VCAM-1 Influences Lymphocyte Proliferation and Cytokine Production during Mixed Lymphocyte Responses

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The allogeneic mixed lymphocyte reaction (MLR) is regarded as an effective model for examining the events which occur during an allospecific immune response. Numerous studies have delineated the role of adherence molecule interactions during the MLR response. In the present study we have identified VCAM-1 as having a contribution to the generation of an allogeneic MLR response. These findings may have broad implications in vivo during antigen-specific and allograft rejection events. RT-PCR analysis was initially used to examine whether VCAM-1 mRNA expression was observed during MLR responses and demonstrated peak expression between 12 and 48 hr of culture. Immunolocalization of VCAM-1 on adherent mononuclear phagocytes, but not non-adherent lymphocytes, from MLR cultures verified its expression during this response. Addition of anti-VCAM-1 mAbs to MLR assays inhibited the proliferative response by over 70%, while addition of anti-VCAM-1 as late as Day 2 of the assay allowed significant inhibition of the proliferative response. This correlated with peak expression of VCAM-1 mRNA observed as late as 48 hr in RT-PCR analyses. In further studies, anti-VCAM-1 significantly inhibited peak expression of IL-2 on Days 3 and 4, while TNF-α production was diminished at 30 min and 1, 96, and 120 hr of culture, compared to control cultures. The production of macrophage inflammatory protein-1α (MIP-1α), a chemotactic cytokine which has an important role in vivo for the recruitment of leukocytes to a site of inflammation, was also significantly inhibited during peak production on Days 4 and 5 of the MLR assay. This study demonstrates novel findings of VCAM-1 expression during an allogeneic MLR response. The expression of VCAM-1 may have important implications during allospecific immune responses for the activation and proliferation of T lymphocytes as well as cytokine production.

INTRODUCTION

The initiation of antigen-dependent responses begin with cell-to-cell adherence and subsequent activation via specific cell surface receptors. In the mixed lymphocyte reaction (MLR) cells recognize and respond to non-self-MHC molecules and is considered to be representative of responses which take place in vivo during allograft rejection (1, 2). The cascade of in vivo events which culminate in the injury of foreign,

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non-self-expressing cells consists of several steps. One of the most critical steps is the initial cell-to-cell interaction resulting in recognition and activation of alloreactive T lymphocytes. Subsequent events consist of the expression of early response cytokines (IL-1 and TNF), accompanied by IL-2 generation (3, 4), and finally in the generation of chemotactic cytokines which are important for the recruitment of leukocytes to the inflammatory site.

Several studies have elucidated multiple cell surface adhesion molecules and their counterreceptors which are involved in cellular adherence and lymphocyte activation in the MLR response (5–8). These molecules include lymphocyte functional antigen-3 (LFA-3 or CD58):CD2 (8, 9), intercellular adhesion molecule-1 (ICAM-1 or CD54): lymphocyte functional antigen-1 (LFA-1 or CD11a/CD18) (5), and B7/BB1:CD28 interactions (6). An additional adhesion molecule, termed vascular cell adhesion molecule-1 (VCAM-1), was first discovered on the surface of endothelial cells (10). Recent studies have demonstrated that the costimulation of T lymphocytes via VCAM-1 and CD3 pathways can provide the necessary signals for proliferation and cytokine production (11, 12). The expression of VCAM-1 by endothelial cells appears to be one of the major mechanisms involved in the recruitment of specific leukocyte populations from the peripheral blood to an area of inflammation via interaction with its counterreceptor, very late antigen-4 (VLA-4) (13), found on mononuclear cells (14), basophils, and eosinophils (15). Recent evidence also has demonstrated the expression of VCAM-1 on fibroblasts (16) and neural cells (17). Therefore, VCAM-1 appears to serve dual roles as a molecule involved in both cellular extravasation and activation.

In the following study, we have demonstrated the importance of VCAM-1 expression in the induction of lymphocyte proliferation and the production of specific cytokines during MLR responses. Our findings also demonstrated that the temporal expression of VCAM-1 during allogeneic responses can play an important role in T cell proliferation, cytokine production, and therefore the induction of an allospecific immune response.

MATERIALS AND METHODS

Mononuclear cell isolation and mixed lymphocyte reaction. Peripheral blood was drawn into a heparinized syringe from healthy volunteers and diluted 1:1 in normal saline, and mononuclear cells were separated by density gradient centrifugation. The recovered cells were washed three times with RPMI 1640. Donors were classified as either responder or stimulator depending upon their responses in the MLR assay. MLR was set up in 96-well flat-bottom tissue culture plates for proliferative studies and in 6-well plates for cytokine elicitation studies. Responder cells were mixed 1:1 with irradiated (2000 rad) stimulator cells in a total volume of 200 μl for 96 wells and 1 ml for 6-well plates. RPMI 1640 supplemented with 1 mM l-Glu, 10 mM Hepes, antibiotics, and 10% fetal calf serum was used in the assay. Dilution studies of both the responder and stimulator cells determined that a 1:1 ratio provided optimal proliferative response. Cells were cultured at 10^5 cells/well for proliferative responses and 3 × 10^6 cells/well for cytokine elicitation. For determination of proliferation, cultured cells were pulse with 0.5 μCi of [3H]thymidine 12–18 hr prior to harvest on Day 6. Cells were harvested and [3H]thymidine incorporation was determined. For cytokine determination, culture supernatants were harvested from 35-mm plates, centrifuged,
and stored at −20°C until cytokine concentrations were assessed. Parallel cultures were performed for both proliferation and cytokine determination using monoclonal antibodies (mAb; 4B9) to VCAM-1 (blocking antibodies provided by Dr. Roy Lobb, Biogen Inc.) at 0.1–5 μg/ml. The optimal antibody concentration was determined to be 5 μg/ml by dilution studies for the VCAM-1 mAb by inhibitory effects in the MLR. Preliminary studies were performed to determine the best responder and stimulator donors for the subsequent MLR assays. Two different sets of donors were observed to give similar proliferative responses. These two donor pairs were used throughout the study for determination of MLR inhibition.

Total RNA isolation and reverse transcriptase (RT)-PCR amplification. Total cellular RNA from cultures was isolated by homogenation of cells in a solution containing 25 mM Tris, pH 8.0, 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, the suspension was added to a solution containing an equal volume of 100 mM Tris, pH 8.0, 10 mM EDTA, and 1.0% SDS. The mixture was then extracted two times each with phenol–chloroform and chloroform–isoamyl alcohol. The RNA was alcohol precipitated and the pellet dissolved in DEPC water. Total RNA was determined by spectrophotometric analysis at 260 nm. Five micrograms of total RNA was reverse transcribed into cDNA utilizing a BRL reverse transcription kit (BRL, Grand Island, NY) and oligo(dT)12-18 primers. The cDNA was then amplified using specific primers for cyclophilin as control (18) and human VCAM-1 (19). The primers used were 5'-CAT-CTG-CAC-TGC-CAA-GAC-TG-3' (sense) and 5'-CTG-CAA-TCC-AGC-TAG-GCA-TG-3' (anti-sense) for cyclophilin and 5'-GGG-AAG-ATG-GTC-GTG-ATC-CTT-3' (sense) and 5'-CCA-GCC-TCC-AGA-GGG-CCA-CTC-3' (anti-sense) for VCAM-1, giving amplified products of approximately 300 and 350 bp, respectively. The amplification buffer contained 50 mM KCl, 10 mM Tris–HCl (pH 8.3), and 2.5 mM MgCl. Specific oligonucleotide primer was added (200 ng/sample) to the buffer, along with 1 μl of the reverse-transcribed cDNA samples. The cDNA was amplified after determining the optimal number of cycles. The mixture was first incubated for 5 min at 94°C and then was cycled 30 times at 95°C for 30 sec, 55°C for 45 sec, and elongated at 72°C for 75 sec. This format allowed optimal amplification with little or no nonspecific amplification of contaminating DNA. After amplification the sample (20 μl) was separated on a 2% agarose gel containing 0.3 μg/ml (0.003%) of ethidium bromide and bands were visualized and photographed using an uv transilluminator.

Immunohistochemical localization of surface VCAM-1. Cytospin preparations of MLR cultures were fixed with 4% paraformaldehyde and then were blocked with normal equine serum for 30 min. The cells were covered with the mouse anti-human VCAM-1 mAb (BBA5, R&D) diluted in PBS (1:1000) for 30 min at 37°C. After rinsing three times with PBS the sections were overlaid for 20 min with biotinylated equine anti-mouse IgG (Biogenex, San Ramon, CA supersensitive reagent 1:10). After rinsing three times with PBS the cell preparations were incubated for 20 min with streptavidin–peroxidase (Biogenex, 1:1000) at 37°C. The slides were rinsed with PBS and overlaid with AEC solution containing 0.3% hydrogen peroxide until color development was observed (15–30 min). Sections were rinsed and counterstained with Mayer’s hematoxylin.

IL-2 assay. Culture supernatants were assayed for IL-2 using the CTL-L2 cell line (ATCC, TIB 214) maintained in RPMI 1640 (Gibco, Gaithersburg, MD) supplemented
with 10% FCS, penicillin, and streptomycin (100 units and 100 μg/ml, respectively), 2 mM L-glutamine, 20 mM Heps buffer, and 100 units of recombinant human IL-2 (Genzyme, Cambridge, MA). The IL-2 concentration in the supernatants was determined by comparison to a standard 1/2 log dilutions of rhIL-2. The serially diluted supernatants were added to 10⁶ cells/well in 96-well plates in a total volume of 200 μl of CTLL medium without rIL-2. Detection threshold for IL-2 was above 0.1 units/ml.

Cytokine ELISAs. Extracellular immunoreactive cytokine levels were quantitated using a modification of a double ligand method as previously described (20). Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F, Denmark, Netherlands) were coated with 50 μl/well of rabbit anti-cytokine antibody (1 μg/ml in 0.6 M NaCl, 0.26 M H₃BO₄, and 0.08 N NaOH, pH 9.6) for 16 hr at 4°C and then washed with phosphate-buffered saline (PBS), pH 7.5, 0.05% Tween 20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer and diluted (1:2 and 1:10) cell-free supernatants (50 μl) in duplicate were added, followed by incubation for 1 hr at 37°C. Plates were washed four times, followed by the addition of 50 μl/well biotinylated rabbit anti-cytokine antibody (3.5 μg/ml in PBS, pH 7.5, 0.05% Tween 20, and 2% FCS), and plates were incubated for 30 min at 37°C. Plates were washed four times, streptavidin–peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) added, and the plates were incubated for 30 min at 37°C. Plates were again washed four times and chromogen substrate (Bio-Rad Laboratories, Richmond, CA) added. The plates were then incubated at room temperature to the desired extinction, and the reaction was terminated with 50 μl/well of 3 M H₂SO₄ solution. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant cytokine from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected respective cytokine concentrations above 10 pg/ml and did not cross-react with other cytokines.

RESULTS

Expression of VCAM-1 mRNA during MLR. To determine whether VCAM-1 expression was upregulated during immune responses, RT-PCR was performed on total RNA from one-way MLR cultures using VCAM-1-specific primers (Fig. 1). The temporal expression of VCAM-1 mRNA during the MLR was highly expressed between

![Fig. 1. Identification of VCAM-1 mRNA during MLR responses by RT-PCR. Total mRNA was isolated from one-way MLR cultures at specified intervals and reversed transcribed into cDNA. The cDNA was then amplified using specific primers for VCAM-1 or cyclophilin (nonspecific control). Control cultures of only responder cells consistently demonstrated little or no constitutive VCAM-1 expression after 30 amplification cycles.](image-url)
12 and 48 hr and then declined at Days 3 and 4 of culture. These results demonstrate that VCAM-1 mRNA was expressed during an allogeneic immune response.

**Immunolocalization of VCAM-1 on mononuclear phagocytes in MLR.** In order to determine the cell population(s) responsible for VCAM-1 expression, MLR cultures were set up in Lab-Tek (Nunc, Inc., Naperville, IL.) chamber slides. Because RT-PCR results demonstrated peak mRNA expression at Day 2, at the end of 2 days of culture, both adherent and nonadherent cellular populations were fixed and immunostained for VCAM-1. Immunolocalization of VCAM-1 was detected only with the adherent mononuclear phagocytes (Fig. 2) and not with nonadherent lymphocytes (data not shown), suggesting that monocytic cells were the predominant cell-type expressing VCAM-1 during the MLR response.

Neutralizing antibodies to VCAM-1 can inhibit proliferation in the MLR. Previous studies have identified that other adhesion molecules are involved in the activation of T lymphocytes in the MLR response (5, 6, 9), while the specific role of VCAM-1 during DTH responses is not known. To ascertain the role of VCAM-1 during an allogeneic response, one-way MLR assays in the presence or absence of neutralizing VCAM-1 mAb were used as a model. Treatment of MLR cultures at Day 0 with anti-VCAM-1 mAb significantly inhibited the proliferative response by 70–80% (Fig. 3). Interestingly, VCAM-1 mAb treatment could be delayed until Day 2 of the MLR culture and still cause a significant attenuation of proliferation (data not shown). Control, subclass-matched IgG1 antibody failed to alter the proliferative response of the MLR.

**Inhibition of cytokines by anti-VCAM-1 during MLR.** The generation of cytokines during the evolution of an immune response plays an important role in the allospecific proliferation of T lymphocytes. Previous results have indicated that costimulation via both VCAM-1 and T cell receptor (TCR) was sufficient for activation of IL-2 production and T cell proliferation (11, 12). Thus, IL-2 production was assayed from supernatants harvested daily from MLR that were cultured in the presence or absence of anti-VCAM-1 mAb. Peak IL-2 production was observed at Days 3 and 4 and ranged from 1 to 2 units/ml on Days 3 and 2 to 4 units/ml on Day 4. Treatment of MLR cultures with anti-VCAM-1 mAb (5 μg/ml) on Day 0 of cell culture resulted in 40–50% reduction in IL-2 production at Day 3 and 60–70% reduction of IL-2 at Day 4 (Fig. 4).

TNF-α has also been demonstrated to play an important role in MLR proliferation and the generation of subsequent cytotoxic T cell activity (3, 21). Supernatants were harvested daily from MLR cultures and TNF-α was assayed using a specific ELISA. Detectable levels of TNF-α were found as early as 30 min postinitiation of the MLR (0.25 ± 0.12 ng/ml). The first peak in TNF-α levels occurred at 1 hr (0.69 ± 0.4 ng/ml) prior to a decline at 24 hr. This was followed by a continual rise in TNF-α levels at 48 hr (0.08 ± 0.05 ng/ml), 72 hr (0.37 ± 0.14), and 96 hr (1.9 ± 0.39 ng/ml), which finally peaked at 120 hr (3.2 ± 0.52 ng/ml) of culture (Fig. 5). The addition of anti-VCAM-1 mAb (5 μg/ml) at Day 0 of the MLR resulted in a significant reduction in TNF-α production, which was detected at 30 min and 1, 96, and 120 hr of culture (0, 0, 0.56 ± 0.106, and 0.86 ± 0.31 ng/ml, respectively).

The production of chemokines in vivo plays an important role for the elicitation of leukocytes to an area of inflammation or allograft rejection (22). Macrophage inflammatory protein-1 (MIP-1α) is chemotactic for monocytes and lymphocytes and can induce inflammatory responses and fever when injected in vivo (24–27). The mea-
FIG. 2. Immunohistochemical localization of VCAM-1 during MLR cultures. Cells from Day 3 MLR cultures were fixed with 4% paraformaldehyde and immunoperoxidase stained using normal mouse IgG (A) or mouse anti-human VCAM-1 mAb (B, C). The figure depicts VCAM-1 localization on monocytes from 3-day MLR cultures. Nonadherent lymphocytes were fixed onto glass slides by cytospin preparation but were devoid of VCAM-1 localization (data not shown). A and B were photographed at 316× and C at 790× magnification.
measurement of MIP-1α in supernatants from one-way MLR cultures demonstrated a temporal expression of MIP-1α. Substantial levels of MIP-1α were detected as early as 30 min and 1 hr (3.4 ± 2.1 and 1.8 ± 0.68 ng/ml, respectively) of cell culture. These levels decline to near background at 24 hr. A second peak of MIP-1α was detected beginning at 72 hr (2.1 ± 0.63 ng/ml), rising at 96 hr (4.4 ± 0.65 ng/ml), and finally peaking at 120 hr (12.9 ± 3.3 ng/ml) of culture (Fig. 5). The addition of anti-VCAM-1 mAb to the MLR significantly inhibited MIP-1α production at 30 min and 1, 72, 96, and 120 hr (0.5 ± 0.17, 0.15 ± 0.07, 0.29 ± 0.14, 1.26 ± 0.76, and 4.7 ± 3.1 ng/ml, respectively) of culture (Fig. 6). Thus it appears that an intimate relationship exists in the MLR between VCAM-1 and subsequent generation of inflammatory and chemotactic cytokines. This may be important in vivo as an activation step during alloreactivity which leads to the generation of cytokines necessary for cellular elicitation that maintains the inflammatory/immune reaction. A control subclass-matched antibody failed to alter the MLR cytokine responses (data not shown).

DISCUSSION

This study demonstrates that expression of VCAM-1 plays an important role in inducing proliferation and cytokine production during MLRs. The RT-PCR amplification of VCAM-1 mRNA during the MLR demonstrated a temporal expression peaking between 12 and 48 hr and corresponded with the ability to block the MLR-induced T cell proliferation when delaying the addition of neutralizing anti-VCAM-1 mAbs for 2 days of culture. Furthermore, the immunolocalization of VCAM-1 protein on adherent monocytes and not lymphocytes during the MLR response suggests its expression is monocyte-derived. The functional importance of VCAM-1 expression was established by inhibition of the MLR proliferative response with a specific neu-
Fig. 4. Inhibition of IL-2 production by anti-VCAM-1 mAbs in MLR. Culture supernatants were harvested daily and assayed for IL-2 levels using the CTLL-2 IL-2-dependent cell line. Peak IL-2 levels were found in the Days 3 and 4 culture supernatants and ranged from 1 to 2 units/ml and 2 to 4 units/ml, respectively. Data are presented as percentage of control culture supernatants and are the means ± SEM of three separate experiments.

In addition, the mAb directed against VCAM-1 significantly suppressed inflammatory cytokine production, IL-2 and TNF-α. Both of these cytokines have important activating roles during alloantigen-specific responses (2–4). The inhibition of VCAM-1 interaction also led to the decreased production of the chemotactic cytokine MIP-1α. Generation of MIP-1α may be important in vivo for the elicitation of leukocytes to an area of inflammation (23). MIP-1α has been shown to be important in monocyte/macrophage activation, cytokine (IL-1β, IL-6, and TNF-α) production, and can cause a prostaglandin-independent fever response when injected subcutaneously (23–25). Recent studies have demonstrated that MIP-1α can induce in vitro chemotaxis of CD8+ T lymphocytes (26), while neutralization of MIP-1α in vivo can suppress inflammatory granuloma formation (27). Taken together, these findings indicate that VCAM-1 interactions during allospecific responses are early events involved in the initiation of a cascade leading to T lymphocyte proliferation, cytokine production, and possibly leukocyte recruitment.

VCAM-1 was first discovered on the surface of vascular endothelial cells (10, 14) and subsequently reported to be involved in the activation of T cells (11, 12) and localization of leukocytes to sites of inflammation (10, 14). The present study provides novel data that verify the involvement of VCAM-1 in allospecific T cell activation, leading to proliferation and cytokine production. This observation may be important during initial stimulation of T cells at a local site of inflammation, as well as within
Fig. 5. Temporal expression and inhibition of TNF-α with mAbs to VCAM-1. TNF-α levels were measured in culture supernatants at various time points during one-way MLR assays by a rabbit anti-human polyclonal sandwich ELISA. Anti-VCAM-1 mAb was added a concentration of 5 μg/ml. Data represent the means ± SEM from five experiments at each time point.

Regional lymph nodes where antigen presentation is an important event. Earlier reports suggested that macrophage-like cells in inflamed human tissue stained positive for VCAM-1 (previously termed INCAM-110) (28). In addition, these studies suggested that dendritic cells, potent antigen-presenting cells (APCs), also express VCAM-1 in vivo. Thus, VCAM-1 expression on APCs not only allows for increased cell-to-cell contact through adhesive interaction but also may have a role in costimulation of T cells probably via its interaction with VLA-4 (11, 12).

Other adhesion molecules have also been shown to be involved in MLRs. Monoclonal antibodies to intercellular adhesion molecule (ICAM)-1 and its counterreceptor, lymphocyte functional antigen-1 (LFA-1 or CD11a/CD18), have been found to block T lymphocyte proliferation (5). The CD28 molecule on the surface of T cells has also been shown to provide a costimulatory signal for the activation of T cells via interaction with its ligand B7/BB1 (6). In addition, the T cell surface molecule, CD2, can provide a costimulatory signal with T lymphocyte proliferation and cytokine production through binding to its counterreceptor, LFA-3 (9). Taken together these studies would suggest that multiple adhesion interactions between APCs and T cells are required to provide the proper signals necessary for the full activation of T lymphocytes. The expression and interaction of various adhesion molecules may provide a complex regulatory pathway for controlling the pattern, magnitude, and fidelity of the immune response.
The data in the present study support an increasingly important role for VCAM-1, not only for cell-to-cell adhesive interactions, but also as a molecule directly involved in antigen-specific activation of T lymphocytes and the subsequent initiation of cytokine production. Future studies are underway to delineate the dual role of VCAM-1 in vivo for both the elicitation and activation of leukocytes at a site of alloantigen-induced inflammation.

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