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Virulent *Treponema pallidum* 47 kDa Antigen Regulates the Expression of Cell Adhesion Molecules and Binding of T-Lymphocytes to Cultured Human Dermal Microvascular Endothelial Cells

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Abstract

Perivasculitis and endothelial cell abnormalities are prominent histopathologic features of syphilis. Various cutaneous lesions are the main clinical features of syphilis. We examined whether *Treponema pallidum* 47 kDa antigen regulates the expression of cell adhesion molecules on human dermal microvascular endothelial cells (HDMEC) and the regulation of T-lymphocytes binding to HDMEC. Using immunofluorescence flow cytometry and enzyme-linked immunosorbent assay (ELISA), we demonstrated that *T. pallidum* upregulated the expression of adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin. The 47 kDa antigen of *T. pallidum* also activated endothelium as measured by the upregulation of the expression of adhesion molecules on HDMEC, and it also promoted an increased adherence of T-lymphocytes to HDMEC. The expressions of ICAM-1 and VCAM-1 on HDMEC and the adherence of T-lymphocytes to HDMEC were inhibited by treatment with anti-TNF-α antibody or anti-IL-1α antibody. These results show that *T. pallidum* or *T. pallidum*-specific 47 kDa antigen are capable of stimulating HDMEC to increase the expression of ICAM-1, VCAM-1 and E-selectin and thereby, promote the adherence of T-lymphocytes. The whole process may play an important role in the immunopathogenesis of syphilis and it is likely that TNF-α and IL-1α are involved.

Key Words: Syphilis, *Treponema pallidum* 47 kDa antigen, cell adhesion molecules, T lymphocytes

INTRODUCTION

Syphilis is a chronic, systemic sexually-transmitted disease caused by a spirochetal bacterium *Treponema pallidum* subsp. *Pallidum*, with clinical features including various cutaneous lesions and systemic involvement.[1] Although the immunopathogenic mechanism is still uncertain, placentitis of congenital syphilis and many of the clinical complications of syphilis appear to be the consequence of vascular changes. Histopathologically, endothelial cell swelling, mural edema, perivascular and interstitial lymphohistocytic infiltrate, and thrombosis can be found.[1-3] Therefore, determining the cellular and molecular bases for vascular changes may be essential for understanding the immunopathogenesis of syphilis.

Adhesion to the endothelium and stromal infiltraton of *T. pallidum* and inflammatory cells may be the key steps initiating the vascular damage associated with syphilis.[4] Adhesion processes are required for cells to interact and to migrate to their intended destinations. Adhesion molecules allow cells to adhere to other cells or to extracellular matrix molecules. There are five structural families of adhesion molecules that have been described to date: the selectins, the mucins, the integrins, the immunoglobulin superfamily (IGSF), and the cadherins. The selectins are a group of cell-surface glycoproteins found on the endothelium, platelets, and leukocytes.[5,6] The selectins are important in several lymphocyte-endothelial interactions, including the initial intra-vascular

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arrest of circulating lymphocytes and the subsequent rolling on intravascular endothelial cells. E-selectin is expressed primarily on activated endothelial cells and transcriptionally up-regulated by multiple stimuli, including interleukin (IL)-1, tumor necrosis factor (TNF)-α, and lipopolysaccharide (LPS).[5] Several members of the IGSF are involved in T-lymphocyte/endothelial cell interactions, namely CD31 on T-lymphocytes and intercellular adhesion molecule 1 (ICAM-1, CD54), ICAM-2 (CD102), VCAM-1 (CD106), CD31, and MAdCAM-1 on endothelial cells.[5,7] ICAM-1 is expressed on endothelial cells, thymic and mucosal epithelial cells, mononuclear cells, and fibroblasts. ICAM-1 can be transcriptionally up-regulated by TNF-β, IL-1, and LPS, but has a longer time-course of expression.[5] Vascular cell adhesion molecule 1 (VCAM-1) is not expressed on normal endothelium, but can be transcriptionally up-regulated by the same inflammatory stimuli as listed above for E-selectin and ICAM-1.[8-10]

T. pallidum has been identified in a complicated array of up to 80 immunogenic proteins. Syphilitic lesions may occur due to *T. pallidum* itself, but the host immune defense against a pathogenic antigen is a more likely cause.[11-13] By analyzing antibodies to different *T. pallidum* antigens by Western blot, the 47 kDa antigen is believed to be the major immunodominant antigen of *T. pallidum*. Although the characteristics of this antigen on humoral immunity have been studied, there is little evidence for its role on cellular immunity.[14-16]

In this study, we examined whether *T. pallidum* 47 kDa antigen regulates the expression of cell adhesion molecules on human dermal microvascular endothelial cells (HDMEC) and the regulation of T-lymphocytes binding to HDMEC. We used immunofluorescence flow cytometry and enzyme-linked immunosorbent assay (ELISA), which allowed the detection of the expression of ICAM-1, VCAM-1, and E-selectin on human dermal microvascular endothelial cells (HDMEC), following their incubation with *T. pallidum* or the 47 kDa antigen of *T. pallidum*. In addition, we used an *in vitro* model of the vascular wall consisting of HDMEC monolayers to study the binding ability of T-lymphocytes to HDMEC after stimulation with the 47 kDa antigen of *T. pallidum*.

MATERIALS AND METHODS

Isolation and culture of HDMECs

HDMECs were isolated from human neonatal foreskins by trypsinization and Percoll (Sigma Chemical Co., St. Louis, MO) gradient centrifugation as described previously.[17,18] Cells were cultured in endothelial basal media (Clonetics Corps., San Diego, CA) with 5 ng/ml of epidermal growth factor (Clonetics), 1 mg/ml hydrocortisone acetate (Sigma), 5×10^{-5} M dibutyryl cyclic AMP (Sigma), 2×10^{-9} M glutamine (Sigma), 100 U/ml penicillin (Gibco Laboratories, Grand Island, NY), 100 μg/ml streptomycin (Gibco), 250 μg/ml amphotericin B (Sigma), and 30% human serum. The experiments were conducted with endothelial cells at passages 2−6.

Isolation and culture of treponemes

T. pallidum Nichols strain (CDC, Atlanta, GA) was maintained and passaged by intra-testicular inoculation of adult male New Zealand White rabbits as described earlier.[19,20] Ten days after inoculation, the rabbits were euthanized and the testes were aseptically removed. Each pair of testes was minced with sterile scissors and organisms were extracted from the tissue in 10 ml of extraction medium per testis for 10 min on a rotary shaker. Heat-inactivated normal rabbit serum (Gibco) in normal saline was used as an extraction medium. Gross debris and whole cells were removed from the extract by two centrifugations at 1,000×g for 5 min and then 8 ml of the supernatant was gently overlayed onto a 16 ml cushion of 43% Percoll (Pharmacia Biotech., Uppsala, Sweden). In situ density gradients were formed by centrifugation at 34,000×g for 20 min at 4°C. Grossly visible bands were carefully harvested with a Pasteur pipet. Percoll was removed by centrifugation at 100,000×g for 1 h at 4°C. Treponemes were counted and observed for morphologic integrity and motility by dark-field microscopy. *T. phagedenis* biotype Reiter (CDC, Atlanta, GA), *T. refringens* biotype Noguchi (ATCC, Rockville, MD) and *Treponema denticola* biotype MRB (ATCC) were used as the representative nonpathogenic treponemes. These were maintained and passaged in thioglycolate medium with 10% heat-inactivated normal rabbit serum at 37°C. In some experiments, *T. pallidum* was inac-

tivated by heating at 56°C for 30 min. The 47 kDa antigen of *T. pallidum* was a gift from Tim White (Biomira Diagnostics Inc, Ontario, Canada).

Flow cytometric analysis

HDMECs were plated in 96-well flat-bottomed microtiter plates and allowed to grow to confluence over 24 h at a concentration of 4×10^4 cells per well. HDMECs were incubated with either 100 μl of 1.5×10^6/ml *T. pallidum* or 100 ng/ml 47 kDa antigen of *T. pallidum* for 16 h at 37°C, at an atmosphere of 4.5% O_2 and 5% CO_2 condition, and were removed by incubation with 1% bovine serum albumin (Gibco) and 5 mM EDTA. HDMECs were then washed with Hank's balanced salt solution (HBSS, Gibco), counted and aliquotted for immunofluorescence staining. HDMECs were then incubated with a total of 10 μl of either anti-ICAM-1 antibody (84H10, Immunotech Inc., Westbrook, ME), anti-VCAM-1 antibody (51-10C9, Pharmingen, San Diego, CA), or anti-E-selectin antibody (1.2B6, Immunotech) at concentrations ranging from 1−10 μg/ml for 30 min on ice, washed and then incubated with 100 μl of FITC-conjugated goat anti-mouse IgG (Sigma), diluted 1 : 20 for 30 min. After another 30 min incubation, cells were washed and resuspended in phosphate buffered saline (PBS, pH 7.2) with 0.5% BSA. A total of 10 ul of 50 μg/ml of propidium iodide was added immediately before flow cytometric analysis to sort dead cells. Fluorescence was measured with a fluorescence activating cell sorter (FACStar, Becton- Dickinson, Lincoln, NJ).

ELISA

HDMECs were plated in 96-well flat-bottomed microtiter plates and allowed to grow to confluence over 24 h at a concentration of 4×10^4 cells per well. They were then incubated with either TNF-α (Amgen Biologicals, Thousand Oaks, CA), spirochetal preparations including *T. pallidum*, *T. phagedenis*, *T. refrigens*, *T. denticola* and heat-inactivated *T. pallidum* or the 47 kDa antigen of *T. pallidum* at 37°C, at an atmosphere of 4.5% O_2 and 5% CO_2. Following co-incubation, the HDMECs were washed three times with HBSS and incubated with a total of 100 μl of either anti-ICAM-1 antibody, anti-VCAM-1 antibody, or anti-E-selectin antibody at concentrations ranging from 1−10 μg/ml for 1 h at 37°C. Cells were then washed and incubated with 1 : 1,000 diluted (diluent, 5% fetal bovine serum) peroxidase-conjugated goat anti-mouse IgG for 1 h at 37°C. After washing, the expression of each adhesion was quantitated colorimetrically by the addition of 100 μl of tetramethylbenzidene (Sigma). One ml of 100 mg/ml stock solution of tetramethylbenzidene in acetone was added to 100 ml of distilled water and 10 μl of 30% H_2O_2 was added immediately prior to use. The chromogenic reaction was stopped with 25 μl of 8 N H_2SO_4 and read spectrophotometrically at 450 nm with an ELISA reader. In some experiments HDMECs were pre-incubated with anti-TNF-α antibody (R&D system, Minneapolis, MN), anti-IL-1α antibody (R&D system) or both for 1 h at 37°C, to test for their ability to inhibit the expression of adhesion molecules on HDMECs. HDMECs were then incubated with the 47 kDa antigen of *T. pallidum* and the surface expression of ICAM-1, VCAM-1, and E-selectin were quantitated as described above.

Separation of peripheral blood mononuclear cells and T-lymphocytes

Peripheral blood monocytes from healthy normal donors were separated using Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation.[21] Adherent mononuclear cells were removed by allowing them to stick to plastic at 37°C for 1 h. To eliminate cells other than T-lymphocytes, complement-mediated cytotoxicity was performed with a total of 20 μl each of low-tox rabbit complement (Cedarlane Lab., Westburg, NY) and monoclonal antibodies CD19 (Leu 12, Becton-Dickinson, San Jose, CA), CD11c (Leu-M5, Becton-Dickinson), anti-Mo-1 (Mac-1, Coulter Immunology, Hialeah, FL), and anti-HLA-DR (Becton-Dickinson) at 10 μg/ml of concentrations. The cocktail of monoclonal antibodies was added to the cells and incubated at room temperature for 30 min. After the addition of T-lymphocyte medium, consisting of RPMI 1640 (Gibco), 2 mM L-glutamine, 10% fetal bovine serum (Gibco), 50 μM 2-mercaptoethanol (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin, they were then pelleted. The pellets were re-suspended in the complete T-lymphocyte media.

T-lymphocyte-HDMEC adherence assay

HDMECs were plated in 96-well flat-bottomed microtiter plates and allowed to grow to confluence over 24 h at a concentration of 4×10^4 cells per well. They were then preincubated with either culture media alone or the 47 kDa antigen of *T. pallidum* at 37°C. T-lymphocytes were labeled with ^{51}Cr (535 mCi/mg, Dupont, Boston, MA) by incubating 100 mCi per 10^6 cells for 2 h at 37°C. After washing, 100 μl of cell suspension was added to each well and incubated for a further 4 h at 37°C. After washing, 100 μl of 1% Triton-X (Sigma) was added to each well, the contents of each well were then harvested and counted in a gamma counter. In some experiments, HDMEC monolayers were preincubated with either anti-TNF-α antibody, anti-IL-1α antibody, or monoclonal antibodies against adhesion molecules, including anti-ICAM-1 antibody, anti-VCAM-1 antibody, anti-E-selectin antibody, and anti-major histocompatibility complex (MHC) class I antibody (W6/32, Becton-Dickinson, Mountain View, CA) for 1 h at 37°C. After pre-incubation with antibodies, the adherence assay was performed as described above in the continuous presence of antibodies. The percentage of bound T-lymphocytes was calculated as follows:- Percent of T-lymphocytes binding = (adherent counts − background counts) / (counts added per well − background counts) × 100

Adherent counts are remaining cpm after washing and adding Triton-X, background counts are cpm in well without any treatment, and counts added are total cpm of ^{51}Cr -labelled T-lymphocytes added per well.

Statistical analysis

Mean values and standard deviations were calculated in all experiments. Data was analyzed using Repeated Measures Analysis of Variance and the Wilcoxon signed rank test. P values less than 0.05 were considered significant.

RESULTS

Expression of adhesion molecules on HDMECs after incubation with *T. pallidum*

Flow cytometry was employed to analyze the expression of ICAM-1, VCAM-1, and E-selectin on HDMECs in response to *T. pallidum.* Unstimulated control cultures of HDMEC displayed low levels of constitutive ICAM-1 expression, but they did not express VCAM-1 or E-selectin. HDMECs incubated with *T. pallidum* increased their expression of ICAM-1 and induced the expression of VCAM-1 and E-selectin on their surfaces (Fig. 1). ELISA showed that the expression of ICAM-1, VCAM-1 and E-selectin was upregulated or induced on HDMECs stimulated with *T. pallidum.* These expressions were increased in a dose-dependent manner by increasing the cell num-

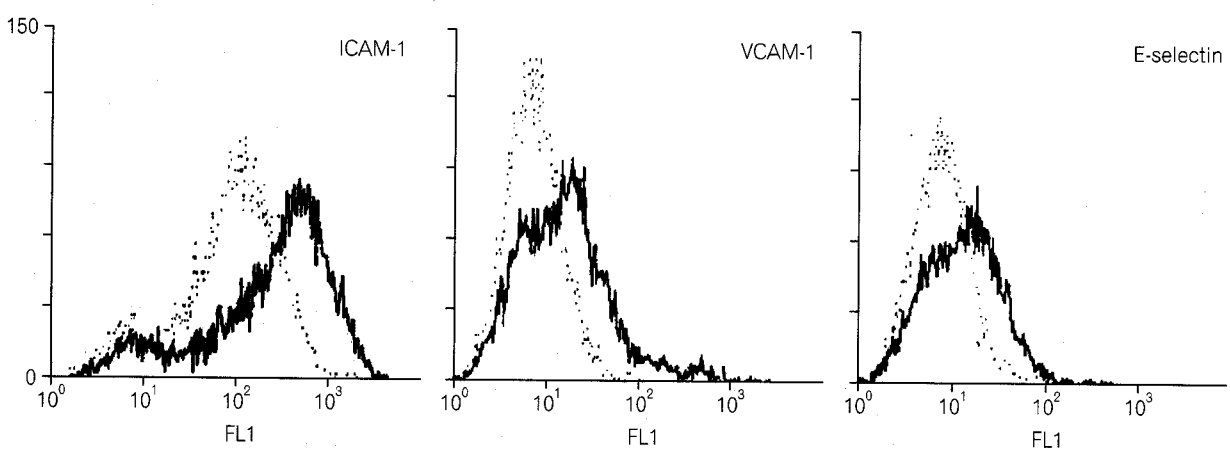

Fig. 1. Flow cytometric analysis of the expression of ICAM-1, VCAM-1, and E-selectin on HDMECs incubated with T. pallidum. HDMECs were incubated with 1.5×10^6/ml T. pallidum for 16 h and the surface expressions of ICAM-1, VCAM-1 and E-selectin were measured by flow cytometric analysis using monoclonal antibody to each molecule. ·····: unstimulated HDMEC, —: T. pallidum-stimulated HDMEC.

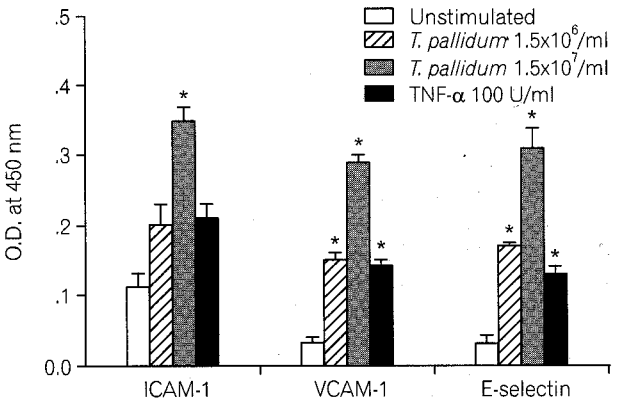

*Fig. 2. Dose dependent expression of ICAM-1, VCAM-1, and E-selectin on HDMECs in response to T. pallidum. Confluent HDMEC monolayers in 96-well tissue culture plates were incubated with T. pallidum and the expressions of ICAM-1, VCAM-1 and E-selectin were measured by ELISA using monoclonal antibody to each molecule. Bars represent the mean ±SD of three replicate samples per experimental group. *p <0.05, compared to an unstimulated control.*

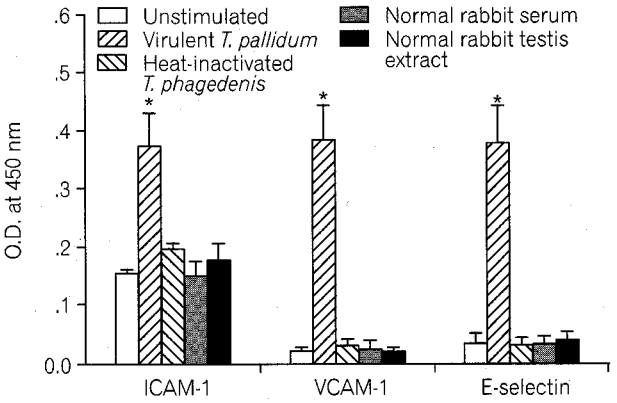

*Fig. 4. Expression of ICAM-1, VCAM-1, and E-selectin on HDMECs incubated with heat-inactivated T. pallidum. Confluent HDMEC monolayers in 96-well tissue culture plates were incubated for 16 h with T. pallidum heat-inactivated at 56°C for 30 min and the expressions of cell surface adhesion molecules were measured by ELISA using monoclonal antibody to each molecule. Bars represent the mean ±SD of three replicate samples per experimental group. *p <0.05, compared to an unstimulated control.*

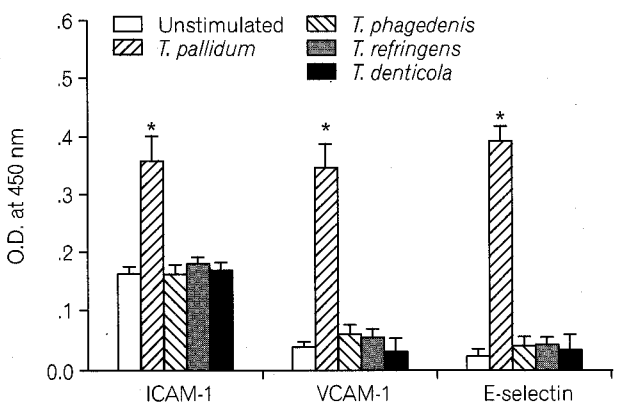

*Fig. 3. Expressions of ICAM-1, VCAM-1, and E-selectin on HDMECs incubated with non-pathogenic treponemes. Confluent HDMEC monolayers in 96-well tissue culture plates were incubated with T. phagedenis, T. refringens or T. denticola for 16 h and the expressions of cell surface adhesion molecules were measured by ELISA using monoclonal antibody to each molecule. Bars represent the mean ±SD of three replicate samples per experimental group. *p <0.05, compared to an unstimulated control.*

bers of *T. pallidum*. These expression were also up-regulated or induced after stimulation with TNF-α, but the expressions after stimulation with TNF-α were lower than the expressions after stimulation with 1.5×10^7/ml of *T. pallidum* (Fig. 2). Non-pathogenic treponemes including *T. phagedenis, T. refringens* and *T. denticola* did not induce significant changes in the expression of ICAM-1, VCAM-1, or E-selectin on HDMECs (Fig. 3). Heat-inactivated *T. pallidum*, normal rabbit serum or normal rabbit testis extract also did not promote a significant expression change of ICAM-1, VCAM-1, and E-selectin on HDMECs (Fig. 4). These results demonstrated that *T. pallidum* induced the expression of ICAM-1, VCAM-1, or E-selectin on HDMEC and pathogenecity and viability are important for the activation of HDMEC by *T. pallidum*.

Expression of adhesion molecules on HDMECs after incubation with the 47 kDa antigen of *T. pallidum*

ELISA was used to analyze the expression of the ICAM-1, VCAM-1, and E-selectin on HDMECs in response to the 47 kDa antigen of *T. pallidum*. The expression of ICAM-1 on HDMECs was increased significantly after incubation with 50 ng/ml of the 47 kDa antigen of *T. pallidum*. Expressions of VCAM-1 and E-selectin were also induced by 10 ng/ml of the 47 kDa antigen of *T. pallidum* ($p < 0.05$). The up-regulation of expression was increased in a dose-dependent manner by increasing the concentration of the 47 kDa antigen of *T. pallidum* (Fig. 5). Time course experiments identified the duration of incubation required to achieve maximal expression of

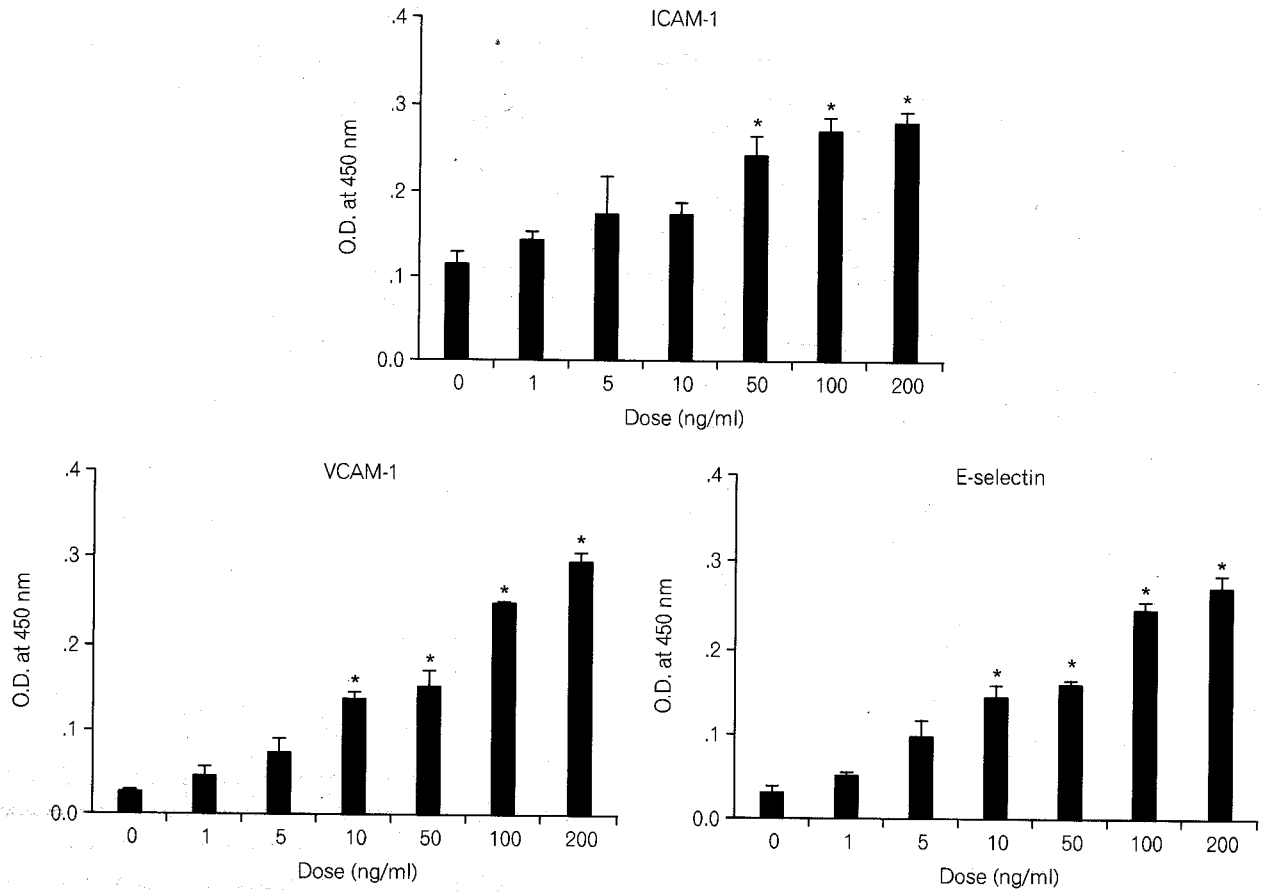

*Fig. 5. Dose response of the 47 kDa antigen of T. pallidum-induced increases of the expression of ICAM-1, VCAM-1, and E-selectin expression on HDMECs. Confluent HDMEC monolayers were incubated with various doses of the 47 kDa antigen of T. pallidum from 1 −200 ng/ml. Expressions of ICAM-1 and VCAM-1 were measured by ELISA after 16 h incubation with monoclonal antibody to each molecule, respectively. Levels of E-selectin were assessed after 4 h of incubation with monoclonal antibody to E-selectin. Bars represent the mean ±SD of three replicate samples per experimental group. *$p < 0.05$, compared to an unstimulated control.*

adhesion molecules on HDMECs. The expression of E-selectin was significantly induced at 1 h, peaked at 4 h and returned to near-basal amounts at 48 h ($p < 0.05$). In contrast, the expression levels of ICAM-1 and VCAM-1 were significantly increased after 4 h, peaked at 24 h and remained elevated at 48 h ($p < 0.05$, Fig. 6).

To assess whether TNF-α and IL-1α played a role in the regulation of the expression of adhesion molecules on HDMECs stimulated with the 47 kDa antigen of *T. pallidum*, HDMECs were pretreated with anti-TNF-α antibody or anti-IL-1α antibody. Pretreatment of anti-TNF-α antibody significantly inhibited the upregulation of the expression of ICAM-1 and VCAM-1 on HDMECs stimulated with the 47 kDa antigen of *T. pallidum* ($p < 0.05$). Pretreatment of IL-1α antibody significantly inhibited the upregulation of the expression of ICAM-1 on HDMECs ($p < 0.05$), and slightly inhibited the expression of VCAM-1, but there was no statistical significance (Fig. 7). Pretreatment of anti-TNF-α antibody or anti-IL-1α antibody inhibited partially the upregulation of the expression of E-selectin, but there was no statistical significance. The blocking assay with monoclonal antibodies showed that HDMEC activated by the 47 kDa antigen of *T. pallidum* secrete TNF-α or IL-1α and this may lead to an increase of the expression of cell adhesion molecules on HDMEC.

Effect of the 47 kDa antigen of *T. pallidum* on T-lymphocyte-HDMEC adherence

In order to determine whether the expression of

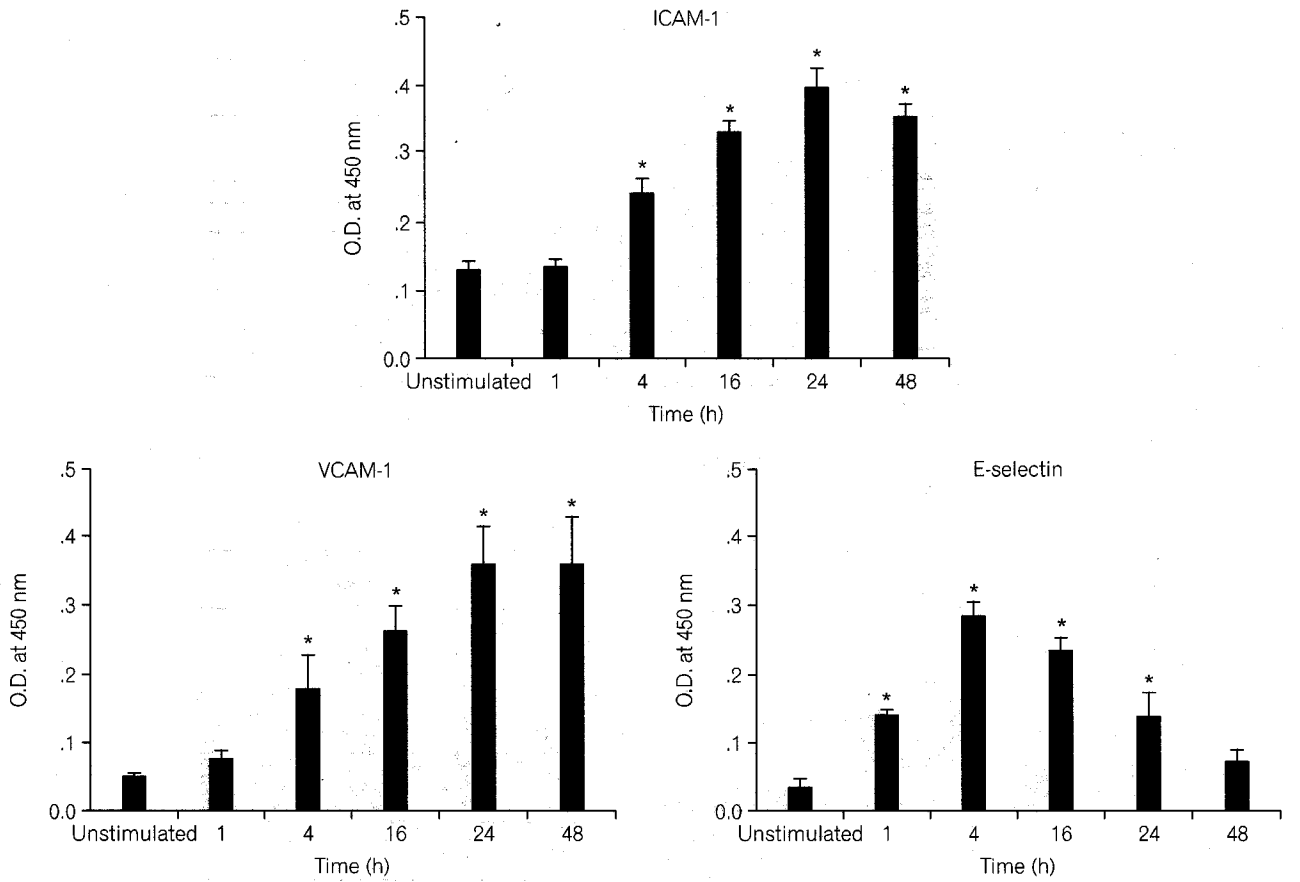

*Fig. 6. Time course of the 47 kDa antigen of T. pallidum-induced increases of the expression of ICAM-1, VCAM-1, and E-selectin on HDMECs. Confluent HDMEC monolayers were incubated with 100ng/ml of the 47 kDa antigen of T. pallidum for various time intervals. Expressions of ICAM-1, VCAM-1 and E-selectin were measured by ELISA with monoclonal antibody to each molecule. Bars represent the mean ±SD of three replicate samples per experimental group. *$p < 0.05$, compared to an unstimulated control.*

adhesion molecules on HDMECs affects T-lymphocyte binding to endothelial cells, we examined the binding of T-lymphocytes to HDMECs after incubation with the 47 kDa antigen of *T. pallidum*. Binding of T-lymphocytes to HDMECs significantly increased from a baseline of 18.2±0.8% to 33.9±1.6% after 16 h of treatment with 100 ng/ml of 47 kDa antigen ($p < 0.05$). In order to elucidate which pathways are functional in the case of T-lymphocyte-HDMEC adhesion, blocking assay was done by use of the monoclonal antibodies against ICAM-1, VCAM-1, E-selectin, class I MHC molecules and cocktail of each antibody. The T-lymphocyte binding to HDMECs stimulated with the 47 kDa antigen for 16 h was inhibited significantly by antibodies which recognized E-selectin, ICAM-1, and VCAM-1, but not by anti-class I MHC molecule antibody (Fig. 8). To assess whether cytokines played a role in the adhesion of T-lymphocytes to HDMECs stimulated with 47 kDa antigen of *T. pallidum*, HDMECs were pretreated with anti-TNF-α antibody or anti-IL-1α antibody. Pretreatment with anti-TNF-α antibody and anti-IL-1α antibody significantly inhibited any increase in T-lymphocyte-HDMEC binding (Fig. 9). This data suggested that the 47 kDa antigen of *T. pallidum* can promote the adhesion of T-lymphocytes to HDMEC probably through the regulation of cell adhesion molecules on HDMEC by TNF-α or IL-1α.

DISCUSSION

The role of cellular and humoral immunity in the pathogenesis and protection against *T. pallidum* in-

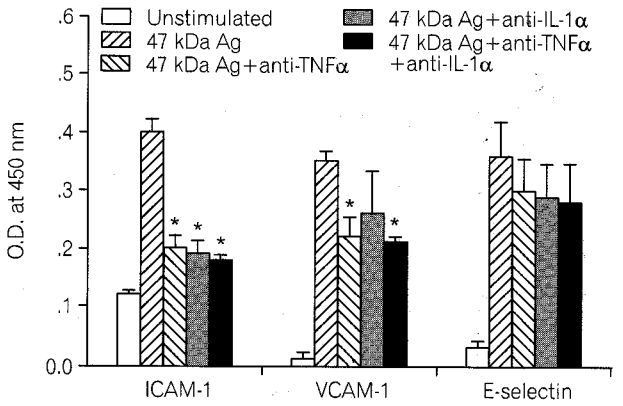

*Fig. 7. Effect of anti-TNF- α antibody and anti-IL-1 α antibody on the 47 kDa antigen of T. pallidum-induced expression of adhesion molecule on HDMECs. Confluent HDMEC monolayers were preincubated with anti-TNF- α antibody or anti-IL-1 α antibody for 1 h and HDMECs were then incubated with the 47 kDa antigen of T. pallidum. Expressions of ICAM-1 and VCAM-1 were measured by ELISA after 16 h incubation with monoclonal antibody to each molecule. Levels of E-selectin were assessed after 4 h incubation with monoclonal antibody to E-selectin. Bars represent the mean ±SD of three replicate samples per experimental group. *p < 0.05, compared to an unstimulated control.*

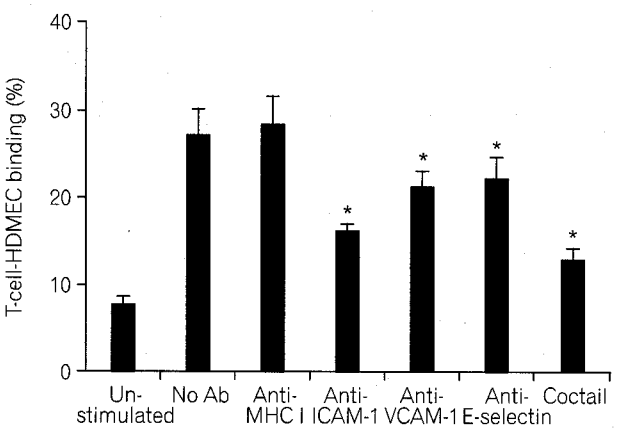

*Fig. 8. Effect of anti-ICAM-1, anti-VCAM-1 and anti-E-selectin MAbs on the adherence of T lymphocytes to HDMECs stimulated with 47 kDa antigen of T. pallidum. Confluent HDMEC monolayers were incubated with the 47 kDa antigen of T. pallidum for 16 h. HDMECs were then incubated with anti-ICAM-1, anti-VCAM-1 or anti-E-selectin MAbs for 1 h. T-lymphocytes labeled with ^{51}Cr were then added and incubated for 1 h in the continuous presence of antibody. Binding of T-lymphocyte to HDMECs was measured using a gamma counter. The percentage of bound T-lymphocytes was calculated as percent of T-lymphocytes binding=(adherent counts-background counts)/(counts added per well-background counts) ×100. Bars represent the mean ±SD of three replicate samples per experimental group. *p <0.05, compared to an unstimulated control.*

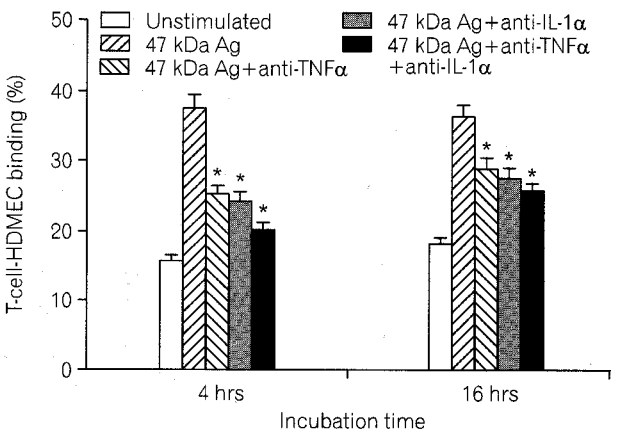

*Fig. 9. Effect of anti-TNF- α antibody and anti-IL-1 α antibody on the adherence of T-lymphocytes to HDMECs stimulated with 47 kDa antigen of T. pallidum. Confluent HDMEC monolayers were incubated with the 47kDa antigen of T. pallidum and either anti-TNF- α antibody or anti-IL-1 α antibody simultaneously for 16 h. Subsequently-added T-lymphocytes labeled with ^{51}Cr were incubated for 1 h in the continuous presence of antibody. Binding of T-lymphocytes to HDMECs was measured using a gamma counter. The percentage of bound T-lymphocytes was calculated as percent of T-lymphocytes bindings=(adherent counts-background counts)/(counts added per well-background counts) ×100. Bars represent the mean ± SD of three replicate samples per experimental group. *p <0.05, compared to an unstimulated control.*

fection has been the subject of study and debate for some time. It has been known from the serological complexity of the humoral immune response during the syphilitic process, that sera from infected rabbits or humans consist of a very large and complex array of both specific and cross-reacting antibodies directed against a multitude of treponemal determinants.[12,14,22,23] However, there are new convincing evidences that cellular immunity is the major immune mechanism in the destruction of infecting organisms. In both testicular and cutaneous lesions induced by inoculation of *T. pallidum* into rabbits, early stage features showed increased T-lymphocyte infiltration and increased number of structurally-intact *T. pallidum* organisms in the lesions, whereas macrophage infiltration occurs during the recovery stage, to eliminate the virulent *T. pallidum* organism.[24-26] T-lymphocytes reactive to *T. pallidum* antigens appear in the draining lymph nodes and spleen within a few days of primary infection and a marked paracortical hyperplasia occurs in the lymph nodes during the immune response.[24,26,27] After inoculation of *T. pallidum* into the skin of rabbits, infecting organisms transiently avoid

cellular immunity by migration into hair follicles, erector pili muscles and the cutaneous nerve, indicating that antibodies have little effect on *T. pallidum* in tissues.[25,28] Thus, it might be considered that effective immunity to syphilis is mediated by T-lymphocytes which secrete lymphokines that in turn activate macrophages to destroy infecting organisms.

Previous studies have demonstrated that *T. pallidum* and *Borrelia burgdorferi* activated cultured human umbilical vein endothelial cells (HUVEC) to upregulate the expression of adhesion molecules in a dose and time-dependent manner; ICAM-1 by *T. pallidum*, ICAM-1, VCAM-1, and E-selectin by *B. burgdorferi*.[29,30] These findings raise the possibility that *T. pallidum* and *B. burgdorferi* induce a host inflammatory response.

The microvascular endothelial cells are phenotypically and functionally distinct from large vessel endothelial cells.[31,32] In this study, we used cultured HDMECs isolated from human neonatal foreskins to examine whether the endothelial cells originated from skin affect by *T. pallidum* 47 kDa antigen in inflammatory process of syphilis. Because *T. pallidum* invades the mucosal surfaces and abraded skin and because the main clinical manifestations of syphilis are mucocutaneous lesions. In this study, *T. pallidum* induced an upregulation in the expression of ICAM-1, VCAM-1, and E-selectin on the HDMEC's surface and that these changes in adhesion molecules are thought to promote the adhesion of T-lymphocytes to endothelial cells. However, non-pathogenic treponemes or heat-inactivated *T. pallidum* did not promote a significant increase in the expression of adhesion molecules on HDMECs, which indicates that pathogenicity and viability are important for the activation of HDMECs by *T. pallidum*.

T. pallidum does not contain LPS, a component of gram-negative bacterial outer membranes, which can induce the expression of a number of endothelial cell surface proteins or immune effector molecules.[13,33] However, it has recently been found that the major membrane immunogens of *T. pallidum* are lipoproteins.[34,35] Studies on the prototype prokaryotic lipoprotein, the murine lipoprotein of *Escherichia coli*, have shown that this molecule is a potent B-cell mitogen and induces IL-1, IL-6 and TNF production by macrophages.[36-38] The treponemal lipoproteins activate macrophages as determined by TNF biosynthesis and the expression of class II MHC molecules.[29,39,40] Acylation is essential for macrophage activation by spirochectal lipoproteins because non-acylated forms of these lipoproteins did not induce cell activation.[39] Recent reports have demonstrated that the 47 kDa integral membrane lipoprotein purified from *T. pallidum* increases the expression of ICAM-1 on the HUVEC surface.[29] Our results showed that the 47 kDa antigen of *T. pallidum* not only promoted a similar increased expression of ICAM-1 on the HDMEC surface but also induced the expression of VCAM-1 and E-selectin, whereas heat-inactivated *T. pallidum* did not induce the expression of adhesion molecules. It has been supposed that heat-inactivated *T. pallidum* loses its ability to acylate membrane-bound lipoproteins,[40] therefore heat-inactivated *T. pallidum* did not activate HDMEC.

The expression of ICAM-1 and VCAM-1 on HDMECs can be increased by biological response modifiers (BRM) such as IL-1 α, TNF-α or IFN-γ, and the onset of increase begins 4 to 6 h after stimulation, and reaches to the maximal level at 16 to 24 h which may persist for up to 48 h.[41,42] The expression of E-selectin on HDMECs can also be induced by IL-1 α and TNF-α. The expression of E-selectin is rapid and a transient peak at 4 to 8 h after BRM stimulation and disappears within 48 h.[18,31] In this study, the time course of adhesion molecule expression on HDMECs stimulated by the 47 kDa antigen of *T. pallidum* correlates well with the upregulation of adhesion molecules' expression by IL-1 α or TNF-α. Evidence for the involvement of BRM in the upregulation of the expression of adhesion molecules on HDMECs by the 47 kDa antigen of *T. pallidum* is provided by the results of our blocking assay with monoclonal antibodies. Pretreatment of HDMECs with anti-TNF-α or anti-IL-1 α antibody inhibited the upregulation of ICAM-1 expression. Anti-TNF-α antibody significantly inhibited the upregulation of VCAM-1 expression on HDMECs, and anti-IL-1 α antibody partially inhibited the upregulation of VCAM-1 expression, but this was without significance. These results suggest that HDMECs activated by the 47 kDa antigen of *T. pallidum* secrete TNF-α or IL-1 α and this may lead to an increased expression of ICAM-1 and VCAM-1 on the HDMECs' surface. However, inhibition of the upregulation of E-selectin expression on HDMECs was not observed in response to anti-TNF-α antibody or anti-IL-1 α

antibody.

The adhesion of T-lymphocytes to endothelial cells is increased by IL-1 α, TNF- α or LPS, and the onset of increase begins 4 h after cytokine stimulation, reaching a maximal level at 24 h which may persist for up to 72 h.[43] Virulent *T. pallidum* promotes increased adhesion of T-lymphocytes and monocytes to HUVECs and *T. pallidum* and *B. burgdorferi* lipoproteins activate monocytes.[40,44] In this study, an increase in the adhesion of T-lymphocytes to HDMECs was observed after treating HDMECs with the 47 kDa antigen of *T. pallidum*. This finding suggests that the 47kDa antigen of *T. pallidum* can stimulate endothelial cells to bind with T-lymphocytes and provides an important insight into the immunopathogenetic mechanism of syphilis. Evidence that adhesion molecules are involved in the regulation of T-lymphocyte-HDMEC binding is provided by the results of our blocking assay with monoclonal antibodies. Anti-ICAM-1 antibody, anti-VCAM-1 antibody and anti-E-selectin antibody inhibited the adhesion of T-lymphocytes to HDMECs. A similar pattern of inhibition was seen when anti-TNF- α antibody and anti-IL-1 α antibody were used to suppress the adhesion of T-lymphocytes to HDMECs.

Our results indicate that *T. pallidum* is capable of stimulating HDMECs to upregulate the expression of ICAM-1, VCAM-1 and E-selectin. The 47 kDa antigen of *T. pallidum* can also stimulate the expression of adhesion molecules on HDMECs, and thereby, promote the adhesion of T-lymphocytes. This process may play an important role in the immunopathogenesis of syphilis and it is likely that TNF- α and IL-1 α are involved.

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