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# The Production of Chemotactic Cytokines in an Allogeneic Response

## The Role of Intercellular Adhesion Molecule-1 and Lymphocyte Function-Associated Antigen-3

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***The* in vitro *mixed lymphocyte reaction (MLR) is regarded as a model of responsiveness to allogeneic major histocompatibility complex antigens and has historically been used to elucidate the pathway of T lymphocyte proliferation. In addition, the MLR response may reflect activation pathways relevant in acute allograft rejection. In the present study, we have applied the MLR to examine the role of adhesion molecules intercellular adhesion molecule-1 and lymphocyte function-associated antigen-3 in the induction of tumor necrosis factor-α (TNF-α) as well as chemotactic cytokines, interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α). Monoclonal antibodies to the adhesion molecules (5 μg/ml) were added to one-way human MLR cultures and supernatants collected at various time points. The monoclonal antibodies to the adhesion molecules significantly suppressed the proliferative response by 50 to 80%. Cytokine production, TNF-α (3.2 ± 0.5 ng/ml), MIP-1α (12.9 ± 3.3 ng/ml), MCP-1 (18.8 ± 3.4 ng/ml), and IL-8 (57 ± 18 ng/ml) peaked on day 5 of the assay. The addition of anti-intercellular adhesion molecule-1 to the cultures suppressed TNF-α, MIP-1α, MCP-1, and IL-8 production by 68% (1.05 ± 0.29 ng/ml), 85% (2.0 ± 1.2 ng/ml), 63% (6.8 ± 2.9 ng/ml), and 47% (30.3 ± 3.7 ng/ml), respectively. Likewise, the addition of anti-lymphocyte function-associated antigen-3 monoclonal antibody suppressed the cytokines by 78% (0.71 ± 0.34 ng/ml), 66% (4.5 ± 2.2 ng/ml), 52% (8.8 ± 2.2 ng/ml), and 73% (15.7 ± 4.4 ng/ml), respectively. Immunohistochemical staining indicated that monocytes were the primary source of the chemokines IL-8, MCP-1, and MIP-1α. The addition of exogenous recombinant TNF-α (5 ng/ml) or recombinant IL-2 (5 units/ml) to the anti-intercellular adhesion molecule-1-treated cultures allowed the recovery of the proliferative response as well as restoration of IL-2, TNF-α, and IL-8, but not MCP-1 or MIP-1α, indicating that both soluble and adhesion molecule signals are required for the production of the C-C family of chemokines in allogeneic responses. Thus, the events resulting in cellular proliferation and chemokine production were dependent on adhesion molecule interactions. (Am J Pathol 1993, 143:1179–1188)***

The recognition of foreign major histocompatibility complex antigens induces the activation of T lymphocytes and the subsequent release of lymphokines and cytokines.[1,2] The *in vitro* mixed lymphocyte reaction (MLR) has been used to elucidate the pathway of cytokine activation and T lymphocyte proliferation and is regarded as a model of responsiveness to allogeneic major histocompatibility complex antigens, which simulates responses in allograft rejection. Previous studies have shown that several cytokines can influence the proliferative response in the MLR.[2–6]

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Lymphokines such as interleukin-2 (IL-2) and interferon-$\gamma$ are important in regulating proliferation of T lymphocytes and the development of cytotoxic T lymphocytes (CTLs), whereas IL-4 has been shown to inhibit the IL-2-driven effects in primary MLR.[2–4] Tumor necrosis factor-$\alpha$ (TNF-$\alpha$), a cytokine secreted by activated T lymphocyte and macrophage/monocytes, has also been shown to play an important role in both the proliferative response and development of cytotoxic T cells.[5,6] Although a MLR *in vitro* is characterized by its proliferative response to an alloantigen, analogous responses *in vivo* not only depend upon proliferation but also the recruitment of additional leukocytes to the inflamed area. In the present study, we were interested in utilizing the MLR to examine chemotactic cytokines released during an inflammatory response that are essential in the recruitment and activation of effector cells at a site of inflammation. IL-8 is a member of the C-X-C gene family of chemotactic cytokines and is a potent chemoattractant for neutrophils and T lymphocytes at nmol/L and pmol/L concentrations, respectively.[7,8] Monocyte chemoattractant protein-1 (MCP-1) a member of the C-C gene family of chemotactic cytokines, is specific for monocytes with optimal chemoattractant activity at nmol/L concentrations.[9] In addition, another member of the C-C supergene family of chemotactic cytokines, macrophage inflammatory protein-1$\alpha$ (MIP-1$\alpha$), has been shown to exert proinflammatory effects in delayed type hypersensitivity responses that correlates with TNF-$\alpha$ production.[10]

Adhesion molecules expressed on the surface of various cell types belonging to the immunoglobulin (Ig) gene superfamily are important in cell-to-cell interactions.[11] Intracellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-3 (LFA-3) are involved in cell-specific extravasation into inflamed tissue and antigen-specific activation of T cells.[11–18] ICAM-1 interacts with LFA-1 and MAC-1 antigen, whereas LFA-3 interacts with the CD2 molecule. These adhesion molecules can be present alone or in combination on antigen-presenting cells, as well as resident endothelial, epithelial, and fibroblast cells, and the binding with their respective ligand/receptor may influence the outcome of an immune response.[11]

In the present study, we have examined the function of adhesion molecules ICAM-1 and LFA-3, utilizing the MLR assay to elucidate the temporal production of TNF-$\alpha$ and the chemotactic cytokines MIP-1$\alpha$, MCP-1, and IL-8. Our results demonstrate that multiple adhesion interactions involved in antigen-specific responses play an important role in cytokine production. The response is dependent upon cell-to-cell adhesion molecule interaction that leads to the production of IL-2 and TNF-$\alpha$, followed by the production of chemotactic cytokines. The reconstitution of cytokines (TNF-$\alpha$, IL-2) in the presence of anti-ICAM-1-treated MLR cultures reestablished the production of C-X-C family member, IL-8, but not C-C family members, MCP-1 or MIP-1$\alpha$. This indicates that both soluble and adhesion molecule signals are required for the full expression of the C-C family of chemokines.

## Materials and Methods

### Monoclonal Antibodies

The following monoclonal antibodies (MAbs) were used: c78.5 anti-ICAM-1 (CD54), isotype IgG1 (generously provided by Miles Scientific, Inc., New Haven, CT); and anti-LFA-3 (CD58), isotype IgG1 (generously provided by Biogen, Boston, MA). The control antibody used for comparison was a nonblocking BBA-5 anti-ICAM-1 (CD54), isotype IgG1 (R & D Scientific, Minneapolis, MN). All antibodies were concentrated from culture supernatants, and lipopolysaccharide concentrations were below the detectable limit using Endotect (ICN, Costa Mesa, CA), with a sensitivity level between 0.06 and 0.1 ng/ml.

### Mononuclear Cell Isolation

Peripheral blood was drawn into a heparinized syringe from healthy volunteers, diluted 1:1 in normal saline, and mononuclear cells separated by density gradient centrifugation. The recovered cells were washed three times with Roswell Park Memorial Institute 1640 medium (RPMI 1640). Donors were classified as either responder or stimulator depending upon their responses in the MLR assay.

### Mixed Lymphocyte Reaction

MLR was set up in 96-well flat-bottom tissue culture plates for proliferative studies and in 6-well plates for cytokine analysis. Responder cells were mixed 1:1 with irradiated (2,000 rads) stimulator cells in a total volume of 200 µl for 96 wells and 1 ml for 6-well plates. RPMI 1640 supplemented with 1 mmol/L L-Glu, 10 mmol/L HEPES, antibiotics, and 10% fetal calf serum was used in the assay. Dilution studies of both the responder and stimulator cells determined that an 1:1 ratio provided optimal proliferative response. Cells were cultured at $10^5$ cells/well for proliferative responses and $3 \times 10^6$ cells/

well for cytokine elicitation. For determination of proliferation, cultured cells were pulsed with 0.5 μCi of [$^3$H]thymidine 12 to 18 hours before harvest on day 6. Cells were harvested and [$^3$H]thymidine incorporation 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 proliferative and cytokine determination using antibodies to the adhesion molecules at 5 μg/ml. The optimal antibody concentration was determined by dilution studies for each antibody, based on inhibition of the MLR proliferation and cytokine production. 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 levels of proliferative responses. These two donor pairs were used throughout the study for determination of MLR inhibition. In addition, as a control, donor cells stimulated with their own irradiated cells failed to mount a proliferative response or significant peak cytokine production.

## *Cytokine Enzyme-Linked Immunosorbent Assays (ELISAs)*

Extracellular immunoreactive TNF-α, MIP-1α, MCP-1, and IL-8 were quantitated using a modification of a double ligand method as previously described.[19] 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 mol/L NaCl, 0.26 mol/L $H_3B0_4$, and 0.08 N NaOH, pH 9.6) for 16 hours 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% bovine serum albumin in PBS and incubated for 90 minutes 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 hour 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% fetal calf serum), and plates incubated for 30 minutes at 37 C. Plates were washed four times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) added, and the plates incubated for 30 minutes at 37 C. Plates were again washed four times and chromogen substrate (Bio-Rad Laboratories) added. The plates were then incubated at room temperature to the desired extinction, and the reaction terminated with 50 μl/well of 3 mol/L $H_2SO_4$ 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.

## *IL-2 Assay*

Culture supernatants were assayed for IL-2 using the CTLL-2 cell line (ATCC, TIB 214) maintained in RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum, penicillin, and streptomycin (100 units and 100 μg/ml, respectively), 2 mmol/L L-glutamine, 20 mmol/L HEPES buffer, and 100 units of recombinant human IL-2 (Genzyme, Cambridge, MA). The IL-2 concentrations in the supernatants were determined by comparison to a standard log 1/2 dilutions recombinant human IL-2. The serially diluted supernatants were added to $10^4$ cells/well in 96-well plates in a total volume of 200 μl of CTLL media without recombinant IL-2. Detection limits for IL-2 within the assay were above 0.2 units/ml.

## *Immunohistochemical Localization of Chemokines*

Cytospin and adherent cell preparations of 5-day MLR cultures were fixed with 4% paraformaldehyde and then were blocked with normal goat serum for 30 minutes. Nonadherent cells were differentially stained (Diff Quik, Baxter, McGaw Park, IL) and examined morphologically. Adherent cells were almost entirely monocytic, whereas, nonadherent cells were predominantly lymphocytic. The sections were covered with the rabbit anti-human MIP-1α serum diluted in PBS (1:1,000) for 30 minutes at 37 C. After rinsing three times with PBS, the sections were overlaid for 20 minutes with biotinylated goat anti-rabbit IgG (Biogenex, San Ramon, CA, supersensitive reagent 1:10). After rinsing three times with PBS, the cell preparation was incubated for 20 minutes with streptavidin-peroxidase (Biogenex, 1:1,000) at 37 C. The slides were rinsed with PBS and overlaid with 3'-amino-9-ethylcarbazole (AEC) solution containing 0.3% hydrogen peroxide, until color development was observed (15 to 30 minutes). Sections were rinsed and counter stained with Mayer's hematoxylin.

### *Statistical Analysis*

Data are expressed as means ± SEM. Data that seemed statistically significant were compared by analysis of normal variance for comparing the means of multiple groups and considered significant if P values were less than 0.05.

## *Results*

### *Inhibition of Proliferation and IL-2 Production in MLR Assays with MAb to Adhesion Molecules*

Five-day one-way MLR studies were performed in the presence or absence of MAb to ICAM-1 and LFA-3. The magnitude of inhibition of the MLR was >75% in the presence of LFA-3 MAb, whereas the MAb to ICAM-1 was less inhibitory, >50% (Figure 1). Examination of peak IL-2 levels on day 3 of the MLR cultures demonstrated 83 ± 4.3% and 74 ± 0.7% reduction, respectively, which corresponded to the decreased proliferative response. MAb to either ICAM-1 or LFA-3 could also inhibit MLR proliferation when added to culture as late as day 2 of the assay. In parallel experiments, a nonblocking MAb to ICAM-1 demonstrated little or no inhibition of the proliferative response.

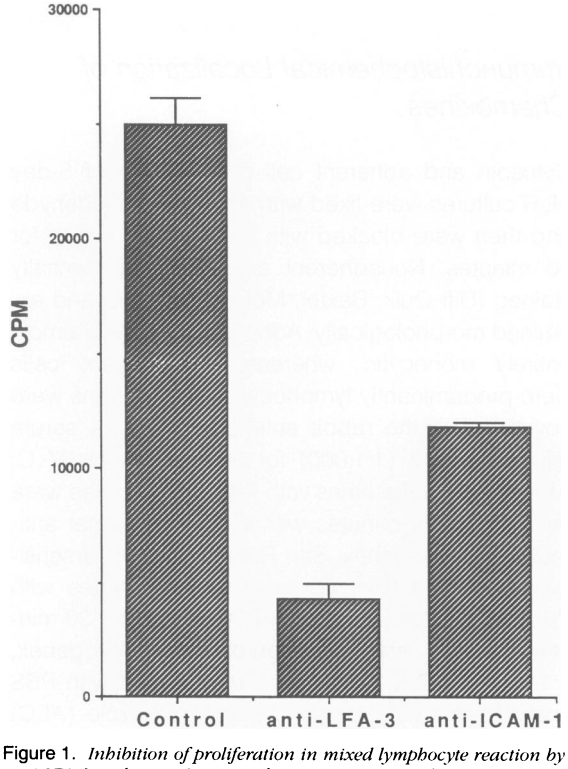


Figure 1. *Inhibition of proliferation in mixed lymphocyte reaction by anti-LFA-3 and anti-ICAM-1 MAbs. One-way MLR cultures were incubated with 5 μg/ml of MAbs to the adhesion molecules and proliferation measured on day 6 of culture. Each set of data represents three individual repeat experiments.*

### *Inhibition of TNF-α production by MAbs to ICAM-1 and LFA-3*

TNF-α production has previously been demonstrated to contribute to the proliferative response of MLR.[5,6] Antigenic levels of TNF-α were measured in a time-dependent manner during the MLR (Figure 2). The production of TNF-α was bimodal in character. The early (first) peak in production of TNF-α was seen within the first 4 hours of the assay, followed by a decline at 24 hours, and a second increase in production beginning at 48 to 72 hours and peaking at 120 hours of culture. The addition of either MAb to ICAM-1 or LFA-3 resulted in a significant decrease in TNF-α production at 1, 96, and 120 hours of culture as compared to control MLR. By 96 and 120 hours of culture, the production of TNF-α in the control cultures was 1.9 ± 0.39 and 3.2 ± 0.52 ng/ml, respectively, whereas the cultures treated with MAbs to ICAM-1 and LFA-3 produced 0.53 ± 0.07 and 0.75 ± 0.42 ng/ml at 96 hours and 1.05 ± 0.29 and 0.71 ± 0.34 ng/ml at 120 hours, respectively.

### *Inhibition of MIP-1α Production by MAbs to Adhesion Molecules*

MIP-1α is a member of the C-C chemokine family and its production has recently been shown to influ-

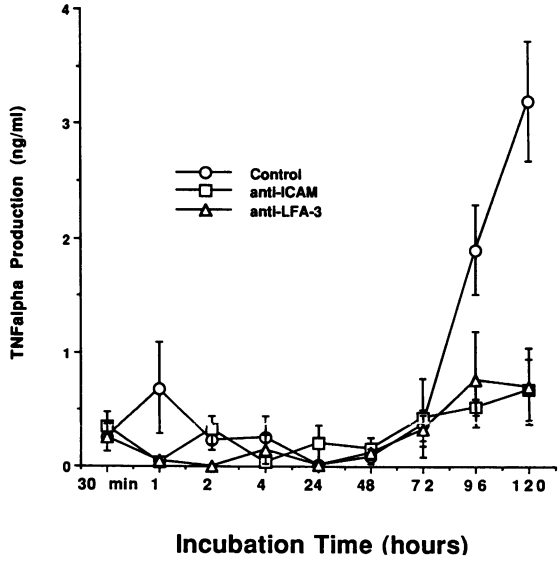


Figure 2. *Temporal expression and inhibition of TNF-α with MAbs to LFA-3 and ICAM-1. TNF-α levels were measured in culture supernatants at various time points during one-way MLR assays by a rabbit anti-human polyclonal sandwich ELISA. Data represents the mean ± SEM from four to five individual experiments at each time point.*

ence TNF-$\alpha$ generation, accompanies enhanced macrophage-mediated tumoricidal activity, and is chemotactic for neutrophils and monocytes.[10] In this study, we determined the magnitude of production of MIP-1$\alpha$ during a MLR and whether its generation was inhibited with the MAbs to the adhesion molecules. The temporal production of MIP-1$\alpha$ was bimodal in character and paralleled the production of TNF-$\alpha$ (Figure 3). MIP-1$\alpha$ was initially detected within the first 30 minutes to 1 hour of culture, decreased by 48 hours, followed by a second peak at 72 hours to 120 hours of culture. The addition of MAb to either ICAM-1 or LFA-3 significantly inhibited MIP-1$\alpha$ production at 1, 72, 96, and 120 hours of incubation as compared to the MLR control. Early MIP-1$\alpha$ production at 30 minutes to 1 hour peaked at 3.4 $\pm$ 2.1 and 1.8 $\pm$ 0.68 ng/ml, respectively, followed by a decline at 24 to 48 hours, and a second peak at 72 to 120 hours of culture produced 2.1 to 12.9 ng/ml. The addition of the MAb to ICAM-1 and LFA-3 resulted in an inhibition of MIP-1$\alpha$ product at 96 hours to 0.28 $\pm$ 0.11 and 2.35 $\pm$ 0.69, respectively, and at 120 hours to 2.0 $\pm$ 1.2 and 4.5 $\pm$ 2.2 ng/ml, respectively.

## *Inhibition of MCP-1 Production by MAbs to ICAM-1 and LFA-3*

MCP-1 is also a member of the C-C chemokine family and a potent monocyte chemotactic factor *in vitro* and *in vivo* that may serve as an important chemotactic cytokine for the elicitation of mononuclear phagocytic cells during allogeneic responses *in vivo*.[9] In the presence of MAbs to ICAM-1 and LFA-3, the production of MCP-1 was inhibited early at 1 hour and later at day 5 of the MLR (Figure 4). The control MLR assay demonstrated an initial peak in MCP-1 at 1 hour of culture (4.9 $\pm$ 3.1 ng/ml), a decline in production at 4 to 24 hours, and a second increase in production beginning at 48 to 72 hours of culture peaking at 120 hours (18.3 $\pm$ 3.4 ng/ml). The addition of ICAM-1 and LFA-3 MAbs to the cultures decreased the production of MCP-1 at 1 hour to 0 and 0.8 $\pm$ 0.56 ng/ml, respectively, and at 120 hours of culture to 6.8 $\pm$ 2.9 and 8.8 $\pm$ 2.2 ng/ml, respectively. Interestingly, MAbs to ICAM-1 induced elevated levels of MCP-1 at 48 hours of incubation. This finding was not observed with the nonblocking MAb to ICAM-1 (data not shown), indicating an increased expression of the MCP-1 at 48 hours, possibly induced by activation via the direct binding of anti-ICAM-1 MAb to the its specific cell-surface protein.

## *Inhibition of IL-8 Production by MAbs to ICAM-1 and LFA-3*

IL-8 is a member of the C-X-C gene superfamily of chemotactic cytokines and is chemotactic for neutrophils and lymphocytes *in vitro* at nmol/L and pmol/L concentrations, respectively. Similar to the previously measured cytokines, IL-8 production in

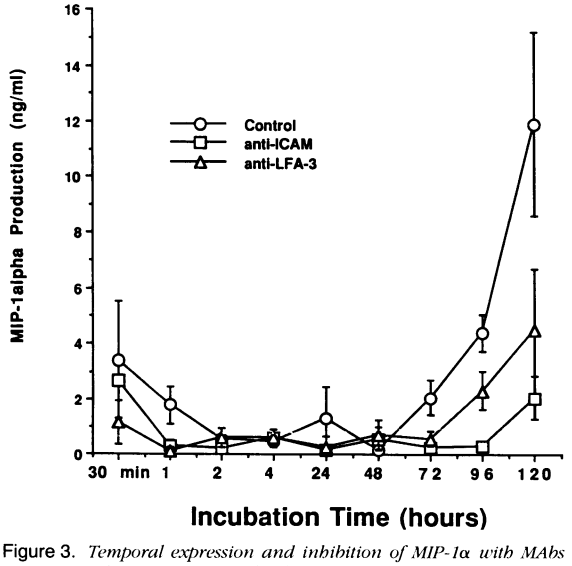


Figure 3. *Temporal expression and inhibition of MIP-1α with MAbs to LFA-3 and ICAM-1. MIP-1α levels were measured in culture supernatants at various time points during one-way MLR assays by a rabbit anti-human polyclonal sandwich ELISA. Data represents the mean ± SEM from four to five individual experiments at each time point.*

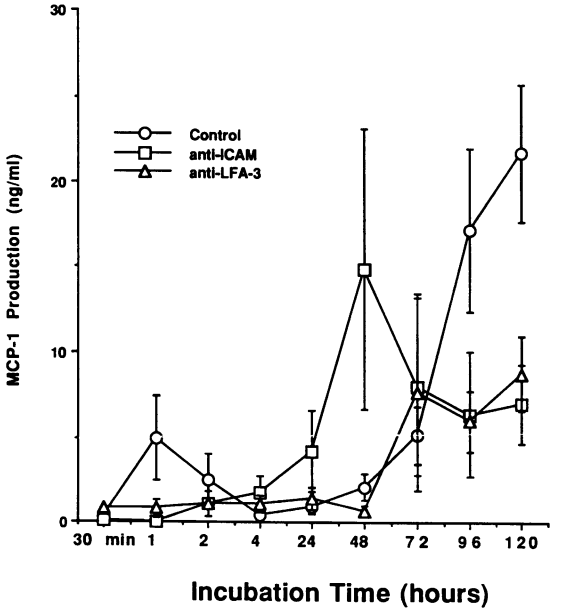


Figure 4. *Temporal expression and inhibition of MCP-1 with MAbs to LFA-3 and ICAM-1. MCP-1 levels were measured in culture supernatants at various time points during one-way MLR assays by a rabbit anti-human polyclonal sandwich ELISA. Data represents the mean ± SEM from four to five individual experiments at each time point.*

control cultures peaked as early as 1 hour (9.7 ± 6.8 ng/ml), declined by 2 to 4 hours, then steadily increased, peaking at 120 hours of culture (57 ± 18 ng/ml; Figure 5). IL-8 levels were significantly reduced by the addition of anti-ICAM-1 (48%) and anti-LFA-3 (73%) only at 1 hour (0.265 ± 0.18 and 1.25 ± 0.106 ng/ml, respectively) and 120 hours (30.3 ± 3.7 and 15.7 ± 4.4 ng/ml, respectively) in culture.

## *Reconstitution of Proliferative Response of the MLR by the Addition of Exogenous TNF-α and IL-2*

Because TNF-α and IL-2 are important mediators of the MLR, we next sought to determine whether the proliferative response could be reconstituted by addition of recombinant cytokines in the presence of anti-ICAM-1-inhibited MLR cultures (Table 1). The addition of TNF-α (5 ng/ml) or IL-2 (5 units/ml) resulted in the recovery of the MLR proliferative response. In contrast, the addition of either IL-8, MCP-1, or MIP-1α failed to reconstitute the MLR proliferative response (data not shown). Likewise, neutralizing polyclonal antibody directed against these chemokines failed to block MLR responses (data not shown). IL-2 production was reestablished with addition of exogenous TNF-α, whereas TNF-α production was reestablished with the addition of exogenous IL-2. However, the levels of all three chemotactic cytokines were differentially reexpressed in the presence of exogenous TNF-α or IL-2. IL-8 (C-X-C family member) production was restored, whereas MCP-1 and MIP-1α (C-C family members) production failed to increase in the presence of either exogenous TNF-α or IL-2. Interestingly, MIP-1α levels actually decreased significantly when TNF-α or IL-2 were added.

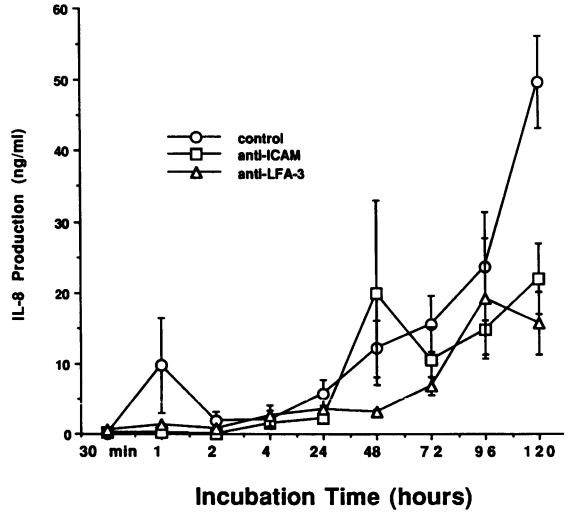


Figure 5. *Temporal expression and inhibition of IL-8 with MAbs to LFA-3 and ICAM-1. IL-8 levels were measured in culture supernatants at various time points during one-way MLR assays by a rabbit anti-human polyclonal sandwich ELISA. Data represents the mean ± SEM from four to five individual experiments at each time point.*

## *Immunohistochemical Localization of Chemokines*

To determine the cell population(s) responsible for the chemokine production, MLR cultures were set up in Lab-Tek (Nunc, Inc., Naperville, IL) chamber slides. At the end of 5-day cultures, both adherent cells and nonadherent cells (cytospin preparations) were paraformaldehyde-fixed (4%) and stained using an anti-chemokine- (MIP-1α, MCP-1, or IL-8) specific polyclonal sera. In all cases when the cells were stained with the individual anti-sera, the monocyte/macrophages were the only cell population identified with localized protein (Figure 6). Thus, the predominant production of the three chemokines, MIP-1α, MCP-1, and IL-8, was from the mononuclear phagocytes.

## *Discussion*

In the present study, we have utilized MAbs to adhesion molecules ICAM-1 and LFA-3 to evaluate the production of TNF and chemokines during an allospecific immune response. These findings establish that cellular adhesion is an initial event responsible for the production of TNF-α, and the chemotactic cytokines MIP-1α, MCP-1, and IL-8. The release of these cytokines in control cultures indicated TNF-α and the chemotactic cytokines IL-8, MIP-1α, and MCP-1 had both a lesser early and greater late peak in production at 1 to 2 hours and at day 5 of culture. The early (first) peak of TNF-α, MIP-1α, MCP-1, and IL-8 production may have resulted from initial nonspecific cell adhesion to plastic or specific surface molecules on surrounding cells. A similar early peak of chemokines was observed in control cultures incubated with syngeneic irradiated cells (data not shown). A specific binding/activation event was substantiated by the inhibition of the production of these cytokines in the presence of MAbs to ICAM-1 and LFA-3. The second peak in cytokine production (days 3 to 5) was much greater in magnitude, presumably due to adhesion molecule interaction via alloantigen specific responses, because a

**Table 1.** *Reconstitution of the Anti-Adhesion Molecule Inhibited MLR with Exogenous Cytokines, IL-2, and TNF*

| Group | Proliferation | IL-2 | TNF | MIP-1α | MCP-1 | IL-8 |
|---|---|---|---|---|---|---|
| Anti-ICAM-1 | 52 ± 1.4 | 18 ± 12 | 64 ± 6.8 | 14.5 ± 5.3 | 51 ± 6.4 | 65 ± 8.5 |
| Anti-ICAM-1+TNF | 89 ± 11 | 172 ± 60 | NA | 1.6 ± 0.8 | 61 ± 9.1 | 95 ± 11 |
| Anti-ICAM-1+IL-2 | 121 ± 20 | NA | 120 ± 23 | 1.4 ± 1.0 | 56 ± 1.1 | 84 ± 29 |

Data are standardized to the MLR in the absence of neutralizing anti-adhesion molecule antibody and are expressed as the percent of control MLR.

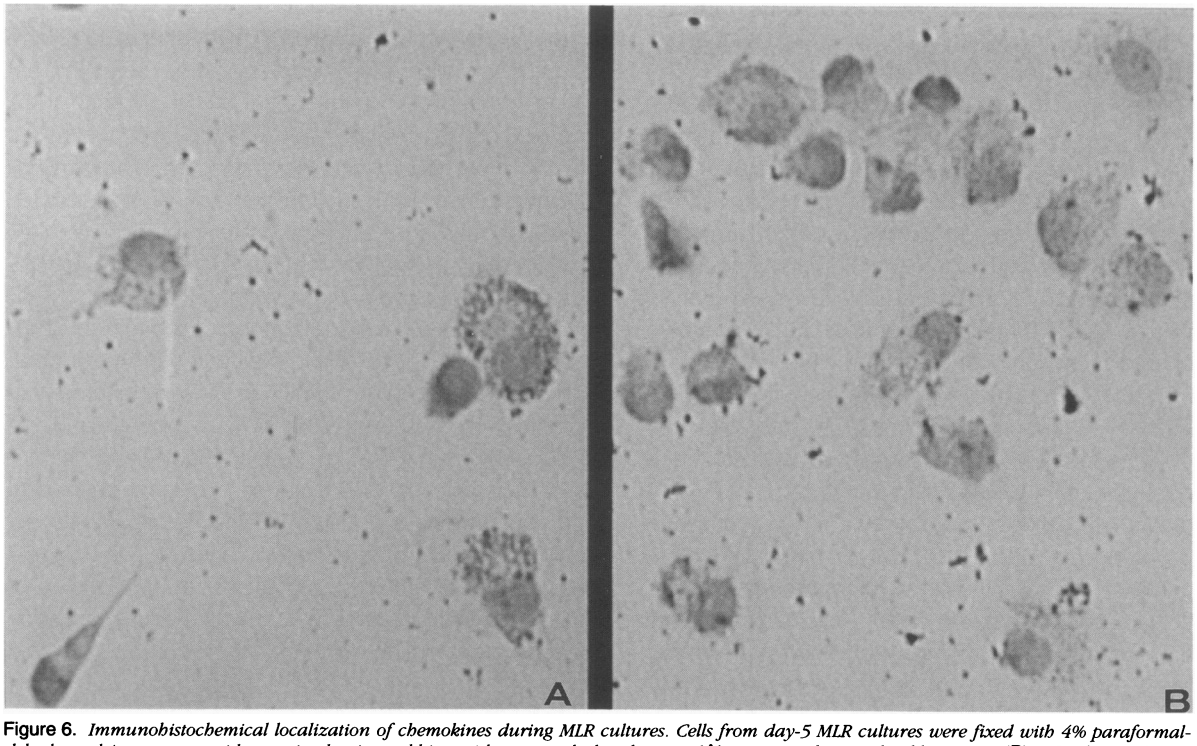


**Figure 6.** *Immunohistochemical localization of chemokines during MLR cultures. Cells from day-5 MLR cultures were fixed with 4% paraformaldehyde and immunoperoxidase-stained using rabbit anti-human polyclonal serum* (**A**) *or normal control rabbit serum* (**B**). *The figure depicts MIP-1α localization in monocytes from day-5 MLR cultures. Nonadherent lymphocytes were fixed onto glass slides by cytospin preparation, but were devoid of chemokine localization (data not shown). Staining of cells for MCP-1 and IL-8 depicted similar localization patterns in adherent monocytes with no staining in nonadherent lymphocytes.*

similar peak was not observed in control syngeneic cultures (data not shown). Although MAbs to either ICAM-1 or LFA-3 were potent inhibitors of the MLR, addition of exogenous TNF-α or IL-2 in the presence of anti-ICAM-1 resulted in the reconstitution of proliferation. The significance of TNF-α in the MLR assay has been well-established in eloquent studies that have demonstrated exogenous TNF-α augments MLR, increases production of IL-2, as well as IL-2, interferon-γ, and TNF-α receptor expression. In addition, TNF-α increases the induction of class I specific CTLs and reverses transforming growth factor-β inhibitory effects on the generation of CTL.[20,22,23] TNF-α has also been shown to be a key effector molecule in mediating acute graft-versus-host disease in skin and gut lesions.[24] In the present study, the addition of exogenous TNF-α to cultures containing MAb to ICAM-1 reestablished not only T lymphocyte proliferation but also IL-2 production to near baseline levels. The fact that IL-2 could also restore the proliferative response and TNF-α production is consonant with previous reports demonstrating the importance of IL-2 in MLR, TNF-α production, and the development of CTLs.[3,23,25] TNF-α has been shown to be a potent cytokine for the induction of IL-8 and MCP-1 from both immune and nonimmune cells.[26–28] However, the ability for TNF-α or IL-2 to reconstitute the production of the chemotactic cytokines in the presence of anti-ICAM-1 was variable. Levels of C-X-C family member IL-8 were increased in the presence of either exogenous IL-2 or TNF-α. However, levels of C-C family members MIP-1α and MCP-1 were not restored by addition of either IL-2 or TNF-α. These data would indicate the necessity of cellular interaction via specific ligand/receptor interactions along with cytokine cascades for the com-

plete generation of C-C family members, MIP-1$\alpha$ and MCP-1. In support of the concept for the necessity of both adhesive and soluble signals for chemokine production are recent data generated in our laboratory that demonstrate that anti-TNF-$\alpha$ antibodies administered at day 0 blocked both IL-8 and MCP-1 production in MLR assays (data not shown). Taken together, these data indicate that inhibition of an alloantigen-specific response at any point in the activation cascade can result in significant attenuation of subsequent production of chemokines.

Previous studies have established that ICAM-1 and LFA-3 are important in both cellular activation and proliferation associated with allospecific immune responses.[14,15,18,31] Our results would support and extend these findings, by demonstrating the importance of adhesion molecule interaction in the induction of IL-2, TNF-$\alpha$, and chemotactic cytokine production. Immunohistochemical localization of the chemokines demonstrated they were produced by monocytes in culture. However, because decreased production of the chemokines correlated with T lymphocyte function (ie, proliferation), it is likely that induction of the cytokines is directed by the T lymphocytes. Interestingly, exogenous TNF-$\alpha$ reconstituted T lymphocyte function but did not restore the monocyte-derived C-C family of chemokines. These results suggest that adhesion molecule interaction may be important for both activation of T lymphocytes and for accessory cell function. In support of this concept are recent studies demonstrating that activation of monocytes via adhesion molecules can induce the production of cytokines.[32]

Chemotactic cytokines IL-8, MCP-1, and MIP-1$\alpha$ are potentially responsible for the elicitation and activation of leukocytes at a focus of alloantigen-specific inflammation. The inhibition of these chemotactic factors through the interruption of adhesion events would ultimately decrease the influx and activation of leukocytes. The adhesion molecules examined in the present study have been identified on multiple cell types.[11,12,16] The interaction of T lymphocytes, as well as other leukocytes, with these molecules allow the migration of cells to a site of inflammation and may participate in activating one or both of the interacting cells. Consequently, blockage of adhesion molecule interaction would result in the interference of two separate but closely related mechanisms for cell extravasation; 1) activation and production of chemotactic cytokines via an alloantigen-specific response and 2) extravasation of cells via specific adhesion molecules up-regulated during inflammation. The ability to control cellular movement into a site of inflammation would be important in chronic inflammatory diseases, such as idiopathic pulmonary fibrosis, sarcoidosis, as well as during transplantation rejection episodes. Therefore, the use of MAb to the adhesion molecules may be attractive from a therapeutic standpoint.

The allospecific inhibitory effect of anti-adhesion molecule MAbs has recently been demonstrated in models of kidney allograft rejection[33] and heterotopic heart transplantation.[34] The latter study demonstrated allospecific protection from rejection using anti-LFA-1 and anti-ICAM-1 MAbs. This inhibition of allograft rejection was allospecific, as third-party skin grafts were rejected. The simultaneous use of MAbs to multiple adhesion molecules may induce substantially greater protection and be instrumental in down-regulating transplantation rejection and chronic inflammation.

The findings of our present study begin to delineate the role of adhesion molecules in the generation of chemotactic cytokines. The requirement of cellular interaction via ICAM-1 and LFA-3 and the subsequent generation of inflammatory and chemotactic cytokines may point to a distinct avenue of immunoregulation. It is likely that future *in vivo* experiments will define the efficiency of using blocking MAbs to adhesion molecules for inhibition of local and systemic immune effects in transplantation and chronic inflammation.

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