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T-Cell Expression Cloning of *Porphyromonas gingivalis* Genes Coding for T Helper-Biased Immune Responses during Infection

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Exposure of the mouse oral cavity to *Porphyromonas gingivalis* results in the development of gingivitis and periapical bone loss, which apparently are associated with a Th1 response to bacterial antigens. We have used this infection model in conjunction with direct T-cell expression cloning to identify bacterial antigens that induce a preferential or biased T helper response during the infectious process. A *P. gingivalis*-specific CD4 T-cell line derived from mice at 3 weeks postchallenge was used to directly screen a *P. gingivalis* genomic expression library. This screen resulted in the identification of five genes coding for previously identified proteins and three other putative protein antigens. One of the identified proteins, *P. gingivalis* thiol peroxidase, was studied in detail because this molecule belongs to a protein family that is apparently involved in microbial pathogenesis. Infection of mice with *P. gingivalis*, either via the subcutaneous route or after exposure of the animal's oral cavity to viable bacteria, resulted in the induction of a strong thiol peroxidase-specific immune response characterized by the production of high titers of specific serum immunoglobulin G2a antibody and the production of gamma interferon by antigen-stimulated lymphoid cells, a typical Th1-biased response. Thus, the use of a proven T-cell expression cloning approach and a mouse model of periodontal disease resulted in the identification and characterization of *P. gingivalis* proteins that might be involved in pathogenesis.

Periodontal disease affects 70% of adults in the United States, and ca. 20% have severe disease, with an estimated annual cost of treatment of approximately 6 billion dollars. *Porphyromonas gingivalis* is a consensus periodontal pathogenic bacterium that has been associated with various periodontal disease syndromes, particularly adult forms of disease (38). *P. gingivalis* is more frequently isolated, and is present in elevated levels, in subgingival pockets from patients with periodontitis compared to gingivitis or periodontally healthy subjects. Its numbers are further increased at sites of active tissue destruction and are reduced in successfully treated sites but commonly remain high in sites with disease recurrence (6, 15, 43). Implantation of *P. gingivalis* leads to periodontal disease initiation in monkeys (16) and in mice (4, 35). Together with *Tannerella forsythia* and *Treponema denticola*, *P. gingivalis* comprises the "red complex" of bacteria that are highly implicated as causative agents in periodontal diseases in humans (39).

P. gingivalis elicits adaptive immune responses in infected hosts, albeit these appear to be weak relative to other oral pathogens. In terms of antibody responses, the frequency of positive responders and the levels of anti-*P. gingivalis* antibody are higher in serum and gingival crevicular fluid in adult periodontitis patients than in patients with gingivitis or localized juvenile periodontitis or periodontally healthy controls (12, 27–29). In addition, some studies also report elevated responses to *P. gingivalis* in rapidly progressive disease in young adults (13, 25, 46).

In mouse models, the disease caused by *P. gingivalis* is markedly influenced by cytokines produced in the local milieu. For example, interleukin-1 (IL-1) is responsible for most bone resorption in this model (40, 44). However, IL-1 expression and activity are regulated by a network of other mainly T-cell-derived cytokines, predominantly of the Th1-type (23). Animals deficient in IL-10, and to a lesser extent in IL-6-deficient mice, had significantly increased bone resorption, whereas IL-4 deficiency unexpectedly had no effect (5, 35). Surprisingly, deficiencies in Th1-type cytokines IL-12 or gamma interferon (IFN-γ) also had minimal effect on resorption, suggesting that inflammatory pathways are redundant and are mainly controlled by IL-10 (5, 18). However, adoptive-transfer experiments have more consistently shown an active role for Th1 cytokines in disease exacerbation and Th2 cytokines as disease protectors. Thus, transfer of antigen-specific Th1 clones in rats exacerbates periodontal bone resorption, whereas Th2 clones are protective (11, 22, 41, 42). A similar result has recently been reported in mice, in which animals vaccinated and protected against *P. gingivalis*-induced lesions had vigorous Th2 cytokine responses, whereas the predominant cytokine in mice with disease was the proinflammatory Th1 cytokine IFN-γ (30). Therefore, it is possible that priming a host by using defined protocols to primarily induce a Th2 response against dominant *P. gingivalis* antigens should prevent or have therapeutic value in treating periodontal disease and bone resorption caused by this organism.

Using this principle, we have been successful in developing a vaccine against leishmaniasis (7, 32). Resistance to the para-

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sites that cause leishmaniasis, in contrast to periodontal disease, is mediated by the Th1 response, whereas Th2 cytokines favor the disease. Using an adjuvant formulation that induces a strong Th1 (e.g., monophosphoryl lipid A with squalene oil emulsion or the cytokine IL-12) response in combination with a dominant parasite antigen, which normally induces a Th2 response during disease, we achieved excellent protection against challenge with virulent parasites (33). Therefore, the discovery and identification of both Th1/Th2 inducing antigens of *P. gingivalis* is of great interest for understanding the periodontal inflammation caused by this organism, as well as for the development of immunotherapeutics including vaccines.

We have developed a novel T-cell expression cloning approach to selectively clone genes associated with resistance to *Mycobacterium tuberculosis* infection in the mouse model (37). A protective $CD4^+$ T-cell line, generated from spleen cells of C57BL/6 mice, harvested at a time point coinciding with the early control of the infection, was used to screen a genomic *M. tuberculosis* library. This led to the identification of several polypeptides of immunological interest. More recently, we have used this approach to identify antigens of *P. gingivalis*. Eight genes that encode for antigens involved in Th1/Th2 responses have been identified and cloned, one of which, a *P. gingivalis* thiol peroxidase, has been more extensively studied and is reported here. This antigen strongly stimulates a Th1 response in mice challenged either systemically or orally with viable *P. gingivalis* thus, validating the antigen discovery approach to identify microbial antigens that might be involved in the pathogenesis of this important oral microorganism.

MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). The mice were maintained under specific-pathogen-free conditions and used at 8 to 12 weeks of age. The Animal Care and Use Committee of the Forsyth Institute approved all experiments.

Generation of anti-*P. gingivalis* murine cell line. For the generation of the murine anti-*P. gingivalis* specific T-cell line, standard procedures were followed. In brief, C57BL/6 mice were initially infected intraperitoneally with ca. 10^9 CFU of *P. gingivalis* strain W83 grown under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) in synthetic medium to avoid contaminating proteins which could be antigenic (47). Mice were sacrificed 4 weeks later, and spleen cells were obtained by using conventional procedures. Mononuclear spleen cells (5×10^6/ml) suspended in complete RPMI medium containing gentamicin (50 μg/ml) were stimulated with 10^8 viable *P. gingivalis*/ml for 4 to 5 days and then with recombinant human IL-2 (2 ng/ml) for approximately 7 days. Cells were then restimulated with syngeneic antigen-presenting cells (APC; i.e., adherent spleen cells) treated with mitomycin C (to prevent APC division) plus 10^8 viable *P. gingivalis* as an antigen. This cycle of stimulation was repeated for two times, after which the cells were tested for antigen specificity by proliferation/IFN-γ production assays. Surface markers were analyzed by fluorescence-activated cell sorting scan for expression of CD4 or CD8 T-cell surface antigens using the following specific fluorescein isothiocyanate-labeled monoclonal antibodies: anti-CD4 clone H129.19, anti-CD8 clone 53-6.7, and anti-TCR (anti-CD3) clone 17A2 (all from Pharmingen, San Diego, CA).

Construction of the plasmid expression library. Genomic DNA from *P. gingivalis* W83 was randomly sheared to an average size of 2 kb, blunt ended with Klenow polymerase, and followed by the addition of EcoRI adaptors. The inserts were subsequently ligated into the λScreen phage vector predigested with EcoRI (Novagen, Madison, WI) and packaged in vitro by using the PhageMaker extract (Novagen). The phage library (Erd λScreen) was amplified, and an aliquot was converted into a plasmid expression library. The *P. gingivalis* Erd λScreen phage library was converted into a plasmid library (pScreen) by autosubcloning using the *Escherichia coli* host strain BM25.8 as suggested by the manufacturer (Novagen). Plasmid DNA was purified from BM25.8 cultures containing the pScreen recombinants and used to transform competent cells of the expressing host strain BL21(DE3). Transformed cells were divided into aliquots into 96-well microtiter plates, with each well containing a pool size of ca. 20 to 30 recombinant colonies. Replica plates of the 96-well plasmid library format were induced with IPTG (isopropyl-β-D-thiogalactopyranoside) to allow recombinant protein expression. After induction, the plates were centrifuged to pellet the *E. coli*, and the bacterial pellet was suspended in 200 μl of phosphate-buffered saline.

Screening of *P. gingivalis* expression library with specific T-cell line. A *P. gingivalis* expression library covering approximately 10 times the bacterial genome was initially divided into ~1,000 pools containing approximately 20 to 30 transformants/well distributed in 96-well microtiter plates and stored in a replica plate manner. Adherent spleen cells were fed with the *E. coli* pools and incubated for processing for 2 h. After washing, APC were exposed to a specific T-cell line in the presence of gentamicin (50 μg/ml) to inhibit the bacterial growth. T-cell recognition of pools containing *P. gingivalis* recombinant antigens was then detected by using a proliferation assay (^{3}[H]thymidine incorporation). The clones present in the wells that scored positive were then redistributed in 96-well plates at one colony/clone per well, followed by induction and rescreening with the specific T-cell line. The clones present in the positive wells of this second screening were expanded, followed by plasmid purification for sequencing of the *P. gingivalis* DNA inserts. Sequences were then used to search for homology with *P. gingivalis* open reading frames using the TIGR website (http://www.tigr.org).

High-level expression and affinity purification of a recombinant *P. gingivalis* antigen. Oligonucleotide PCR primers were designed to amplify the full-length sequence of *P. gingivalis* thiol peroxidase using genomic DNA of the virulent W83 strain as a template. The following oligonucleotide primers were used: forward primer (5′-CAATTACAT<u>ATG</u>*AGTCTAAAAATATTTCTAACA*-3′) and reverse primer (5′-CATGGGATCC*<u>TTA</u>***GTGATGGTGATGGTGATG**CTTCAGTGCAGCGATAGCAG-3′). The forward primer contains an NdeI restriction site preceding the ATG initiation codons (underlined), followed by sequences derived from the gene (italics). The reverse primer contains a BamHI restriction site followed by a stop codon (underlined), a nucleotide sequence encoding six histidines (boldface), and the protein coding sequence. The resultant PCR product was digested with NdeI and BamHI and cloned into the pET17b vector similarly digested with NdeI and BamHI for directional cloning. Ligation products were subsequently transformed into *E. coli* BL21(DE3)/pLysS host cells (Novagen) for expression. Recombinant thiol peroxidase attached with His tag at its C terminus was purified from 500 ml of IPTG-induced batch cultures by affinity chromatography using the one-step QIAexpress Ni-NTA agarose matrix (QIAGEN, Chatsworth, CA) as we previously described (37). The yield of recombinant protein varied from 25 to 50 mg per liter of induced bacterial culture, and the purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining. The recombinant protein was then passed through a column with immobilized polymyxin B (Detoxi-Gel; Pierce, Rockford, IL) and assayed for endotoxin contamination (<100 endotoxin units/mg of protein) using the *Limulus* amebocyte assay (BioWhittaker, Walkersville, MD).

Proliferation and cytokine assays. Lymphoid cells were obtained by conventional procedures from popliteal lymph nodes harvested from mice inoculated subcutaneously with 10^8 live *P. gingivalis*. Cells were cultured in the presence of either medium only (RPMI with 10% fetal calf serum and gentamicin [50 μg/ml]) or medium containing specific antigens at the indicated concentrations. For proliferation assay, plates were cultured for 3 days at 37°C in 5% CO_2 and were pulsed with 1 μCi of [^{3}H]thymidine (Amersham) for an additional 18 h. Cells were harvested onto filter mats, and the radioactivity incorporated into lymphocytes was determined by liquid scintillation counting. The results are expressed as the stimulation index, which is the ratio of the counts per minute (cpm) of [^{3}H]thymidine incorporation by cells cultured in the presence of antigens to the cpm incorporation by cells cultured in the presence of medium alone (nonstimulated cells). For cytokine analysis, spleen cells at 10^6 cells/well (24-well tissue culture plates) were incubated with or without antigens for 72 h (45). Supernatants were harvested and analyzed for IFN-γ and IL-4 by a double sandwich enzyme-linked immunosorbent assay (ELISA) using a specific monoclonal antibody (Pharmingen, San Diego, CA) as described previously (8).

Animal colonization with *P. gingivalis*. Specific-pathogen-free BALB/c mice were kept on a 12-h light-dark cycle and received distilled water and food ad libitum. The animals (eight per group) were age-matched females, 8 to 10 weeks old, at the start of experiments. Mice were given 1.2 mg of sulfamethoxazole and 0.24 mg of trimethoprim (Sulfatrim Pediatric; Alpharma, Baltimore, MD)/ml, 10 ml per pint in deionized water, ad libitum for 10 days, followed by a 3-day antibiotic-free period. Mice were then infected with 10^9 CFU of live *P. gingivalis* W83 in 100 μl of phosphate-buffered saline with 2% carboxymethyl cellulose directly placed into the oral cavity of the animals under general anesthesia (ketamine/xylasine). Animals were kept sedated for at least 1 h to prevent the rapid elimination of the bacteria. This procedure was repeated three times at

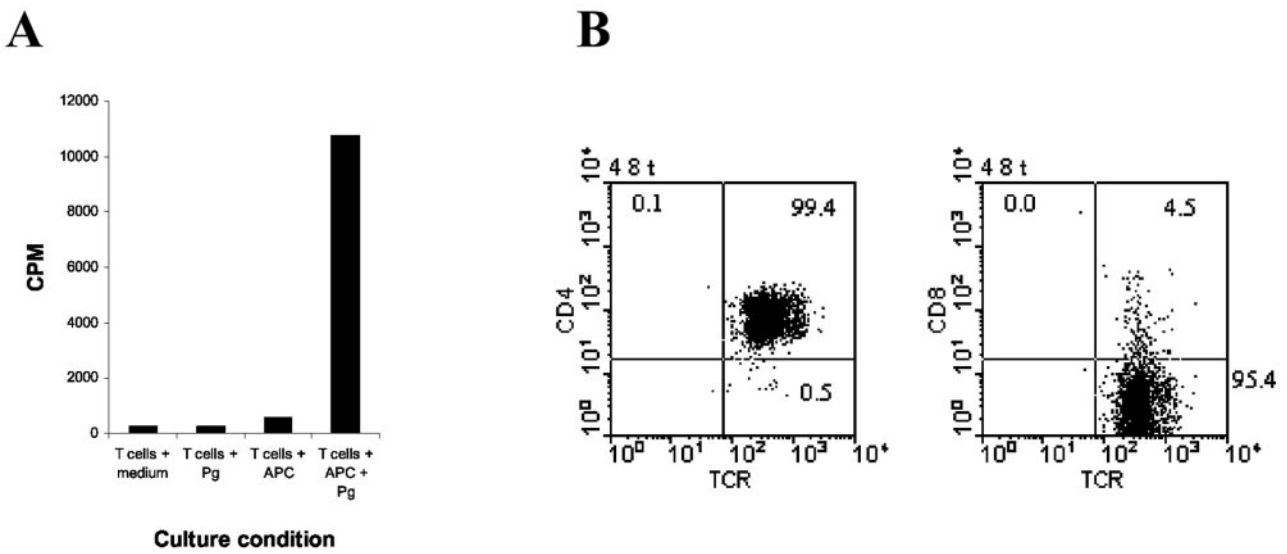

FIG. 1. Characterization of the $CD4^+$ *P. gingivalis*-specific cell line. The T-cell line was generated from the spleens of C57BL/6 mice infected intraperitoneally with viable *P. gingivalis*. (A) For antigen specificity, the T cells were cultured with or without APC and stimulated with either medium or *P. gingivalis* lysate antigens (10 μg/ml). Cultures were incubated for 72 h, and proliferation was assessed by measuring the incorporation of [^{3}H]thymidine. (B) For cell surface analyses, an aliquot of the cells was harvested approximately 10 days after in vitro restimulation with APC plus *P. gingivalis* antigens and analyzed by FACS using specific monoclonal antibodies to CD4, CD8, and T-cell receptor (TCR) cell surface antigens or isotype-matched monoclonal mouse control immunoglobulin.

2-day intervals (4). Controls included sham-infected mice, which received the antibiotic pretreatment and the carboxymethyl cellulose but without *P. gingivalis*. At 47 days after the first mouth exposure, the mice were bled for assessment of the immunoglobulin G1 (IgG1)/IgG2a serum-specific antibody response, followed by euthanasia by CO_2 inhalation.

IgG isotype ELISA. Mice were bled before and 8 weeks after infection with *P. gingivalis*, and sera were stored at −20°C until use. The specific serum IgG isotype antibody response was measured by conventional enzyme-linked immunosorbent assay (ELISA). Wells of ELISA plates (Costar, Cambridge, MA) were coated with recombinant thiol peroxidase protein at a concentration of 100 ng/well. Sera were added at twofold serial dilutions, followed by washes and the addition of biotinylated isotype-specific rat anti-mouse IgG1 (clone A85-1) and anti-mouse IgG2a (clone R19-15) monoclonal antibodies (BD Biosciences/Pharmingen, San Diego, CA). Both antibodies, at 2 μg/ml, have comparable reactivities with their respective antigens in an ELISA (manufacturer information). Wells were then washed and incubated with streptavidin-conjugated horseradish peroxidase (HRP; Zymed), after which the substrate and chromogen were added and the absorbance was read on an ELISA plate reader (Dynatech, Chantilly, VA) at 490 nm.

RESULTS

Generation and specificity of a $CD4^+$ T-cell line from splenocytes of *P. gingivalis*-infected C57BL/6 mice. An anti-*P. gingivalis* $CD4^+$ T-cell line was generated from spleen cells of C57BL/6 mice infected for approximately 4 weeks with viable *P. gingivalis* in the absence of adjuvant. We chose to infect the mice using the intraperitoneal route to maximize the immunization protocol and to prevent the T helper bias exerted by adjuvants. In addition to generating the T-cell line, the spleen cells were stimulated in vitro with viable *P. gingivalis* to favor the development of T-cell clones that are specific for antigens actively produced by the bacteria internalized by the APC. This protocol was facilitated by the fact that *P. gingivalis* is an anaerobic microorganism and therefore incapable of growing under the aerobic conditions of the T-cell culture. The resulting cell line was specific and strongly reactive with *P. gingivalis* antigens and comprised exclusively $CD4^+$ T cells (Fig. 1). This cell line was then used for the T-cell expression cloning experiments.

$CD4^+$ T-cell expression cloning and molecular characterization of antigens from *P. gingivalis*. This strategy has been previously described by one of us (A. Campos-Neto) for rapid cloning of *M. tuberculosis* (37) and is diagrammed in Fig. 2. Its general principle is based on the direct recognition by the T cells of antigens presented by APC that have internalized a library of *E. coli*-containing expressed recombinant antigens. The *P. gingivalis* library was initially divided into pools containing approximately 20 to 30 recombinant colonies per well in a 96-well microtiter plate format. Adherent spleen cells from naive C57BL/6 mice were fed with the library containing pools of *E. coli* expressing *P. gingivalis* antigens, followed by incubation with the $CD4^+$ T-cell line. The recognition of specific *P. gingivalis* antigens was detected by proliferation of the T cells ([^{3}H]thymidine incorporation). The screening of the 96-well microtiter master plates identified at least eight positive pools. For the selection of these pools, we used the same criteria that we used in our former studies (37). Basically, only pools with cpm 5 to 10 times higher than the control nontransformed *E. coli* pools were selected. The cpm of the selected clones varied from 1,500 to 8,000, while the cpm of the control nontransformed *E. coli*, as well as of the majority of the pools in the master plates, varied from 85 to 500 (results not shown). The subsequent cloning of the genes coding for *P. gingivalis* proteins that stimulated the specific $CD4^+$ T-cell line resulted in the characterization of eight ORFs of both previously known *P. gingivalis* proteins and novel putative protein antigens (Table 1). Interestingly, all of the previously known proteins are molecules that are actively involved in bacterial physiology, a finding which supports the initial idea of generating the specific T line using viable *P. gingivalis* to stimulate the spleen

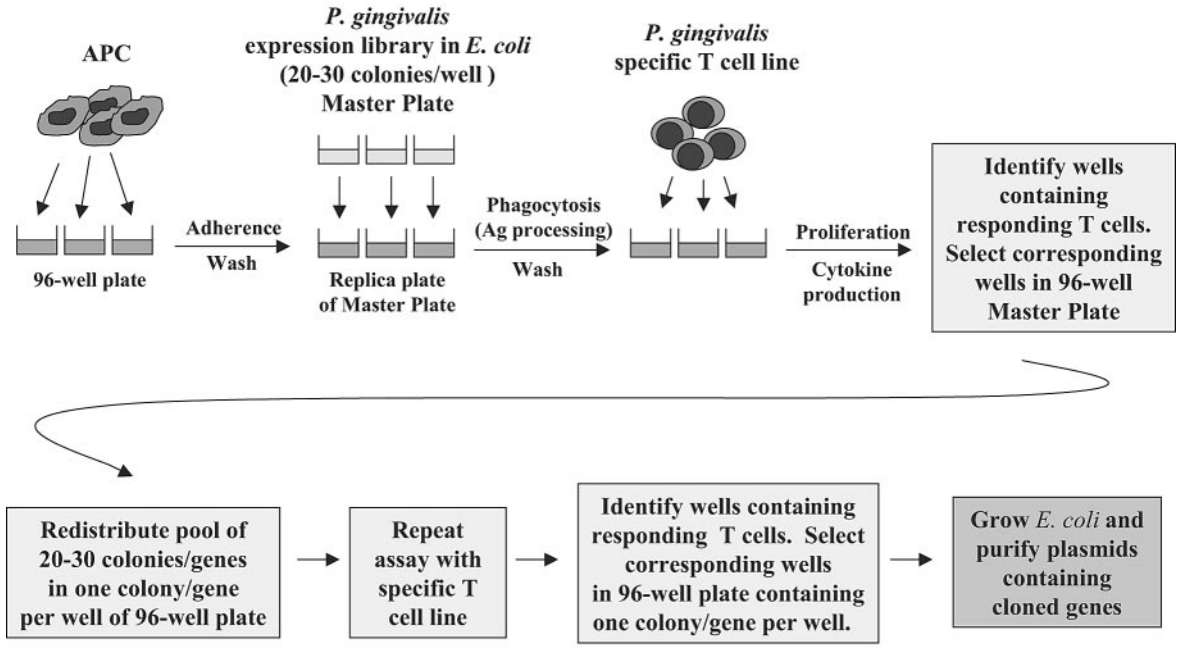

FIG. 2. Schematic illustration of T-cell expression cloning strategy for the identification of *P. gingivalis* genes encoding proteins involved in biased T-helper cell responses.

cells from immunized mice. In addition, the deduced sequence of two of these proteins (PG1236 and PG2024) revealed the presence of strong signal peptide sequences, which suggests that these proteins are either membrane-associated molecules or are bacterial secreted molecules. No signal peptide sequences could be identified in the other six proteins. Furthermore, the protein thiol peroxidase (PG1729) has been recently described to be upregulated during infection of HEp-2 human epithelial cells with *P. gingivalis* (17). Because of this latter property and because thiol peroxidase is a protein possibly involved in bacterial escape mechanisms (cellular detoxification) from host defense (10, 20, 24, 34), this molecule was the first among the identified proteins to be selected for further studies.

Expression and purification of recombinant thiol peroxidase protein. The open reading frame of the full-length gene of thiol peroxidase was amplified by PCR with 5′ and 3′ specific oligonucleotides and cloned into the pET17b expression vector. The construct was designed to contain six C-terminal histidine residues for ease of purification by affinity chromatography over the Ni-NTA matrix. The recombinant protein was purified from the soluble phase of the bacterial lysate with yields ranging from 25 to 50 mg of purified protein per liter of induced culture. Figure 3 shows Coomassie blue-stained SDS-PAGE gel of the *E. coli* cultures before and after induction and the respective purified recombinant antigens. The recombinant thiol peroxidase migrated with a molecular size of ~18 kDa, which is slightly below the predicted 19.2-kDa size of the native molecule.

Immunogenicity studies with thiol peroxidase. To verify whether thiol peroxidase is a protein that is actively produced by *P. gingivalis* during infection, C57BL/6 mice were initially infected subcutaneously with approximately 10^8 CFU of live *P. gingivalis* two times, 4 weeks apart. Both anti-thiol peroxidase antibody responses and T-cell responses were evaluated 1 week after the second inoculation. The antibody response was

TABLE 1. *P. gingivalis* genes coding for proteins identified by T-cell expression cloning

Putative identification	TIGR annotation	TIGR cellular role(s)	Gene length (bp)	Protein size (kDa)	pI
Thiol peroxidase	PG1729	Cellular processes: detoxification	540	19.2	4.98
Conserved putative protein antigen	PG1841	Not known	924	34.9	6.18
Putative protein antigen	PG0471	Not known	1,065	39.1	7.57
Putative protein antigen	PG1236	Not known	711	27.47	6.51
Transporter protein	PG2185	Transport and binding proteins; unknown substrate	1,317	49.3	9.66
Pyruvate ferredoxin/flavodoxin oxidoreductase family protein	PG0548	Energy metabolism: electron transport	3,582	131.9	6.45
DNA topoisomerase I	PG0754	DNA metabolism: DNA replication, recombination, and repair	2,367	89.5	8.99
Hemagglutinin protein (HagE)	PG2024	Cellular processes: pathogenesis	5,121	185.6	4.74

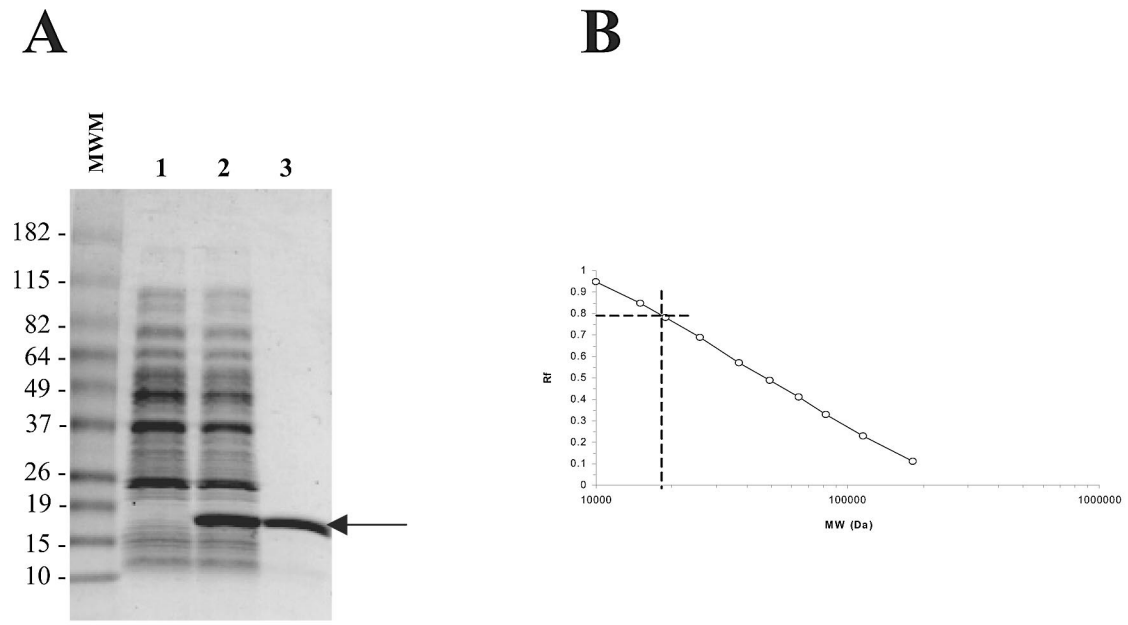

FIG. 3. Expression of *P. gingivalis* thiol peroxidase gene as purified recombinant protein. *E. coli* BL21/pLysS transformed with the expression vector pET17b containing the thiol peroxidase gene was grown and induced with IPTG. The cells were lysed, and the thiol peroxidase protein was purified by affinity chromatography using the one-step QIAexpress Ni-NTA agarose matrix. (A) Expression and purification were evaluated by SDS-PAGE (4 to 20%) under reducing conditions, and the gel was stained with Coomassie blue. Lane 1, noninduced *E. coli* lysate; lane 2, induced *E. coli* lysate; lane 3, purified recombinant thiol peroxidase protein (1 μg). Numbers on the left side indicate the molecular sizes of the markers in kilodaltons. The arrow points to a 18-kDa band calculated using the standard molecular mass curve obtained for the 4 to 20% gel (B).

evaluated by ELISA using specific rat anti-mouse IgG1 and IgG2a isotype monoclonal antibodies (21). Both IgG1 and IgG2a specific anti-thiol peroxidase antibodies were present in the sera of infected mice (Fig. 4). Of note was the observation that the titer of specific IgG2a antibody was much higher than the titer of IgG1. In addition, it is important to mention that none of these sera reacted with Mtb41, an *M. tuberculosis* recombinant His-tagged protein (37), thus excluding possible recognition of this epitope by the sera of *P. gingivalis*-challenged mice (results not shown). Because the production of IgG2a isotype antibodies has been considered to be a surrogate of the Th1 response, these observations suggest that the infection of mice with *P. gingivalis* results in a Th1 response to the microorganism's thiol peroxidase protein.

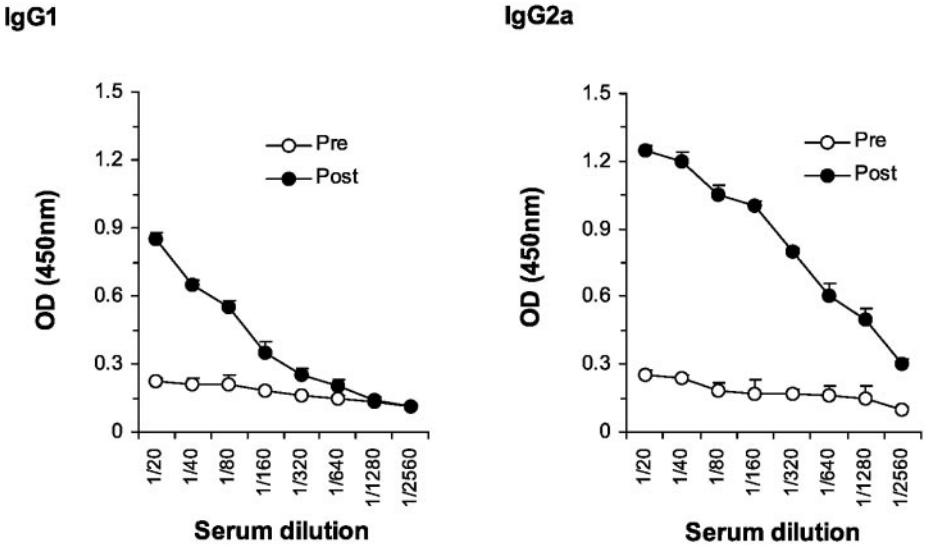

FIG. 4. Isotype-specific antibody response of mice infected subcutaneously with *P. gingivalis*. Anti-*P. gingivalis* thiol peroxidase antibody responses (IgG1 and IgG2a isotypes) were tested by ELISA using specific HRP-labeled goat anti-mouse immunoglobulin isotypes. Sera were obtained from mice prior to infection (Pre) and 30 days after subcutaneous inoculation with 10^8 viable *P. gingivalis* (Post).

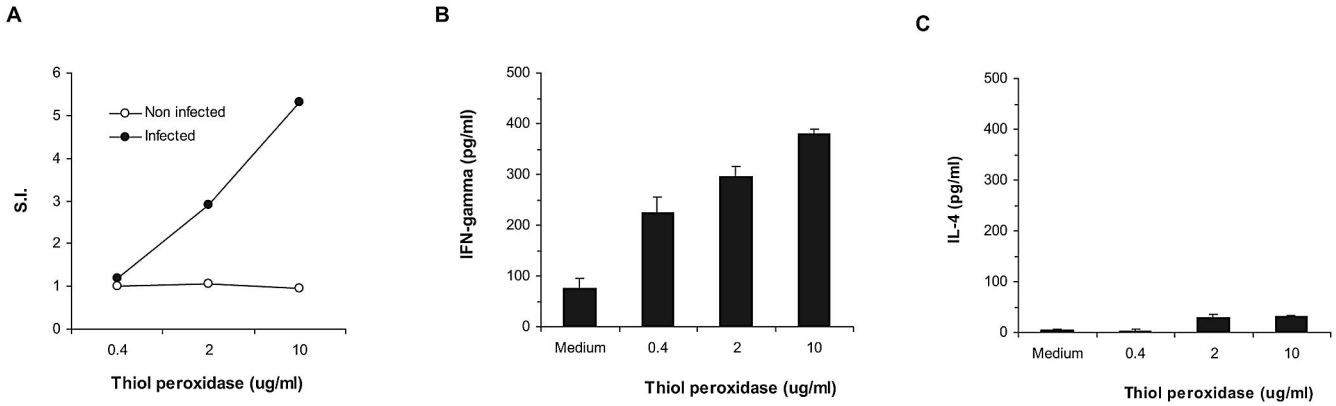

FIG. 5. Proliferative response and production of cytokines by lymph node cells of mice infected with *P. gingivalis*. C57BL/6 mice were infected subcutaneously with 10^8 viable *P. gingivalis*. Mice were sacrificed 3 weeks later, and lymphocytes were obtained from popliteal lymph nodes and cultured for 3 days in the presence of medium, 0.4, 2, and 10 μg of purified recombinant *P. gingivalis* thiol peroxidase protein/ml. Proliferation (A) was assessed by determining the incorporation of [^{3}H]thymidine and is expressed as the stimulation index (S.I.), and the production of both IFN-γ (B) and IL-4 (C) was assayed by ELISA in the culture supernatants.

For evaluation of the T-cell response, lymphoid cells from infected mice were obtained and stimulated in vitro with purified recombinant thiol peroxidase. Cells were cultured in 96-well plates in the presence of 10, 2, or 0.4 μg of the antigen/ml or with medium alone. Proliferation was measured by measuring the incorporation of [^{3}H]thymidine, and the production of the cytokines IFN-γ and IL-4 was analyzed by sandwich ELISA in the culture supernatants using cytokine-specific monoclonal antibodies. Figure 5A shows that lymph node cells obtained from mice infected with *P. gingivalis* proliferate upon stimulation with recombinant thiol peroxidase protein and also produce predominantly IFN-γ (Fig. 5B). Little or no IL-4 could be detected in the culture supernatants of the antigen-stimulated cultures (Fig. 5C). These results, in conjunction with the high serum IgG2a antigen specific antibody titers, suggest that infection of mice with *P. gingivalis* results in the development of an anti-thiol peroxidase T-cell response of the Th1 phenotype.

Recognition of recombinant thiol peroxidase by mice orally infected with *P. gingivalis*. The former experiments were performed using the artificial parenteral route of infection with *P. gingivalis* in order to facilitate the evaluation of the T-cell response present in the lymph nodes draining the infection site. Unfortunately, mice exposed to *P. gingivalis* using the natural oral route of infection do not develop a detectable T-cell response in their spleen nor are there reliable draining lymph nodes available to obtain these cells. However, mice orally exposed to viable *P. gingivalis* develop systemic antibody responses to the microorganism (3). In view of these facts, immunogenicity studies to verify the antibody response to recombinant thiol peroxidase in animals orally exposed to *P. gingivalis* were performed. Approximately 10^8 CFU of *P. gingivalis* (100 μl) were deposited in the sublingual area of each animal. The exposure was repeated 1 month later. At 4 weeks after the second exposure, the animals were bled, sera were obtained, and the IgG1 and IgG2a antibody responses were evaluated by ELISA with specific anti-mouse isotype antibodies. The results indicated that mice exposed to live *P. gingivalis* in the oral cavity developed both IgG1 and IgG2a serum antibody responses against thiol peroxidase recombinant protein (Fig. 6). These observations suggest that the infectious process caused by exposing the oral cavity of mice with *P. gingivalis* results in a systemic anti-thiol peroxidase antibody response compatible with a Th1-biased phenotype.

DISCUSSION

Most evidence suggests that infection or immunization with intact *P. gingivalis* elicits a predominantly Th1-type response in experimental animals and humans (19, 22). In addition, adoptive-transfer experiments have indicated an active role for Th1 cytokines in disease exacerbation and for Th2 cytokines in protection from disease (11, 22, 41, 42). In fact, opposite effects of cytokine response associated with disease protection or exacerbation have been described for a variety of infectious diseases and has been used as a guiding parameter for the development of vaccines and immunotherapeutics. Leishmaniasis is perhaps the foremost example of such a pattern. In these diseases, the effects of Th1 and Th2 cytokines are opposite those observed in periodontal diseases, i.e., Th1 cytokines are protective and Th2 cytokines are disease promoters. Paradoxically, we (7, 32) have observed that antigens that induce strong Th1 cytokine responses during the natural evolution of the disease usually do not induce protection if they are used in vaccination protocols. In contrast, antigens that induce strong Th2 response during the disease can be highly protective if they are used in vaccination protocols that induce strong Th1 responses to them (1, 9, 26). Our hypothesis that emerges from those studies is that subverting the infectious agent's ability to elicit Th1 or Th2 responses to survive within the host can be an efficient strategy in vaccine development against microbial pathogens (7, 32). In other words, a successful immunotherapeutic candidate to halt the inflammation mediated by *P. gingivalis* is a protein that during infection induces a potent Th1 response (disease promoter) provided that this antigen is used in a vaccination formulation that induces a potent and antigen-specific Th2 response. Using this counterintuitive paradigm, we have developed a highly protective anti-*Leishmania* vaccine

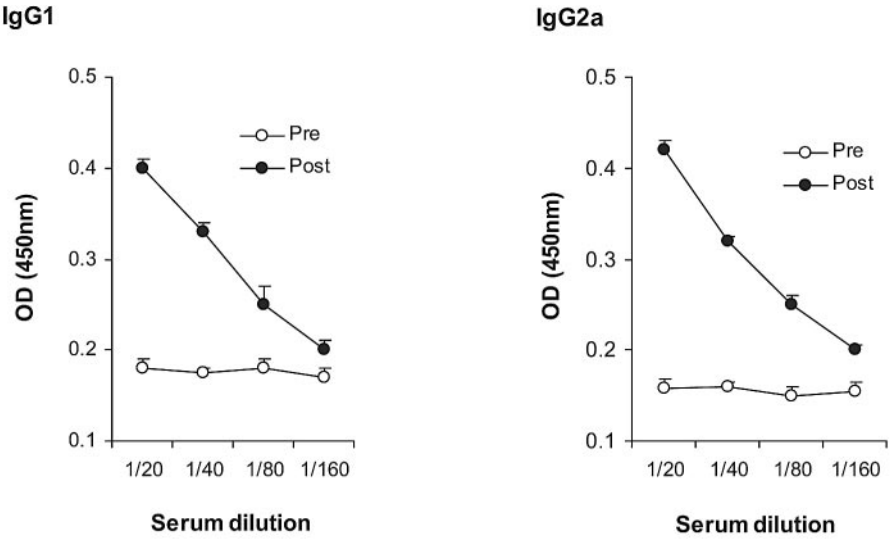

FIG. 6. Isotype-specific antibody response of mice orally infected with *P. gingivalis*. Anti-*P. gingivalis* thiol peroxidase antibody responses (IgG1 and IgG2a isotypes) were tested by ELISA with specific HRP-labeled goat anti-mouse immunoglobulin isotypes. Sera were obtained from mice prior to infection and 30 days after mice were orally exposed three times (every other day) with 10^8 viable *P. gingivalis*.

that has been recently and successfully tested in a phase I clinical trial (U.S. Food and Drug Administration Office of Vaccines, BB-IND #10116). In the present study the same rationale was used to select for *P. gingivalis* antigens that during infection are associated with the induction of either Th1 or Th2 responses.

This proposal was facilitated by the T-cell expression cloning approach to discover microbial antigens that we and others have recently developed and validated (2, 31, 37). This approach has basically two major advantages. (i) It allows for rapid cloning of a gene without any previous information about the structural properties of a potential protein antigen. (ii) It can be used to define and select antigens associated with either a Th1 or a Th2 phenotype of immune responses during the infectious process. Using this approach, we were able to identify eight *P. gingivalis* genes (Table 1) coding for proteins that are potential candidate molecules associated with the inflammation induced by this oral pathogen. Five of these genes code for known *P. gingivalis* proteins, and three code for putative protein antigens. One of the known proteins, thiol peroxidase, was chosen to be the first candidate to be studied in detail because this family of molecules is apparently involved in microbial pathogenesis (10, 20, 24, 34). Coincidentally, our earlier antigen discovery strategy in leishmaniasis led us to find the antigen thioredoxin peroxidase that shares ~50% homology (protein level) with *P. gingivalis* thiol peroxidase. Interestingly, the leishmania parasite molecule was found to be an antigen that induces excellent protection against the challenge of both mice and monkeys with the virulent *Leishmania major* (9). Because of these findings, the parasite thioredoxin peroxidase is one of the three antigen components of the antileishmania vaccine under the clinical trial mentioned above. Despite being only circumstantial, this interesting analogy, which is associated with the recent demonstration that thiol peroxidase is upregulated during *P. gingivalis* adherence and/or infection of human epithelial cells (17), led us to prioritize this molecule for further studies.

Similar to our former studies with the leishmanial thioredoxin peroxidase, the expression and purification of the *P. gingivalis* thiol peroxidase was easily achieved, yielding high concentrations of recombinant protein (25 to 50 mg/liter of *E. coli* broth culture) obtained from the soluble phase of the bacterial lysate. The fact that the protein was purified from the soluble phase was a great advantage in that no harsh procedures (e.g., the addition of denaturing agents such as urea to solubilize inclusion bodies) were needed to obtain the recombinant molecule.

The in vivo experiments were important to validate thiol peroxidase as an antigen that is actively produced by *P. gingivalis* during infection. This conclusion is based on the facts that the inoculation of mice using the subcutaneous route with viable *P. gingivalis* resulted in both strong humoral and cellular responses to the purified recombinant antigen. In addition, although the subcutaneous route of inoculation of the viable bacteria results in an ectopic and unconventional site of infection, the immune response induced by the *P. gingivalis* specific for thiol peroxidase was a typical Th1 response. Thus, at the humoral response, high titers of both IgG1- and IgG2a-specific antibodies were generated and detected in the sera of the infected animals. It is important to mention that IgG1 has been used in the past as a surrogate of Th2 response because the immunoglobulin class switch to generate this immunoglobulin isotype was earlier shown to be induced by the Th2 cytokine IL-4. However, recent evidences (14) have demonstrated that IgG1 antibodies are divided in two distinct families of molecules: one that is dependent on IL-4 (Th2 associated) and another that is dependent of IL-12 and IFN-γ (Th1 associated). Therefore, the presence of high titers of anti-thiol peroxidase IgG1 is not necessarily an indication of a Th2 response to the recombinant antigen after infection with *P. gingivalis*. In

contrast, because the class switch to generate IgG2a is solely dependent on IFN-γ, a high-titer IgG2a immune response has been generally accepted as a strong surrogate of a typical Th1 response. Indeed, the in vitro recall experiments confirmed that the infection with *P. gingivalis* induced this phenotype of immune response specific for thiol peroxidase. This conclusion was supported by the observation that the draining lymph node cells from the infected mice, when stimulated in vitro with the thiol peroxidase, produced large quantities of IFN-γ and only barely detected IL-4.

Moreover, the conclusion that the infection of mice with *P. gingivalis* results in a preferential Th1 response to thiol peroxidase was further substantiated by the results obtained from mice exposed in their oral cavity with viable bacteria. This mode of inoculation of *P. gingivalis* results in oral infection characterized by moderate gingival lesions and bone loss, thus mimicking human periodontal disease (4, 36). Although at lower titers than mice inoculated subcutaneously with *P. gingivalis*, these animals developed both IgG1 and IgG2a antibody responses to thiol peroxidase, a phenotype compatible with Th1-biased response.

We are currently evaluating a possible role of thiol peroxidase in the inflammation caused by *P. gingivalis* using both a mouse model of periapical bone loss and a rabbit model of periodontitis. The hypothesis for these experiments is to correlate the phenotype of immune response specific for antigens such as thiol peroxidase and others such as those identified in the present study (Table 1) with possible mechanisms of lesion development in the periodontitis caused by *P. gingivalis*.

Finally, these results support the premise of the approach used in these studies, i.e., the use of a $CD4^+$ T-cell line to directly screen a pathogen expression library to clone genes encoding for microbial antigens associated with Th1 and potentially Th2 responses during the infectious processes.

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