a]	Whole Cells[b]	Outer Membranes[a]
C57BL/6	M9	- -	- -	- -	- -
	M10	- -	- -	- -	- -
	M11	- -	- -	- -	- -
	M12	- -	- -	- -	- -
Balb/c	M13	- -	- -	- -	- -
	M14	- -	- -	- -	.219
	M15	- -	- -	- -	.239
	M16	- -	- -	- -	- -
C57BL/6	M17	.504	- -	- -	- -
	M18	- -	- -	- -	- -
	M19	.371	.368	- -	- -
	M21	- -	- -	- -	- -
Balb/c	M27	-	- -	.733	.385
	M28	1.332	.673	.461	.296
	M29	1.810	1.132	1.110	.513
	M30	- -	- -	- -	- -
C57BL/6	M31	1.311	.970	1.210	.665
	M32	.754	- -	- -	- -
C57BL/6	M33	2.98	1.585	2.369	1.305
	M34	- -	- -	- -	- -
	M35	.368	- -	- -	- -

a - All values <0.200 not included in Table
b - All values <0.400 not included in Table

Table 4.15 ELISA ASSAY OPTICAL DENSITY READINGS AT 450 NM. USING MOUSE SERA AGAINST *PREVOTELLA INTERMEDIA* BH18/23 WITH *PREVOTELLA MELANINOGENICA 25845* AS ANTIGEN.

Mouse strain	Mouse serum	IgG - 1:100		IgM - 1:50	
		Whole Cells[a]	Outer Membranes[a]	Whole Cells[b]	Outer Membranes[a]
C57BL/6	M9	- -	- -	.500	- -
	M10	- -	- -	.406	- -
	M11	- -	- -	.522	- -
	M12	- -	- -	.546	- -
Balb/c	M13	- -	- -	.936	- -
	M14	- -	- -	1.067	- -
	M15	- -	- -	1.228	- -
	M16	- -	- -	1.076	- -
C57BL/6	M17	- -	- -	- -	- -
	M18	- -	- -	.705	- -
	M19	- -	- -	.702	- -
	M21	- -	- -	.521	- -
Balb/c	M27	-	- -	1.275	- -
	M28	- -	- -	.964	- -
	M29	- -	- -	1.480	.231
	M30	- -	- -	1.077	- -
C57BL/6	M31	- -	- -	.609	- -
	M32	- -	- -	.542	- -
C57BL/6	M33	.542	.408	1.347	.418
	M34	- -	- -	.558	- -
	M35	- -	- -	.572	- -

a - All values <0.200 not included in Table
b - All values <0.400 not included in Table

Table 4.16 ELISA ASSAY OPTICAL DENSITY READINGS AT 450 NM. USING MOUSE SERA AGAINST *PREVOTELLA INTERMEDIA* BH18/23 WITH *BACTEROIDES FRAGILIS 8560* AS ANTIGEN.

Mouse strain	Mouse serum	IgG - 1:100		IgM - 1:50	
		Whole Cells[a]	Outer Membranes[a]	Whole Cells[b]	Outer Membranes[a]
C57BL/6	M9	- -	- -	.426	- -
	M10	- -	- -	.434	- -
	M11	- -	- -	.448	- -
	M12	- -	- -	- -	- -
Balb/c	M13	- -	- -	.676	.213
	M14	- -	- -	.579	.209
	M15	- -	- -	.727	.232
	M16	- -	- -	.619	.233
C57BL/6	M17	- -	- -	- -	- -
	M18	- -	- -	- -	.211
	M19	- -	- -	.412	- -
	M21	- -	- -	- -	- -
Balb/c	M27	-	- -	.808	.295
	M28	- -	- -	.470	.315
	M29	- -	- -	.524	.344
	M30	- -	- -	.614	.466
C57BL/6	M31	- -	- -	- -	- -
	M32	- -	- -	- -	- -
C57BL/6	M33	- -	- -	.458	- -
	M34	1.030	.574	.616	.314
	M35	- -	- -	- -	.229

a - All values <0.200 not included in Table
b - All values <0.400 not included in Table

Table 4.17 ELISA ASSAY OPTICAL DENSITY READINGS AT 450 NM. USING MOUSE SERA AGAINST *PREVOTELLA INTERMEDIA* BH18/23 WITH *PORHYROMONAS ASACCHAROLYTICUS* 25260 AS ANTIGEN.

Mouse strain	Mouse serum	IgG - 1:100		IgM - 1:50	
		Whole Cells[a]	Outer Membranes[a]	Whole Cells[b]	Outer Membranes[a]
C57BL/6	M9	- -	- -	.608	- -
	M10	- -	- -	.477	- -
	M11	- -	- -	.763	- -
	M12	- -	- -	.697	- -
Balb/c	M13	- -	- -	1.161	- -
	M14	- -	- -	1.310	- -
	M15	- -	- -	1.580	- -
	M16	- -	- -	1.323	- -
C57BL/6	M17	- -	- -	- -	- -
	M18	- -	- -	.620	- -
	M19	- -	- -	.721	- -
	M21	- -	- -	.573	- -
Balb/c	M27	-	- -	1.420	- -
	M28	.241	- -	1.114	- -
	M29	- -	- -	1.538	- -
	M30	- -	- -	1.222	- -
C57BL/6	M31	- -	- -	.522	- -
	M32	- -	- -	.452	- -
C57BL/6	M33	.253	- -	.566	- -
	M34	.248	- -	.515	- -
	M35	- -	- -	.578	- -

a - All values <0.200 not included in Table
b - All values <0.400 not included in Table

Table 4.18 ELISA ASSAY OPTICAL DENSITY READINGS AT 450 NM. USING MOUSE SERA AGAINST *PREVOTELLA INTERMEDIA* BH18/23 WITH *PORHYROMONAS GINGIVALIS* AS ANTIGEN.
(HRP ANTI-MOUSE IgG AND IgM CONJUGATE ABSORBED WITH *P. GINGIVALIS* WHOLE CELLS)[c]

Mouse strain	Mouse serum	IgG - 1:100		IgM - 1:50	
		Whole Cells[a]	Outer Membranes[a]	Whole Cells[b]	Outer Membranes[a]
C57BL/6	M9	- -	- -	- -	- -
	M10	- -	- -	- -	- -
	M11	- -	- -	- -	- -
	M12	- -	- -	- -	- -
Balb/c	M13	- -	- -	- -	- -
	M14	- -	- -	- -	- -
	M15	- -	- -	- -	- -
	M16	- -	- -	- -	- -
C57BL/6	M17	- -	- -	- -	- -
	M18	- -	- -	- -	- -
	M19	- -	- -	- -	- -
	M21	- -	- -	- -	- -
Balb/c	M27	-	- -	- -	- -
	M28	- -	- -	- -	- -
	M29	- -	- -	- -	- -
	M30	- -	- -	- -	- -
C57BL/6	M31	- -	- -	- -	- -
	M32	- -	- -	- -	- -
C57BL/6	M33	- -	- -	- -	- -
	M34	- -	- -	- -	- -
	M35	- -	- -	- -	- -

a - All values <0.200 not included in Table
b - All values <0.400 not included in Table
c - see details- page 104

results indicate that Balb/c mice may carry higher levels of IgG and IgM natural antibody to antigens on whole cells of *P. melaninogenica* and *P. asaccharolyticus*, than C57BL/6 mice.

4:1) Comparison of Antibody Reactivity of Individual Mice Immunized with Chronic Low Doses of *P. intermedia* BH18/23 using ELISA (Groups M36-37)

A group of 10 C57BL/6 mice were immunized with 6 low doses (10^3-10^4 viable cells) of *P. intermedia* over a period of 7 weeks, with 5 mice representing the control group (M37). Sera obtained from each individual mouse were kept separate and an ELISA assay against outer membrane of the homologous organism performed.

Table 4.19 shows the optical density readings (450 nm.) of immunoglobulin response to the homologous organism. The IgG levels were assayed at a 1:100 concentration, while the IgM levels were assayed at a 1:50 dilution.

The average value of O.D. reading for M36 - IgG (1:100) was 0.885 units, with a range of 0.662 to 1.194 units for the individual animals. The M37 - control group gave significantly lower values averaging 0.043 (range of 0.021 to 0.062). The IgM response (1:50) by the experimental groups M36 produced an average reading of 0.751 (range of 0.574 to 0.972), with the control group - M37, reading an average value of 0.016 (range 0.00 to 0.024). The 2 sample student t-test analysis of the data showed that the difference in optical density between the immunized and non-immunized mice was statistically significant ($p < 0.001$).

Comparison of the O.D. values with Western blots developed by the same mouse sera (M36) showed that the highest IgG O.D. values (mice #1 and 6) did not match the reactivity of the serum on Western blots, except for mouse #6(M36) (Figure 4.7), suggesting that in individual mice there may not always be a correlation between reactivity on blots and levels of antibody assayed by ELISA.

Table 4.19 ELISA ASSAY OPTICAL DENSITY READINGS AT 450 NM. USING SERA FROM INDIVIDUAL C57BL/6 MICE (GROUPS M36-37) AGAINST *PREVOTELLA INTERMEDIA* BH18/23 OUTER MEMBRANE

	IgG - 1:100		IgM - 1:50	
Mouse number	Group 36	Group 37	Group 36	Groups 37
1	1.194	.021	.822	0
2	.781	.036	.766	.022
3	.736	.062	.862	.024
4	.662	.038	.854	.019
5	.655	.060	.680	.016
6	1.198	-	.574	-
7	.712	-	.671	-
8	.983	-	.716	-
9	.977	-	.597	-
10	.950	-	.972	-

$p < 0.001$ (2 sample student t-test)

CHAPTER 5
DISCUSSION

Antibodies to a specific organism detected or measured in "normal" healthy humans or patients with periodontal disease may arise from stimulation by that organism, or by stimulation by organisms among the resident flora which carry similar antigens. Antibodies promoted by this second mechanism, which are present in the serum of normal animals are termed "natural antibody". The role that these antibodies or sensitization of the host by members of the resident flora, play in disease is not known. However, it has been shown that there is considerable potential for the generation of cross-reactive antibody to *Prevotella*, *Bacteroides* and *Porphyromonas*, when sera from hyperimmunized animals is used to analyze the antigenic profiles of species of these genera (Bowden and Nolette, 1991). It is not known however, if what may be called "natural" immunization by the host's resident flora generates "natural" antibodies as diverse or reactive as those in the sera from hyperimmunized experimental animals.

There is no doubt that animals including humans, which are regarded as "normal" carry serum antibody to organisms found in their oral flora. Studies by Ebersole et al. (1991) on the sera from humans and non-human primates have shown antibodies to *Prevotella*, *Bacteroides* and *Porphyromonas*. In their examination of sera from non-human primates Ebersole et al. (1991) found IgG antibodies to *Prevotella* and *Porphyromonas* and antibodies of the IgM class to *Bacteroides fragilis*. The level of antibody to *Prevotella* exceeded that to *Porphyromonas* by a factor of four, possibly reflecting the differences in numbers and habitats of these species in the host. This result is not unexpected as non-human primates are known to be colonized by species of these genera. However, as far as is known, the oral cavity of the mouse is not colonized

by *Prevotella* and *Porphyromonas* (Trudel et al., 1986), although these or similar organisms may be present in the gut.

Given the lack of colonization of the mouse oral cavity by these bacteria, is the mouse a useful animal to use to study the development of cross-reactive antibody between *Prevotella*, *Bacteroides* and *Porphyromonas*? Does the mouse recognize similar antigens in the outer membranes from these bacteria as humans, and is it possible to produce as diverse a range of antibodies, given sufficient stimulation of their humoral immune system?

The studies described here were intended to answer these questions and also to explore the degree of immunization necessary to stimulate the production of antibodies cross-reactive with bacteria, closely related to the immunizing strain. An important aspect of the studies was the selection of an immunizing strain and a standardized antigen to test for specific and cross-reactive antibody in sera from immunized and control mice.

The immunizing strain selected was a representative of *P. intermedia* genospecies 2, BH18/23, isolated from a periodontal pocket of a patient with periodontitis. This species was chosen because of its demonstrated cross-reactivity with other *Prevotella* and *Porphyromonas*. Two genomic species of *P. intermedia* are known: genospecies 1 has a limited habitat and seems to be more closely related with periodontal disease than is genospecies 2, which is found widely distributed in supragingival and subgingival plaque. The wide distribution of genospecies 2, which would make it a good candidate as a strain stimulating the generation of cross-reactive antibodies in humans, was selected as the immunizing strain. Very often cells used as antigens from immunizing animals are treated before they are used for injection. In many cases with pathogenic bacteria, this is necessary to protect the experimental animal. It was decided to use viable untreated cells of *P. intermedia* in the present studies to avoid causing modification of

the surfaces of the cells and to present antigens to the immune system in as natural a form as possible.

Cross-reaction between *Prevotella* and *Porphyromonas* has been demonstrated using whole cells and cell extracts. In addition, outer membranes have been shown to be a useful source of antigen for use in Western blotting of species of these genera (Bowden and Nolette, 1990). Outer membranes were selected as a standard antigen in these studies, as they carry cell surface antigens to which the immune system of the host would be most likely to be exposed. A single preparation of outer membranes from each of the species was aliquoted into small amounts and stored frozen. A separate aliquot was taken for each blot or ELISA assay, to ensure standardized antigen throughout the entire study.

Another technical difficulty in using mice as an experimental animal was the small volumes of serum that can be obtained from each animal, approximately 200-300 µl. While this volume is sufficient for several ELISA assays, it limits the numbers of Western blots that can be run, unless the sera are of very high titre. In the present studies the titres of the sera were likely to be low, especially those of natural antibody in the control mice. It was for this reason that the sera from individual mouse groups were pooled in the initial survey of responses. Although not ideal, it was thought that pooling of the sera in this way was legitimate, as the mice were inbred strains and theoretically individual animals should give closely similar responses. Pooled sera would not reveal variations in response introduced as a result of small differences in technique of injection and cell numbers. After the initial screening of different protocols using pooled sera, a selected protocol was examined using sera from individual mice in a group.

Within the limitations and decisions mentioned above, it has been possible to obtain useful data on the response of mice to *P. intermedia* and on the generation of cross-reactive antibody, which provides a firm basis for further studies. The results of

comparison between immunized mice, adult human patients and children suggest that the mouse will respond to the same antigens from these bacteria as humans. Serum from immunized mice (Section 4:2) recognized 7 of the 10 antigens detected in the outer membranes of *P. intermedia* by sera from children, and 7 of 12 detected by sera from adult patients (Table 4.5) The more valid comparison would be to the childrens' sera, as they, unlike adults, were free of periodontal disease. In contrast, hyperimmune rabbit sera detected a wider range of antigens and more closely resembled the adult human sera.

One difference between mouse and human sera was seen when they were screened for natural antibody to the organisms. The human sera, even from the children, detected a minimum of 10 antigens to *P. intermedia*, while non-immune mouse sera detected 3. The non-immune mouse sera did however, detect more antigens in the membranes from other species such as *P. corporis* and *P. gingivalis* (Table 4.2). It is clear from the data presented in Tables 4.2 and 4.5 however, that the human sera recognized a wider range of antigens from the species tested than did the non-immune mouse sera. The results almost certainly reflect the colonization of humans by these bacteria and increased stimulation of the humoral immune system of the adult patients. Colonization of a host by organisms which would be used as antigens, to determine if cross-reacting antibody was generated by immunization with a related organism, would make interpretation of the results difficult. These colonizing bacteria would cause a specific response to their own antigens, which would be difficult or impossible to distinguish from cross-reacting antibody produced by immunization with related bacteria. In this respect, the mouse with its relatively simple flora and the absence of oral colonization by *Prevotella*, *Bacteroides* and *Porphyromonas* provides a useful experimental animal for detection of cross-reacting antibody to heterologous strains following immunization by a related organism.

Both C57BL/6 and Balb/c mice carried natural antibody that reacted with outer membrane antigens of several of the test organisms. Analysis of these responses has to

take into account the presence of natural antibody in the goat anti-mouse peroxidase labelled antibody (Table 4.1) and the comparisons drawn in Table 4.4, which show that natural antibody in C57BL/6 mouse sera recognize antigens in the outer membranes of *P. intermedia* genospecies 2, *P. melaninogenica*, *P. loescheii*, *P corporis* and *P. gingivalis*, which were not detected by the goat antibody. These natural antibodies occurred as both IgG and IgM. In many cases where the antigen was recognized by the non-immune mouse sera and the goat antibody, the reaction produced on the blot by the mouse sera was visibly more intense. A similar situation occurred with Balb/c mice, although the range of antigens detected varied from those of C57BL/6 (Figure 4.2) indicating an impact of the genetic make-up of the animal on the response. As might be expected, analysis of sera from germ-free mice showed very few natural antibodies except to *P. gingivalis* and *P. asaccharolyticus* and with one exception of antibody to a 24 kDa antigen in the outer membrane of *P. gingivalis*, all antigens were equivalent to those detected by the goat anti-mouse antibody, although they reacted with greater intensity on the blot containing germ-free mouse sera. The germ-free mouse sera which reacted on the blots with the goat anti-mouse sera, acted as a control for the normal mouse sera and the negative results for six of the eleven test organisms (Table 4.4) compared to the sera from normal mice, reinforces the suggestion that mice carry natural antibody against *Prevotella*, although fewer antigens are detected compared to sera from normal human children. Sera from germ-free mice detected antigens in the outer membranes of *Porphyromonas gingivalis* (24 kDa) and *Prevotella oralis* (27, 45, 52, kDa), which were not detected by the goat anti-mouse serum, suggesting that antibody to these antigens might be stimulated by environmental or food antigens.

Both mouse strains (C57BL/6 and Balb/c) used in the experiments carried fewer natural antibodies, both IgG and IgM to the test organisms than human sera, and both were capable of responding to a range of antigens similar to those recognized by human sera. Given this information and having characterized the patterns of natural

antibody carried in the two mouse strains, they could now be used to examine the degree of immunization necessary to promote not only a specific response to the homologous organism used as antigen, but also a response generating antibodies cross-reactive with outer membrane antigens of related bacteria.

It was apparent that single low doses of 10^3-10^4 viable cells of *P. intermedia* injected intravenously into 2-3 or 4-5 week old mice generally did not cause the production of antibodies recognizing antigens in the homologous outer membranes that were not detected by natural antibodies in their serum. In addition, this immunization protocol did not stimulate the production of antibodies cross-reactive with antigens in the outer membranes of the other related test organisms. There was one exception to this and that was when serum from young mice had received a booster dose of *P. intermedia* cells, where one antigen of 65 kDa in the outer membrane of *P. asaccharolyticus* was detected.

A more dramatic response was seen when the dose of *P. intermedia* viable cells was increased to 10^6-10^7. Immunization of C57BL/6 and Balb/c mice with this amount of cells with or without boosting caused the production of antibodies recognizing antigens (26, 34, >100 kDa) in the outer membranes of *P. intermedia*, which were not detected by natural antibody (Table 4.9, 4.10., Figure 4.3). The responses between the two mouse strains varied: C57BL/6 mice only produced antibody recognizing antigens in the homologous outer membranes, no cross-reacting antibody was detected. In contrast the sera from Balb/c mice not only recognized more antigens (26, 34, 76, >100 kDa) within the homologous profile, but also cross-reacted with membrane from the second genospecies of *P. intermedia* (strain BH20/30) and *P. denticola* (Table 4.10). Sera from these mice also gave enhanced responses to antigens recognized by natural antibodies (Table 4.10). These results suggest that immunization of mice with a relatively high dose of cells will stimulate production of antibody specific for the immunizing strain. However, as evidenced here with the Balb/c mice stimulation of

antibody cross-reactive with heterologous organisms is likely to be dependent on the genetic make-up of the mouse selected for immunization. Another characteristic demonstrated by these immunization protocols was the increase in reactivity of the natural antibody after challenging the mice with low or high doses of viable cells.

A stronger stimulus to the immune system of the mice was provided by intramuscular injection of 10^6-10^7 viable cells of *P. intermedia* together with Freund's Complete Adjuvant (FCA). Both strains of mice produced antibodies to new antigens in the outer membrane of *P. intermedia* and also to antigens from heterologous bacteria (Figure 4.5, Table 4.11). Mice were also immunized with *P. intermedia* in FCA and given an intravenous booster dose of cells. This protocol produced sera of equivalent activity to hyperimmune rabbit sera which cross-reacted widely with heterologous bacteria (Figure 4.6, Table 4.13). The cross-reacting antibodies were of the IgG class and recognized between 9-14 antigens in the outer membranes of *P. melaninogenica*, *P. denticola*, and *P. loescheii*.

It is apparent from the results with FCA that the mouse has the capacity to produce antibodies cross-reactive with heterologous bacteria. It is unlikely that the heterologous reactions are simply the result of enhancement of the natural antibody produced in the mice as a response to the resident flora, as different antigens are recognized by hyperimmune sera compared to sera from control mice. The results with hyperimmune mouse sera confirm the observations using hyperimmune rabbit sera, that *Prevotella* and *Porphyromonas* carry antigens with common epitopes.

Comparisons of hyper-immune mouse sera to human adult sera shows that the mouse developed fewer antigenic bands on immunoblots, for example: 6 bands in the outer membrane of *P. denticola* were developed by the mouse IgG (1:100), compared to 8 with human adult IgG(1:100), although the reactivity of mouse serum to these individual bands was visibly greater on Western blotting, demonstrating an increased quantitative difference. Human adult serum from periodontal patients generated in

response to colonization of the periodontal pocket - a considerably weaker stimulation of the host immune system compared to FCA, carries antibody capable of recognizing a larger number of outer membrane antigens of *Prevotella, Porphyromonas* and *Bacteroides*. A possible explanation for the more extensive recognition of antigen bands by the human adult in comparison to the mouse, may be the result of genetic constitution of the host (the mouse consistently representing a more simplified immune response compared to the human).

Although the mouse responded well to immunization with FCA, it seemed unlikely that a host would receive such stimulation from its resident bacteria or from an increase of an organism in the flora of a periodontal pocket. It was thought that in the former situation one might expect that there would be a constant immunization with low levels of cells. Six weekly doses of low numbers of viable cells (10^3-10^4) were given intravenously in order to mimic long term low dose immunization. The results of this immunization were specific reactions limited to *P. intermedia*. There was no evidence that antibodies to antigens other than those detected in control sera had been produced, but two of these antigens (34, 44 kDa - IgG) showed definitely stronger reactions (Figure 4.6) when reacted with the immune serum. Chronic low dose immunization apparently only stimulated an enhanced response to selected antigens in the outer membranes of the homologous organism. However, this result did not show that there was selectivity in the response, in that there was not a generalized increase in reactivity of all the antigens in the profile. This suggests some variation in the immunogenicity of the outer membrane antigens of *P. intermedia*. Similar enhanced response to antigens in the outer membranes was detected in C57BL/6 and Balb/c mice following a single low dose of *P. intermedia* cells which included the 44 kDa antigen. The same held true for C57BL/6 mice after a single high dose immunization, but high dose immunization of Balb/c mice promoted a more diverse response (Table 4.10).

Including booster doses into immunization protocols with high doses of cells stimulated the production of antibodies recognizing new antigens in the homologous outer membrane profiles and cross-reacting antigens in profile of some heterologous species (Tables 4.9, 4.10). Although chronic immunization did not stimulate the production of antibodies other than those in the control sera, boosting after chronic immunization may cause stronger and more diverse responses. Testing this possibility using a local booster injection (sublingual) after chronic immunization showed results for *P. intermedia* IgG antibodies equivalent to those for chronic immunization without boosting (Table 4.12, Section 3:VI). However, boosting with a localized injection after chronic immunization caused stimulation of IgM antibodies against *P. corporis*.

Studies to this point using pooled sera had revealed that stimulation of antibodies recognizing outer membrane antigens generally required high dose immunization with booster doses. Antibodies cross reacting with strains of related species could also be generated in response to high dose immunization depending on the strain of mouse, however, the most extensive cross-reactions were produced using FCA and booster doses.

Two aspects of the immune response had not been considered using the pooled sera. Firstly, the responses of individual mice within a group was not known. Although both strains were inbred, variation in response could result from differences in the number of cells injected or other technical variations. Secondly, differences in response seen in Western blots had not been translated into quantitative values. In order to explore these possibilities, three examinations of the antibody in sera from the mice were made.

The first was to examine the response of individual mice in a group by Western blotting, although it was not possible to do this for each protocol. Consequently, a single protocol, low dose chronic immunization which, perhaps most closely represented the natural situation of the response to components of the resident flora was selected. The small volumes of serum obtained from individual mice restricted the numbers of

analyses that could be made and only IgG responses to *P. intermedia* was determined. Clear differences between the individual mice in group 36 were seen (Figure 4.7). These differences were not in the range of outer membrane antigens detected, but were one of degree of reactivity. Three of the ten mice (#2, 6, 8) gave the strongest response, mice 3 and 7 also showed increase reactivity with a 34 kDa antigen. These results suggest that individuals within a group, apparently receiving the same immune stimulation may vary in their degree of response. The differences detected could reflect differences in the amount of antibody in the sera or individual differences in the degree of reactivity of antibodies with epitopes on the outer membrane antigens.

Studies of the quantitative aspects of the immune response to *P. intermedia* were initiated by ELISA of the pooled mouse sera using selected strains as antigen. In these assays both whole cells and outer membranes were used as antigen. The former were included because very often whole cells are used as antigen in ELISA of antibody levels to *P. intermedia* and related bacteria in human sera. Reference to Table 4.14 shows that increases in antibody levels in C57BL/6 mice to both whole cells and outer membranes measured by O.D. were stimulated by chronic low doses of cell (M31) and with high doses of cells with FCA (Hyperimmune serum - M33). Balb/c mice immunized with a high dose of cells IV and boosting (M29) also produced increased amounts of antibody. The sera identified above that had high levels of antibody tested in Western blots did not always show increases in the diversity of antigens detected or increased cross-reactivity. Low dose chronic immunization of C57BL/6 (M31) only gave sera that showed increased reactivity to those antigens recognized by natural antibody and did not cross-react with related organisms (Figure 4.6: lane L). This indicates that low doses of cells may increase the antibody activity towards antigens to which the mouse is already sensitized and that low doses do not stimulate antibody recognizing "new" antigens in the outer membranes. Thus, low doses of cells are most likely to promote a secondary IgG response to selected antigens in the membranes. The IgM response to

whole cells of sera from group M31 (Table 4.14) probably represents a primary response, perhaps to lipopolysaccharide and other antigens of high molecular weight (Fig 4.6; Lanes L and C).

The hyperimmune serum (M33) might have been expected to show increased amounts of antibody to the immunizing strain and such was the case. In addition, this antiserum recognized "new" antigens in the homologous strain and cross-reacted widely with related strains (Fig. 4.6, Table 4.13). This is reflected in the O.D. readings for the ELISA (Table 4.14) for the homologous strain and in Tables 4.15, 4.16 and 4.17 for *P. intermedia*, *B. fragilis*, and *P. asaccharolyticus* respectively. In the case of the hyperimmune serum, the results of the Western blots were reflected by the quantitative results of the ELISA.

One area of interest in the reactivity of the pooled sera from C57BL/6 mice was the relatively high O.D. values for IgM antibody when whole cells of *P. melaninogenica*, *B. fragilis* and *P. asaccharolyticus* were used as antigen (Tables 4.15, 4.16, 4.17). In these cases the O.D. values for the control groups were equivalent to those of the immune mice. This was not the case with *P. intermedia* (Table 4.14), where the reactions to whole cells were generally below the threshold (O.D. - 0.4). This difference in reactivity suggests that while C57BL/6 mice carry natural IgM antibody to *P. melaninogenica*, *B. fragilis*, and *P. asaccharolyticus*, such is not the case with *P. intermedia.* In the former organisms, the antigens recognized by IgM appear to be associated with whole cells and not cell membranes, as the ELISA O.D. values were below the threshold of 0.2, when the latter were used as antigens (Tables 4.15, 4.16, 4.17).

Balb/c mice were immunized with high doses of cells and boosting produced high levels of antibody (IgG - 0.0673-1.81 0.D., Table 4.14) to *P. intermedia* (M28, 29). However, although increased response to *P. melaninogenica* and *P. denticola* were seen in Western blots (Figure 4.4, Table 4.10) this was not apparent from the O.D. readings in ELISA (Tables 4.15, 4.16, 4.17). In common with C57BL/6, Balb/c mice showed

relatively high levels of IgM antibody to whole cells of *P. melaninogenica*, *B. fragilis* and *P. asaccharolyticus* (Tables 4.15, 4.16, 4.17), however in contrast to C57BL/6, their sera also reacted (ELISA O.D. - 0.209-0.466) with outer membranes of *B. fragilis* (M13-16).

Taken together, these results indicate that quantitative changes in antibody to strains related to *P. intermedia* will only be stimulated by hyper-immunization of the mice. Immunization with *P. intermedia* without FCA and boosting increased the level or reactivity of antibodies to antigens specific for the immunizing strains as measured by ELISA. Low dose chronic immunization reflected this situation and indicated that sera with increased reactivity may only recognize antigens to which the animals are "naturally" sensitized, that is, antigens detected on Western blots by control non-immunized mouse sera. Increased amounts of antibody, both IgG and IgM were produced against antigens on whole cells of some cross-reacting strains and it was apparent that the mice also carried natural antibody (IgM) to these organisms. Comparing the reactivity of sera in Western blots to the O.D. readings in ELISA, it was apparent that in the case of the immunizing strain with high dose and chronic doses of cells, and with hyperimmune serum with related strains, increased reactivity on blotting was reflected by higher O.D. levels in ELISA.

A final quantitative analysis of the response was made by assay of the ELISA values for individual mice in a single group. The sera from mice analyzed by Western blot (Figure 4.7) was assayed by ELISA using outer membranes as antigen. The results are shown in Table 4.19 and increases in O.D. of both IgG and IgM for the immunized (M36) versus the control group (M37) were detected. There was variation in the O.D. levels between the mice, but the means and standard deviations were reasonable: IgG (mean 0.886, S.D. 0.205), IgM (mean 0.757, S.D. 0.132) and it was clear that the immunized mice had significantly higher reactivity to the outer membrane antigens than the controls. However, the ELISA O.D. levels for the individual sera did not match the

reactivity of the serum on Western blots, except for mouse 6 (Figure 4.7), suggesting that in individual mice there may not always be a correlation between reactivity on blots and levels of antibody assayed by ELISA.

These results obtained from the immunization of mice with a single organism may provide further information about development of an immune response in humans with periodontal disease. Several studies have demonstrated an increased level of antibody to organisms isolated from disease-active periodontal pockets (Ebersole, et al., 1982; Ebersole et al., 1984; Ebersole et al., 1987), while serological studies of *Bacteroides* (*Prevotella*, *Porphyromonas* and *Bacteroides*) using hyperimmunized rabbit sera has demonstrated extensive cross-reactivity among these genera (Bowden and Nolette, 1990). In mice, however, little cross-reactive response was generated by single low dose immunization or chronic low dose immunization, which could be analogous to situations encountered in periodontal disease in humans.

Low chronic dose immunization of mice (M31) produced serum with increased reactivity of specific antibodies, which were already present in the animal's "natural" repertoire (control mice). In 1990, Deslauriers and Mouton showed that specific antibody to an immunodominant hemagglutinating adhesin of *P. gingivalis*, which is required for the successful colonization by this bacteria was present in both healthy and chronically diseased periodontitis patients. Together, these results suggest that part of the response to organisms involved in periodontal disease progression, may involve the production of antibodies reactive with antigens to which the host has already been sensitized through colonization by the resident flora (antibodies present in "normal" patients), rather than by the generation of specific or true cross-reacting antibodies.

In contrast, extensive IgG cross-reactivity with related species of *Prevotella, Bacteroides* and *Porphyromonas* was evident only with hyperimmune mouse serum (M33), suggesting that the production of cross-reactive antibodies in humans may require an excessive stimulation of the immune system. Such a situation could be

encountered in patients with severe periodontal breakdown, i.e. the late stages of periodontal disease, or possibly with rapidly progressing periodontitis. As periodontal disease in most individuals is a chronic, slowly progressing disease with bursts of activity, followed by periods of remission, antibody production in disease most likely involves the production of antibody to antigens already recognized by the host's natural antibody repertoire, specific antibody formation in response to immunization or a combination of both.

Both children and disease-free healthy adults possess a "natural" antibody repertoire capable of recognizing antigens present in organisms isolated from the mouth. In order to further understand the humoral response to microorganisms in periodontal disease in humans, a study of changes in the pattern of "natural" antibodies in humans compared to experimental gingivitis would be beneficial. Colonization of the periodontal pocket by anaerobic Gram-negative organisms may result in the production of specific IgG and IgM antibody directed at the new colonizers, or activation of antibody clones to which the host has been previously sensitized. To determine the role of host sensitization and antibody formation, future mouse studies involving the sensitization of the animal with a related microorganism, such as *P. melaninogenica*, followed by immunization with *P. intermedia* could provide useful information.

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APPENDIX A

Growth Media Used in Experiments

B. M. Medium (for bulk growth of fastidious bacteria)

Tryptone or Trypticase Peptone	1%
Proteose Peptone or Tryptose	1%
Yeast Extract	0.5%
Glucose	0.5%
Sodium Chloride	0.5%
L-cysteine HCl	0.075%
Neutralized liver digest	0.25%

Dissolve ingredients in distilled in 1 liter of distilled water and adjust pH to 7.4
Haemin (1ml/100 ml) and menadione/Vitamin K (0.02ml/100 ml) are added
Autoclave at 121°C for 30 minutes.

Supplemented Blood Agar

Blood Agar Base No. 2 4%

Dissolve ingredient in 1 liter of distilled water and autoclave at 121°C for 30 minutes.
Allow media to coll to approximately 50°C, before adding the following ingredients aseptically:

Haemin	1ml/100 ml
menadione or Vitamin K	0.02 ml/100 ml

Haemin solution

Dissolve 50 mg. haemin in 1 ml/1N NaOH; make up to 100 ml. with distilled water.
Autoclave at 121°C for 15 minutes.

Vitamin K_1 Stock solution-Menadione

Dissolve 0.15 ml. of Vitamin K_1 in 30 ml of 95% ethanol. Do not sterilize.
Keep Vitamin K_1 stock solution under refrigeration.

EDTA Buffer (2x)

Dibasic Sodium Phosphate (Na_2HPO_4) (0.05M)	14.2 g.
Sodium Chloride (NaCl)	17.4 g.
Sodium EDTA	7.44 g.
Distilled water	made up to 1 liter
pH 7.4	

Hepes Buffer (10 mM)

Dissolve 2.381 grams of Hepes in 1 liter of distilled water. pH to 7.4

APPENDIX B

Reagents for ELISA Assay

Coating Buffer - 0.05 M carbonate buffer, pH 9.6 + 0.02% NaN_3

1.6 g. $Na_2 CO_3$ (anhydrous)
2.9 g $NaHCO_3$
0.2 g NaN_3
make up to 1 liter volume with distilled water
pH -9.6
Discard after 2 weeks

Blocking Reagent
0.1 % Bovine Serum Albumin
0.02% NaN_3
Make up to 1 liter with Phosphate Buffered Saline
pH 8.0

Phosphate Buffered Saline
1 tablet Phosphate Buffered Saline(OXOID Ltd., England) dissolved in 100 mls. of distilled H_2O

Phosphate Buffered Saline-Tween
0.1% Tween 20
0.02% NaN_3
Make up to 1 liter with Phosphate Buffered Saline
pH-8.0

Horse Radish Peroxidase - Diluent
0.1% Bovine Serum Albumin
Make up to 1 liter with Phosphate Buffered Saline
pH 8.0
No NaN_3 added

Citrate-Phosphate Buffer
Mix 4 parts 0.1 M citric acid with 6 parts of 0.2 Na_2HPO_4.
Adjust pH to 4.5, if necessary with either solution.

Substrate
1mg/ml of ortho-phenylenediamine in citrate-phosphate buffer (pH4.5)
Add 0.012% H_2O_2 (4 µl of 30% H_2O_2/10 ml of buffer)
**Substrate is light sensitive