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INDUCTION BY IL 1 AND INTERFERON-γ: TISSUE DISTRIBUTION, BIOCHEMISTRY, AND FUNCTION OF A NATURAL ADHERENCE MOLECULE (ICAM-1)

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ICAM-1 is a cell surface glycoprotein originally defined by a monoclonal antibody (MAB) that inhibits phorbol ester-stimulated leukocyte aggregation. Staining of frozen sections and immunofluorescence flow cytometry showed intercellular adhesion molecule-1 (ICAM-1) is expressed on non-hematopoietic cells such as vascular endothelial cells, thymic epithelial cells, certain other epithelial cells, and fibroblasts, and on hematopoietic cells such as tissue macrophages, mitogen-stimulated T lymphocyte blasts, and germinal center dendritic cells in tonsils, lymph nodes, and Peyers' patches. ICAM-1 staining on vascular endothelial cells is most intense in T cell areas in lymph nodes and tonsils showing reactive hyperplasia. ICAM-1 is expressed in low amounts on peripheral blood leukocytes. Phorbol ester-stimulated differentiation of myelomonocytic cell lines greatly increases ICAM-1 expression. ICAM-1 expression on dermal fibroblasts is increased threefold to fivefold by either interleukin 1 (IL 1) or interferon-γ at 10 U/ml over a period of 4 or 10 hr, respectively. The induction is dependent on protein and mRNA synthesis and is reversible. ICAM-1 displays M, heterogeneity in different cell types with a M, of 97,000 on fibroblasts, 114,000 on the myelomonocytic cell line U937, and 90,000 on the B lymphoblastoid cell line. ICAM-1 biosynthesis involves a M, ~73,000 intracellular precursor. The non-N-glycosylated form resulting from tunicamycin treatment has a M, of 55,000. ICAM-1 isolated from phorbol myristic acetate (PMA) stimulated U937 and from fibroblasts yields an identical major product of M, = 60,000 after chemical deglycosylation. ICAM-1 MAB interferes with the adhesion of phytohemagglutinin blasts, and the adhesion of the cell line SKW3 to human dermal fibroblast cell layers. Pretreatment of fibroblasts but not lymphocytes with ICAM-1 MAB, and of lymphocytes but not fibroblasts with lymphocyte function-associated antigen 1 MAB inhibits adhesion.

Intercellular adhesion is increased by prior exposure of fibroblasts to IL 1, and correlates with induction of ICAM-1.

Immune responsiveness of T lymphocytes to antigens and several effector activities of leukocytes requires cell-cell contact and adhesion [1–3]. Adhesion to both hematopoietic and non-hematopoietic cells is an obligate step in antigen presentation (4–7) and effector cell functions, such as cytolytic T lymphocyte-mediated killing (1). Molecules have been characterized that are involved in both antigen-specific and nonspecific contributions to these adhesion processes (8–10). One antigen nonspecific adhesion molecule, the lymphocyte function-associated-1 (LFA-1) antigen, is thought to strengthen adhesion to cells bearing specific antigens, perhaps by binding to unidentified molecules on the antigen-presenting cell or target cell (11, 12), thereby increasing the range of avidities over which antigen-specific interactions can be effective (13). Alternatively, LFA-1 may regulate adhesion without itself engaging in ligand-receptor interactions (13). Understanding the molecular nature and regulation of these antigen nonspecific interactions is important for an understanding of adhesion interactions in the immune response, inflammation, and other aspects of leukocyte biology.

As a model for leukocyte adhesion we recently studied lymphocyte self-aggregation [14, 15]. Lymphocytes stimulated with specific antigen or with phorbol esters become adherent and form large cell clusters (16, 17). This aggregation is completely inhibited by anti-LFA-1 monoclonal antibody (MAB) [18]. In further support of the importance of LFA-1 in this adherence reaction, lymphocytes from LFA-1-deficient patients fail to self-aggregate. LFA-1-deficient lymphocytes, however, can form mixed aggregates with normal (LFA-1+) lymphocytes, suggesting the involvement of additional surface molecules (15).

To identify additional intercellular adhesion molecules (ICAM), MAB were prepared against LFA-1-deficient lymphocytes and were screened for their ability to inhibit...
aggregation of LFA-1+ lymphocytes. A MAb, RR 1/1, was obtained to an antigen distinct from LFA-1 that inhibited the phorbol ester-stimulated self-aggregation of a B lymphoblastoid and a myelomonocytic cell line. This antigen has been designated ICAM-1. Here we report that ICAM-1 is present on non-hematopoietic and hematopoietic cells. ICAM-1 surface expression on dermal fibroblasts is rapidly up-regulated by interleukin 1 (IL 1) and interferon-γ in a process that is dependent on de novo mRNA and protein synthesis. ICAM-1 regulates the natural adherence of lymphocytes to dermal fibroblasts. Furthermore, the M, of mature ICAM-1 glycoprotein, its intracellular precursor, and the polypeptide backbone have been characterized in hematopoietic and non-hematopoietic cells.

MATERIALS AND METHODS

Reagents. Recombinant mouse IL 1 (6 × 10^6 U/mg) was a gift of Dr. P. Lomedico, Hoffmann-LaRoche Inc., Nutley, NJ. Recombinant human IFN-γ (10^6 U/mg) and IFN-β (10^6 U/mg) was a gift of Dr. D. Novick, Virology Department, Weizmann Institute, Rehovot, Israel. The monoclonal antibodies 3D.2.6.4.1.1 (1) was a gift of Dr. R. Hamill, Eli Lilly, Indianapolis, IN. Affinity-purified goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-goat anti-mouse IgG was purchased from Zymed, South San Francisco, CA. Actinomycin D was purchased from Calbiochem, San Diego, CA. Tissue culture reagents were purchased from GIBCO, Grand Island, NY. Radiochemicals were obtained from New England Nuclear, Boston, MA. Protein A Sepharose and Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. All other reagents were of the highest grade available and were obtained from Fisher, Fair Lawn, NJ, or Sigma Chemical Co., St. Louis, MO.

fibrosities (MAH) Mouse fibroblasts were grown in RPMI 1640 or DMEM plus 20% bovine serum, 50 μg/mL streptomycin, 50 μg/ml gentamicin, and 5% or 10% fetal bovine serum (FBS). The supernatants from post-log cultures were collected and were complement inactivated by heating to 56°C for 30 min. Preparation of the hybridomas used was described. RR 1/1.11.7 1/21.21.19 (19), TS 2/1/1 (19), and W6/32 (20). Control supernatants were from P3X63Ag8.6.1-producing myeloma cell line.

Purification of human monocyte (HM) IL 1. Human platelets were by-products were used as a source of mononuclear cells. The adherent cell population was stimulated with opsonized heat-killed Staphylococcus albus in the presence of methionine-free medium containing 50 μCi/ml of [35S]methionine and was incubated for 36 hr. The supernatant was purified by sequential immunosorbation, gel-filtration, and chromatofocusing. Details of the purification are described elsewhere. The antibody used to make the immune complexes is described in another paper. The antibody used to make the immune complexes was 1/21.22. IL 1 was isolated by using the co-stimulator assay (23). The specific activity of HM IL 1 was approximately 1 U/μg as estimated from the gel. One U/μl is defined as doubling the mitogen response.

Human recombinant (HR) IL 1. The IL 1 cDNA was expressed in E. coli by isolating the 1112 bp Ncol-XmnI fragment (bp 295-1407) from the IL 1 cDNA plasmid pcD 12/1-18 (24). The HR-IL 1 included 46 amino acids of the IL 1 precursor peptide that are present before the alanine at position 117. This alanine is the N-terminus of the processed IL 1 found in the supernatants of stimulated human blood monocytes. IL 1, 1 μg/ml, in 115 ml of 80 μl of EDTA buffer containing 5 μg of FITC-goat anti-mouse IgG and 100 μCi/ml of [35S]methionine and was incubated for 36 hr. The supernatant was purified by sequential immunosorbation, gel-filtration, and chromatofocusing. Details of the purification are described elsewhere. The antibody used to make the immune complexes is described in another paper. The antibody used to make the immune complexes was 1/21.22. IL 1 was isolated by using the co-stimulator assay (23). The specific activity of HM IL 1 was approximately 1 U/μg as estimated from the gel. One U/μl is defined as doubling the mitogen response.

Preparation of IL 1-containing U937 supernatant. U937 cells were incubated in Teflon beads at 4 × 10^6 cells/ml with 2 μg/ml of antiserum. The beads were dialyzed against Hank's balanced salt solution (HBSS) and 1% bovine serum albumin for 3 days with three dialysis changes to remove free phorbol myristic acid (PMA). This source of IL 1 activity was used for biochemical characterization and other procedures requiring large volumes of cells. It has been demonstrated that IL 1 from U937 has identical effects to IL 1 in an enzyme-linked immunosorbent assay (ELISA). The supernatant probably contains other materials, such as tumor necrosis factor, which may also have IL 1 like activities. An anti-IL 1 antiserum inhibits IL 1 activity in the ELISA.

Cells and cell culture. In general, cells were maintained in RPMI 1640, 20 μM l-glutamine, 50 μg/mL streptomycin, 50 μg/ml gentamicin, and 10% FBS at 37°C in a 5% CO2, 95% air humidified atmosphere. Human fibroblasts derived from normal foreskin were obtained from Dr. J. Rheingold, Dana Farber Cancer Institute, Boston, MA. The premalignant leukemia-derived cell line HL-60 and the erythroleukemia cell line K562 were obtained from Dr. J. Stromberg, Dana-Farber Cancer Institute. The T cells were obtained from Dr. P. Cresswell, Duke University, Durham, NC. An anti-JY CTL clone was obtained from Dr. S. Meltzer, Dana-Farber Cancer Institute, Boston, MA. The SV-40-transformed fibroblast cell line M126 (26) was obtained from Dr. C. Terhorst, Dana-Farber Cancer Institute. Epstein-Barr virus (EBV)-transformed human B lymphocytes from whole blood were prepared as described (27). Phytohemagglutinin (PHA) blasts were prepared from isolated peripheral blood mononuclear cells (28). Briefly, mononuclear cells isolated from whole blood by dextran sedimentation and Ficol-Hyphaque (d = 1.08) centrifugation were incubated for 4 days in complete medium (CM) plus 10% FBS and 1,000 U/ml IL2. The cells were washed and were resuspended in CM plus IL 2 (28) and were always maintained between 0.5 and 5 × 10^6 cells/ml. The blasts were used between 8 and 12 days.

Immunohistochemical staining. Frozen tissue sections (4 μm thick) of normal human tissues (thymus, lymph nodes, tonsil, kidney, liver, small and large intestine, and skin) were fixed in acetone and allowed to air-dry. The sections were then incubated with biotinylated horse anti-mouse IgG and avidin-biotinylated peroxidase complex. The sections were then incubated in a solution containing 3-amin-9-ethyl-carbazole (Aldrich Chemical Co., Inc., Milwaukee, WI) and hydrogen peroxide. The sections were then washed, and formaldehyde for 5 min and were counterstained with hematoxylin. Controls included sections incubated with unrelated MAb instead of RR 1/1 antibody.

Immunofluorescence flow cytometry. Nonadherent cells were isolated by centrifugation, were washed twice at 4°C with HBSS plus 10 mM HEPES, no Ca++ or Mg++, 2 g/l EDTA, 0.05% sodium azide, and 10% heat-inactivated FBS (EDTA buffer), and were resuspended in the same to 10^6 cells/ml. Fibroblast cell layers were washed once with HBSS, no Ca++ or Mg++, 10 mM HEPES, and 2 g/l EDTA and were incubated for 15 min at 37°C in the same. Cells were suspended in the same medium and clumps were dispersed by using a rotating Teflon pestle homogenizer at 50 rpm for 30 sec at 4°C. The suspension was passed underlayed with Ficol-Hyphaque (d = 1.08) and was centrifuged at 10,000 × g for 25 min. The cells at the interface were collected and resuspended in the EDTA buffer. The cells were washed three times and were resuspended in 10 to 20% of the initial cells were recovered as a single cell dispersion with >95% viability. Trypsin was avoided because the binding of RR 1/1 (anti-ICAM-1) is reduced by trypsin. Once collected and washed, the cells were aliquoted into wells of 96-well V-bottomed microliter plates at 10^4 cells/well. For indirect immunofluorescence staining, the cells were pelleted by centrifugation at 200 g for 2 min at 4°C, resuspended in 30 μl of EDTA buffer and 50 μl of the appropriate hybridoma supernatant including one well with P3X63Ag8.6.1 (X63) supernatant as a negative control. Cells were incubated for 30 min at 4°C with vigorous agitation. The cells were pelleted, washed twice, and were resuspended in 80 μl of EDTA buffer containing 5 μg of FITC-goat anti-mouse IgG that had been centrifuged at 12,000 × g for 10 min to remove aggregated IgG. Cells were incubated for 30 min at 4°C, and then were washed twice with CM and once with CM plus 30 μl of 0.5% NaCl (ICAM-1) or 30 μl of HBSS (4°C, EDTA buffer) at 4°C. To each well was added 50 μl binding buffer and 50 μl of the appropriate hybridoma supernatant with X63 and W6/32 as the negative and positive controls, respectively. After incubation for 30 min at 4°C with gentle agitation, the wells were washed twice with binding buffer, and the second antibody, 1.971-goat anti-mouse IgG, was added at 50 nCi in 100 μl. The 1.971-goat anti-mouse IgG was prepared by using iodogen (Pierce) (30). After 30 min at 4°C, the
CELL ATTACHMENT PHA-blasts and SKW3 cells were labeled with 51Cr for quantitation of attachment. Cells were pelleted and resuspended in 0.5 ml of complete medium to which 200 μCi of 51Cr had been added. The suspension was incubated for 2 hr at 37°C and then was washed three times with complete medium. Spontaneous release of 51Cr during subsequent incubations was evaluated by running parallel 37°C incubations and counting cell-free supernatants. None of the MAb used enhanced spontaneous release under these conditions and the spontaneous release did not exceed 5%. Human dermal fibroblasts were grown to confluency in 2.01 cm² wells. The cell layers were washed three times with CM and 10 mM Hepes, 50 mM NaCl, 5 mM EDTA, and 50 mM NaHCO₃ was added in a total vol of 1 ml which also contained a 1/10 dilution of the appropriate hybridoma supernatant. After a 1-hr incubation at 37°C, the wells were washed four times with 1 ml of phosphate-buffered saline by running the buffer down one side of the well (~1 ml/sec) and aspirating from the opposite side with each successive wash. Attached 125I was released by addition of 200 μl of 0.1 N NaOH. This and a 200-μl wash were counted in the gamma counter.

RESULTS

Tissue distribution of ICAM-1. Immunohistochemical studies were performed on frozen tissue sections of normal human organs to determine distribution of ICAM-1 in thymus, lymph nodes, intestine, skin, kidney, and liver. ICAM-1 was found to have a distribution most similar to that of major histocompatibility complex (MHC) class II antigens (Table 1). Most of the blood vessels (both small and large) in all tissues showed staining of endothelial cells with ICAM-1 antibody. The vascular endothelium staining was more intense in the interlobar (paracortical) areas in lymph nodes, tonsils (Fig. 1), and Peyer's patches as compared with vessels in kidney, liver, and normal skin. In the liver, the staining was mostly restricted to sinusoidal lining cells; the hepatocytes and the endothelial cells lining most of the portal veins and arteries were not stained.

In the thymic medullary diffuse staining of large cells and a dendritic staining pattern was observed. In the cortex, the staining pattern was focal and predominantly dendritic. Thymocytes were not stained. The staining pattern most likely represented reactivity with thymic epithelial cells. In the peripheral lymphoid tissue, the germinal center cells of the secondary lymphoid follicles were intensely stained (Fig. 1). The staining pattern most likely reflected reactivity with dendritic reticulum cells.

| Table 1 |
| Distribution of ICAM-1 in normal human tissues* |

| Vascular endothelium |

| Germinal center cells (dendritic reticulum cells, B cells), interdigitating reticulum cells, and macrophages in lymphoid tissue (tonsil, lymph node, Peyer’s patches) |

| Fibroblast-like cells and dendritic cells in all organs including skin, intestine, kidney, liver, and thymus |

| Epithelial cells (thymic epithelial cells, mucosal epithelium in tonsil and sometimes tubular epithelial cells in kidney) |

* Organs studied: skin, kidney, liver, thymus, tonsil, lymph node, and intestine.
as well as B cells. In some lymphoid follicles the staining pattern was mostly dendritic with no recognizable staining of lymphocytes. Faint staining of cells in the mantle zone was also observed. In addition, dendritic cells with cytoplasmic extensions (interdigitating reticulum cells) and a small number of lymphocytes in the interfollicular or paracortical areas stained with ICAM-1 antibody.

Cells resembling macrophages were stained in the lymph nodes and lamina propria of small intestine. Fibroblast-like cells (spindle shaped cells) and dendritic cells scattered in the stroma of most of the organs studied stained with ICAM-1 antibody. However, there was no recognizable staining of Langerhans/indeterminate cells in the epidermis. Smooth muscle did not stain.

The staining of epithelial cells was consistently seen in the mucosa of the tonsils. Although hepatocytes, bile duct epithelium, intestinal epithelial cells, and tubular epithelial cells in kidney did not stain in most instances, sections of normal kidney tissue obtained from a nephrectomy specimen with renal cell carcinoma showed staining of many proximal tubular cells for ICAM-1. Interestingly, the tubular epithelial cells in this case also stained with an anti-HLA-DR antibody.

**Immunofluorescence flow cytometry.** Flow cytometry analysis of tumor cell lines and peripheral blood leukocytes supported the results obtained in frozen tissue sections (Table II). Peripheral blood leukocytes had low but significant expression of ICAM-1. ICAM-1 expression on cell lines followed the trend: EBV-transformed B lymphoblastoid lines > erythroleukemia cell line K562 > anti-JY CTL line > PHA blasts. The T cell lymphoma cell line SKW3 expressed ICAM-1 at approximately the same level as peripheral blood mononuclear cells. The myelomonocytic cell lines U937 and HL-60 can be induced to express monocyte/macrophage-like characteristics by incubation with PMA over a period of 3 days. This induction of more differentiated properties that may be analogous to the normal differentiation of monoblasts to monocyte/macrophages (38) was accompanied by a dramatic increase in ICAM-1 expression. HL-60 expression of ICAM-1 went from negative to strongly positive, and U937 expression was increased 15-fold.

A human dermal fibroblast explant from a normal donor and a SV-40-transformed fibroblast cell line M1 were also analyzed by flow cytometry (Table II). Expression on the dermal fibroblast varied from 28 to 49 fluorescence units depending on cell density, with cells in log phase growth expressing less ICAM-1 than quiescent cells (see below Fig. 2, profile A3 and B3). In at least five experiments, there was a positive correlation between cell density and ICAM-1 expression (not shown). ICAM-1
expression on the cell-line M1 was very low but was reproducibly detectable by both immunofluorescence and binding of 125I-labeled ICAM-1 MAb (not shown).

Modulation of ICAM-1 expression by IL 1 and IFN-γ. The effects of inflammatory and immune cytokines on ICAM-1 expression were investigated, because several surface molecules have been shown to be modulated by these factors (39–41) and staining of frozen tissue sections showed increased ICAM-1 expression in reactive lymphoid tissue and in delayed type hypersensitivity inflammatory sites (data not shown). Human dermal fibroblasts were used as targets, because these cells were readily available, had been used in these kinds of studies previously (40), and may play a significant role in inflammatory and immune responses (40, 42). Incubation of human dermal fibroblasts with IL 1-containing U937 supernatant for 5 hr (Fig. 2A) increased ICAM-1 expression 4.3 ± 1.1-fold (four experiments with cells both in log and stationary growth). Incubation with 100 U/ml recombinant human IFN-γ for 18 hr (Fig. 2B) resulted in a fivefold increase in ICAM-1 expression. Incubation with IL 1-containing U937 supernatant resulted in a small increase in HLA-A, B, C expression, whereas IFN-γ increased HLA-A, B, C expression by 2.5-fold and resulted in detectable expression of HLA-DR (not shown). Forward angle light scattering was not affected by incubation with IL 1 or IFN-γ, suggesting that no significant change in cell size or cytoplasmic contents occurred. Although basal ICAM-1 expression was density dependent, cell density did not affect the level to which ICAM-1 could be induced by a given activity of IL 1 or IFN-γ. IFN-β (10 to 1000 U/ml) and prostaglandin E2 (PGE2) (0.28 to 28 μM) had no effect on fibroblast ICAM-1 expression at 24 hr (not shown). The concentration for IFN-γ that was used was sufficient to increase HLA-A, B, C expression at 24 hr and had no effect on HLA-DR expression, which remained negative (not shown). IFN-γ induced a threefold increase in ICAM-1 on the K562 erythroleukemia cell line that accompanied HLA class I antigen induction (Table II).

Time course of HM IL 1 and IFN-γ effects. The kinetics of HM IL 1 and IFN-γ effects on ICAM-1 expression on dermal fibroblasts were determined using a 125I goat antimouse IgG binding assay. The effect of IL 1 with a t½ for ICAM-1 induction of 2 hr was more rapid than that of IFN-γ with a t½ of 3.75 hr (Fig. 3). No significant change in HLA-A, B, C expression was seen with HM IL 1, whereas IFN-γ increased expression of these antigens by approximately twofold at 10 hr (not shown). The time-course of return to resting levels of ICAM-1 appears to depend on the cell cycle or rate of growth. In quiescent cells, the HM IL 1 and IFN-γ effects are stable for 2 to 3 days, whereas in proliferating cultures, ICAM-1 expression is near baseline 2 days after removal of IL 1 or IFN-γ (not shown).

Concentration dependence of IL 1 and IFN-γ effect. The dose response curves for induction of ICAM-1 by recombinant mouse and human IL 1, for purified HM IL 1 and for recombinant human IFN-γ were compared (Fig. 4). IFN-γ and HM IL 1 have similar concentration dependencies with nearly identical effects at 1 ng/ml. The human and mouse recombinant IL 1 also have similar curves but are much less effective than the HM IL 1. Both recombinant IL 1 were purified under denaturing conditions, and contain sequences that are removed by processing in HM IL 1. The recombinant human IL 1 is presently 100-fold less effective than the HM IL 1 in the endogenous pyrogen assay (Dinarello, unpublished ob-
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IFN-β has no effect at concentrations up to 10 ng/ml.

Requirement for protein and mRNA synthesis. Cycloheximide, an inhibitor of protein synthesis, and actinomycin D, an inhibitor of mRNA synthesis, abolished the effects of both IL 1 and IFN-γ on ICAM-1 expression on fibroblasts (Table III). Furthermore, tunicamycin, an inhibitor of N-linked glycosylation, only inhibited the IL 1 effect by 43%. These results suggest that protein and mRNA synthesis, but not N-linked glycosylation, are required for IL 1 and IFN-γ-stimulated increases in ICAM-1 expression.

Molecular characterization of ICAM-1. ICAM-1 was isolated from different cell types by using MAB coupled to Sepharose, subjected to SDS-PAGE, and visualized by silver-staining (Fig. 5). ICAM-1 from fibroblasts is

TABLE III

Effects of cycloheximide, actinomycin D, and tunicamycin on ICAM-1 induction by IL-1 and IFN-γ on human dermal fibroblasts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (4 hr)</th>
<th>+ cycloheximide</th>
<th>+ actinomycin D</th>
<th>+ tunicamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1254 ± 140</td>
<td>1521 ± 210</td>
<td>1290 ± 46</td>
<td>1461 ± 176</td>
<td></td>
</tr>
<tr>
<td>11928 ± 600</td>
<td>10678 ± 471</td>
<td>12276 ± 608</td>
<td>12340 ± 940</td>
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IL 1 (10 U/ml) (4 hr)

<table>
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<tr>
<th>Treatment</th>
<th>4264 ± 249</th>
<th>1619 ± 381</th>
<th>1613 ± 88</th>
<th>3084 ± 113</th>
</tr>
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<tr>
<td>12155 ± 510</td>
<td>12676 ± 446</td>
<td>12294 ± 123</td>
<td>13434 ± 661</td>
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</table>

IFN-γ (10 U/ml) (18 hr)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4659 ± 109</th>
<th>1461 ± 59</th>
<th>1326 ± 186</th>
</tr>
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<tbody>
<tr>
<td>23675 ± 500</td>
<td>10675 ± 800</td>
<td>12089 ± 550</td>
<td></td>
</tr>
</tbody>
</table>

*Human fibroblasts were grown to a density of 8 x 10^4 cells/0.32 cm² well. Treatments were carried out in a final vol of 50 μl containing the indicated reagents. Cycloheximide, actinomycin D, and tunicamycin were added at 20 μg/ml, 10 μM, and 2 μg/ml, respectively, at the same time as the cytokines. All points are means of quadruplicate wells ± SD.

Figure 5. Isolation of ICAM-1 by immunoprecipitation from fibroblasts, U937 cells, and JY cells. Lane 1, untreated fibroblasts immunoprecipitated with control Sepharose CL-4B. Lane 2, untreated fibroblasts with anti-ICAM-1 Sepharose. Lane 3, IL 1-treated fibroblasts with control Sepharose. Lane 4, IL 1-treated fibroblasts with anti-ICAM-1 Sepharose. Lane 5, untreated U937 with control Sepharose. Lane 6, untreated U937 with anti-ICAM-1 Sepharose. Lane 7, PMA treated (3 days) U937 with anti-ICAM-1 Sepharose. Lane 8, PMA-treated (3 days) U937 with control Sepharose. Lane 9, JY with anti-ICAM-1 Sepharose. Lane 10, JY with control Sepharose. Immunoprecipitates from 5 x 10⁶ fibroblasts, from 10⁶ U937 cells, and from 5 x 10⁶ JY cells were subjected to nonreducing SDS 9% PAGE and silver staining. The faint, sharp band at ~160,000 M₈ in lanes 2, 4, 6, 7, and 9 is ICAM-1 MAB that dissociated from the Sepharose. The high M₈ material at the top of Lane 7 is probably aggregated monomer, because this material is not seen on reducing gels (not shown).

Figure 6. Pulse-chase metabolic labeling of dermal fibroblast ICAM-1 and TFMS treatment of ICAM-1 from dermal fibroblasts and U937 cells. Fibroblasts were grown to 2 x 10⁶ cells/25 cm² flask. Fibroblasts were treated with 1 × 10⁵ conditioned media for 3 hr before labeling and were in methionine-free media for 1 hr before labeling. Labeling conditions: lanes 1 and 2, 10 min pulse; 10 min chase; lanes 3 and 4, 10 min pulse; 20 min chase; lanes 5 and 6, 10 min pulse; 40 min chase; lanes 7 and 8, 10 min pulse; 80 min chase; lanes 9 and 10, 60 min pulse with 2 μg/ml tunicamycin present and added 1 hr before labeling. Lysates were immunoprecipitated with ICAM-1 MAb-Sepharose in oval lanes and control Sepharose in even lanes. For TFMS treatment, dermal fibroblasts (lane 11) or PMA-stimulated U937 cells (lane 12) were labeled by pulsing 1 hr and chasing 1 hr. ICAM-1 was immunoprecipitated with ICAM-1 MAB-Sepharose, was eluted, and was treated with TFMS. Precipitates were subjected to reducing SDS 9% PAGE and fluorography.
an \( M_2 = 55,000 \) (Fig. 6, lane 9). To evaluate the contribution of glycosylation to the heterogeneity of ICAM-1 from different cell types, we made use of trifluoromethane sulfonic acid, which removes both N- and O-linked oligosaccharides [32]. This treatment results in essentially identical banding patterns for fibroblast and PMA-stimulated U937 ICAM-1, with a major band at an \( M_2 = 60,000 \) and a minor band at an \( M_2 = 47,000 \) (Fig. 6, lanes 11 and 12). This result shows that dermal fibroblast and U937 ICAM-1 have polypeptides with identical \( M_2 \), suggesting the polypeptides are identical and carbohydrate processing leads to the different \( M_2 \) of the mature proteins in these two cell types. The presence of two species after TFMS modification makes it unclear whether or not ICAM-1 contains O-linked oligosaccharides. Incomplete removal of O-linked oligosaccharides could account for the two bands. Alternatively, a site-specific cleavage of the polypeptide chain occurring to a similar extent in both samples could also yield two bands.

**Natural attachment assay.** The adhesion of in vitro activated T lymphocytes to dermal fibroblasts in a non-antigen-specific manner has been described and is referred to as natural attachment [43–45]. This system has been used here to study lymphocyte adhesion to cells on which ICAM-1 expression can be modulated and for which adhesion can be readily assayed by measuring attachment of radiolabeled lymphoid cells to fibroblast cell layers. To obtain activated T lymphocytes, peripheral blood lymphocytes were stimulated with PHA, washed, and then cultured in the absence of PHA for 3 to 6 days. Under these conditions, surface-bound PHA is internalized and would not contribute to the adhesion reaction. Figure 7 shows the adhesion of fluorescently labeled PHA blasts to sparsely plated fibroblasts. This demonstrates the nature and specificity of the interaction. Very few PHA blasts were bound to the plastic slide, whereas those bound to fibroblasts were often seen to spread out on the fibroblast apparently increasing the area of contact between cell surfaces.

MAb to ICAM-1 and other cell surface molecules were evaluated for their effect on the adhesion of \(^{51} \text{Cr} \)-labeled PHA-blasts to continuous layers of dermal fibroblasts (Table IV). Natural attachment of PHA blasts was inhibited 70% by anti-ICAM-1 MAb. The T lymphoma cell line SKW3 was also studied because homotypic adhesion by this cell line was inhibited by anti-LFA-1 but not anti-ICAM-1 MAb. It was therefore an important question to determine whether heterotypic adhesion between SKW3 and ICAM-1 fibroblasts would be inhibited by anti-ICAM-1 MAb. Natural attachment of SKW3 cells to fibroblasts was inhibited 78% by anti-ICAM-1 MAb. Attachment of PHA blasts and SKW3 cells was inhibited 66 to 76% by MAb to LFA-1 \( \alpha \) and \( \beta \) subunits. In contrast, MAb to HLA-A,B,C, LFA-2, and LFA-3 had no effect. Anti-HLA-A,B,C binds to fibroblasts at greater density than any of the effective antibodies (Table II), which suggests that the inhibition seen with anti-ICAM-1 and anti-LFA-1 MAb is not due to nonspecific effects of surface-bound IgG. Purified anti-ICAM-1 IgG and Fab' half-maximally inhibited attachment of SKW3 at 0.2 \( \mu \text{g} / \text{ml} \) and 0.5 \( \mu \text{g} / \text{ml} \), suggesting that anti-ICAM-1 is very efficient at inhibiting attachment and inhibition is not dependent on bivalency of the MAb (Fig. 8).

Preincubation of one cell population with MAb followed by natural attachment assay was used to identify the cell on which ICAM-1 and LFA-1 are required for adhesion in this heterotypic system (Table V). When fibroblasts were preincubated with anti-ICAM-1 MAb, adhesion was inhibited by 66 and 73%. Despite the presence of ICAM-1 on the lymphoid cells, preincubation of these cells with anti-ICAM-1 MAb had no effect on attachment. Preincubation of the lymphoid cells with anti-LFA-1 MAb inhibited attachment, but preincubation of fibroblasts with anti-LFA-1 MAb had no effect.

The effect of IL 1 pretreatment of dermal fibroblasts on natural attachment was determined to additionally evaluate the role of ICAM-1 in this adhesion process. There was a significant correlation between ICAM-1 surface expression and natural attachment when the dose responses for HM IL 1 were compared (Fig. 9). Furthermore, the amount of natural attachment that was inhabitable by anti-ICAM-1 showed a similar increase. Binding not inhabitable by anti-ICAM-1 changed little over the range of IL 1 concentrations used. A significant increase in SKW3 binding of twofold to threefold is also

Figure 7. PHA-blasts binding to human dermal fibroblasts. PHA blasts (10 days) were labeled with carboxyfluorescein diacetate (40 \( \mu \text{g} / \text{ml} \)) by incubation in CM with 1% serum. The PHA blasts were washed and added to plastic slides (Tiintersection) with sparsely plated fibroblasts at >10 PHA blasts: 1 fibroblast. After 1 hr at 37°C, the unattached cells were washed out, and the slide was observed immediately by using a combination of visible wavelength phase illumination and fluorescence without fixation. Bar = 20 \( \mu \).
INTERCELLULAR ADHESION MOLECULE-1 CHARACTERIZATION

TABLE IV

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cells Bound x 10^-4</th>
<th>PHA blasts</th>
<th>SKW3</th>
</tr>
</thead>
<tbody>
<tr>
<td>X63</td>
<td>2.4 ± 0.2</td>
<td>2.1 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>aICAM-1</td>
<td>0.7 ± 0.1</td>
<td>0.47 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>aLFA-1</td>
<td>0.8 ± 0.1</td>
<td>0.50 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>aLFA-1, b</td>
<td>0.65 ± 0.1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>aHLA-1, A,B,C</td>
<td>2.6 ± 0.1</td>
<td>2.01 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>aLFA-3</td>
<td>2.6 ± 0.2</td>
<td>2.30 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>aLFA-2</td>
<td>2.4 ± 0.1</td>
<td>2.21 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

* Human dermal fibroblasts were grown to 5 x 10^6 cells/2.01 cm^2 well. PHA blasts or SKW3 (2 x 10^6) cells labeled with ^51Cr were added for 1 hr at 37°C. PHA blasts had an activity of 5.600 cpm/10^6 cells and SKW3 of 120,000 cpm/10^6 cells. Hybridoma supernatants were added at a dilution of 1/10 just before addition of the labeled cells. The final vol was 1 ml. The results are means of quadruplicate determinations ± SD.

**ND. not done.**

Figure 8. Effect of anti-ICAM-1 IgG and Fab' on PHA blast binding to human dermal fibroblasts. Fibroblasts were grown to 2.5 x 10^6 cells/2.01 cm^2 well. PHA blasts (2 x 10^6) and the MAb (a) or MAB fragment (n) were added and were incubated 1 hr at 37°C. Final volume was 1 ml.

Table V

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Lymphoid cell or fibroblast pretreatment with MAB</th>
<th>Cells Bound x 10^-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>2.57 ± 0.00</td>
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<tr>
<td>None</td>
<td>aICAM-1</td>
<td>0.88 ± 0.08</td>
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<tr>
<td>None</td>
<td>aLFA-1</td>
<td>2.52 ± 0.09</td>
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<tr>
<td>None</td>
<td>aHLA-1, A,B,C</td>
<td>2.40 ± 0.21</td>
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<tr>
<td>None</td>
<td>aICAM-1</td>
<td>2.84 ± 0.18</td>
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<tr>
<td>None</td>
<td>aLFA-1</td>
<td>0.73 ± 0.16</td>
</tr>
<tr>
<td>None</td>
<td>aHLA-1, A,B,C</td>
<td>2.40 ± 0.10</td>
</tr>
</tbody>
</table>

* Human dermal fibroblasts were grown to 2 x 10^6 cells/2.01 cm^2 well. Fibroblasts or T lymphoid cells were incubated with a 1/10 dilution of hydridoma supernatant for 30 min at 24°C and then were washed three times with CM. PHA blasts or SKW3 (2 x 10^6) were added to wells in a final vol of 1 ml of CM with no more addition of hybridoma supernatant. The results are the mean of quadruplicate determinations ± SD.

seen with fibroblasts incubated with 10 U/ml HM IL 1 for 4 hr (not shown).

DISCUSSION

ICAM-1 is a glycoprotein expressed on the surface of both hematopoietic and non-hematopoietic cells. Although ICAM-1 expression is uniformly low on peripheral blood leukocytes, it is expressed to higher levels on EBV-transformed B lymphocyte blasts, mitogen-stimulated T lymphocyte blasts, a cloned CTL line, macrophage-like cells in several organs, and dendritic cells and possibly B lymphocytes in germinal centers. ICAM-1 is also induced on the myelomonocytic cells lines U937 and HL-60 after treatment with PMA for 3 days. PMA treatment causes maturation of these cells to a monocyte/macrophage phenotype. These observations suggest that increased ICAM-1 expression is associated with activation of lymphocytes and with maturation of macrophage-like cells.

The overall distribution of ICAM-1 in thin tissue sections is similar to HLA-DR. ICAM-1 is present on non-lymphoid cells including vascular endothelial cells, thymic epithelial cells, mucosal epithelial cells, and dendritic cells in germinal centers and T cell areas in lymphoid tissue. These cells are recognized for their role in immune and inflammatory responses, particularly endothelial and dendritic cells, which may be antigen-presenting cells (3, 6, 7). Furthermore, mucosal epithelial cells and fibroblasts are important potential targets of invading viruses and may also be targets for lymphocyte functions involving adhesion.

These studies establish that IL 1 and IFN-γ increase ICAM-1 expression on fibroblasts and suggest that ICAM-1 may have a role in inflammatory and immune responses. IL 1 and IFN-γ have been reported to have other effects on dermal fibroblasts. For example, IL 1 stimulates proliferation (45), PGE₂, synthesis, and collagenase production (42, 47), whereas IFN-γ decreases collagen synthesis, causes a late inhibition of proliferation (40), increases PGE₂, elaboration (49), and increases expression of class I and II MHC antigens (40). IFN-β, which does not increase ICAM-1 expression on dermal fibroblasts, also decreases collagen synthesis, causes growth inhibition (48), and increases expression of class I, but not class II MHC antigens (40). The regulation of ICAM-1 expression on dermal fibroblasts is novel in that it is the only antigen expression modulation system in which IL 1 and IFN-γ have the same effect. It is also notable that non-immune IFN-β, which shares several effects on dermal fibroblasts with IFN-γ, does not up-regulate the expression of ICAM-1.

IL 1 and IFN-γ also increase ICAM-1 expression on endothelial cells. A comparison of IL 1-induced surface expression of ICAM-1 and another antigen that is specific

for endothelial cells and is identified by the MAb H4/18 (41) has been made on cultured umbilical vein endothelial cells. ICAM-1 expression was increased threefold to fourfold on endothelial cells by IL 1 and eightfold by tumor necrosis factor. The expression of the antigen recognized by H4/18 was induced by IL 1 on endothelial cells but was not detected on resting endothelial cells. The antigen recognized by H4/18 also shows different kinetics of induction with a peak at 4.5 hr followed by decay of expression down to almost background levels by 24 hr even in the presence of IL 1 or tumor necrosis factor. In contrast, ICAM-1 expression continues to increase for 24 hr and remains stable for 72 hr.

The ICAM-1 glycoprotein displays M, heterogeneity in different cell types. On the basis of the results of chemical deglycosylation with TFMS, it appears that this heterogeneity is based on differential glycosylation of common polypeptide, although it remains to be rigorously demonstrated that the polypeptides from dermal fibroblasts and U373 are identical. Pulse-chase metabolic labeling of fibroblast ICAM-1 shows that a precursor of M, = 73,000 is converted to a mature form of M, = 97,000 in 20 to 30 min. If maturation in the Golgi complex is followed by transport to the cell surface within a few minutes, then this data would be consistent with the rapid mRNA and protein synthesis-dependent up-regulation of ICAM-1 by IL 1, which is apparent within 2 hr. The MAb RR 1/1 appears to bind to a protein epitope on ICAM-1, because RR 1/1 can immunoprecipitate ICAM-1 from cells labeled in the presence of tunicamycin. This is notable because the dermal fibroblast form of ICAM-1 is 45% carbohydrate as estimated by the mobilities of the glycosylated and nonglycosylated forms in SDS-PAGE. The product synthesized in the presence of tunicamycin was M, = 55,000. Estimating approximately 2,000 to 3,000 M, U per high mannose oligosaccharide on the intracellular precursor of M, = 73,000, ICAM-1 would contain approximately 6 to 9 such oligosaccharide units per molecule.

Previous studies demonstrated the importance of ICAM-1 and LFA-1 in PMA-stimulated self-adhesion of several leukocyte cell types (15). PHA blasts and the T lymphoma cell line SKW3 were used here to study the binding of lymphocytes to non-hematopoietic ICAM-1-positive cells, which lack leukocyte markers such as LFA-1. The natural attachment assay in which activated but not resting T lymphocytes have been shown to adhere to fibroblasts in a species-specific manner (45). We found that both T lymphocyte blasts and SKW3 T lymphoma cells bind to dermal fibroblasts, and this binding is inhibitable by either anti-LFA-1 or anti-ICAM-1 MAb. Furthermore, it has been established here that ICAM-1 is required on the dermal fibroblast, whereas LFA-1 is required on the lymphocyte. On incubating dermal fibroblasts with IL 1 natural attachment was significantly increased in an IL 1 concentration-dependent manner that was correlated with ICAM-1 surface expression.

The importance of ICAM-1 and LFA-1 and the regulation by IL 1 suggest natural attachment is a functionally significant assay. Natural attachment may represent part of the non-antigen-specific component of leukocyte adhesion in functions such as antigen presentation (50), and cytotoxic T lymphocyte-mediated killing (13). ICAM-1 modulation by IL 1 and IFN-γ at sites of monocyt or T lymphocyte activation might increase the tendency of T lymphocytes to adhere to connective tissue cells such as fibroblasts on which ICAM-1 expression is stimulated and increase the probability of afferent or effector T lymphocyte functions.

A possible role for ICAM-1 in lymphocyte diapedesis is suggested by immunoperoxidase staining of sections of tonsils and lymph nodes reacting to inflammation that show very strong ICAM-1 expression on vascular endothelial cells in T lymphocyte areas. Furthermore, areas of delayed hypersensitivity reaction in skin show more intense staining of ICAM-1 on vascular endothelial cells as compared with vessels in normal skin (unpublished observation). ICAM-1 induction on endothelial cells by inflammatory mediators may facilitate margination and extravasation of T and possibly B lymphocytes at sites of inflammation or a localized inflammatory immune response. It could be speculated that because ICAM-1 upregulation on endothelial cells by IL 1 is rapid, increased adhesion of lymphocytes mediated by ICAM-1 might be an event mediating lymphocyte influx into inflammatory foci. Subsequent production of IFN-γ by activated lymphocytes at the inflammatory site might mediate additional amplification of the local inflammatory immune response.

ICAM-1 has some properties in common with the papa- and trypsin sensitive molecule proposed to be LFA-1 ligand (11, 12). ICAM-1 on JY cells is trypsin sensitive under the same conditions as those used to define the hypothetical ligand (unpublished observations). Furthermore, ICAM-1 appears to be required in two LFA-1-dependent adhesion systems, leukocyte self-aggregation and natural attachment. These results suggest that ICAM-1 could be a cell surface molecule that interacts with LFA-1 on other cells.

The work reported here suggests that ICAM-1 is important in leukocyte adhesion and is regulated in a manner consistent with its being an important molecule in inflammatory and immune responses. Additional work is needed to define the receptor for ICAM-1 and the exact molecular interactions involved in ICAM-1-dependent cell-cell adhesion.

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