Modulation of Gamma Interferon-Induced Major Histocompatibility Complex Class II Gene Expression by *Porphyromonas gingivalis* Membrane Vesicles

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Gamma interferon (IFN-γ)-induced endothelial cells actively participate in initiating immune responses by interacting with CD4⁺ T cells via class II major histocompatibility complex (MHC) surface glycoproteins. Previously, *Porphyromonas gingivalis* membrane vesicles were shown to selectively inhibit IFN-γ-induced surface expression of HLA-DR molecules by human umbilical cord vascular endothelial cells. In this study, we demonstrated an absence of HLA-DRα mRNA from IFN-γ-induced cells in the presence of *P. gingivalis* membrane vesicles by using reverse transcriptase-PCR and Southern blotting. Vesicles also prevented transcription of the gene encoding class II transactivator, a transactivator protein required for IFN-γ-induced expression of MHC class II genes. In addition, the effects of vesicles on IFN-γ signal transduction involving Jak and Stat proteins were characterized by using immunoprecipitation and Western blot analyses. Jak1 and Jak2 proteins could not be detected in endothelial cells treated with membranee vesicles. Consequently, IFN-γ-induced phosphorylation of Jak1, Jak2, and Stat1α proteins was prevented. The class II-inhibitory effect of the membrane vesicles could be eliminated by heating vesicles at 100°C for 30 min or by treating them with a cysteine proteinase inhibitor. This indicates that the cysteine proteinases were most likely responsible for the absence of Jak proteins observed in vesicle-treated cells. The observed increased binding of radiolabeled IFN-γ to vesicle-treated cells suggests that vesicles may also modulate the IFN-γ interactions with the cell surface. However, no evidence was obtained demonstrating that vesicles affected the expression of IFN-γ receptors. Thus, *P. gingivalis* membrane vesicles apparently inhibited IFN-γ-induced MHC class II by disrupting the IFN-γ signaling transduction pathway. Vesicle-inhibited class II expression also occurred in other IFN-γ-inducible cells. This suggested that the ability of *P. gingivalis* membrane vesicles to modulate antigen presentation by key cells may be an important mechanism used by this particular bacterium to escape immunosurveillance, thereby favoring its colonization and invasion of host tissues.

*Porphyromonas gingivalis*, a gram-negative anaerobic bacterium, has been shown elsewhere to be a major etiologic agent of destructive adult periodontitis (37). Recent epidemiological studies have suggested that adult periodontitis may cause conditions more serious than tooth loss. These include cardiovascular disease (1, 28) and preterm labor and low birth weight (27). Like other gram-negative bacteria, *P. gingivalis* produces outer membrane vesicles (OMVs), by the outgrowth of its outer membranes, which are subsequently released into the surrounding environment (12). It has been proposed elsewhere that such vesicles are liberated from the bacteria by a mechanism involving cell wall turnover (46). Consequently, vesicles normally retain a full complement of outer membrane constituents of the bacterial cell wall, including proteins, lipopolysaccharide, muramic acid, capsule, and fimbriae (8, 12, 46). Many of these components play a crucial role in the pathogenicity of *P. gingivalis*. In addition, vesicles exhibit adhesion properties and strong proteolytic activity, which, together with their small size (20 to 500 nm), enable them to penetrate the intact oral mucosa and enter the underlying gingival tissue (24). By this activity, vesicles are able to deliver and release the numerous virulence factors that they contain into the surrounding tissue.

It is well documented that venular endothelial cells actively participate in immunological responses by expressing certain adhesion and major histocompatibility complex (MHC) molecules in response to specific cytokines. For example, endothelial cells can participate in acquired immune responses by the induction and expression of MHC class II genes in response to gamma interferon (IFN-γ) (6), which enables them to function as antigen-presenting cells (APCs) (36, 43). As such, endothelial cells have the ability to stimulate antigen-specific CD4⁺ T cells, resulting in their activation and proliferation. Thus, the regulation of MHC class II synthesis is of considerable biological importance. Studies have shown that the induction of MHC class II molecules by IFN-γ requires the de novo synthesis of the class II transactivator (CIITA), which interacts with ubiquitous DNA binding proteins at MHC class II promoters (2, 3, 22, 40). The activation of CIITA occurs following the binding of IFN-γ to its specific receptor, which triggers the tyrosine phosphorylation of signaling components, i.e., the receptor-associated tyrosine kinases, Jak1 and Jak2, and Stat1α (11). The phosphorylated Stat1α forms a homodimer which functions as a transcriptional factor that translocates to the nucleus and binds to the gamma activation sequence in IFN-γ-stimulated genes, including the CIITA gene (16, 34).

Previous studies in this laboratory demonstrated that treatment of endothelial cells with *P. gingivalis* membrane vesicles can inhibit the surface expression of IFN-γ-induced HLA-DR molecules, whereas other cytokine-induced molecules, such as
tumor necrosis factor alpha-induced E-selectin or interleukin-1-induced intercellular adhesion molecule 1, were not affected (39). This suggests that the inhibitory effect observed may suppress specific immune responses, enabling the bacteria to survive and colonize host tissues. Therefore, the present study was undertaken to determine the molecular target of inhibition by P. gingivalis membrane vesicles. For this purpose, the effects of vesicles on IFN-γ-induced expression of both the HLA-DR gene and the CIITA gene as well as on the IFN-γ signaling cascade involving Jak and Stat proteins were examined.

MATERIALS AND METHODS

Cells, media, and reagents. Human umbilical vascular endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC: Manassas, Va.; CRL 1730), and the purity was determined as previously described (19). HUVECs were cultured as previously described (19), with cultures of 19 to 24 passages used in all experiments. Other cell lines used, obtained from ATCC, included human embryonic lung fibroblasts (MRC-5; ATCC CCL 171), 143B human osteosarcoma cells (ATCC CRL 8304), and THP-1 monocytic cells (ATCC TIB 202). The fibroblast and osteosarcoma cells were grown to confluency in six-well plates and subconfluent monolayers of HUVECs (5 × 10^4 cells per ml). Treatments with THP-1 cells, media, and reagents. Human umbilical vascular endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC; No. TIB 202). The other cell lines used, obtained from ATCC, included human embryonic lung fibroblasts (MRC-5; ATCC CCL 171), 143B human osteosarcoma cells (ATCC CRL 8304), and THP-1 monocytic cells (ATCC TIB 202). The fibroblast and osteosarcoma cells were grown to confluency in six-well plates and subconfluent monolayers of HUVECs (5 × 10^4 cells per ml). Treatments with THP-1 cells, media, and reagents.

Preparation of P. gingivalis membrane vesicles. The P. gingivalis strain SW50 was obtained from ATCC (ATCC 53978). Growth of bacteria and preparation of membrane vesicles were previously described (20). Proteolytic enzyme activity was determined as described by Smallley and Birss (38) using Nα-benzoyl-L-arginine-p-nitroanilide (BAPNA; Sigma) as a chromogenic substrate. The enzymatic release of p-nitroanilide was measured at an absorbance of 410 nm by using a Beckman DU-60 spectrophotometer. The proteolytic activity of vesicles was abolished either by preincubation with a cysteine proteinase inhibitor, Nε-tosyl-L-lysine chloromethyl ketone (TLCK, Sigma), at a final concentration of 2 mM for 1 h at 37°C or by heating at 100°C for 30 min.

Monoclonal and polyclonal antibodies. A mouse anti-HLA-DR monoclonal antibody (mAb) and a mouse anti-HLA-ABC MAb, conjugated to phycocerythrin, were purchased from Becton Dickinson (Mountain View, Calif.) and Caltag Laboratories (Burlingame, Calif.), respectively, and used for the detection of cell surface MHC molecules. A mouse immunoglobulin G2a (IgG2a) MAb (Becton Dickinson) was used as an isotype-matched negative control. A mouse anti-human IFN-γ receptor, a mouse IgG1 MAb, and a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG1, obtained from Genzyme (Boston, Mass.), were used in evaluation of IFN-γ receptor surface expression. An antiphosphotyrosine MAb, 4G10, and rabbit antiserum to Jak1, Jak2, and Stat1 were obtained from Upstate Biotechnology Inc. (Lake Placid, N.Y.) for immunoprecipitation and Western blot analyses.

Flow microfluorimetric analysis. For analysis of cell surface membrane expression of class II HLA-DR or class I HLA-ABC molecules, cells were stained with either phycoerythrin-conjugated mouse anti-HLA-DR MAb or anti-HLA-ABC MAb, or an isotype-matched mouse IgG2a MAb, as previously described (19). In the case of THP-1 cells, nonspecific binding through Fc receptors was prevented by preincubating the cells with human gamma globulin (Sigma). For analysis of surface IFN-γ receptor expression, cells were stained with either a mouse anti-human IFN-γ receptor MAb or an isotype-matched mouse IgG1 MAb at 4°C for 30 min and then incubated with FITC-conjugated goat anti-mouse IgG at 4°C for an additional 30 min. Flow cytometric analysis and cell viability testing were previously described (19).

RNA isolation, RT-PCR, and Southern blot analysis. Total cellular RNA was isolated from confluent monolayers of HUVECs (5 × 10^6 cells) that were stimulated for 3 days with IFN-γ (250 U/ml), in the absence or presence of vesicles (30 μg of protein/ml), as described previously (19). Sequences of oligonucleotide primers used in reverse transcriptase PCR (RT-PCR) and probes for Southern blot analysis were also previously described (19). RT-PCR was performed using a SuperScript One-Step RT-PCR with the PLATINUM Taq System (GIBCO-BRL). Reactions were performed in a total volume of 50 μl, which contained 2.4 mM MgSO4, 0.4 mM (each) deoxynucleoside triphosphate, 1 μl of RT-PLATINUM Taq mix (Taq DNA polymerase and SUPERSCRIPT II H-RT), 0.2 μM sense and antisense primer, and 0.5 μg of template RNA, using a 2400 DNA Thermal Cycler (Perkin-Elmer). The RT-PCR amplification program was set as follows. First, cDNA synthesis was achieved in one cycle of 50°C for 30 min. The RNA-cDNA hybrid was denatured at 94°C for 2 min. The PCR was performed for 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min, followed by 1 cycle of 72°C for 10 min. The β-actin mRNA detection was used as an internal control for the amount of cDNA synthesized. Absence of genomic DNA contamination in the RNA preparation was verified by omitting the RT-Taq mix and substituting 2 U of Taq DNA polymerase in the reaction mixtures. PCR products were analyzed on a 2% agarose gel containing ethidium bromide and subsequently transferred onto nitrocellulose for Southern blot analysis. All RT-PCR amplifications were done at least twice using RNA obtained from two separate extractions.

Southern hybridization was carried out according to the method of Sambrook et al. (32) under moderately stringent conditions in which the blot was incubated with γ-32P-labeled probes for 12 h at 44°C (20). End labeling of the probe was performed according to the methods previously described by Richardson (31) and Maxam and Gilbert (23). T4 polynucleotide kinase was obtained from GIBCO-BRL as part of an end-labeling kit, and γ-32P[ATP] (5,000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, N.J.). Immunoprecipitation and Western blot analysis. HUVECs (5 × 10^6 cells) were incubated for 3 days with vesicles (30 μg of protein/ml) for 24 h at 37°C, and then cells were rinsed several times with Hanks’ balanced salt solution prior to stimulation with IFN-γ (250 U/ml) for 60 min. Untreated HUVECs or HUVECs treated with vesicles alone for 24 h or with IFN-γ alone for 60 min served as controls. Whole-cell lysates were prepared from HUVECs with ice-cold lysis buffer containing both protease and phosphatase inhibitors, as previously described (20). Immunoprecipitation was performed using the anti-humanJak1, Jak2, or Stat1α antibodies, and proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (20).

IFN-γ binding assay. The IFN-γ binding assay was performed as previously described using 125I-IFN-γ (Dupont NEN, Boston, Mass.) (20). Specific binding was determined by subtracting the difference between total binding in tubes without the unlabeled IFN-γ and the nonspecific binding in tubes containing excess unlabeled IFN-γ.

RESULTS

P. gingivalis membrane vesicles inhibit MHC class II surface expression in IFN-γ-inducible cells in addition to HUVECs. The effects of P. gingivalis membrane vesicles on IFN-γ-inducible cells other than HUVECs, including MRC-5 fibroblasts, 143B osteosarcoma cells, and THP-1 monocytic cells, were examined to determine if the inhibition of class II molecules previously demonstrated in HUVECs was unique. As indicated by the number of HLA-DR-positive cells, cell surface expression of class II glycoproteins induced by IFN-γ (250 U/ml) was clearly inhibited by vesicles (30 μg of protein/ml) in MRC-5 fibroblasts and 143B osteosarcoma cells (class II negative) in a manner similar to that of the HUVECs (Fig. 1). In the case of class II-positive THP-1 cells, the upregulation of class II antigen expression by IFN-γ could also be inhibited in the presence of vesicles. In comparison to IFN-γ-treated control cells, there was a significant reduction in the percent HLA-DR-positive cells of THP-1 cells exposed to vesicles and IFN-γ was not significantly different from constitutive levels expressed by the untreated control cells (Fig. 1). It is quite evident from these results that P. gingivalis membrane vesicles are capable of inhibiting IFN-γ-induced MHC class II antigen expression in cell types other than HUVECs

P. gingivalis membrane vesicles inhibit transcription of HLA-DRα and CIITA genes. To determine if P. gingivalis membrane vesicles could be inhibiting HLA-DR and CIITA...
gene transcription, the effect of vesicles on the HLA-DRα and CIITA mRNA content in IFN-γ-stimulated endothelial cells was analyzed by RT-PCR and Southern blotting. Cells stimulated with IFN-γ (250 U/ml) for times varying from 6 to 72 h showed increased levels of HLA-DRα mRNA (Fig. 3A) in comparison to constitutive levels of β-actin mRNA. HLA-DRα transcription was first detected 12 h following IFN-stimulation, and it increased with prolonged incubation. In a second set of experiments, total RNA was isolated from endothelial cells that had been stimulated with IFN-γ in the absence or presence of vesicles (30 μg of protein/ml) for 3 days. As indicated in Fig. 3B, HLA-DRα mRNA was not detected in those cells stimulated with IFN-γ in the presence of vesicles.

As previously noted, CIITA is necessary for IFN-γ-induced MHC class II synthesis and its gene expression is induced by this cytokine. Therefore, transcription of CIITA genes in the presence and absence of P. gingivalis membrane vesicles was next examined to ascertain whether membrane vesicles could block transcription of the CIITA gene or inhibit HLA-DRα gene expression at some step subsequent to CIITA gene expression. In these experiments, the same mRNA isolated for the HLA-DRα transcription experiments was subjected to RT-PCR using primers complementary to the CIITA gene. IFN-γ induced the synthesis of CIITA mRNA, which appeared several hours earlier than that for HLA-DRα (less than 6 h) (Fig. 4A). The presence of vesicles in cell cultures throughout the period of IFN-γ stimulation resulted in complete inhibition of CIITA mRNA synthesis (Fig. 4B). This suggests that vesicle-mediated blockage of class II expression is secondary to the inhibition of CIITA transcription.

**P. gingivalis membrane vesicles can inhibit the Jak-Stat signaling pathway.** Since the transcription of CIITA is ultimately dependent on upstream signaling events of the IFN-γ signal transduction pathway, the integrity of this signaling pathway in cells pretreated with P. gingivalis membrane vesicles was next investigated. For this purpose, the effects of vesicles on the phosphorylation of Jak1, Jak2, and Stat1α were determined by immunoprecipitation of each protein by its specific MAb, followed by Western immunoblot analyses using a phospho-tyrosine-specific MAb. As shown in Fig. 5, phosphorylated Stat1α, Jak1, and Jak2 proteins were detected in IFN-γ-treated cells (lane 3) but not in control cells (lane 1) or vesicle-treated cells that were unstimulated (lane 2) or subsequently stimulated with IFN-γ (lane 4). Western blot analyses using specific antibodies to Stat1α, Jak1, and Jak2 proteins indicated that vesicles had no effect on Stat1α expression, while Jak1 and Jak2 proteins were missing in the vesicle-treated cells (Fig. 5, lanes 2 and 4). These results revealed that vesicles blocked the IFN-γ signaling pathway by a mechanism(s) involving Jak proteins.

**Effect of vesicle proteolytic activity on the inhibition of HLA-DR surface expression.** Since it was previously shown by others that vesicles contain proteolytic enzymes, a major virulence factor for P. gingivalis (10, 12, 14), there was the possibility that this activity was involved in the inhibition of HLA-DR synthesis. Preliminary experiments indicated that our vesicle preparations possessed proteolytic activity which was diminished after incubation with TLCK (a cysteine protease inhibitor) or heating at 100°C for 30 min. Consequently, the effects of TLCK-treated or heat-treated vesicles on the class II inhibition were compared to those of untreated vesicles. As clearly shown in Fig. 6, both treatments eliminated the ability of vesicles to inhibit IFN-γ-induced HLA-DR synthesis and surface expression. These results strongly suggest that vesicle proteinases play a major role in blocking class II synthesis.

**Effects of P. gingivalis membrane vesicles on MHC class I surface expression in HUVECs.** Since P. gingivalis membrane vesicles act on the IFN-γ signaling pathway, it was reasoned that they should also affect the upregulation of class I antigen expression by this cytokine. In contrast to class II, class I
molecules are constitutively expressed by all nucleated cells including HUVECs. As shown in Fig. 7, incubating HUVECs with 250 U of IFN-γ/ml for 72 h resulted in a significant upregulation of MHC class I antigen expression above that of controls. The mean fluorescence intensity of IFN-γ-stimulated cells in the presence of vesicles (30 μg of protein/ml) was comparable to constitutive levels expressed by untreated control cells. Because the expression of class I MHC genes can also be upregulated by IFN-α, in which case its signaling pathway also involves Jak1 and Stat1, it was of interest to determine if vesicles could also inhibit IFN-α-upregulated expression of class I molecules. Similar to the IFN-γ results, IFN-α-upregulated class I surface expression was also inhibited by vesicles (Fig. 7).

The results of these experiments indicated that *P. gingivalis* membrane vesicles can also inhibit the upregulation of MHC class I synthesis and membrane expression by IFN-γ and IFN-α.

*P. gingivalis* membrane vesicles do not interfere with the surface expression of IFN-γ receptors. To determine if the demonstrated vesicle-induced inhibitory effects were solely due to an intracellular mechanism(s), the influence of vesicles on the binding of IFN-γ to its specific receptor on the surface of HUVECs was examined using 125I-labeled IFN-γ. As shown in Fig. 8, IFN-γ receptor binding was slightly decreased when vesicles were added to the cells at the same time as was IFN-γ.

Unexpectedly, pretreatment of cells with vesicles for 6, 12, or 24 h before addition of the radiolabeled IFN-γ greatly enhanced the attachment of the cytokine to endothelial cell surfaces. Also indicated is that only a limited number of IFN-γ receptors were present on cell surfaces.

To examine the possibility that the vesicle-enhanced 125I-IFN-γ binding was due to upregulation of IFN-γ receptor synthesis and surface expression, HUVECs were incubated in the absence or presence of vesicles at 37°C for 6, 12, or 24 h. By using an anti-human IFN-γ receptor MAb and a fluorescence-conjugated secondary antibody, the surface expression of IFN-γ receptors was determined by flow cytometry. As can be seen in Fig. 9, the mean fluorescence intensity of the vesicle-treated cells stained with the specific antibody to IFN-γ receptor was comparable to that of the normal cells. These results provide good evidence that the vesicles did not affect the synthesis and membrane expression of IFN-γ receptors. Thus, the greatly increased binding of radiolabeled IFN-γ to the vesicle-treated cells was not due to the increase in IFN-γ receptors.

These results, in conjunction with those of earlier studies dem-
indicating that class II synthesis could be blocked by adding vesicles before or simultaneously with IFN-γ, and that inhibitory effects may occur at the membrane level as well as intracellularly.

**DISCUSSION**

MHC class II molecules are essential for the presentation of peptides generated in the endocytic vesicles of endothelial cells as well as other APCs to CD4+ T-helper cells. Recent studies have demonstrated that the treatment with outer membrane vesicles isolated from *P. gingivalis* can inhibit the surface expression of MHC class II molecules on IFN-γ-treated endothelial cells (39). This study demonstrated that, in addition to HUVECs, the treatment with *P. gingivalis* vesicles also blocked IFN-γ-induced MHC class II antigen expression on other unrelated class II-negative cells, including osteosarcoma cells and fibroblasts. Moreover, the vesicles interfered with the up-regulation of class II antigen expression by IFN-γ-stimulated THP-1 monocytic cells. They did not, however, prevent the expression of class II molecules at the constitutive level observed in the absence of vesicles and IFN-γ. These findings indicate that the vesicle-induced inhibitory effect on class II molecules is not cell type specific. The downregulation of class II antigen synthesis and membrane expression can lead to a suppression of local host defense mechanisms against microbial pathogens by affecting the antigen-presenting activity of APCs. Such changes can obviously influence the efficiency of T-cell-mediated immune responses in such a way as to affect the outcome of the infection. Consequently, it is our view that the production and release of membrane vesicles by *P. gingivalis* in situ may be the strategy used by this particular bacterium to prevent or modulate antibacterial immune responses in order to avoid elimination from host tissues.

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**FIG. 5.** Effects of *P. gingivalis* membrane vesicles on IFN-γ-induced tyrosine phosphorylation of Stat1α, Jak1, and Jak2 in endothelial cells. HUVECs were incubated in medium alone (lane 1) or with vesicles (OMVs, 30 μg of protein/ml) alone for 24 h (lane 2) or stimulated with IFN-γ (250 U/ml) for 60 min at 37°C (lane 3). Another set of cells was treated with vesicles for 24 h prior to IFN-γ stimulation (lane 4). Cell lysates were prepared and immunoprecipitated with an antibody specific for Stat1α, Jak1, or Jak2. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antiphosphotyrosine MAb and anti-Stat1α, anti-Jak1, and anti-Jak2 antibodies. Data shown are from one of three experiments that yielded similar results.

**FIG. 6.** Proteolytic activity of *P. gingivalis* membrane vesicles is responsible for class II inhibition. HUVECs were stimulated with IFN-γ (250 U/ml) alone or in the presence of untreated vesicles (OMVs), vesicles that had been treated with TLCK at a final concentration of 2 mM for 1 h at 37°C, or vesicles heated for 30 min at 100°C. After a 3-day incubation, cells were stained with a phycoerythrin-conjugated anti-HLA-DR MAB or an irrelevant isotype-matched MAB. Stained cells were analyzed by flow cytometry. Averages and standard deviations were calculated from three separate experiments.

**FIG. 7.** Effects of *P. gingivalis* membrane vesicles on class I MHC expression. HUVEC cultures were treated with medium alone (control) or with IFN-γ (250 U/ml) or IFN-α (250 U/ml) alone or in the presence of vesicles (OMVs, 30 μg of protein/ml). After a 3-day incubation, cells were stained with a phycoerythrin-conjugated anti-HLA-ABC MAB or an irrelevant isotype-matched MAB. Stained cells were analyzed by flow cytometry. Representative results are presented from one of three separate experiments that yielded similar results.
The involvement of MHC class II molecules and CD4+ T cells in *P. gingivalis* infection is supported by the studies showing that the CD4+/CD8+ T-cell ratio from gingival tissues of patients with adult periodontitis was decreased compared to that of the peripheral blood or cells extracted from normal gingival tissues (5, 41). It is obvious that the decrease in the CD4+ cell population would result in a corresponding reduction in the number of activated CD4+ T cells. Such a reduction would result in decreased migration and proliferation of memory CD4+ T lymphocytes into gingival tissues. Whether the vesicle-induced inhibition of class II synthesis observed in vitro also occurs in situ remains to be determined. However, in advanced periodontitis lesions, very few macrophages were found to express detectable HLA-DR molecules (4). Similar inhibitory effects on IFN-γ-induced class II expression have also been demonstrated by others using macrophages infected with certain intracellular pathogens including *Chlamydia trachomatis* (45), *Ehrlichia chaffeensis* (18), *Listeria monocytogenes* (35), *Mycobacterium avium* (15), *Mycobacterium tuberculosis* (13), *Leishmania donovani* (26), and cytomegaloviruses (25, 30). In the case of *P. gingivalis* infection, we also found that *P. gingivalis* bacteria, in a fashion similar to that of their vesicles, significantly inhibited MHC class II antigen expression (data not shown). This finding, together with the ability of *P. gingivalis* to invade and multiply in epithelial cells and endothelial cells (7, 29, 33), suggests that both the vesicles and intact bacteria may be capable of interfering with the expression of class II MHC molecules in situ.

Since HUVECs are class II-negative cells which respond well to IFN-γ stimulation and thereby act like macrophages in antigen processing and presentation, they were used in this study to critically examine the cellular and molecular mechanisms by which *P. gingivalis* membrane vesicles modulate the synthesis and surface expression of MHC class II molecules. The impaired responses to IFN-γ in inducing MHC class II antigen expression due to the effects of vesicles on both HLA-DR and CIITA gene expression were indicated from the RT-PCR results. This suggested the possibility that earlier events involving in the intracellular signaling pathway, such as the phosphorylation of Stat1α, Jak1, and Jak2 proteins, were being affected by the vesicles. Indeed, immunoprecipitation and Western blot analyses demonstrated that vesicle-treated endothelial cells expressed Stat1α but were unable to initiate IFN-γ-induced phosphorylation of Stat1α. Surprisingly, Jak1 and Jak2 proteins were found to be absent in vesicle-treated cells, which obviously negated any possibility that these proteins as well as Stat1α would be phosphorylated. Jak1 and Stat1α proteins are also involved in the signaling pathway of IFN-α-induced class I genes, along with Tyk2 and Stat2 proteins. Consequently, it was assumed that the absence of Jak1 in vesicle-treated cells would also affect the biosynthesis and membrane expression of class I molecules induced by IFN-α. This proved to be the case in that the upregulation of class I antigen expression by IFN-γ as well as IFN-α was clearly inhibited by the vesicles. Thus, the inhibitory effects of vesicles on IFN-γ were not limited to just class II molecules. An obvious mechanism by which *P. gingivalis* membrane vesicles could inhibit the IFN-γ signaling pathway is the proteolytic degradation of Jak proteins. As previously noted, *P. gingivalis* membrane vesicles possess strong proteolytic activity (12). The major proteinases involved are cysteine proteinases, Arg-gingipain (Rgp) and Lys-gingipain (Kgp), which hydrolyze peptide bonds after arginyl or lysyl residues, respectively (10). The fact that class II-inhibitory effects could be eliminated by heating the vesicles at 100°C for 30 min or by pretreating them with a cysteine proteinase inhibitor, TLCK, strongly suggests that these enzymes were responsible for the degradation of the Jak proteins. Since the antibodies used for the immunoprecipitation
tation experiments were directed against the carboxy-terminal sequences of Jak1 and Jak2 proteins, the absence of Jak proteins was most likely due to cleavage by the vesicle proteinases within the carboxy-terminal domain of the Jaks. The 21-amino-acid peptide from human Jak1 used as an immunogen contains five potential cleavage sites. These include three sites for Rgp (R792, R797, and R799) and two sites for Kgp (K785 and K789). The Jak2 immunogen, which was a 19-amino-acid peptide, contains four cleavage sites (R761, R769, K762, and K776). Other domains of both Jak molecules containing these residues may also have been degraded by the vesicle proteinases. In the case of Stat1α, the absence of arginyl residues and the presence of only one lysyl residue (K742) in the carboxy-terminal sequence could account for the lack of Stat1α sensitivity to the vesicle proteinases. In addition, there is the possibility that the folding and location of Stat1α in the cytoplasm may make it inaccessible for protease interactions.

The degradation of the Jak proteins most likely occurs following the uptake, accumulation, and subsequent release of bacterial vesicles into the endothelial cell cytosol. This possibility is supported by our electron microscopic studies demonstrating that vesicles were readily taken up by endothelial cells, which were initially sequestered in endosomes (unpublished observation). Some of these endosomes had ruptured, releasing the bacterial vesicles into the cell cytosol. It is also possible that these vesicles are capable of fusing with the host cell membrane (17). This would result in the introduction of the contents of the bacterial vesicles directly into the cytosol of the cells, in which case interactions of vesicle proteases with Jak proteins could occur. It should be noted that Jak1 proteins have been found elsewhere to be degraded in fibroblasts and endothelial cells infected with human cytomegalovirus (25). A proteasome-dependent mechanism was believed to be responsible for the degradation observed. Recently, C. trachomatis has been reported to suppress IFN-γ-induced MHC class II synthesis by degrading upstream stimulatory factor 1, a transcription factor required for class II synthesis (45). Inhibition of class II antigen expression may occur by other mechanisms. For example, previous studies in this laboratory have demonstrated that dextran sulfate inhibited class II antigen expression by preventing the phosphorylation of Jak1 and Stat1α without protein degradation (20).

The Jak-Stat signaling pathway can also be disrupted by simply preventing the binding of IFN-γ to its receptor (9, 20, 44). Results obtained from the IFN-γ binding studies suggested that vesicles could affect ligand-receptor interactions. For example, pretreatment of HUVECs with vesicles caused a significant increase in IFN-γ binding to cells in a time-dependent manner. It is highly unlikely that this increase was due to an enhancement of IFN-γ receptor synthesis and membrane expression, because vesicles exerted no effect on IFN-γ receptors on endothelial cell surfaces as shown by flow cytometric analysis. Thus, the increased IFN-γ binding was not indicative of the functional interaction of IFN-γ with its specific receptor. A possible explanation for the increase in nonreceptor binding of IFN-γ to cell surfaces is that it was sequestered by electrostatic interactions between its basic amino acid residues (9, 21, 42) and the cell membrane-bound vesicles. Interactions between the vesicles and cell membrane might also have produced changes that allowed for IFN-γ binding to nonspecific cell surface components, such as proteoglycans. It should be noted that IFN-γ binding to the cell membrane decreased if the cytokine and vesicles were added to cell cultures at the same time. These results, together with the fact that there is a limited number of specific receptors on cell membranes, further suggest that this class II inhibition could also partially result from direct or indirect physical interactions of vesicles with IFN-γ or its receptor. The significance of these observations relates to the importance of P. gingivalis membrane vesicles in modulating a key component of the immune system.

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