JUL 10 2006

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	5.Jul.06	THESIS

4. TITLE AND SUBTITLE
THE T-CELL RESPONSE ACTINOBACILLUS ACTINOMYCETEMCOMITANS.

5. FUNDING NUMBERS

6. AUTHOR(S)
CAPT MCDONOUGH PATRICK M

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
UNIVERSITY OF TEXAS HSC AT SAN ANTONIO

8. PERFORMING ORGANIZATION REPORT NUMBER
CI04-1817

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
THE DEPARTMENT OF THE AIR FORCE
AFIT/CIA, BLDG 125
2950 P STREET
WPAFB OH 45433

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Unlimited distribution
In Accordance With AFI 35-205/AFIT Sup 1

DISTRIBUTION STATEMENT A
Approved for Public Release
Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS

15. NUMBER OF PAGES
58

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

The T Cell Response to *Actinobacillus actinomycetemcomitans*

A

THESIS

Presented to the Faculty of

The University of Texas Health Science Center at San Antonio

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

DISTRIBUTION STATEMENT A
Approved for Public Release
Distribution Unlimited

By

Patrick Michael McDonough, D.D.S.

San Antonio, Texas

May 2006

The T Cell Response to *Actinobacillus actinomycetemcomitans*

Publication No. _______________________

Patrick Michael McDonough, D.D.S., M.S.

The University of Texas Health Science Center at San Antonio

Graduate School of Biomedical Sciences

Supervising Professor: Ellen B. Kraig, Ph.D.

The association between *Actinobacillus actinomycetemcomitans* (*Aa*) and aggressive periodontitis is well documented. Although the humoral immune response to *Aa* has been thoroughly studied, little is known concerning the *Aa* proteins that elicit a T cell response. Heat shock proteins (HSPs) are known to be immunodominant in some oral and nonoral diseases. The purpose of our study was to characterize more fully the T cell response to the *Aa* HSPs GroEL, GroES, and DNAJ in mice using T cell hybridoma technology and in human aggressive

periodontitis patients via stimulation of peripheral blood T lymphocytes (PBCs) to individual *Aa* HSPs.

Genes coding *Aa* HSPs GroEL, GroES, and DNAJ were amplified by polymerase chain reaction (PCR), cloned into a prokaryotic expression vector, and recombinant proteins were produced. These HSPs were then screened for the ability to stimulate *Aa*-specific murine T cell hybridomas previously generated from orally inoculated BALB/c and C57Bl/6 mice. In addition, the recombinant HSPs were tested for the ability to either stimulate or inhibit a proliferative response of PBCs from patients diagnosed with aggressive periodontitis. *Aa* infection in the human subjects was assessed by PCR analysis of subgingival plaque samples using *Aa*-specific primers.

Of the 36 murine T cell hybridomas tested, 3 recognized GroES and 1 was stimulated by GroEL. Thus, mice inoculated with viable *Aa* do generate a T cell response to certain HSPs. None the less, these recombinant HSPs were unable to stimulate proliferation of human T cells from periodontitis patients. T cell proliferation was observed in one control subject in response to *Aa* HSPs, although PCR analysis did not indicate the presence of *Aa* in the sites sampled. This validates our experimental model and shows that T cell proliferation is seen to certain *Aa* HSPs. The *overall* lack of human T cell reactivity may have been due to the absence of active *Aa* in the periodontium of these particular individuals in this study. A Western blot analysis of subject sera indicated cross-reacting antibodies to *Aa* in all subjects (particularly C4), suggesting a possible history of prior exposure. Efforts are currently underway to repeat the analysis using PBCs from localized aggressive periodontitis patients where *Aa* is likely to have been involved and is still present.

The current study does, however, clearly demonstrate that recombinant proteins, including GroEL, were not inhibitory. The proliferative response of normal human T cells to a mitogen, phytohemaglutinin (PHA), was compared in the presence of normal, heat-killed, and UV irradiated HSPs and whole cell *Aa* extracts. The human T cells proliferated well to mitogenic stimulus, even in the presence of *Aa* GroEL. In addition, the inhibitory effect of *Aa* on human T cells was heat labile, suggesting the existence of a toxic factor in *Aa* that is likely to be a protein. The HSPs GroES and GroEL were identified as T cell epitopes in our murine hybridoma model. The lack of proliferation to the same *Aa* antigens in human subjects may indicate variability in host T cell responses or that recent *Aa* exposure is necessary. Importantly,

recombinant GroEL alone was not inhibitory, and the observed heat labile inhibitory effect may indicate the presence of an as yet unidentified *Aa* T cell suppressive factor.

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Acknowledgements

I would like to thank my research mentor, Dr. Ellen Kraig, for her support, patience, and understanding over the last couple years. An excellent teacher, Ellen has the unique ability to guide students into a true understanding of science not attainable on one's own. I will forever count myself lucky to pursue my graduate studies under her tutorship.

I would also like to thank every member of the Kraig lab, particularly Kendra Johnson, for her willingness to help and selfless devotion of time and effort into this project. Without the technical support and guidance of so many this research would not have been possible.

Finally, I am very grateful to my committee members, Dr. Ellen Kraig, Dr. David Cappelli, Dr. Howard "Mick" McDonnell, Dr. Michael Mills, and Dr. Judy Teale for their patience and understanding.

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I. Introduction and Background

A. Periodontal Disease

Periodontitis is defined as "an inflammatory disease of the supporting tissues of the teeth caused by specific microorganisms or groups of microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession, or both" (1). It is well recognized that periodontal disease may present as several distinct conditions, including chronic periodontitis, aggressive periodontitis, necrotizing forms of periodontal disease, and/or as a manifestation of systemic disease. Although numerous risk factors are known to contribute to disease susceptibility and severity, the primary etiology of all forms is a shift in the bacterial flora towards mostly gram-negative organisms. In addition, certain species appear to be more heavily involved in the initiation and progression of the disease, including *P. gingivalis*, *E. corrodens*, *T. denticola*, and *A. actinomycetemcomitans* (2-3). Periodontal destruction most likely results from the combined actions of periodontopathic microorganisms and by the inflammatory mediators released by the host immune system in response to infection (4). Thus, pathogenic microorganisms are required, but not sufficient, to cause disease; a susceptible host is inherently necessary.

The progression of periodontal disease begins with the colonization of the gingival sulcus by periodontopathic microorganisms. In the model proposed by Page and Schroeder, this colonization leads to the formation of the initial lesion (sub-clinical gingivitis) within 2 to 4 days (5). This stage is characterized by vasculitis, exudation of fluid from the sulcus, increased migration of polymorphonuclear leukocytes (PMNs), alteration of the junctional epithelium at the base of the pocket, and the dissolution of perivascular collagen (5). The initial lesion is followed by the early lesion within 4 to 7 days following bacterial plaque accumulation. The early lesion is associated with leukocyte accumulation at the site of acute inflammation, cytopathic alterations in resident fibroblasts, increased collagen loss within the marginal gingiva, and proliferation of the basal cells of the junctional epithelium (5). If untreated, the inflammatory reaction persists to form the established lesion within 2 to 3 weeks following the onset of plaque accumulation. Plasma cells predominate in the established lesion, and the presence of immunoglobulins within the connective tissue and junctional epithelium is noted (5). Collagen destruction continues along with proliferation, apical migration, and lateral extension

of the junctional epithelium. Early pocket formation may or may not be present at this stage. The disease may then remain confined indefinitely to the marginal and coronal portion of the gingiva, or it may progress to the advanced lesion (5). This stage shares many features with the established lesion but includes the loss of alveolar bone, periodontal ligament and the formation of periodontal pockets. Unlike the relatively innocuous initial, early and established lesions, the advanced lesion and associated bone destruction may progress until eventual tooth loss (5).

The most common form of periodontal disease is chronic periodontitis. The bacterial species typically implicated in chronic periodontitis are *P. gingivalis, T. denticola,* and *T. forsythia* (4). The prevalence of chronic periodontitis increases with age, and the disease usually becomes clinically significant only in adults. The diagnosis of periodontitis is determined through a complete clinical examination and the measurement of the clinical attachment level (CAL) around teeth. This measurement combines the probing pocket depth and recession measurement from a fixed reference point, usually the cemento-enamel junction (CEJ), to determine the severity of disease. The prevalence in a population, however, depends greatly on the threshold chosen, ranging from as high as 99% with >1mm of attachment loss to a low of 7% for a threshold of >7mm (6). At a threshold of 3mm, the prevalence of attachment loss in at least one site in the mouth was 53.1%. The prevalence of periodontitis increases steadily with age; 35.7% for adults aged 30-39 to 89.2% for those in the 80-90 year old group (6).

In a study by Brown, which evaluated the prevalence, severity, and extent of periodontitis in an employed U.S. adult population, loss of attachment (LOA) >1mm was found in 99% of individuals (7). Approximately 44% of persons aged 18-64 exhibited LOA >3mm at 1 or more sites, with an average of 3.4 affected sites per person. The number and percentage of sites affected was found to increase with age, and on average, African-Americans had slightly more LOA than whites, and males were slightly more at risk than females (7).

Without intervention, the periodontal lesion usually progresses over time. In a series of studies by Löe, the natural history of periodontal disease was studied in two populations; a Norwegian group which received regular dental care and a Sri Lankan population without access to any dental care (8). By age 40, the Norwegian population exhibited a mean clinical attachment loss (CAL) of approximately 1.5mm, while the Sri Lankans showed a mean CAL of 4.5mm. Over time, the rate of interproximal attachment loss was 0.08mm/year and 0.3mm/year, respectively (8). Further analysis of the Sri Lankan population showed that not all individuals

lost attachment at the same rate. Although virtually all gingival surfaces showed severe inflammation, LOA varied considerably and three different populations emerged: those with rapid progression (RP) of periodontal disease (8%); those with moderate progression (MP) of disease (81%); and those who remained stable, or non-progressing (11%) (9). By age 45, the mean LOA for the RP group was 13mm, while the MP group was 7mm. Thus, 89% of the Sri Lankan population had severe periodontitis that progressed at a much more rapid rate than the Norwegian population in the absence of proper therapy and maintenance (9). However, the possibility of genetic variations within this population cannot be dismissed.

B. Aggressive Periodontitis

Aggressive periodontitis is a form of periodontal disease characterized by rapid and severe loss of attachment. The rate of periodontal destruction can be three to four times faster than in chronic periodontitis (10). Similar to chronic periodontitis, aggressive periodontitis may be classified as localized (occurring around certain teeth in the mouth) or generalized (following a widespread distribution), and both forms are associated with particular bacterial microbiota. The localized form (formerly Localized Juvenile Periodontitis) is typically circumpubertal in onset, affects females more than males, often presents with an incisor-first molar distribution, and is associated with a robust antibody response (11). A prominent feature of localized aggressive periodontitis is the lack of clinical gingival inflammation despite the presence of deep periodontal pockets. In addition, the amount of bacterial plaque often seen on the teeth is inconsistent with the extensive periodontal destruction characteristic of the disease (11). Other clinical features of the disease include deep pain on mastication, first molar mobility, distolabial migration of maxillary incisors, and occasional periodontal abscess formation with concomitant regional lymph node enlargement (12). The prevalence of localized aggressive periodontitis in the United States is estimated to be less than 1%, and most reports suggest an even lower figure near 0.2% (13). African Americans appear to be at a much higher risk for localized aggressive periodontitis, and somewhat surprisingly, black males were 2.9 times more likely to present with the disease than black females. White females, however, are more likely to have the disease than white males (14). An individual is most likely to develop the disease between the onset of puberty and 20 years of age (15).

The generalized form of aggressive periodontitis has many of the same features, but tends to afflict slightly older individuals (<30 years old) with a more widespread distribution in the oral cavity (16). The disease affects at least three permanent teeth in addition to the incisors and first molars. As with the localized form, patients present with extensive loss of periodontal attachment with seemingly small amounts of plaque on the teeth (11). Patients with generalized aggressive periodontitis may also exhibit systemic manifestations of the disease, such as weight loss and psychological depression (17). In a study of U.S. adolescents aged 14 to 17, the prevalence of generalized aggressive periodontitis was 0.13%. African Americans were at much higher risk than whites for all forms of the disease, and males were more likely to have generalized aggressive periodontitis than females (13). In another study of Sri Lankan tea workers, Löe and colleagues reported that 8% of the population exhibited an annual loss of attachment consistent with generalized aggressive periodontitis (18). Interestingly, aggressive periodontitis is sometimes seen in individuals with immune deficiency disorders, suggesting that a normal immune system may provide some protection from periodontal disease (16).

C. Immune Defects Associated with Aggressive Periodontitis

Various defects in the immune response have been implicated in the pathogenesis of aggressive periodontitis. Certain human leukocyte antigens (HLAs) have emerged as likely candidate markers for periodontal disease susceptibility due to their role in immune regulation. HLAs are cell surface molecules which are critical for T cell recognition of antigen. Over 40 diseases, most of which are autoimmune in nature, have been associated with particular haplotypes of HLAs (19). While over 150 different HLA haplotypes have been identified, the two most consistently associated with aggressive periodontitis are HLA-A9 and B15. The risk of disease in subjects with HLA-A9 and B15 was roughly 1.5 to 3.5 times greater than in subjects lacking these molecules (19). However, these haplotypes are seen in only 31% and 21% of affected patients, respectively, so other factors are likely involved (19).

Several studies have shown that aggressive periodontitis patients exhibit defects in their polymorphonuclear leukocytes (PMNs), monocytes, or both (20-22). In some patients, the chemotactic abilities of PMNs are impaired, while in others, the ability to phagocytose and kill microorganisms at the site of infection is decreased. Such defects in PMNs, which contribute to the early "first response" of cellular immunity, can severely impair the host's ability to neutralize

the pathologic microbial challenge (20-22). Patients with localized aggressive periodontitis have also shown hyperresponsiveness of monocytes and elevated prostaglandin E_2 (PGE_2) production in response to lipopolysaccharide (LPS) from periodontopathic bacteria, such as *A. actino-mycetemcomitans* and *P. gingivalis* (23). Excessive production of inflammatory mediators such as PGE_2 can lead to tissue destruction and bone loss. In addition, the receptor for IgG2 antibodies on monocytes, FcγRII, is poorly functioning in a subset of patients with localized aggressive periodontitis, which can impair the antibody response to the disease (24). Finally, polymorphisms in the interleukin (IL)-1 gene may influence one's susceptibility to aggressive periodontitis (25). IL-1 is an important inflammatory cytokine produced by activated monocytes. Elevated levels of IL-1 can lead to increased inflammatory destruction, as seen in one genetic variant which results in a four-fold increase in IL-1β production (26). This variant is seen in approximately 35% of Northern European Caucasians, compared to <3% of first generation Chinese-Americans (25, 26).

D. *Actinobacillus actinomycetemcomitans* (*Aa*) and Aggressive Periodontitis

Although numerous microorganisms are found in patients with aggressive periodontitis, *Actinobacillus actinomycetemcomitans* (*Aa*) has been implicated as the primary pathogen associated with the initiation and progression of one form of the disease, localized aggressive periodontitis (LAP) (28). A nonmotile, gram-negative, capnophilic coccobacillus, *A. actinomycetemcomitans* is also associated with forms of chronic periodontitis and other nonoral human infections (27). The link between *A. actinomycetemcomitans* and aggressive periodontitis, specifically the localized form of the disease, is based on several lines of evidence summarized by Tonetti and Mombelli (27): 1) *A. actinomycetemcomitans* is found in over 90% of lesions characteristic of localized aggressive periodontitis (29-33); 2) sites that show evidence of disease progression are more likely to have elevated levels of *A. actinomycetemcomitans* (29,31-33); 3) the majority of patients with systemic manifestations of localized aggressive periodontitis have significantly elevated serum antibody titers to *A. actinomycetemcomitans* (32,34-37); 4) several studies have shown a correlation between reduction in the bacterial load of *A. actinomycetemcomitans* and a favorable clinical response (31, 32, 38-40); and 5) *A. actinomycetemcomitans* is known to produce a variety of virulence factors which may impact the disease process.

Virulence factors are attributes of a microorganism that allow it to "colonize a particular niche in its host, overcome the host defenses, and initiate a disease process" (41). The virulence factors of *A. actinomycetemcomitans*, as summarized by Fives-Taylor, include factors that promote colonization and persistence in the oral cavity, such as adhesins, bateriocins, and invasins (42-44). Other attributes of *Aa* include factors that interfere with host defenses, such as leukotoxin (which kills PMNs), immunosuppressive factors, and chemotactic inhibitors (45-49). *Aa* also produces cytotoxins, collagenases, proteases, and LPS which mediate bone resorption and stimulate the production of inflammatory mediators from host immune cells (50-54). Other factors enable *Aa* to attach to extracellular matrix proteins and invade epithelial cells; thereby making them resistant to non-surgical debridement or locally delivered chemotherapeutics (42, 51, 52, 55-58).

Typically, *A. actinomycetemcomitans* infections are successfully treated with aggressive therapy involving surgical intervention in conjunction with systemic antibiotics, including metronidazole, amoxicillin, or tetracycline family derivatives (38-40). The increasing resistance of *A. actinomycetemcomitans* to antibiotics is prompting concern among the dental community, as treatment failures for oral conditions could make a periodontal patient more susceptible to systemic infection involving *Aa* (56, 59). Thus, current research is focusing on the development of new antibiotics to counter this threat. Another, and perhaps more important approach, is to develop vaccines that could afford protection against *Aa* infections.

E. Heat Shock Proteins

Resident microorganisms in the periodontal pocket are subjected to a wide range of environmental conditions and must adapt to ensure survival. Even the consumption of hot and cold foods can cause significant variations in gingival temperatures (60). Although not as dramatic, the inflammatory process in diseased periodontal sites leads to an increase in temperature relative to that seen in healthy sites (60). The pH can also be affected by the presence of periodontal pathogens. For example, bacterial by-products, such as organic acids and proteases, influence the pH of the oral cavity (61). The metabolic activities of asaccharolytic bacteria such as *P. gingivalis* and *F. nucleatum* cause an increase in the pH of gingival crevicular fluid to 8.5, compared to 6.9 in a healthy gingival sulcus (61). Variations in oxygen levels and the availability of nutrients also significantly influence the survival and growth of the oral flora.

In an effort to cope with such a wide range of environmental stressors, prokaryotic and eukaryotic cells respond by synthesizing stress proteins, including heat-shock proteins (HSPs). Many are expressed constitutively under normal physiologic conditions, but are up-regulated during periods of stress. This induction is extremely rapid, and represents an emergency response of the microorganism (62). Even slight changes in pH, temperature, redox potential, and nutrient availability can induce their synthesis (63). The function of HSPs is to act as molecular chaperones in the assembly and folding of proteins, and also as proteases in the degradation of damaged or toxic proteins (64-66). In this way they protect cells from the harmful effects of non-physiologic, stressful conditions. Even sub-lethal exposure to stress can impart immunity to lethal levels of the same (adaptive response) or different (cross-protection response) stress via an increase in HSP synthesis (66, 67). In a recent study, Goulhen and colleagues showed that *Aa* cells pre-stressed at 43°C were transiently protected against a lethal temperature stress (68).

Heat shock proteins are highly conserved, and commonly grouped into families based on molecular weight (64). These include GroES-homologue or HSP10 (~ 10kDa), DnaJ-homologue proteins or HSP40 (~ 40 kDa), GroEL-homologue proteins or HSP60 (~ 60 kDa), and others families with molecular weights of approximately 70, 90, and 100 kDa (63-66, 69). HSPs that belong to the same family share strong similarities in amino acid sequence. HSPs are also highly immunoreactive, although various factors influence the resultant host immune response (70). As summarized by Shinnick, the immune system targets HSPs because: 1) they are highly conserved across bacteria, 2) they are abundant cellular proteins, 3) they are presented to host immune cells frequently, and 4) they are likely an independent virulence factor (71). HSPs are also known to be immunodominant antigens of many different human pathogens, including the etiologic agents of malaria, syphilis, Lyme disease, and leprosy (71, 72).

F. Role of Heat Shock Proteins in *Aa* Pathogenicity

HSPs are known to be expressed in periodontal diseases. In a study by Ando, gingival homogenate samples from patients with periodontal disease showed strong reactivity with anti-human HSP60 antibodies, while gingival tissues from healthy controls did not (73). Tabeta et al. reported that gingival crevicular fluid (GCF) from both healthy and diseased periodontal pockets contained antibodies to GroEL, but that experimental samples reacted more strongly to *P.*

gingivalis GroEL than did controls (74). These studies suggest that the production of bacterial HSPs in diseased pockets may lead to the formation of immunocomplexes with specific antibodies. In addition to the antibody response to HSP60, Yamakazi found that peripheral blood mononuclear cells (PBMC) from patients with periodontal disease have a higher proliferative response to HSP60 compared to PBMC from healthy controls. Interestingly, this study also found that HSP60-reactive T cells accumulate in diseased periodontal pockets (75).

Several studies have showed that *Aa* GroEL is an immunodominant antigen in humans. One study found that sera from ten healthy patients did not react to *Aa* GroEL, while sera from 9 of 29 periodontal patients had antibody titers to this HSP (76). Mice immunized with *Aa* extracts showed a primary immune response against a 65 kDa protein, which is likely GroEL (77). Goulhen et al. found that GroEL was externally localized by stressed *Aa* cells, and that this native GroEL was extremely toxic to human periodontal ligament cells (78). Other investigators hypothesize that the recognition of specific epitopes on highly conserved antigens, such as HSPs, contribute to one's protective immunity (79). Alternatively, this reaction could lead to a pathological autoimmune reaction (70, 71, 74). Evidence of cross-reactivity of host and pathogen HSPs may be a factor in such reactions, where "molecular mimicry" between bacterial HSPs and human HSP60 or other proteins may lead to autoimmunity (79).

G. Immune Response to *Aa* Infection

The immune response to infection can be divided into two basic categories: humoral and cell-mediated. This division is rather arbitrary, for both "categories" function in a cooperative and interrelated manner to protect the host. Another distinction made in the immune response is that between innate and adaptive immunity. Innate immunity includes phagocytes such as macrophages and neutrophils, which can recognize certain bacterial surface molecules, complement proteins, and/or endogenous pyrogens to produce fever. Other nonspecific barriers such as skin and glandular secretions also assist in protecting the host from infection (80). Despite their effectiveness, nonspecific immune responses often fail to completely eliminate infections. In the case of *Aa,* its leukotoxin kills host PMNs, thereby compromising one arm of the innate immune response (28). The process of adaptive immunity includes responses dependent on lymphocytes, which can recognize specific antigens on bacteria, viruses, or other pathogens and efficiently destroy the organisms. Immune memory, which develops during an

adaptive immune response, protects the host against reinfection with the same microorganism (81).

H. Human B Cell Response to *Aa* Infection

Specific antibodies to *A. actinomycetemcomitans* are frequently found in both the serum and gingival crevicular fluid of infected patients (82). Furthermore, increased levels of immunoglobulin G (IgG) antibody to *A. actinomycetemcomitans* serotype b have been shown in 90% of aggressive periodontitis patients (83). Distinct *A. actinomycetemcomitans* proteins are recognized when these antibodies are used as probes on Western blots; these include the leukotoxin, serospecific domains of lipopolysaccharide (LPS), and other outer membrane proteins (OMPs) (84). The presence of certain classes of antibodies, or isotypes, such as IgG and IgA, usually correlates with less severe periodontal destruction, suggesting that they play a protective role in the humoral immune response (85). Among these, IgG2 antibodies appear to be particularly crucial in facilitating phagocytosis of *Aa* by neutrophils (84).

I. Human T Cell Response to *Aa* Infection

The response of T cells and their function in managing an infection of *A. actinomycetemcomitans* is less well understood. T cells play a critical role in regulating the type and specificity of B cell responses, so presumably their role in *A. actinomycetemcomitans* infection is vital in determining the scope of the host response. Nitta and Ishikawa found that genes in the major histocompatability complex (MHC) class II regulated the B cell antibody response to *A. actinomycetemcomitans* in mice (86). T cells, therefore, are likely to be involved, since they recognize antigens in the context of the MHC class II gene products on the cell surface of antigen-presenting cells (86). Studies in animal models provide further evidence that T cells play a critical role in host protection. For example, one study found that nude rats, which lacked functional T cells, exhibited greater bone loss after *A. actinomycetemcomitans* infection (87). In a separate study, adoptive transfer of a Th2 clone specific for outer membrane protein (omp)-34 protected against bone loss in nude rats (88). Th2 T cells help regulate the humoral immune response and typically secrete IL-4, which may play a role in isotype selection.

In contrast, evidence that T cells contribute to disease progression has also emerged. Teng and coworkers found that bone loss increased in non-obese diabetic/severe combined

immunodeficient (NOD/SCID) mice after *A. actinomycetemcomitans* exposure when CD4+ peripheral blood lymphocytes (PBLs) from periodontal patients were adoptively transferred (89). Furthermore, oral inoculation of NOD/SCID mice with *A. actinomycetemcomitans* led to the induction and expression of osteoprotegerin ligand (OPG-L), a key mediator of osteoclastogenesis and osteoclast activation by human periodontal CD4+ T cells (90). Inhibition of OPG-L function reduced alveolar bone destruction and the number of osteoclasts at sites of local periodontal inflammation (90).

The T cell receptor (TCR) Vα and Vβ gene usage of *A. actinomycetemcomitans*-reactive T cells was studied by Gau and Teng (91). The results of PCR analysis showed a heterogenous TCR repertoire in peripheral T cells in patients with localized aggressive periodontitis and in NOD/SCID mice adoptively transferred with T cells from human subjects. In contrast, periodontal T cells expressed clonally dominant Vα and Vβ gene transcripts, suggesting a limited number of T cell specificities at the local site of periodontal inflammation (91). Also, periodontal CD4+ T cells were used as probes to screen the entire *A. actinomycetemcomitans* genomic library. Results showed that a limited number of bacterial antigens, or epitopes, were recognized for immune activation (91).

J. Human T cell Epitopes

The antigens on *A. actinomycetemcomitans* that elicit T cell responses in humans are largely unknown. Recent studies have suggested that *A. actinomycetemcomitans* is a potent activator of T cells, although only a small subset of these activated cells actually differentiated into effector T cells (92). Zadeh et al. reported that only 2-5% of all T cells stimulated with *A. actinomycetemcomitans* expressed any T cell cytokines, such as IL-2, IL-4, INF-γ, and IL-10 (92). This low level of cytokine expression occurred despite the fact that *A. actinomycetemcomitans* activated over half of all T cells in vitro. Such findings suggest that the activation of T cells to *A. actinomycetemcomitans* is incomplete, and that a significant proportion of T cells become unresponsive to restimulation. Zadeh also reported that following this mass activation, large scale apoptosis of the activated human T cells was observed (93,94). In a later study, Zadeh suggested that human T cell inhibition to *Aa* antigens was due to the heat shock protein GroEL, since an antibody directed against *E. coli* hsp60 (GroEL) eliminated the observed T cell apoptosis (95).

The role of T cells specific for individual antigens in the host response is also unclear. To date, one apoptotic effector gene, cagE, has been identified as a human T cell antigen by Teng et al. (96). In that study, all serum samples from *Aa*-infected periodontitis patients, but not from healthy controls, recognized cagE by ELISA and Western blot. Upon secretion, cagE elicited apoptosis on primary human epithelia, endothelia, osteoblasts, and T cells by 4-12 h in vitro. Previous investigators in our lab utilized murine models to characterize the T cell response to *A. actinomycetemcomitans* (see next section). Such characterization of the T cell response to *A. actinomycetemcomitans* is a critical step in the eventual design of a vaccine, and also serves to directly link T cells in the pathogenesis of periodontal diseases.

K. BALB/c and C57Bl/6 *Aa*-Specific T cell Hybridoma Panels

In an effort to elucidate the various T cell antigenic determinants on *A. actinomycetemcomitans*, murine T cell hybridomas were utilized. One such panel was generated by Dr. Paul Ezzo using BALB/c mice orally inoculated with viable *A. actinomycetemcomitans* (97). The lymph nodes were harvested and the activated T cells were immortalized by fusing them with a thymoma line, BWZ.36, creating a panel of T cell hybridomas. These hybridomas were then screened for *A. actinomycetemcomitans* specificity and the sizes of the proteins bearing the T cell epitopes were determined via T cell "Westerns." Interestingly, nine of the fifteen BALB/c hybridomas recognized the 116 kDa leukotoxin (Ezzo, personal communication). An important virulence factor of *Aa*, leukotoxin (Lkt) is a member of the RTX (repeat in toxin) pore-forming toxins, which includes the leukotoxin or hemolysin of pathogenic *Escherichia coli*, *Mannheimia* (*Pasteurella*) *haemolytica*, and *Bordetella pertussis*, the causative agent of whooping cough (98,99). The *Aa* operon consists of four genes: LktA, LktB, LktC, and LktD. LktA encodes for the 116 kDa protein, while the remaining genes encode for proteins involved in processing and transport of the leukotoxin. Early studies showed a high degree of specificity in the cells affected by LktA. These target cells include primarily PMNs and monocytes. Human platelets, fibroblasts, endothelial, and epithelial cells appear to be resistant to LktA (100,101).

In addition, one of the fifteen BALB/c hybridomas was shown to be specific for omp-34, an *Aa* outer membrane protein (Ezzo, personal communication). An earlier study showed that omp-34 elicited the dominant T cell response in a rat model of periodontitis (102). To determine

if *Aa* proteins recognized by one strain were applicable to other mouse strains, a separate T cell hybridoma panel was generated by Michael Hannaman and Christine Gear using lymph node T cells from orally inoculated C57Bl/6 mice (103). The Bl/6 T cell hybridomas were tested for reactivity to *Aa* strains and the LktA and omp-34 knockouts. Only one *Aa*-specific Bl/6 hybridoma recognized leukotoxin, while three were reactive to omp-34 (Gear, personal communication). Thus leukotoxin dominance appears to be strain specific and other antigenic epitopes on *Aa* can elicit a T cell response.

Since neither hybridoma panel was tested for reactivity against heat shock proteins, the next step was to determine whether T cells are elicited to these proteins in mice inoculated with live *Aa*. In addition, we sought to characterize the T cell response to *Aa* in humans with aggressive periodontitis to compare our results with the murine models.

II. Materials and Methods

A. Synthesis of Heat Shock Proteins (GroEL, GroES, and DNAJ)

1. PCR amplification of HSP genes from *Aa* JP2 DNA

To isolate and amplify the individual sequences coding for GroEL, GroES, and DNAJ, PCR (polymerase chain reaction) was performed on genomic DNA from *Actinobacillus actinomycetemcomitans* (strain JP2, serotype B). These DNAs had been previously isolated using standard procedures and were already available in our laboratory (75, 81). A Perkin Elmer Cetus 480 DNA Thermal Cycler (Perkin Elmer Inc., Wellesley MA) was utilized for all PCRs. The primers and PCR conditions are summarized in Table 1. All primers used were synthesized by the University of Texas Health Science Center at San Antonio (UTHSCSA) core facility and employed without further purification. Following the PCRs, 10 μl of each sample was electrophoresed on a 1% agarose gel at 75V for 1 hour (GibcoBRL Model H5, Life Technologies Inc., Gaithersburg, MD), stained with ethidium bromide, and visualized under ultraviolet (UV) light.

2. Insertion in TOPO Cloning Vector

Following PCR amplification, the products were cloned into pCR-TOPO 2.1 (InVitrogen, Inc., Carlsbad, CA) and transformed into one shot chemically competent *E. coli* TOP-10 (InVitrogen). 40 μl from each transformation was grown at 37°C overnight on LB plates (containing 100 mg/ml ampicillin, 24 mg/ml IPTG, and 20 mg/ml Xgal in dimethylformamide (DMF)), and individual transformants were selected. The TOPO 2.1 cloning vector was utilized because it allows easy blue/white screening of colonies (white colonies indicate successful incorporation of an insert, which interrupts and inactivates the β-galactosidase gene). Colonies were harvested with sterile toothpicks and they were used to inoculate flasks of LB broth with 100mg/ml ampicillin and grown overnight at 37°C with shaking. Plasmids were isolated from each overnight using a miniprep kit (Qiagen, Inc., Valencia CA). The plasmid DNAs were resuspended in 200 μl TE pH 8.0 (Tris EDTA, 10mM Tris, 0.1mM EDTA). For each transformant, 5 μl of DNA was digested at 37°C overnight with EcoRI and the products were electrophoresed on a 1% agarose gel at 75V for 1 hour to determine transformants in which the

Table 1: Oligonucleotides and Conditions for PCR Analysis of Heat Shock Proteins. The table shows the different primers and conditions used for the PCR reactions to isolate and amplify the sequences encoding for the HSPs GroES, GroEL, and DNAJ in *Aa* strain JP2.

Table 1

Oligonucleotides and Conditions for PCR Analysis of Heat Shock Proteins

PRIMERS	SEQUENCE (5'/3')	PCR CONDITIONS	SIZE
GroES	5'-AACTGCAGCAAATATTCG TCCATTACACG-3' 5'-AAGAATTCGCGTTTTACT TACTCAACGATCG-3'	95°C 2' 95°C 1' ⎫ 58°C 1' ⎬ 30 cycles 72°C 1' ⎭ 4°C overnight	288bp
GroEL	5'-AACTGCAGCAAAAGACGT AAAATTCGGC-3' 5'-AAGAATTCTTACATCATC CCGCCCATGC-3'	95°C 2' 95°C 1' ⎫ 58°C 1' ⎬ 30 cycles 72°C 1' ⎭ 4°C overnight	1639bp
DNAJ	5'-AAGGATCCGATGGCAAAA CAAGACTAC-3' 5'-AAGAATTCTGGTTTACTT CGTCAGATC	95°C 2' 95°C 1' ⎫ 58°C 1' ⎬ 30 cycles 72°C 1' ⎭ 4°C overnight	1125bp

insert had been successfully cloned into pCR-TOPO 2.1 (Figure 1). From these results one sample of each HSP was selected for large scale plasmid preps (see Figure 1 legend). 200 ml of Terrific Broth [180 ml TB (12 g tryptone, 12 g yeast extract, 4 ml glycerol, qs 900 ml w/ ddH_2O) + 20 ml K-salts (23 g KH_2PO_4, 125 g K_2HPO_4, qs 1 L w/ ddH_2O)] and 0.4 ml ampicillin (stock 100mg/ml concentration) was combined in a 2 L flask. The preparation was inoculated with 1 ml of bacteria from the overnight cultures and incubated at 37°C overnight with vigorous shaking (200 rpm). Large scale plasmid preparations were performed as we previously described (104, 105). Phenol: chloroform extractions were performed. The plasmid DNA was recovered by ethanol precipitation, resuspended in 200 μl TE pH 8.0 (10mM Tris, 0.1mM EDTA) and stored at -20°C.

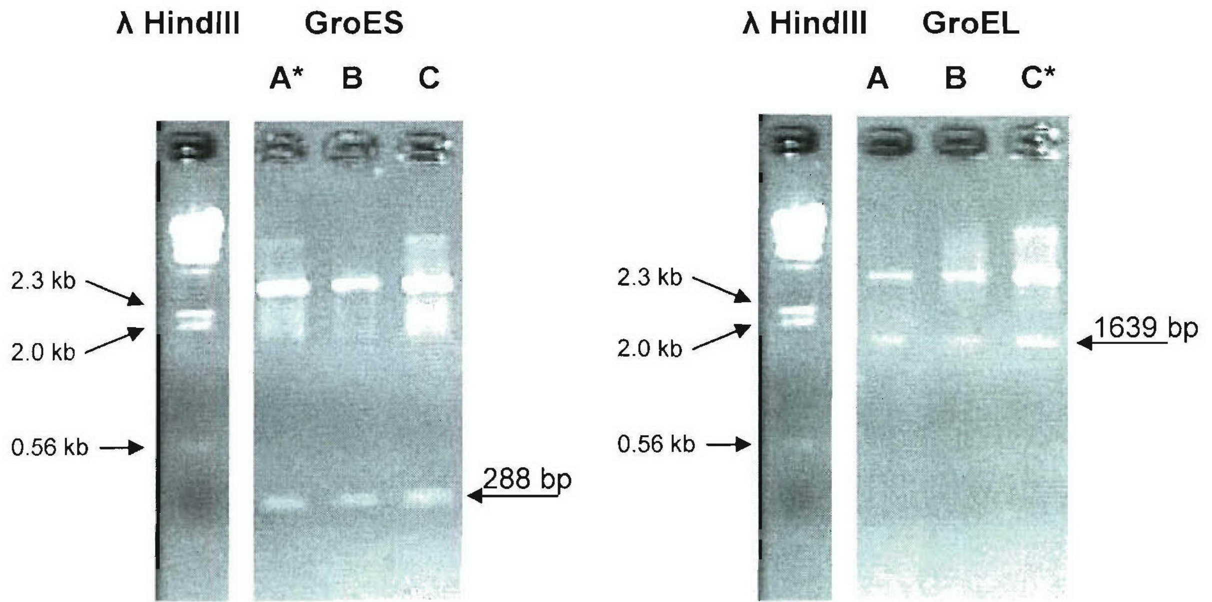
3. Subcloning into pRSETB and Confirmation by Sequencing

Each insert was removed from TOPO 2.1 by digestion with PstI and EcoRI and ligated into the PstI – EcoRI sites in the polylinkers of pRSETB (Invitrogen, Inc), an expression vector. The vector and insert fragments were purified using gels of low melting point agarose (1.2% for the insert and 0.8% for the vector). Both gels were run for approximately 8.5 hrs at 30V. The DNA was stained with ethidium bromide and was then visualized under longwave UV. The DNA bands were cut out, melted at 65°C, and ligated directly in the LMP agarose under standard conditions (NE Biolabs). The ligation proceeded at room temperature for one hour and then at 15°C overnight. Ten microliters of each ligation was then melted, diluted with 100 μl 10mM Tris pH 7.5, and then 200 μl competent TB-1 cells were transformed by the $CaCl_2$ method (106). The transformants were selected on LB plates containing ampicillin. To verify the correct insertion of the HSP DNA into pRSETB, sequencing was accomplished by the Sanger method (dideoxynucleotide chain termination) using Sequenase (US Biochemical, Cleveland OH). The plasmid was recovered via a miniprep kit (Qiagen) following the manufacturer's recommendations. The products were electrophoresed on a 1% agarose gel and mid-size plasmid preparations were completed on appropriate clones (Figure 2).

Following sequencing, large scale plasmid preparations were completed on appropriate clones. The resultant pellets of plasmid DNA were resuspended and samples were electrophoresed on a 1% agarose gel to test for quantity and quality of the inserts.

Figure 1: Cloned HSPs into TOPO 2.1. The following figure shows that the PCR products containing the genes for the HSPs GroES, GroEL, and DNAJ were successfully inserted into TOPO 2.1. Plasmid DNAs were isolated from transformed *E. coli* and digested with a restriction enzyme, EcoRI, to release the inserts. Three independent transformants were tested and all harbored the appropriate inserts. For future experiments, clone A was used for GroES (pEK 872), clone C was used for GroEL (pEK 876), and clone A was used for DNAJ (pEK 877).

Figure 1



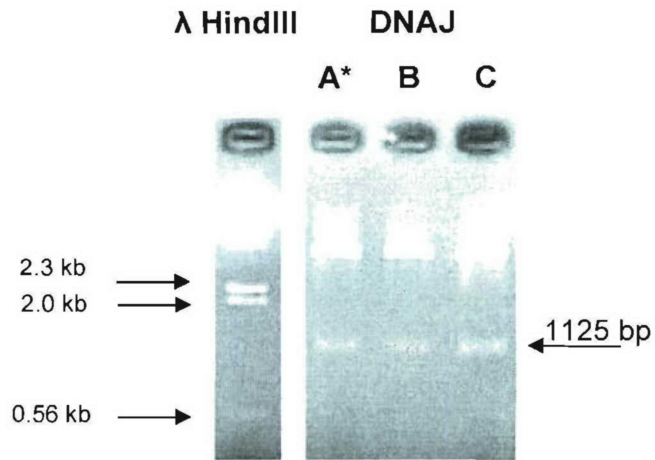
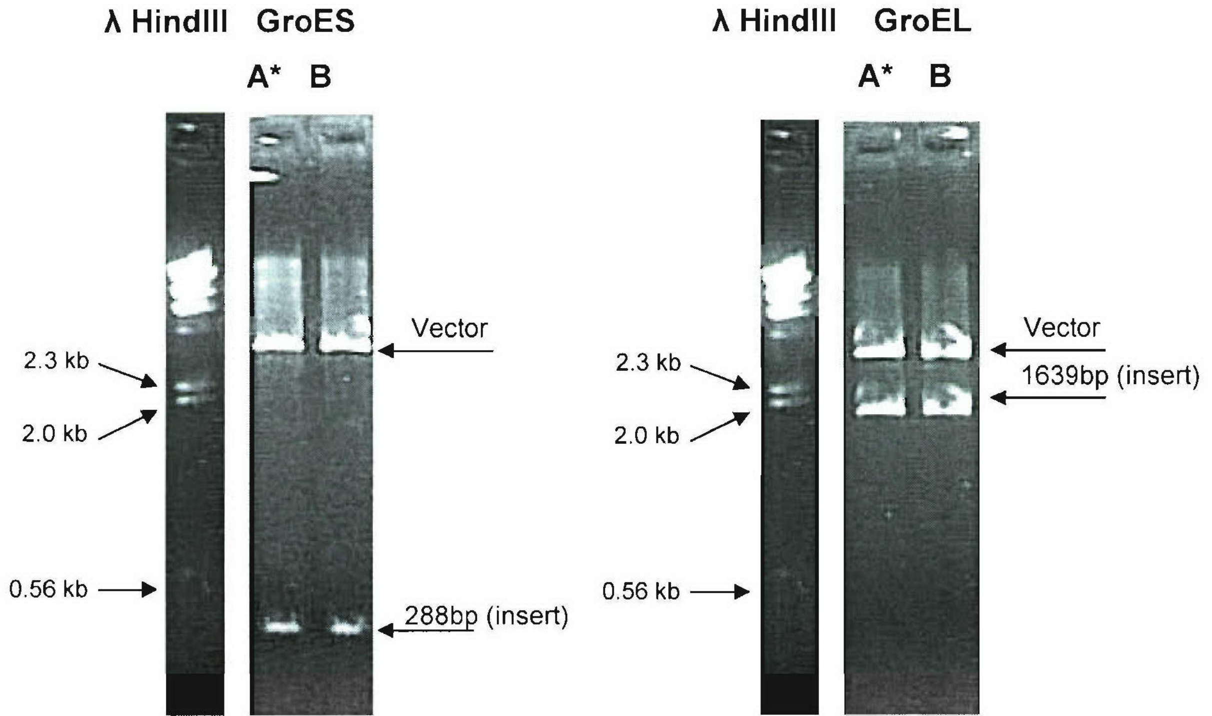
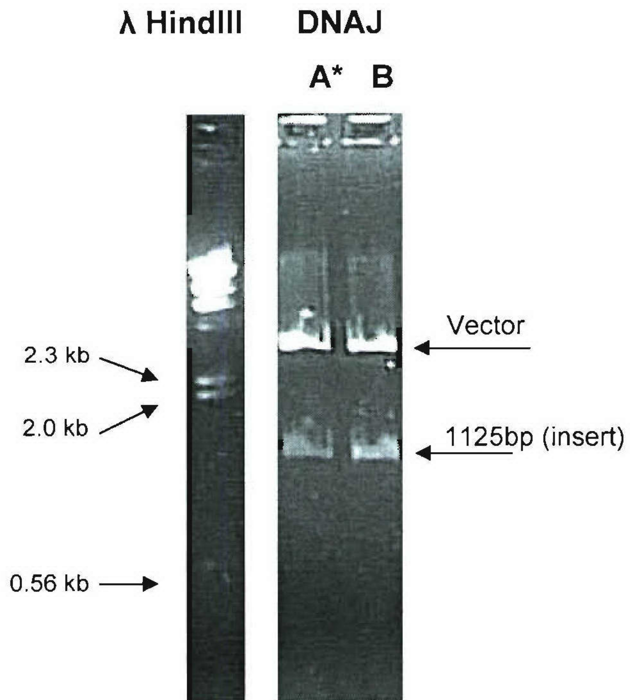


Figure 2: Cloned HSPs into the expression vector, pRSETB. The following figure shows that the PCR products containing the genes for the HSPs GroES, GroEL, and DNAJ had been successfully inserted into pRSETB. (NOTE: All images are not from photographs at the same exposure). Plasmid DNAs were removed from TOPO 2.1 by digestion with the restriction enzymes, PstI and EcoRI, and ligated into the Pst1-EcoRI sites of pRSETB. Two independent transformants were tested and all harbored the appropriate inserts. For future experiments, clone A was used for GroES (pEK 903), GroEL (pEK 905), and DNAJ (pEK 907).

Figure 2





4. Expression of Recombinant Proteins

Once the sequences of the HSP inserts were confirmed, the plasmids were used to transform *E. coli* BL-21 for protein expression. Plated colonies were selected on ampicillin for induction and overnight cultures were grown in liquid LB and ampicillin. Five hundred milliliters of LB broth was inoculated with 10 ml of the overnight preparation and grown at 37°C with shaking until OD_{600} = 0.6. One ml of the pre-induced sample was pelleted and resuspended in 50 μl of 5X SDS-PAGE Sample Buffer (50% glycerol, 0.25M Tris pH 6.8, 5% SDS, 0.0125% Bromophenol blue, 5% β-mercaptoethanol). Expression was induced by the addition of IPTG (Isopropyl b-D-thiogalactopyranoside, Bioline, Canton, MA) to a final concentration of 1mM (5 ml of 100mM stock). The cultures were then incubated at 37°C with shaking for 4 hours. One ml of "induced" sample was collected and resuspended in SDS-PAGE buffer and stored for later use on an SDS-PAGE gel. The 500 ml sample was centrifuged at 9000 rpm for 30 minutes and the pellet stored overnight at -20°C. The pellet was thawed on ice for 15 minutes and resuspended in Buffer A (see Table 2) @ 5 ml/g weight. The solution was then subjected to three cycles of freezing in liquid nitrogen followed by thawing in a 37°C water bath. Finally, the lysate was sonicated at 60% amplitude on constant pulse for 30 seconds on ice (VC100 ultrasonic processor, Sonics & Materials Inc., Newtown, CT). The sample was then centrifuged at 10,000 rpm for 30 minutes to remove all cellular debris and the cleared lysate was frozen at -20°C.

5. Purification of Recombinant Proteins

Where indicated, the recombinant proteins were further purified over Ni-NTA (Nickel-Nichel nitrilotriacetic) resin (Qiagen, Valencia, CA). This enrichment was possible due to the presence of a vector-encoded tag containing 6 histidines located on the amino terminus of the recombinant protein, which facilitated purification over a Ni-NTA column. Ten milliliters of a 50% slurry of Ni-NTA resin was equilibrated with 70 ml Buffer A, 30 ml of Buffer B, 60 ml Buffer C, 25 ml Buffer F, and then followed by another 50 ml of Buffer A. The cleared lysate was added and rocked gently for one hour to facilitate binding of the protein. The mixture was then loaded into an empty column and the cap removed to collect the flow through. The column was washed with 60 ml of Buffer A and 10 ml fractions collected. Next, a wash of 30 ml of Buffer B was completed and 5 ml fractions collected. Washes were completed with 60 ml of

Table 2: Nickel-NTA Column Purification Buffers. The following table lists the composition of all buffers used in the Nickel-NTA column to purify the recombinant *Aa* HSPs.

Table 2

Nickel-NTA Column Purification Buffers

Lysis Buffers

Buffer A (1 L)

100 mM NaH_2PO_4	13.8g NaH_2PO_4-H_2O (MW 137.99 g/mol)
10 mM Tris-Cl	1.2g Tris base (MW 121.1 g/mol)
6 M GuHCl	573g guanidine hydrochloride

Adjust pH to 8.0 using NaOH

Buffer B (1L)

100 mM NaH_2PO_4	13.8g NaH_2PO_4-H_2O (MW 137.99 g/mol)
10 mM Tris-Cl	1.2g Tris base (MW 121.1 g/mol)
8 M urea	480.5g (MW 60.06 g/mol)

Adjust pH to 8.0 using NaOH

Wash Buffer

Buffer C (1L)

100 mM NaH_2PO_4	13.8g NaH_2PO_4-H_2O (MW 137.99 g/mol)
10 mM Tris-Cl	1.2g Tris base (MW 121.1 g/mol)
8 M urea	480.5g (MW 60.06 g/mol)

Adjust pH to 6.3 using HCl

Elution Buffers

Buffer D (1L)

100 mM NaH_2PO_4	13.8g NaH_2PO_4-H_2O (MW 137.99 g/mol)
10 mM Tris-Cl	1.2g Tris base (MW 121.1 g/mol)
8 M urea	480.5g (MW 60.06 g/mol)

Adjust pH to 5.9 using HCl

Buffer E (1L)

100 mM NaH_2PO_4	13.8g NaH_2PO_4-H_2O (MW 137.99 g/mol)
10 mM Tris-Cl	1.2g Tris base (MW 121.1 g/mol)
8 M urea	480.5g (MW 60.06 g/mol)

Adjust pH to 4.5 using HCl

Buffers C and D, with 5 ml fractions collected each time. Finally, a 50 ml wash with Buffer E was performed with collection of 5 ml fractions. The fractions (20 μl) were electrophoresed on an SDS-PAGE gel (Figure 3) and those containing the protein were pooled and dialyzed for two days with two buffer changes (20mL Tris (2M, pH 8.0), 80mL 5M NaCl, QS 4L). Protein concentrations in the pooled fractions were determined using a microtiter plate protocol with the Bio-Rad protein assay (Bio-Rad, Inc., Hercules CA) and read using a microplate reader (MRX Microplate Reader, Dynatech, Inc., Chantilly VA).

B. Human T cell Assays

1. Patient Recruitment

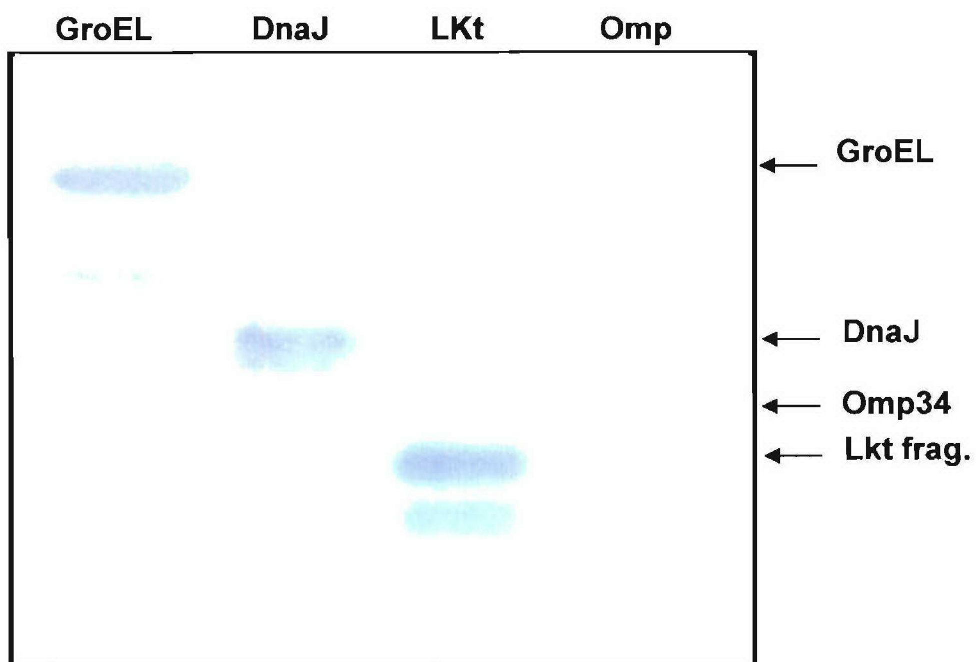
Aggressive periodontitis patients were identified from the patient population of the UTHSCSA Dental School. These patients met the following criteria: 1) diagnosed with aggressive periodontitis as confirmed by medical/dental history and current full mouth charting; 2) no antibiotic, anti-inflammatory, steroid, or oral contraceptive use in the past six months; 3) presence of at least 15 teeth; 4) no history of serious systemic illness (cardiac abnormalities, history of joint replacement, cancer, organ transplant, etc) and; 5) able to give consent (≥18 years old). Four patients meeting the above criteria were identified. In addition, four control subjects were recruited from the student population of the UTHSCSA and verified for periodontal health by full mouth charting and dental history. Informed consent was obtained from all subjects and each was assigned a tracking number. No personal information was included with the sample, and any information linking the sample to the individual was secured in a separate database accessible only by the primary investigator. The control subjects were matched to the aggressive periodontitis patients according to age, gender, and ethnicity, and gave no history of aggressive periodontitis or *A. actinomycetemcomitans* infection. Collection of all blood and plaque samples took place in the graduate periodontics clinic at the University of Texas Health Science Center at San Antonio per IRB approval.

2. Collection of Plaque Samples

Subgingival plaque samples were collected for all experimental and control subjects. Sites sampled in controls included the mesiofacial of teeth #3, 9, 19, and 25, or if these teeth were missing, the nearest mesiofacial site of an adjacent tooth. Three fine endodontic paper

Figure 3: Western blot of recombinant *Aa* proteins. The recombinant HSPs were purified over Ni-NTA resin, facilitated by the presence of a vector-encoded tag containing 6 histidines on the amino terminus of the recombinant protein. Fractions were collected from the Ni column, electrophoresed on an SDS-PAGE gel, and pooled together. The following figure shows a Western blot of the protein fractions eluted from JP2 and knock-out *Aa* strains.

Figure 3



points (Johnson and Johnson, New Brunswick, NJ) were inserted into the gingival sulcus of the test site for approximately 30 seconds and removed. The paper points were placed in 200 μl of standard phosphate buffered saline (PBS) and stored at 4°C for future PCR analysis. Paper points were also used for sample collection in experimental subjects. Unlike the controls, the "worst" sites were sampled based on previous charting or, if data were not available, a full mouth charting was completed to determine the "worst" sites in the mouth based on probing pocket depth. Three paper points were inserted into the diseased sites with the greatest probing pocket depth for approximately 30 seconds and stored in PBS as described above.

3. Isolation of DNA and PCR Analysis

To determine the presence of *Actinobacillus actinomycetemcomitans*, PCR was performed on the GCF samples as previously described. The paper points were removed from the PBS tubes and centrifuged at 13,000 RPM for 7 minutes. The supernatants were removed and the pellet resuspended in 100 μl of lysis buffer (1% Triton x-100, 10mM Tris-HCl, 2mM EDTA, pH 8.0). The sample was boiled for 5 minutes at 100°C and centrifuged again at 13,000 RPM for 7 minutes. The supernatants were collected and utilized for PCR analysis. The primers and PCR conditions are described in Table 3. Following the PCR, 10 μl of each sample was electrophoresed on a 1% agarose gel at 75V for 1 hour (GibcoBRL Model H5, Life Technologies Inc., Gaithersburg, MD), stained with ethidium bromide, and visualized with ultraviolet (UV) light.

4. Collection and Handling of Peripheral Blood

Peripheral blood (approx 43 ml) was collected from both experimental and control subjects using conventional venipuncture techniques (18 guage needle with vacutainer attachment) in the antecubital fossa. One 13 ml vacutainer with SST Gel and Clot Activator (Becton-Dickenson, Franklin Lakes NJ) was used for autologous serum collection. Three 10 ml vacutainers with K_3 EDTA (Becton-Dickenson, Franklin Lakes NJ) were used for T cell harvesting.

Table 3: Oligonucleotides and Conditions for *Aa* PCR Analysis. The following table shows the different primers and conditions used for the PCR analysis of gingival crevicular fluid samples from human subjects with and without aggressive periodontitis. The leukotoxin and *Aa* specific primers (AaF, AaR) were taken from Suzuki and Külecki, respectively (106,107).

TABLE 3

Oligonucleotides and Conditions for *Aa* PCR Analysis

	Primers	Conditions		Size
Aa Leukotoxin	5'-CTAGGTATTGCGAAACAATTTG-3'	95°C 2'		
	5'-CCTGAAATTAAGCTGGTAATC-3'	94°C 15s	35 cycles	262bp
		54°C 30s		
		72°C 1'		
		4°C overnight		
AaF	5'-TCGCGAATCAGCTCGCCG-3'	95°C 2'		
AaR	5'-GCTTTGCCAGCTCCTCACC-3'	94°C 15s	35 cycles	285bp
		54°C 30s		
		72°C 1'		
		4°C overnight		

5. Peripheral Blood Mononuclear Cells (PBMCs) isolation

The 13 ml vacutainer with SST Gel and Clot Activator, or "Tiger Top," was centrifuged at 3800 rpm for 10 minutes at room temperature to allow harvesting of autologous sera. Approximately 400 microliters of sera was collected and stored at -20°C for use in antibody assays. The remainder, approximately 12 mls, was needed for the resuspension and maintenance of T cells *in vitro*; it is our experience that human T cells grow much better in autologous serum than in commercially available pooled human serum (Infante and Kraig, unpublished observation). The remaining sera was pipetted from the top of the tube and placed at room temperature. For each patient, the three 10 ml vacutainers with K_3 EDTA containing peripheral blood were combined and diluted with an equal volume of filter sterilized room temperature PBS. The total volume of the diluted sample was aliquoted into 15 ml sterile conical tubes, at a volume of 10 mls per tube. Three mls of room temperature Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden), or 0.3 volumes of the sample, was slowly added under the blood/PBS layer with a glass pipette to avoid disturbing the interface. The T cells were enriched along the Ficoll gradients by centrifuging the mixture at 2100 rpm for 30 minutes at room temperature in a Sorvall T7 centrifuge. The resultant samples were stratified into 4 distinct layers. The top layer consisted of diluted sera, followed by a thin layer of PBMCs. Underneath the PBMC layer was the Ficoll layer, followed by a bottom layer of red blood cells. The top layer of sera was aspirated off close to the PBMC layer but without touching it. Since the serum layer also contained platelets, it was desirable to remove as much of this layer as possible without removing any PBMCs. The presence of platelets in the final sample could lead to counting difficulties as a result of aggregation, or clumping, of the cells. The PBMCs were gently collected with a pipette and three times this volume of room temperature Hanks balanced salt solution (Sigma-Aldrich Corp., St. Louis MO) was added. The Ficoll layer was carefully avoided during the collection process. The lymphocyte/Hanks solution was centrifuged at 1000 rpm for 10 minutes at room temperature. The supernatant was aspirated and 10 ml of Hanks was added. This spin and wash cycle was repeated two more times, and following aspiration of the supernatant the final pellet was resuspended with 2 mls of complete media (90% RPMI 1640, 10% autologous sera, supplemented with 200mM L-glutamine, 1M HEPES buffer, 50mM β-mercaptoethanol, 200 U/ml penicillin, and 200 μg/ml streptomycin). Ten microliters of PMBCs was collected to determine the concentration of cells in the sample and were stained with 90 μl

(or 1:10) of PBS containing a 1:100 dilution of FDA (fluoracine diacetate, 5 mg/ml stock) in PBS. The sample was then loaded into a hemocytometer (Hausser Scientific 0.1 mm deep). The viable cells were counted as FDA-positive using a fluorescent microscope and the concentration of the sample was calculated in cells per milliliter. The volume was adjusted with complete autologous sera to produce a final concentration of 5 x 10^5 cells/ml.

6. Preparation of Antigens

A list of all *Aa* strains used is shown in Table 4. *Actinobacillus actinomycetemcomitans* (strain JP-2) were grown at 37°C in Trypticase Soy Broth supplemented with yeast extract (TSBYE, 30g Tryptic Soy Broth (DIFCO Laboratories, Detroit, MI) and 6g Yeast Extract (FisherBiotech, Fair Lawn, NJ) in 1 L ddH_20). The culture was maintained in an anaerobic chamber (Coy Laboratory Products, Ann Arbor MI) at 5% CO_2, 10% H_2, and 85% N_2. At log phase growth (O.D. ~0.4), the bacteria were harvested via centrifugation at 4°C for 10 min at 7700 rpm. The *Aa* cells were then washed in PBS, sonicated for 30 seconds, placed in clear centrifuge tubes, and exposed to ultraviolet (UV) light for 30 minutes in a Stratalinker 1800 (Stratagene, La Jolla, CA). A Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA) was used to determine protein concentrations, and the extracts were aliquoted and stored at -20°C until later use. Preparation of *Aa* strains 33384 (serotype c), strain 29523 (serotype a), M68 (leukotoxin knockout), and M71 (omp-34 knockout) proceeded in the same manner. The recombinant heat shock proteins were prepared as previously described. Recombinant leukotoxin (fragment 676/LktA) and omp-34 were generated by Mr. Michael Hannaman using the same protocol. Briefly, recombinant clones of *E. coli* BL21 containing either the leukotoxin fragment or omp-34 in the pRSETA expression vector were grown to an O.D. of 0.4 and induced by the addition of 1.5% IPTG. Protein expression was confirmed by SDS-PAGE and the recombinant proteins were purified over a Ni-NTA column, as previously described.

7. Stimulation of T cells in vitro

Standard protocols for assaying PBMC proliferation in response to antigen were followed (111). The prepared antigens were loaded into a flat-bottom 96 well plate (Costar, Bio-Rad, Hercules, CA). All wells were prepared in duplicate. As a positive control for proliferation, one set of duplicate wells was treated with the non-specific T cell mitogen phytohemaglutinin (PHA,

Table 4: ***Aa*** **Strains and Characteristics.** The following table shows the different *Aa* strains used for experiments and assays. The source and individual characteristics of each strain is also shown. The leukotoxin distributions of Y4 and 33384 were taken from Kolodrubetz et al. and Spitznagel et al., respectively (108,109).

Table 4

Name	Source	Characteristics
JP2	**Human Isolate (provided by Dr. Jeff Ebersole)**	**Serotype B** (Highly leukotoxic strain of *Aa*)
Y4	**Human Isolate (provided by Dr. Jeff Ebersole)**	**Serotype B** (Moderately leukotoxic strain of *Aa*, 10% of JP2) (87)
33384	**Human Isolate (provided by Dr. Jeff Ebersole)**	**Serotype C** (Minimally leukotoxic strain of *Aa*, 2% of JP2) (88)
29523	**Human Isolate (provided by Dr. Jeff Ebersole)**	**Serotype A**
AaM68	**Generated in Kraig/Koludrubetz Laboratories**	**Serotype B** **Isogenic JP2 leukotoxin knockout strain** (Spectinomycin resistant)
AaM71	**Generated in Kraig/Koludrubetz Laboratories**	**Serotype B** **Isogenic JP2 Omp-34 knockout strain** (Spectinomycin resistant)

10μg/ml final concentration). As a negative control, a set of duplicate wells was prepared without antigen. In the final human assay (Experiment 4), the antibiotic polymixin B (25μg/ml final concentration in PBS) was added to all wells to limit any negative effects from bacterial lipopolysacharride (LPS). Briefly, 1 x 10^5 cells per duplicate individual well (200 μl total volume) were loaded onto the plate to be tested against various antigens. The negative control was added first. The plates were then placed in a water-jacketed tissue culture incubator for 24 hours at 37°C and 5% CO_2. At 24 hours, 25μl of supernatant was pipetted from the surface of each well, taking care not to disturb the bottom of the well. The supernatants were stored at -20°C until later analysis and the plate was returned to 37°C. Supernatants were harvested again 48 hours later (72 hours total incubation) in an identical manner without replacing the volume removed. Following removal of the 72 hour supernatants, the cells were pulsed with 1mCi [^{3}H]-thymidine (ICN, Irvine, CA) per well and placed at 37°C. The cells were then harvested 18 hours later with an automated cell harvester (Skatron model 11021), collected onto Filtermat #11731 filter discs, and transferred into small scintillation vials. [^{3}H]-thymidine uptake was measured by liquid scintillation spectrometry and the average counts/minute of duplicate wells was calculated. Results were expressed as the difference between counts/minute with antigen and without antigen.

In one experiment, the role of *Aa* in the inhibition of T cell proliferation was investigated by comparing the effects of normal, heat-killed, and UV irradiated *Aa* on human T cell activation by PHA. The assay proceeded in a similar manner to previous experiments, however all wells received 20μl PHA (stock 0.1μg/μl in PBS). Wells then received *Aa* extract in one of three forms: normal, heat-killed, or UV irradiated. Heat-killed antigen had been placed at 60 degrees for 10 min immediately prior to use. UV irradiated antigen had been subjected to 120 millijoules of ultraviolet light using a Stratalinker 1800 (Stratagene, La Jolla, CA). The in vitro proliferation assays were then performed as previously described.

8. Statistical Analysis

The peripheral blood T cells were stimulated in vitro with *Aa* and various recombinant proteins. In all cases, duplicate wells (triplicate, if there were sufficient cells) were prepared. As a positive control, replicate wells were stimulated with PHA, a human T cell mitogen. T cell proliferation was assessed by the amount of [^{3}H]-thymidine taken up by the cultures.

9. Western Blots using Patient Sera

Five hundred microliters of *Aa* JP2 whole cell extract was combined with 167 microliters of loading dye (50% glycerol, 0.25M Tris HCl, pH 6.8, 5% SDS, 5% β-mercaptoethanol (βME)) and boiled for 5 minutes. Twenty microliters of this mixture was loaded into the each well of an 1.5 mm thick 8% resolving/4% stacking sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel prepared using a 15 well comb. Ten microliters of kaleidoscope marker (Bio-Rad, Hercules, CA) was loaded into one flanking reference well, and the proteins were electrophoresed for 2.5 hours at 100V. Subsequently, the proteins were transferred onto nitrocellulose overnight at 20V on ice using a Trans-blot device (Bio-Rad) in CAPS buffer (2.21g 3-(cyclohexylamino)- 1-propane sulphonic acid, 0.5g DTT, 150 ml MeOH in 1L H_2O). The nitrocellulose was sliced into vertical strips approximately 5 mm wide and "blocked" with 5% BSA (bovine serum albumin, ICN) in TBS (Tris buffered saline, 20mM TRIS pH 7.5, 200mM NaCl, Sigma, St. Louis MO) for one hour at 37°C. The strips were then rinsed with 0.1% BSA in TBS and then each strip was incubated with an individual human serum sample. The serum samples from each subject had been diluted either 1:10 or 1:100 with antibody dilution solution (1% BSA, 0.05% Tween 20, and 0.1% NaN_3). The Western blot strips were incubated with the serum samples for 2 hours and the filters were then rinsed 3 times with 0.1% BSA in PBS. Anti-human IgG (Sigma Immuno Chemicals, St. Louis, MO) was used as the secondary antibody at a 1:15,000 dilution, and the JP2 specific antibodies were visualized upon incubation with NBT/BCIP (5-Bromo-4-chloro-3-indolyl phosphate dipotassium/ nitrotetrazolium blue chloride, Promega, Madison WI). An additional nitrocellulose strip was probed with the secondary antibody alone and developed as above.

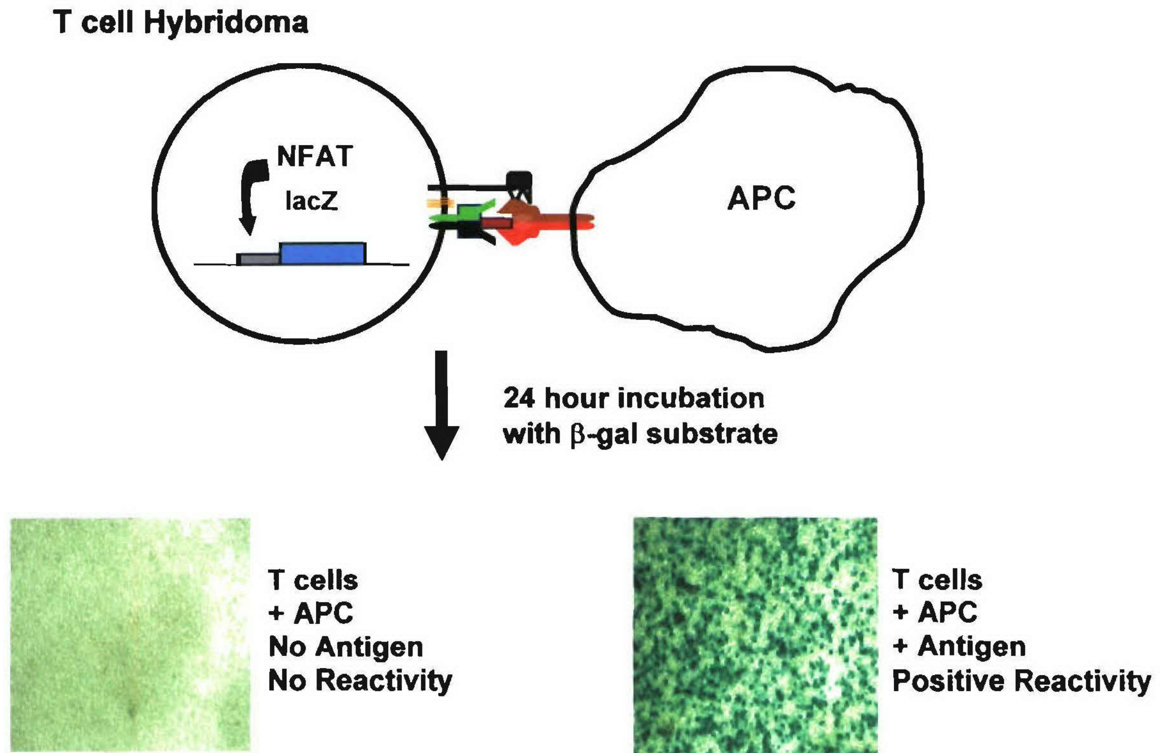
C. Mouse T cell Hybridoma Assays with Recombinant *Aa* Proteins

In order to identify the proteins on *Aa* that elicit a T cell response in mice, T cell hybridomas were screened with recombinant *Aa* proteins. The T cell hybridomas from BALB/c and C57Bl/6 mouse strains were previously generated in our lab by Paul Ezzo, Christine Gear, Michael Hannaman, and Ellen Kraig. The hybridoma cells whose antigens were not already known were expanded in complete RPMI media and plated onto a 96-well flat bottom microtiter plate at approximately 1 x 10^6 cells/well in 100μl complete RPMI. Irradiated (131.7 cGy/min for 29 minutes) splenocytes from the appropriate murine strain were added to each well as antigen

presenting cells (APCs) at approximately 5 x 10^5 cells/100µl complete RPMI medium. Duplicate wells were stimulated with the *Aa* test antigens (5-10µg protein) to a final volume of 200µl. The plates were then incubated for 24 hours at 37°C and 5% CO_2 and centrifuged at 4°C for 10 minutes at 3000 rpm (Sorval RT7 centrifuge, Sorvall Products, L.P., Newton, CN). In order to assess activation of the T cell hybridomas to *Aa* antigens, a β-galactosidase assay was utilized (112). The T cell fusion partner, BWZ.36, is a thymoma which contains a β-galactosidase gene, lacZ. This allows for relatively simple chromatographic screening of hybridoma reactivity. Briefly, when stimulated by the appropriate antigen and antigen presenting cell (APC), the T cell hybridoma will upregulate the transcription factor NFAT (nuclear factor of activated T cells). The lacZ reporter gene is NFAT-responsive, so it becomes transcriptionally active and β-galactosidase is produced, which causes the cells to turn "blue" and allows easy visualization (Figure 4). Thus, T cell hybridomas can be used to analyze responses to individual proteins. The supernatants were aspirated (130µl retained for later use) and the cell pellets were then fixed with 100µl/well of a filter sterilized solution containing 2% formaldehyde/0.2% gluteraldehyde in 1X PBS and incubated for 5 minutes at 4°C. Following incubation, the plates were centrifuged at 4°C for 10 minutes at 3000 rpm. The supernatants were aspirated again and the pellets were washed once with 100µl /well of 1X PBS. The remaining cells were then overlaid with 100µl of filter sterilized 1mg/ml X-gal [5-bromo-4-chloro-3-indolyl-B-D-galactoside (Gibco BRL Products, Life Technologies, Rockville, MD)], 5mM potassium ferrocyanide, 5mM potassium ferricyanide, and 2 mM $MgCl_2$ in PBS as described by Sanderson and Shastri (112). The cells were then incubated for approximately 16 hours at 37°C and stored at 4°C until scored microscopically based on the number of blue cells (reactive T cells) present in the well. The T cell hybridoma was considered positive if there was at least a 5-fold increase in the number of reactive cells over the negative (no antigen) control.

Figure 4: Schematic for β-galactosidase assay to screen T cell hybridomas. Activated T cell hybridomas produce β-galactosidase via the upregulation of an NFAT-responsive lacZ reporter gene. A T cell hybridoma was considered positive when at least a 5-fold increase in the number of blue cells was observed.

Figure 4



Picture courtesy Dr. Paul Ezzo

III. Results

A. Murine T Cells are Elicited to HSPs upon Inoculation with *Aa*

1. T cell hybridoma reactivity in response to HSP inoculation

Previous studies in our lab utilized T cell hybridomas to understand the various antigenic determinants on *Aa* that stimulate a T cell response. Once such panel using BALB/c mice identified leukotoxin (lkt) and outer membrane protein (omp)-34 as antigenic epitopes on *Aa* (97). A similar T cell hybridoma panel using C57Bl/6 mice identified lkt, omp-34, and cagE as antigenic epitopes (103). In order to determine whether HSPs elicited a T cell response, we tested T cell hybridomas from BALB/c and C57Bl/6 mice orally inoculated with viable *Aa*. Those hybridomas whose antigens had not been previously identified were screened for reactivity with a variety of whole cell extracts and recombinant proteins, including *Aa* heat shock proteins (HSPs). Genes encoding the HSPs GroEL, GroES, and DNAJ were amplified by polymerase chain reaction (PCR), cloned into pRSETB, a prokaryotic expression vector, and recombinant proteins were produced. In order to assess activation of the T cell hybridomas, a chromatographic β-galactosidase assay was utilized, as described in Materials and Methods.

Six hybridomas from the BALB/c mouse strain and 24 hybridomas from the C57Bl/6 mouse strain were included. All of these hybridomas were previously shown to be specific for *Aa*, but were not tested for reactivity to any HSPs. The BALB/c T cell hybridomas were tested in duplicate against whole cell extracts from different *Aa* strains and the recombinant heat shock proteins GroEL, GroES, and DNAJ. Table 5 shows the results from the BALB/c hybridomas., with the average number of blue cells per center grid for each stimulation. The 24 hybridomas from the C57Bl/6 mouse strain were tested in a similar manner (Table 6). While none of the BALB/c hybridomas exhibited reactivity to any of the purified heat shock proteins, three T cell hybridomas from the C57Bl/6 panel reacted with GroES: 1C10.1, 5B3.6, and 3F12.5. One hybridoma, 4C11.6, reacted with GroEL. Two hybridomas, 5B5.4 and 5E5.8, reacted strongly with nearly all antigens, strongly suggestive of cross reactivity with *E.coli* proteins. These reactivities should be considered false positives and thus were not scored as positive in the β-galactosidase assay. In conclusion, two *Aa* HSPs, GroEL and GroES, were identified as T cell epitopes in our murine hybridoma model.

Table 5: BALB/c T Cell Hybridoma Panel and Results of β-galactosidase Assay. This table shows the 6 T cell hybridomas from the BALB/c murine strain and their reactivity against *Aa* whole cell extracts and the recombinant heat shock proteins GroEL, GroES, and DNAJ. The panel was tested in duplicate and the average number of blue cells per center grid for each stimulation is shown. The (-) sign indicates an inactive well (no blue cells).

Table 5

	No Ag	JP2	M68	M71	Y4	33384	29523	GroEL	GroES	DNAJ
2CG10.7	0	0	0	0	0	0	0	0	0	0
2CH10.8	0	0	0	0	0	0	0	0	0	0
3CC4.2	11	156	0	0	127	0	0	0	0	0
3CD8.14	35	305	425	148	460	147	163	0	0	0
3CE3.8	23	364	515	110	429	279	0	0	0	0
3CH6.10	4	0	0	0	0	0	0	0	0	0

Table 6: C57Bl/6 T Cell Hybridoma Panel and Results of β-galactosidase Assay. This table shows the 24 T cell hybridomas from the C57Bl/6 strain and their reactivity against *Aa* whole cell extracts and the recombinant heat shock proteins GroEL, GroES, and DNAJ. The panel was tested in duplicate and the average number of blue cells per center grid for each stimulation is shown. The (++) sign indicates a well of high reactivity (>500 blue cells), while (+++) indicates a well with extremely high reactivity (>1000 blue cells).

Table 6

	No Ag	JP2	M68	M71	Y4	33384	29523	GroEL	GroES	DNAJ
1C10.4	45	++	+++	+++	+++	+++	+++	0	**303**	0
2B1.10	0	0	0	0	0	0	0	0	0	0
2G8.5	0	0	0	0	0	0	0	0	0	0
3A10.4	23	++	+++	+++	+++	+++	+++	0	0	0
3B5.4	25	173	172	106	+++	+++	+++	138	0	96
3B9.2	25	++	+++	+++	+++	+++	+++	0	0	0
3B11.6	0	0	0	0	0	0	0	0	0	0
3C7.2	7	90	51	17	51	79	0	0	0	0
3E4.6	0	0	0	0	0	0	0	0	0	0
3F2.2	32	246	+++	+++	+++	+++	+++	0	0	0
3F12.5	22	+++	+++	+++	+++	+++	+++	0	**113**	0
3H1.2	14	132	182	120	149	73	131	0	0	0
3H3.6	0	0	0	0	0	0	0	0	0	0
3H10.1	6	188	128	95	142	91	88	0	0	0
4A7.2	17	+++	+++	+++	+++	+++	+++	0	0	0
4C11.6	7	124	154	70	173	24	75	**104**	0	0
4H1.2	12	45	53	55	43	25	72	38	0	0
4H7.4	0	0	0	0	0	0	0	0	0	0
5B3.6	33	399	++	++	429	350	0	0	**495**	0
5B8.1	25	325	+++	469	568	254	328	0	0	0
5B11.1	27	158	173	0	261	254	0	0	0	0
5E3.1	44	321	147	480	232	491	245	0	107	0
5E5.8	117	+++	+++	+++	+++	+++	+++	+++	+++	+++
5F12.7	32	+++	+++	+++	+++	+++	+++	0	128	68

B. Human T Cells from Non-*Aa* Infected Subjects do not Show Reactivity to Individual *Aa* antigens

1. Human T Cell Proliferation Assays

In a murine hybridoma model, we have shown that several *Aa* proteins elicit a T cell response, including leukotoxin, omp-34, cagE, and most recently the HSPs GroEL and GroES. Our next goal was to determine if these same proteins elicit T cell responses in humans with aggressive periodontitis and disease-free controls. Experimental subjects were diagnosed with aggressive periodontitis based on clinical and radiographic parameters. Once identified, peripheral blood was drawn and T cells and autologous sera were prepared as previously described in Materials and Methods. The T cells were then incubated with a variety of whole cell preparations and recombinant *Aa* proteins and tested for activation using an in vitro proliferation assay. The amount of T cell stimulation was measured by uptake of tritiated thymidine. Due to equipment failures the proliferation results of Experiment 1 are not shown. Figures 5 and 6 show the T cell proliferation results of Experiments 2 and 3, respectively.

In all experiments, human T cells proliferated well to the positive control, the T cell mitogen PHA. However, only T cells from one tested subject, C4, were stimulated by *Aa* or its antigens. To ensure that LPS was not affecting proliferation, cells from subjects C4 and E4 were stimulated with and without polymixin B (Figure 7). The addition of polymixin B had little effect on T cell proliferation, and we still did not see a high response from the experimental subject. We suspected that this might be due to the lack of recent *Aa* exposure in these subjects (with the exception of C4). To test this possibility, Western blots and PCR analyses were utilized.

2. Western Blot Analysis of Human Sera

In an effort to determine the presence of peripheral antibodies to *Aa*, serum samples were tested using a Western Blot analysis, as described in Materials and Methods. A 1:10 dilution was performed on each serum sample and then used to individually incubate a Western blot strip containing the *Aa* JP2. A 1:15,000 anti-human IgG was used as a secondary antibody, and the JP2 specific antibodies were visualized upon incubation with NBT/BCIP. An additional nitrocellulose strip was probed with the secondary antibody alone. Figure 8 shows the results of the western blot analysis. Both control and experimental subjects exhibited the presence of

Figure 5: T Cell Proliferation Assay Results- Experiment 2. T cells from a subject with aggressive periodontitis (E2) and a disease-free control subject (C2) were incubated in vitro with the indicated stimulus for 90 hours. [^{3}H]-thymidine was added for the final 18 hours and uptake of [^{3}H]-thymidine was measured as an indicator of T cell proliferation/activation.

Figure 5

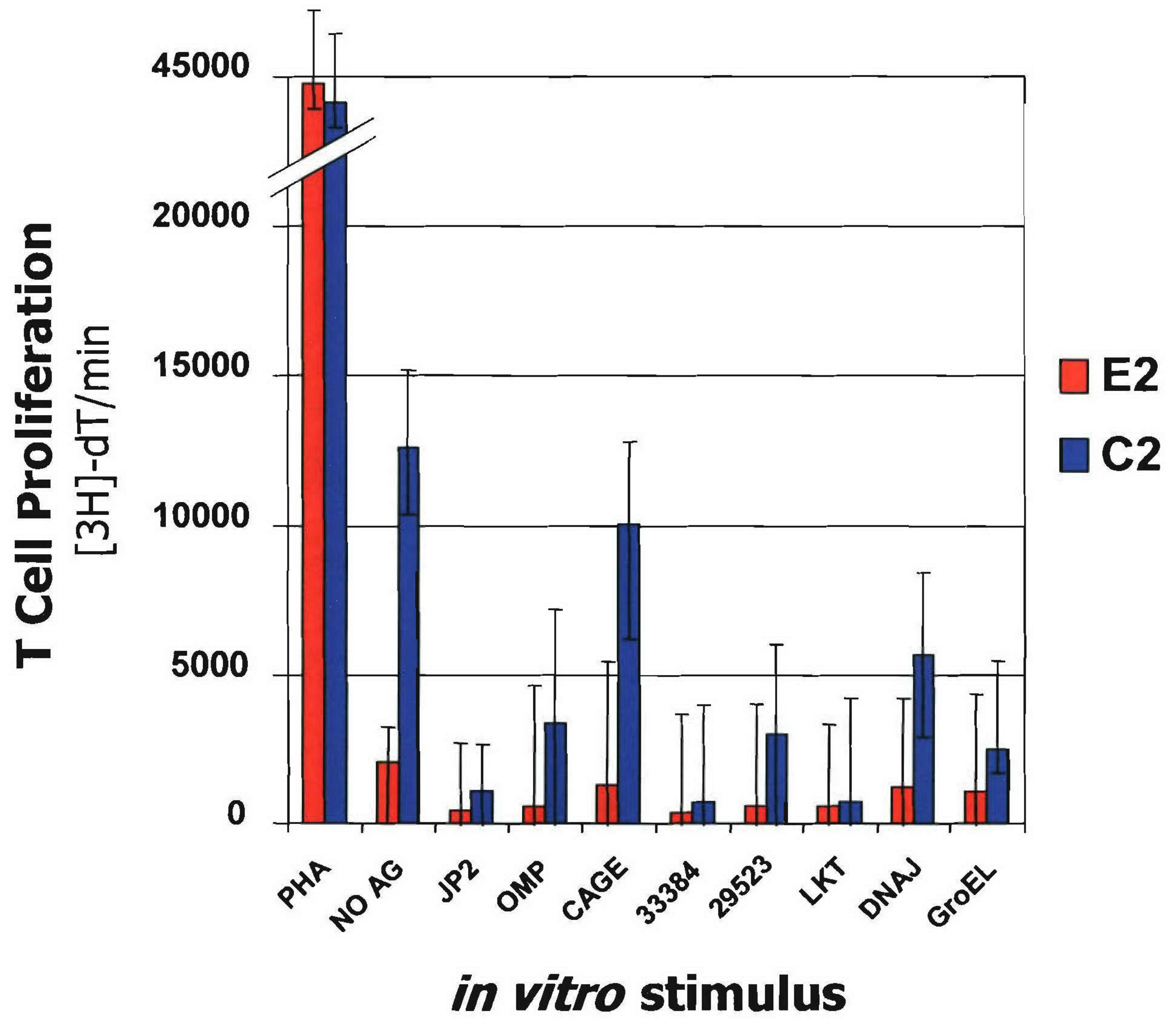


Figure 6: T Cell Proliferation Assay Results- Experiment 3. T cells from a subject with aggressive periodontitis (E3) and a disease-free control subject (C3) were incubated in vitro with the indicated stimulus for 90 hours. [^{3}H]-thymidine was added for the final 18 hours and uptake of [^{3}H]-thymidine was measured as an indicator of T cell proliferation/activation.

Figure 6

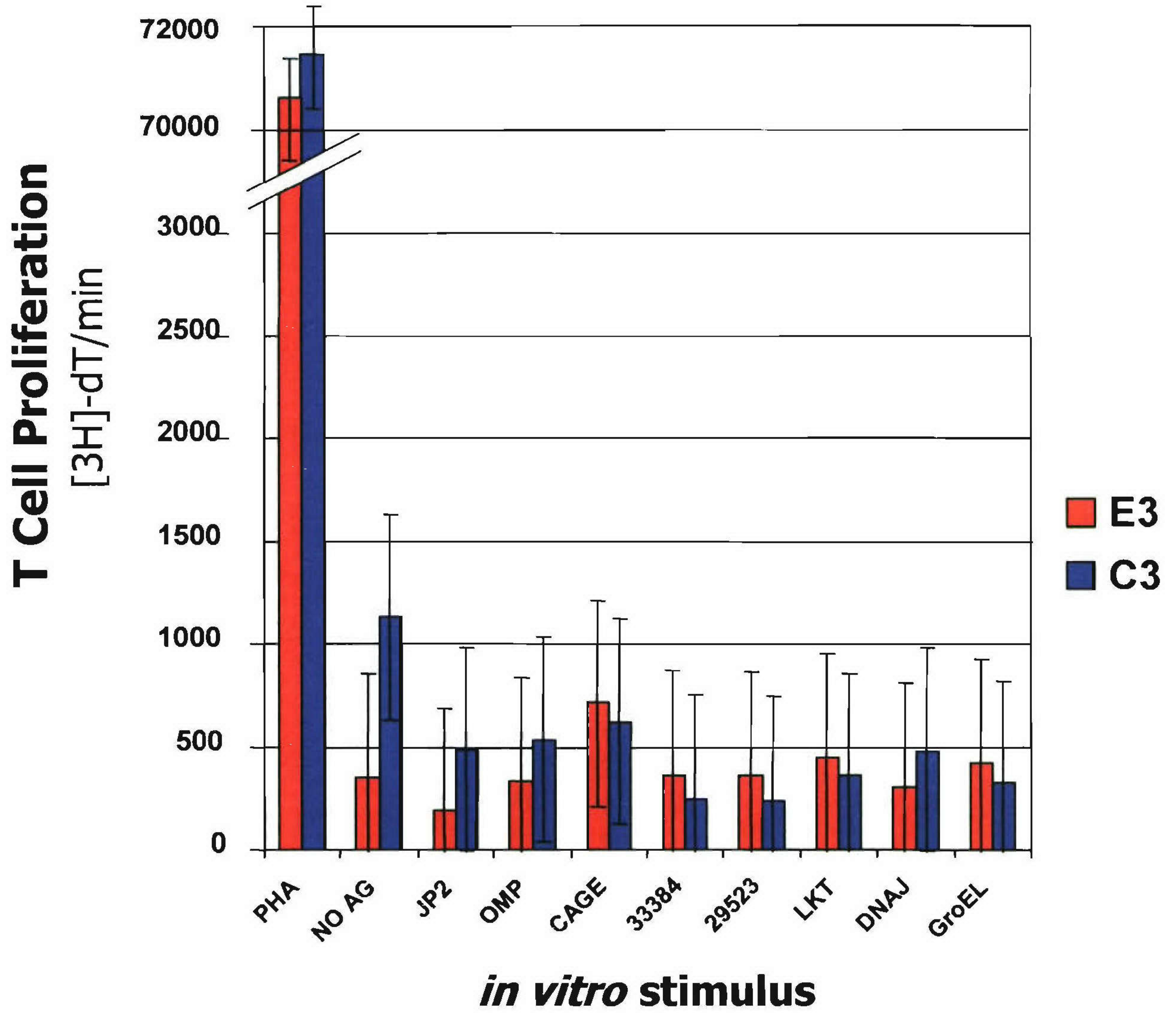


Figure 7: T Cell Proliferation Assay Results- Experiment 4. This graph shows the results of T cell proliferation to various whole cell *Aa* extracts and recombinant *Aa* proteins with and without the antibiotic, polymixin B. T cells from a subject with aggressive periodontitis (E4) and a disease-free control subject (C4) were incubated in vitro with the indicated stimulus for 90 hours. [^{3}H]-thymidine was added for the final 18 hours and uptake of [^{3}H]-thymidine was measured as an indicator of T cell proliferation/activation. Interestingly, T cell proliferation to several *Aa* antigens was seen in the control subject (C4).

Figure 7

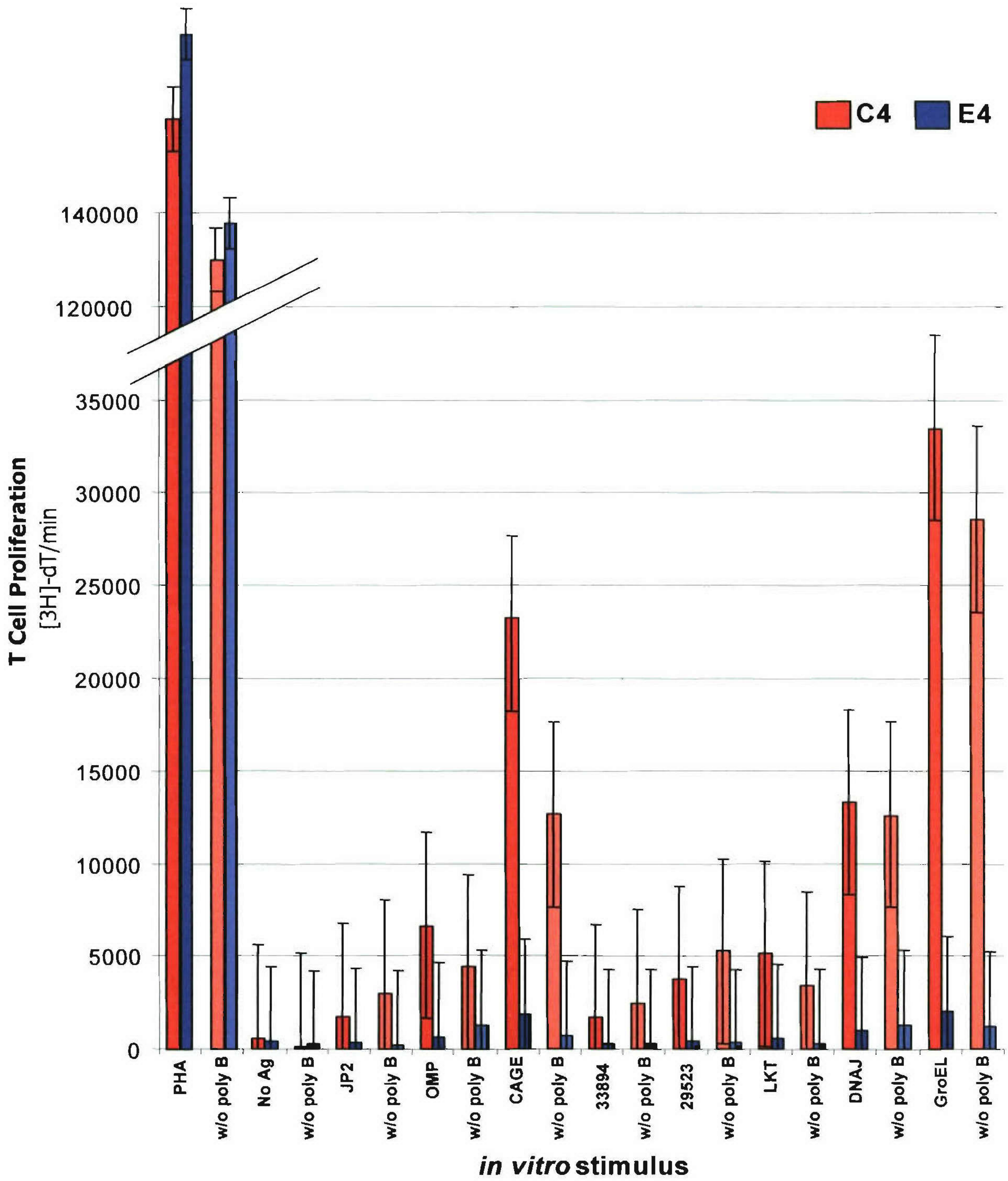
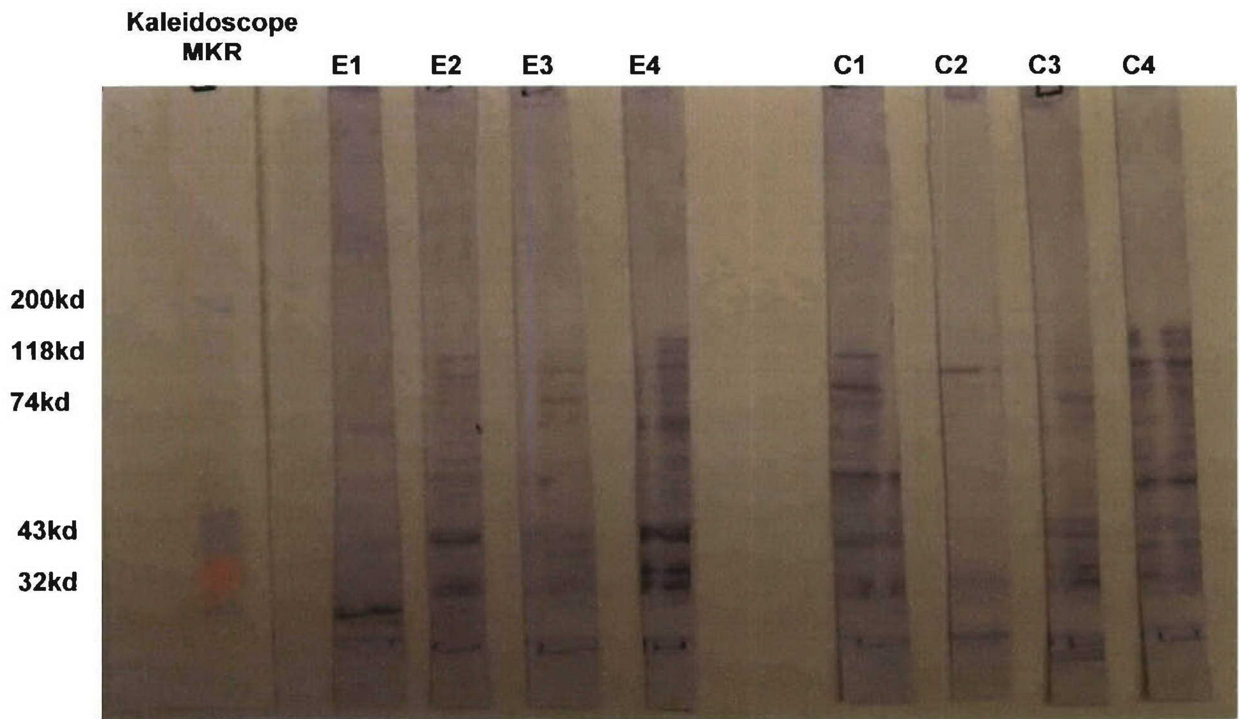


Figure 8: Western Blot Analysis of Human Serum Samples. This picture shows the results of the Western Blot Analysis of the human sera samples from all experimental and control patients. The strips were incubated with an extract of *Aa* JP2. Reactivity (or cross-reactivity) to *Aa* was seen in sera from all subjects.

Figure 8



cross-reacting antibodies to *Aa,* suggesting a history of prior exposure or the presence of some cross-reactive antibodies. However, individual C4, who showed a more pronounced T cell proliferative response to *Aa* also had a higher antibody response, as evidenced by more pronounced bands on the Western blot.

3. PCR Analysis of Subgingival Plaque Samples

In order to determine the presence of an active *Aa* infection in test subjects, subgingival plaque samples were collected from eight human subjects, four of whom had been identified with aggressive periodontitis. The control subjects were matched to the aggressive periodontitis patients according to age, gender and ethnicity, and gave no history of aggressive periodontitis or *Aa* infection. Following informed consent, subgingival plaque samples were collected from all subjects using endodontic paper points. This was accomplished immediately after the peripheral blood draw and clinical exam (if required). Control sites included the mesiofacial of teeth # 3, 9, 19, and 25, or if these teeth are missing, the nearest mesiofacial site of an adjacent tooth. For experimental subjects with aggressive periodontitis, the four "worst" sites were sampled based on probing pocket depth. All paper points were placed in 200 μl of PBS and stored at 4°C. PCR analysis was performed as previously described to determine the presence of *Actinobacillus actinomycetemcomitans* in the samples. Following the PCR, the samples were electrophoresed on an agarose gel, stained with ethidium bromide, and visualized with UV light.

As a positive control, bacterial extracts of *Aa* strain JP2 were utilized. Figure 9 shows the PCR results using two sets of *Aa* specific primers (Table 3) with our positive control. On wells with JP2 DNA, both sets of primers successfully yielded bands of the appropriate sizes.

Figure 10 shows the results of the PCR analysis of Experiment 1, consisting of a human subject with aggressive periodontitis and a periodontally healthy control subject. Using the same primers, the experimental subject is shown positive for *Aa* in the sampled sites, while the control patient is negative (no bands). In addition, our positive control (JP2) clearly shows bands at 262 kDa and 285 kDa, representing our set of primers specific for *Aa*. Thus our selected primers were capable of identifying *Aa* specific DNA in both our positive control and in clinical samples harvested from patients with aggressive periodontitis.

The PCR results for Experiments 2-4 are shown in Figure 11. All primers utilized were identical to previous PCR analyses. From the Figure, no bands are evident in any of the

Figure 9: PCR Results with Positive Control (JP2). This agarose gel shown the PCR results with our positive control, whole cell bacterial extract from *Aa* strain JP2. Our leukotoxin and *Aa* specific primers successfully identified JP2 DNA under these PCR conditions (see Table 3).

Figure 9

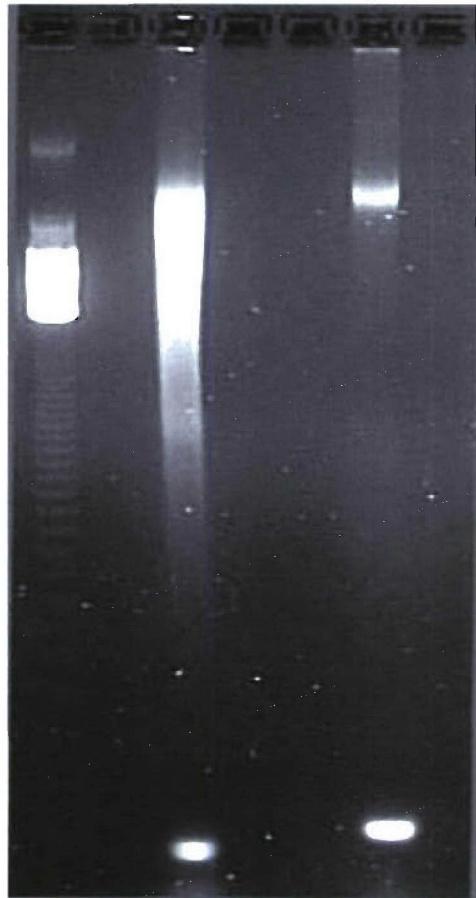


Figure 10: PCR Results from Experiment 1. This agarose gel shows the PCR results from Experiment 1. The experimental patient with aggressive periodontitis is shown at left. Lanes were prepared in duplicate with 1 or 5 μl of sample DNA and the Lkt or *Aa* specific primers; thus bands are lighter or darker based on the quantity of the sample used. Note corresponding bands on the JP2 sample (far right).

Figure 10

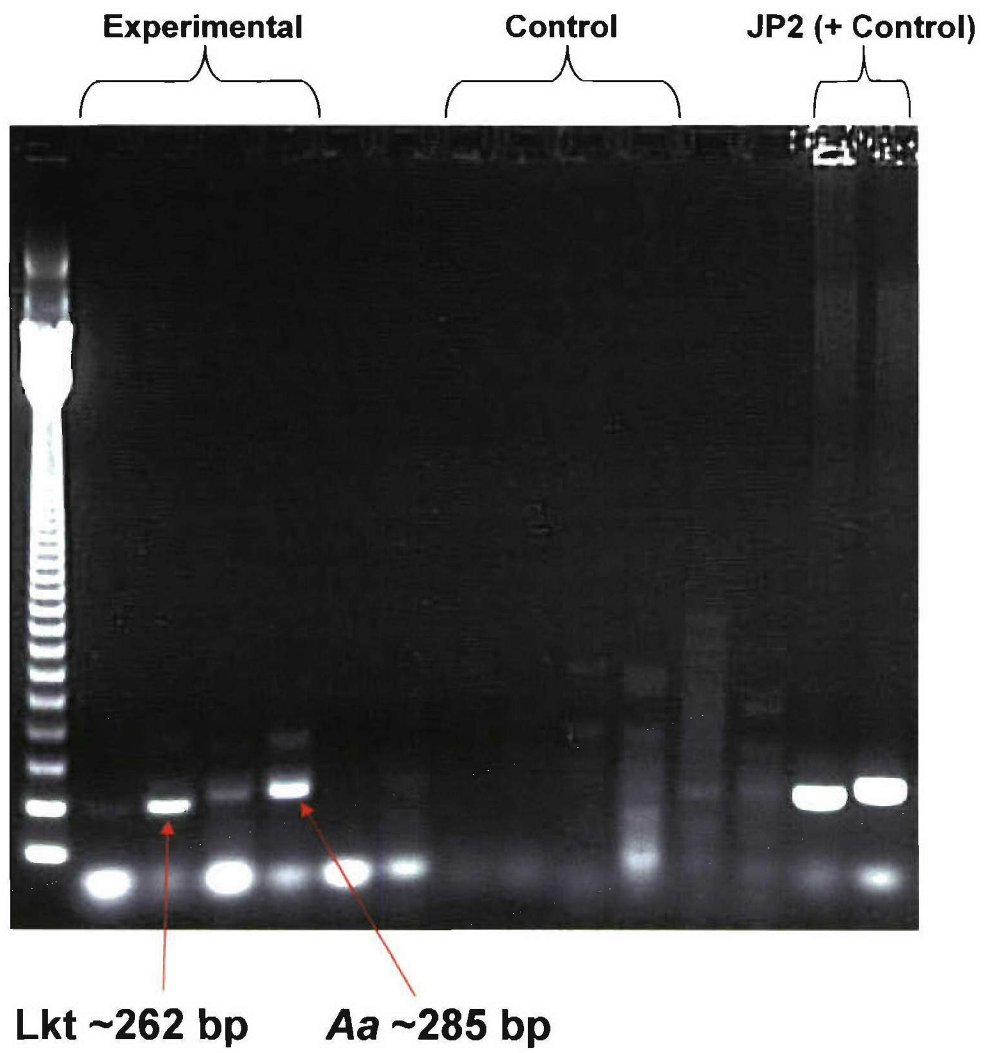
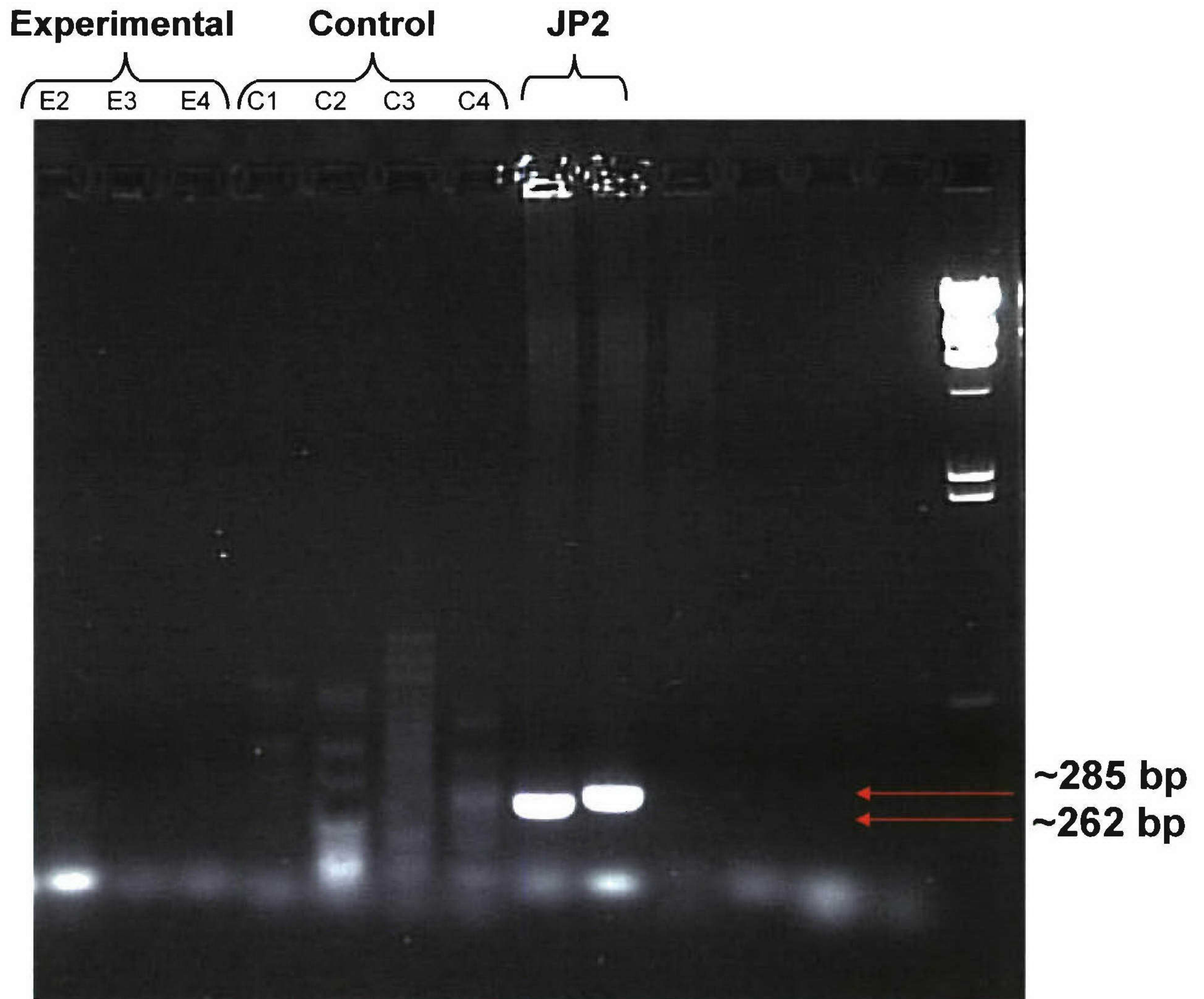


Figure 11: PCR Results from Experiments 2-4. This agarose gel shows PCR results from Experiment 2-4. Lanes were prepared with 5 μl of sample DNA and Lkt or *Aa* specific primers were utilized. No significant bands appeared at either the 262 or 285 bp range for E2-E4 and C1-C3, indicating a negative finding for *Aa*. This is in contrast to C4, where a positive result for *Aa* was seen. This subject also showed T cell reactivity (proliferation) to several *Aa* proteins.

Figure 11



experimental or control patients, with the exception of subject C4. This is the same subject that showed T cell reactivity (proliferation) to *Aa* proteins. We previously tested our primers against a positive control and also yielded a positive finding for *Aa* in our aggressive periodontitis patient in Experiment 1. Thus our primers were capable of identifying *Aa* DNA if it was present and the only individuals shown to harbor Aa were E1 and C4.

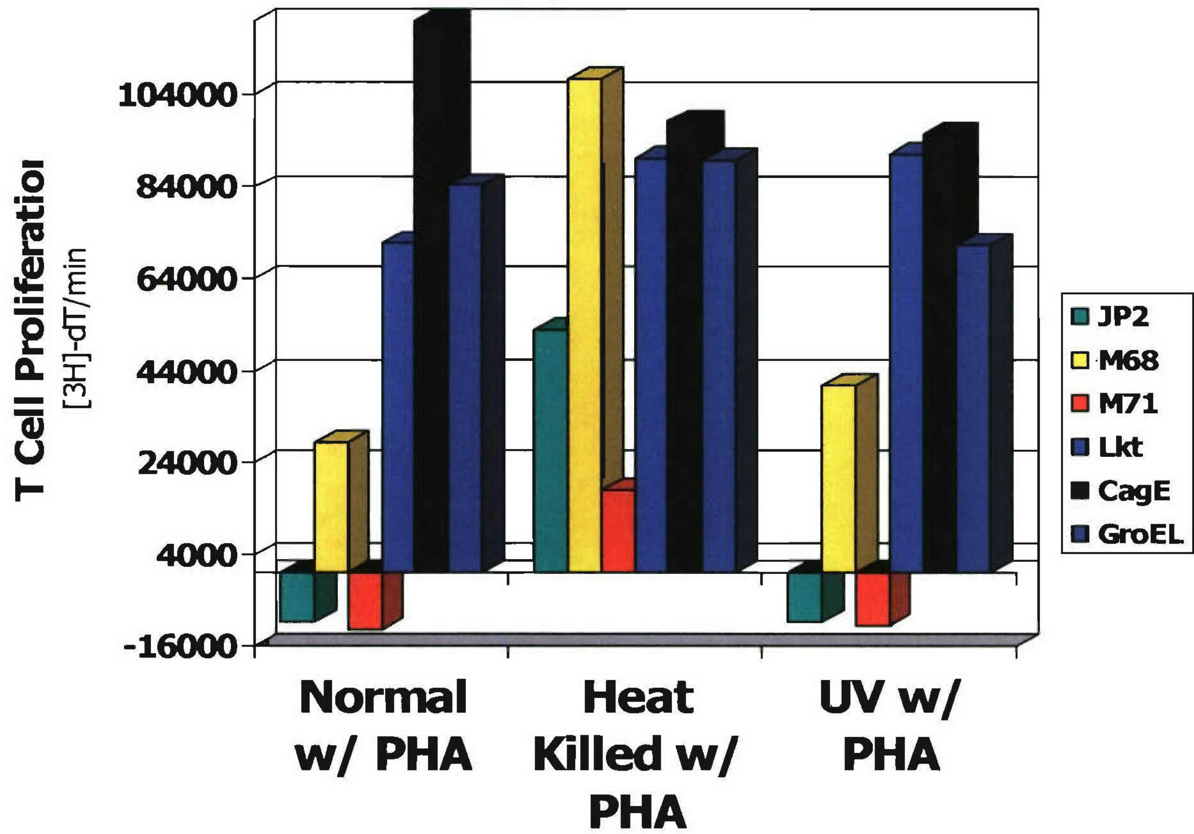
C. Elucidation of a T cell Inhibition Factor

1. Human T cells are inhibited by a Heat Labile *Aa* molecule/protein

From our experiments we found that little T cell proliferation was seen to anything other than PHA, our positive control. We hypothesized that *Aa* was inhibiting T cell proliferation. The question that now remained was how is *Aa* inhibiting our human T cell proliferation? Furthermore, if such inhibition remains in future experiments, will we be able to determine which *Aa* proteins elicit a T cell response? To do this, T cells from normal, healthy subjects were stimulated in vitro with PHA and then *Aa* or treated *Aa* extracts were added (Experiment 5). The *Aa* extracts used were: untreated, heat-killed, or UV irradiated. Recombinant proteins were treated and tested in parallel. As seen in Figure 12, the normal whole cell *Aa* antigen significantly inhibited PHA-induced proliferation, while the recombinant proteins (leukotoxin, cagE, and GroEL) did not. UV treatment did not reverse the inhibitory effect, but heat-killing did reverse the effect. Thus, it seems likely that a protein on *Aa* inhibits human T cell proliferation. The recombinant proteins, as before, showed no proliferative inhibition. Thus our recombinant proteins did not appear to be the causal factor in the inhibition of T cell proliferation that we observed in earlier assays.

Figure 12: Effect of Treated *Aa* Antigens on PHA-Induced Proliferation. This graph shows the effects of normal, heat-killed, and UV treated *Aa* antigens on normal human T cells in the presence of PHA.

Figure 12



IV. General Discussion and Summary

The primary goal of this study was to better characterize the T cell response to *Actinobacillus actinomycetemcomitans (Aa)*. Although *Aa* has been associated with chronic periodontitis and nonoral human infections, such as brain abscesses, it is the primary pathogen associated with the initiation and progression of localized aggressive periodontitis. In light of recent reports involving an increase in antibiotic resistance, current research is focusing on the development of vaccines that could afford protection from *Aa* infections. Ultimately this approach would allow a more specific and rapid response of the host against infection. However, such an endeavor first requires a more complete understanding of how *Aa* interacts with the host immune system.

The T cell response to *Aa* is largely unknown. The type and specificity of the B cell response is largely dependent on T cells, so presumably their role in *Aa* infection is vital in determining the scope and specificity of the immune response. While the human T cell epitopes on *Aa* are largely uncharacterized, recent studies have implicated the role of heat shock proteins (HSPs) in initiating an immune response (73-78). HSPs are known to be expressed in periodontal disease, and several studies show that the HSP GroEL is an immunodominant antigen in humans.

Murine T cell hybridoma technology was previously utilized to determine the immunodominant *Aa* antigens that elicit a T cell response. Using a BALB/c specific hybridoma panel, Ezzo found that nine of fifteen hybridomas recognized leukotoxin, and one hybridoma was specific for omp-34 (97). In a later study with C57Bl/6 specific panels, Gear found that only one from this strain tested positive for leukotoxin, and three were reactive to omp-34 (103). Thus certain antigens appeared to be strain specific in their initiation of a T cell response, and other antigenic epitopes on *Aa* were capable of eliciting a response. Neither hybridoma panel was tested for reactivity with heat shock proteins, so the next step was to determine if reactivity to these proteins was also strain specific.

Genes coding *Aa* HSPs GroEL, GroES, and DNAJ were amplified by polymerase chain reaction (PCR), cloned into a prokaryotic expression vector, and recombinant proteins were produced. T cell hybridoma panels from both BALB/c and C57Bl/6 strains were tested in duplicate against whole cell *Aa* extracts and the HSPs. While none of the BALB/c hybridomas

exhibited reactivity to the recombinant HSPs, reactive T cell hybridomas were seen in the C57Bl/6 hybridoma panel. Three hybridomas reacted with GroES, while one reacted with GroEL. Thus, mice inoculated with viable *Aa* generated a T cell response to certain HSPs, and GroES and GroEL were identified as T cell epitopes in our murine hybridoma model.

The next step was to determine if human T cells responded to HSPs in a similar manner. Peripheral blood and subgingival plaque samples were collected from experimental patients with aggressive periodontitis and from healthy controls. The recombinant HSPs were then tested for the ability to either stimulate or inhibit a proliferative response of peripheral blood T lymphocytes (PBCs). *Aa* infection in the human subjects was assessed by PCR analysis of subgingival plaque samples using *Aa*-specific primers. Although the HSPs GroEL and GroES were capable of initiating a response in the hybridoma model, they were unable to stimulate proliferation of human T cells from periodontitis patients. However, proliferation to *Aa* HSPs GroEL and DNAJ was seen in a control patient, C4. The *overall* lack of human T cell reactivity may have been due to the absence of active *Aa* in the periodontium of these particular individuals in this study. A Western blot analysis of subject sera indicated cross-reacting antibodies to *Aa* in all subjects, suggesting a history of prior exposure. In addition, the aggressive periodontitis patients identified in this study exhibited a generalized level of disease. Since *Aa* is most implicated in the localized form of aggressive periodontitis, it is possible that the type of disease impacted the T cell response to *Aa* antigens in these patients. Efforts are currently underway to repeat the analysis using PBCs from localized aggressive periodontitis patients where *Aa* is likely to have been involved and is still present.

Interestingly, we showed a higher T cell proliferative response from control subject C4, compared to T cells from an experimental subject with aggressive periodontitis. This may be because C4 had a history of recent *Aa* exposure. The PCR analysis showed a faint band in the 262bp range, indicating at least the presence of *Aa* in the periodontium of subject C4. While this does not prove the existence of viable Aa in the site sampled, it does indicate at least a history of prior exposure. Furthermore, the presence of bands on our Western blot analysis were particularly strong for C4, indicating the presence of antibodies to *Aa* proteins.

In contrast to the T cell proliferation seen with C4, we observed an overall inhibitory effect on T cell proliferation from the majority of subjects sampled. To determine why, we then compared the role of *Aa* antigens in the inhibition of PHA-induced proliferation. *Aa* antigens

were prepared as before, although two subsets of antigens were either UV irradiated or heat-killed at 60 degrees for 10 minutes immediately prior to use. The assay then proceeded as before, using PBCs from a normal control in the presence of PHA. The normal whole cell *Aa* antigen significantly inhibited PHA-induced proliferation, while the recombinant proteins (leukotoxin, cage, and GroEL) did not. Similar results were seen using the UV irradiated antigens, where whole cell antigen inhibited PHA-induced proliferation while recombinant proteins did not. Heat-killed antigen exhibited a markedly different effect on PHA-induced proliferation. The heat-killed whole cell *Aa* extracts did not inhibit proliferation as they did in the normal and UV treated samples. As before, no proliferative inhibition was seen using recombinant proteins.

Thus the current study clearly demonstrates that recombinant proteins, including GroEL, were not inhibitory. The human T cells proliferated well to PHA stimulus, even in the presence of *Aa* GroEL. Furthermore, the inhibitory effect of *Aa* antigens on human T cells was heat labile, suggesting the existence of a toxic molecule or protein on *Aa* that is distinct from GroEL. The lack of proliferation to the same *Aa* antigens in most human subjects might indicate variability in host T cell responses, that recent *Aa* exposure is necessary, or that *Aa* is not as immune reactive in the generalized form of aggressive periodontitis . In contrast to other studies, recombinant GroEL alone was not inhibitory to human T cell proliferation, and the observed heat labile inhibitory affect may indicate the presence of an as yet unidentified *Aa* T cell suppressive factor. The proliferation seen in subject C4 supports the use of this model for assessing T cell stimulation to recombinant *Aa* proteins, including HSPs. Thus future studies should endeavor to utilize this model to further identify T cell epitopes on *Aa*.

Although this study did not identify HSPs, such as GroEL, as human T cell epitopes, it did reveal their importance in the T cell response using the murine hybridoma model. Since variations in inhibition were observed following heat treatment, the presence of a heat-labile toxic factor cannot be ruled out. To further characterize the T cell response to *Aa* in humans, future experiments should endeavor to elucidate this toxic factor. The use of a localized aggressive periodontitis patient population will enhance our ability to determine the immune response in patients with a clear history of *Aa* infection. Future vaccine development depends on a thorough understanding of the normal response to infection, and the identification of specific T

cell epitopes so that a discrete, targeted vaccine can be engineered to protect future generations from *Aa* infection.

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VI. VITA

Patrick Michael McDonough was born on [illegible] in Goldsboro, North Carolina. His parents, [illegible], moved the family to Fayetteville, North Carolina in 1980. In 1995, he graduated from Terry Sanford High School with a full scholarship and appointment to the United States Air Force Academy in Colorado Springs, Colorado. As a cadet, he completed a senior research project in guided tissue regeneration through the periodontics department at the Cadet Dental Clinic. He presented his research at the 1999 AADS Annual Meeting in Vancouver, British Columbia and received the Dentsply Award for best student poster presentation. He graduated in June 1999 with academic honors and a B.S. in biology. He was also commissioned as a second lieutenant in the United States Air Force. He received the Health Professions Scholarship through the Air Force Institute of Technology and attended the University of North Carolina at Chapel Hill School of Dentistry in Chapel Hill, North Carolina, where he met and married Lee Chaix Katz of Silver Spring, Maryland in 2001.

While in dental school, he pursued his interest in periodontics and completed a research project with Dr. David Paquette involving host modulation and nitric oxide synthase inhibition. He presented his senior project at the 2002 AADR Annual Meeting in San Diego, California. He received his D.D.S in 2003, graduating with distinction and academic honors. He was commissioned a captain in the United States Air Force and entered the joint program in periodontics offered by Wilford Hall Medical Center (WHMC) and the University of Texas Health Science Center at San Antonio (UTHSCSA). Patrick is scheduled to graduate in June 2006 with an M.S. in periodontics from UTHSCSA and a clinical certificate in periodontics from WHMC. He plans to practice periodontics in the United States Air Force and looks forward to serving his country.

Patrick maintained academic excellence at the Air Force Academy by earning a place on the Dean's List every semester for four years. In dental school, Patrick graduated with distinction in the top 10% of his dental class and was honored by membership in Omicron Kappa Upsilon Honorary Dental Society. Patrick also received the student award in periodontics from the American Academy of Periodontology.