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TUMOR NECROSIS FACTOR COMBINES WITH IL-4 OR IFN-γ TO SELECTIVELY ENHANCE ENDOTHELIAL CELL ADHESIVENESS FOR T CELLS

The Contribution of Vascular Cell Adhesion Molecule-1-Dependent and -Independent Binding Mechanisms[1]

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The adhesion of lymphocytes to vascular endothelium is the first step in their passage from the blood into inflammatory tissues. By modulating endothelial cell (EC) adhesiveness for lymphocytes, cytokines may regulate lymphocyte accumulation and hence the nature and progression of inflammatory responses. IL-1, TNF, IFN-γ, and IL-4 each increase EC adhesiveness for T cells when used alone in adhesion assays in vitro. As cytokines are more likely to act in combination at sites of inflammation in vivo, we have studied the stimulating effect of different combinations of cytokines on EC adhesiveness for T cells and polymorphonuclear leukocytes (PMN). Acting alone IL-1, TNF, IFN-γ, and IL-4 each significantly enhanced EC adhesiveness for T cells ($p < 0.005$), whereas only IL-1 ($p < 0.005$) and TNF ($p < 0.005$) but not IFN-γ or IL-4 significantly enhanced adhesiveness for PMN. When EC were stimulated with optimal concentrations of TNF in combination with IL-4 or IFN-γ, there was a significant further increase in adhesiveness for T cells ($p < 0.003$), but not PMN, over that seen with TNF alone. The additive effect of TNF and IL-4 was more marked than that of TNF and IFN-γ. Although approximately equal proportions of T cells and PMN bound to TNF-stimulated EC, nearly double the proportion of T cells compared with PMN bound EC preincubated with TNF and IL-4 together. A similar interaction with IL-4 or IFN-γ was exhibited by lymphotoxin. mAb-inhibition studies indicated that the extra increase in binding caused by stimulating EC with TNF and IL-4 in combination was mediated by VCAM-1 whereas that caused by stimulating with TNF and IFN-γ in combination was substantially mediated through leukocyte function-associated Ag-1- and VCAM-1-independent mechanisms. These observations suggest that whereas IL-1 and TNF alone are unselective in terms of leukocyte adhesion to EC, the combination of TNF (or LT) with IL-4 or IFN-γ may be of key importance in determining the recruitment of a lymphocyte-predominant infiltrate in immune mediated inflammation, and in initiating the transition from acute to chronic inflammation.

In order to leave the circulation and pass into the tissues lymphocytes must first adhere to the endothelial cell (EC)[4] lining of blood vessels. Within lymphoid organs lymphocyte-EC adhesion occurs in specialized post-capillary venules and is an important step in the normal physiologic recirculation of lymphocytes (1, 2). Elsewhere few lymphocytes leave the circulation, possibly because the endothelial lining of blood vessels is normally poorly adhesive for lymphocytes. However, at sites of inflammation, particularly in immune mediated and chronic inflammation, lymphocytes leave the circulation in large numbers and accumulate in the tissues. Because lymphocyte binding to EC is the first step in this process, modulation of EC adhesiveness is likely to be critically important in regulating the localization, timing, nature, and progression of lymphocyte accumulation in different types of inflammatory response.

Studies in vitro with cultured human vascular EC have shown that IL-1 and TNF-α can each increase the adhesiveness of EC for lymphocytes (3, 4) and PMN (5–7). LT, also called TNF-β, shares this and other properties of TNF, although sometimes with lower potency (8–11) and probably binds the same cell-surface receptor (12). In contrast, IFN-γ and IL-4 each increase the adhesiveness of EC for lymphocytes (13, 14) but do not increase EC adhesiveness for PMN (14, 15). This difference, together with the delayed kinetics of the increased adhesiveness for T cells seen with IL-4 or IFN-γ compared with IL-1 or

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[4] Abbreviations used in this paper: EC, endothelial cells; AET, S-2-aminoethyl-isothiouronium hydrobromide; ELAM-1, endothelial cell leukocyte adhesion molecule-1; HBSS=, HBSS without calcium or magnesium; HS, heat-inactivated human serum; ICAM-1, intercellular adhesion molecule-1 (CD54); LFA-1, leukocyte function associated Ag-1 (CD11a/CD18); LT, lymphotoxin (TNFβ); MNC, mononuclear cells; NAC, nonadherent cells; PM, plain medium; PMN, polymorphonuclear leukocytes; VCAM-1, vascular cell adhesion molecule-1.

TNF, divides these agents into two distinct pairs with respect to their actions on EC adhesiveness for leukocytes. At sites of inflammation in vivo, however, EC are unlikely to be subject to the actions of individual cytokines acting in isolation but are rather more likely to be influenced by the combined or sequential action of several different cytokines acting in concert. This may well result in quite different effects on EC adhesiveness than those seen in vitro with individual agents.

Earlier studies looking at cytokine interactions have failed to show evidence of additive or synergistic effects on EC adhesiveness of optimal concentrations of IL-1 and IFN-γ (3) or IL-1 and TNF (4). However, TNF and IFN-γ are reported to act synergistically on EC to increase expression of class I MHC Ag (16), to alter EC morphology from epithelioid to fibroblastoid (17), and to up-regulate EC ICAM-1 expression (18). In addition, we have recently reported synergy between IL-4 and IL-1 or TNF for induction of the EC activation Ag recognized by mAb 1.4C3 (19, 20) which is now known to be the same as VCAM-1, a recently described adhesion ligand for lymphocytes and monocytes (21–23). IL-4 also exhibited further modulatory effects on the actions of cytokines on EC by partially inhibiting the IL-1 or TNF-stimulated expression of the adhesion molecules ICAM-1 (CD54), which acts as a ligand for LFA-1, and ELAM-1, a selective adhesion ligand for PMN and perhaps monocytes (20, 24–27).

We have now extended these experiments on the effects of combinations of cytokines on the EC surface membrane to an analysis of EC adhesiveness for T cells and PMN, using adhesion assays in vitro. In this paper we present evidence that the action of TNF and IL-4 in combination, and to a lesser extent TNF and IFN-γ, may be critically important in altering the EC lining of blood vessels to facilitate the selective accumulation of lymphocytes in immune mediated inflammatory lesions and the conversion of acute inflammatory infiltrates dominated by PMN into chronic inflammatory infiltrates characterized by the presence of lymphocytes.

MATERIALS AND METHODS

Cytokines and other reagents. Human rIL1-β was obtained from Syntex (Palo Alto, CA) and human rTNF-α (sp. act. of 3.2×10^7 U/mg) was a kind gift from Chiron Corporation, Emeryville, CA. Human rIL-4 was obtained from British Biotechnology (Oxford, U.K.) and human rIFN-γ was obtained from Biogen (Cambridge, MA). Human rLT (TNF-β) was the kind gift of Dr. T. Meager, National Institute for Biological Standards Control, Potters Bar, U.K. mAb 1.4C3 (IgG1) and 1G11 (IgG1) were raised in this laboratory and were used as diluted ascitic fluid. mAb 1.4C3 was first characterized as reacting with a cytokine-inducible Ag on EC (19). This is now known to be VCAM-1 as judged by specific reactivity with COS cells transfected with VCAM-1 cDNA (Dr. A. Shaw and Dr. S. D'Humière (Glaxo, Geneva, Switzerland) personal communication). The hybridoma lines secreting the anti-CD18 mAb TS1/18 (IgG1) and the anti-MHC class I framework determinant mAb BB7.5 (IgG1) were purchased from the American Type Culture Collection (Rockville, MA).

Isolation and culture of EC. EC were obtained from human umbilical cords by collagenase (type II; Sigma, Poole, U.K.) digestion by using a modification of the method of Jaffe et al. (28) as previously described (3). Cells from different cords were cultured separately at 37°C in 5% CO_2 by using EC growth medium consisting of RPMI 1640 with L-glutamine (GIBCO, Paisley, Scotland) supplemented with 15% heat-inactivated FCS (Sera-lab, Crawley Down, U.K.), 10% pooled heat-inactivated HS from normal donors, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) (GIBCO), 10 μg/ml polymixin B sulfate (Sigma), 5 U/ml sodium heparin (Leo, Princes Risborough, U.K.), and 15 μg/ml EC growth factor (Sigma) in tissue culture flasks pretreated with 1% gelatin (Sigma). At confluence, cells were detached by using 0.125% trypsin-EDTA (GIBCO) in Puck's saline A (GIBCO) with 10 mM HEPES and subcultured in EC growth medium at a 1:3 split ratio. At fourth passage confluent EC were removed from culture flasks with 0.125% trypsin-EDTA in Puck's saline A and resuspended in 15% FCS, 10% HS, antibiotics and 10 μg/ml polymixin B sulfate in RPMI 1640 (basic medium) at a concentration of 2×10^5 cells/ml. Aliquots (0.2 ml) were then cultured overnight in flat bottomed, gelatin-coated 96-well microtiter plates (Costar, Cambridge, MA) resulting in confluent EC monolayers. Cytokine stimulation of EC was performed by adding 22 μl of cytokine in basic medium at 10 times the desired final concentration to the appropriate wells for the required time. The wells were then washed with warm RPMI 1640 with 10% FCS and used in the adhesion assays.

The cells were confirmed to be EC by morphologic criteria and by immunofluorescent staining and flow microfluorometry analysis (FACStar, Becton Dickinson, Cowley, U.K.). More than 99% of fourth passage cells stained with the anti-EC mAb EN4 (Sanbio, Uden, Holland) (29) whereas less than 1% stained with the monocyte- and macrophage-specific mAb Mo2 (Coulter, Hialeah, FL) (30).

Isolation and chromium-51 labeling of T cells. T cells were isolated from heparinized peripheral blood collected by venipuncture from normal donors. The blood was diluted with an equal volume of HBSS (GIBCO) and layered over Ficoll/Hypaque ("Lymphoprep", Nycomed, Oslo, Norway). MNC were separated by density gradient centrifugation as described by Boyum (31) at $1250 \times g$ for 20 min. The MNC-rich interface was collected, washed with HBSS and resuspended in RPMI 1640 with 10% FCS (T cell medium) at 10^7 cells/ml and incubated for 45 min at 37°C and 5% CO_2 in plastic Petri dishes (10 ml/9-cm diameter dish). The NAC were harvested by gentle washing and resuspended at 10^7 cells/ml in T cell medium. T cells were obtained by rosetting NAC with AET (Sigma)-treated SRBC (GIBCO). SRBC were washed and incubated with AET at 37°C for 30 min and then washed again. NAC were incubated with AET-treated SRBC for 1 h on ice. After gentle resuspension the cells were separated by density gradient centrifugation over Ficoll/Hypaque (650g for 20 min) into T and non-T cells. T cells purified by this method contained more than 95% CD2-positive cells (mAb OKT11; American Type Culture Collection), less than 1% staining with mAb Leu-M3 (Becton Dickinson, Mountain View, CA) specific for monocytes and macrophages (32) and less than 1% staining with the B cell-specific mAb RFB7 (a kind gift of Dr. L. Poulter, Royal Free Hospital, London, U.K.) as determined by flow cytometry.

T cells were resuspended in 0.2 ml of T cell medium containing 200 μCi of ^{51}Cr, Na_2CrO_4 (Amersham International, Aylesbury, U.K.). After a 90-min incubation at 37°C with intermittent agitation, soluble ^{51}Cr was removed by washing four times in HBSS. Cell viability was greater than 95% as judged by trypan blue exclusion.

Isolation and chromium-51 labeling of PMN. PMN were routinely isolated from heparinized peripheral blood collected by venipuncture from normal donors. E were sedimented for 45 min by mixing in a 5:1 ratio with 6% dextran (average m.w. 110,000) in saline (dextran 110; Fisons Plc, Loughborough, U.K.). The supernatant was then layered over Ficoll/Hypaque and the PMN and MNC fractions were separated by density gradient centrifugation ($350 \times g$ for 30 min). E contaminating the PMN-rich pellet were removed by incubating for 10 min at 5°C with lysing solution (10 mM potassium bicarbonate containing 0.155 M ammonium chloride and 0.1 mM EDTA) and washing with HBSS without calcium and magnesium (HBSS=). The purity of the resulting PMN suspension was >95% as determined by light microscopy of Grünwald- and Giemsa-stained films.

PMN were resuspended in 0.2 ml of HBSS= containing 200 μCi of ^{51}Cr, Na_2CrO_4 (Amersham International). After a 90-min incubation at 37°C with intermittent agitation, soluble ^{51}Cr was removed by washing four times in HBSS=. Cell viability was greater than 95% by trypan blue exclusion.

Adhesion assay to EC monolayers. For the adhesion assays media was removed from the wells containing the confluent EC monolayers and 4×10^5 ^{51}Cr-labeled T cells in 0.2 ml of T cell medium or 2×10^5 ^{51}Cr-labeled PMN in 0.2 ml of HBSS containing 30 mM HEPES (Hanks'/HEPES) were added to each well. Test and control samples were performed in triplicate in each experiment. In most assays T cells were co-incubated with EC for 60 min at 37°C in 5% CO_2. However, in assays in which PMN and T cell adhesion were being directly compared PMN and T cells were co-incubated with EC for 30 min in order to optimise detection of changes in PMN binding (15). The microtiter wells were then washed four times with 0.2 ml of warm medium to remove nonadherent T cells or PMN, and 0.2 ml of an aqueous 1% solution of Nonidet P-40 (BDH Ltd., Poole, U.K.) was added to each well and incubated with agitation for more than 20 min to lyse the cells. The percentage of applied T cells or PMN that bound to the EC monolayer (% adhesion) was then calcu-

lated as follows:

$$\% \text{ Adhesion} = \frac{\text{cpm in 0.1 ml of lysate}}{\text{cpm in 0.1 ml of original T cell/PMN suspension}} \times 100.$$

Immunoprecipitation and SDS-PAGE. EC were metabolically labeled in 175-cm^2 tissue-culture flasks by replacement of culture medium with 5 ml methionine-free RPMI, 10% FCS dialyzed against methionine-free RPMI, 10 ng/ml TNF, and 150 μCi [^{35}S]methionine (Amersham International, Amersham, U.K.). After 18 h, EC were washed in the flasks with HBSS and then lysed for 30 min in situ with ice-cold buffer consisting of 4 mM EDTA, 50 mM Tris in 150 mM sodium chloride, pH 7.4, with 0.5% NP-40, 1 μM pepstatin A, 2 μg/ml Leupeptin, and 1 mM PMSF (all from Sigma). After centrifugation to remove nuclear debris, radiolabeled EC lysates were precleared with rabbit anti-mouse Ig agarose (Sigma) and then immunoprecipitated with specific mAb and rabbit anti-mouse Ig agarose. Immunoprecipitates were analyzed by SDS-7.5% PAGE under reducing conditions followed by autoradiography, using ^{14}C-methylated protein standards (Amersham International). For quantification, the autoradiograph was overlaid onto the gel to identify the relevant immunoprecipitated bands. These were cut from the gel, minced with fine scissors and suspended in scintillation fluid for counting. Specific band activity was calculated by subtracting the activity of the equivalent area of a parallel lane immunoprecipitated by irrelevant control mAb.

Statistical analysis. Differences between the results of experimental treatments were evaluated by means of the Student's two-tailed *t*-test.

RESULTS

Effect of optimal stimulation with different cytokines and different cytokine combinations on EC adhesiveness for T cells. EC were optimally stimulated for 18 h with IL-1, TNF, IFN-γ, or IL-4, or combinations of any two of these cytokines and adhesion assays carried out with peripheral blood T cells (Table I). As previously reported, stimulation with IL-1, TNF, IFN-γ, or IL-4 alone significantly enhanced EC adhesiveness for T cells ($p < 0.005$ in each case). When EC were stimulated with combinations of TNF + IL-4 or TNF + IFN-γ there was a significant further enhancement of EC adhesiveness for T cells over and above that resulting from TNF stimulation alone (TNF + IL-4 $p < 0.001$; TNF + IFN-γ $p < 0.003$). In each of three experiments the increase in adhesion resulting from TNF + IL-4 stimulation was greater than that seen with TNF + IFN-γ stimulation (Table II). Interestingly, however, combinations of optimal concentrations of TNF + IL-1 or IL-4 + IFN-γ were no more effective than the more potent of each pair at stimulating an increase in EC binding of T cells. Furthermore, stimulation of EC with combinations of IL-1 with IL-4 or IFN-γ did not result in any further enhancement of adhesiveness. In two separate T cell-EC adhesion experiments LT (1000 U/ml) was also found to synergize with IL-4 and IFN-γ in a manner similar to TNF (data not shown).

The effect of combinations of cytokines at suboptimal concentrations on EC adhesiveness for T cells. In order to establish if the effects of cytokine combinations observed on EC adhesiveness for T cells occurred at suboptimal as well as optimal concentrations, dose responses were performed. EC were stimulated for 18 h with different concentrations of cytokine in each of the different combinations and the effect on T cell-EC adhesion was quantified. When used at suboptimal concentrations, combinations of IL-1 + TNF (Fig. 1*a*), IL-1 + IL-4 (Fig. 1*b*), IL-1 + IFN-γ (Fig. 1*d*), and IL-4 + IFN-γ (Fig. 1*f*) all exhibited some enhancement of adhesion compared with

TABLE I

T cell adhesion to EC stimulated with optimal concentrations of different cytokines alone and in combination[a]

Stimulus	Mean ± Adhesion	± SD	Δ[b]	*p*[c]	*p*[d]
PM	25.25	4.10			
TNF	50.45	2.56	25.20	<0.001	
IL-1	48.28	2.29	23.03	<0.001	
IL-4	39.90	2.04	14.65	<0.005	
IFN-γ	39.86	1.94	14.61	<0.005	
TNF + IL-4	68.16	1.06	42.91	<0.001	<0.001
TNF + IFN-γ	63.36	2.34	38.11	<0.001	<0.003
IL-1 + TNF	49.78	5.56	24.53	<0.004	ns[e]
IL-1 + IL-4	47.53	3.72	22.28	<0.002	ns
IL-1 + IFN-γ	47.90	1.60	22.65	<0.001	ns
IL-4 + IFN-γ	43.21	2.67	17.96	<0.005	ns

[a] EC were stimulated for 18 h with PM, IL-1 (20 U/ml), TNF (320 U/ml), IFN-γ (250 U/ml), IL-4 (200 U/ml), or combinations of any two cytokines and the % adhesion of T cells to EC quantified in a 60-min adhesion assay. The mean % adhesion ± SD of triplicates is shown for one experiment out of three similar experiments.

[b] Δ represents the increase in % adhesion (i.e., the % adhesion to stimulated EC − % adhesion to unstimulated EC).

[c] *p* values for the % adhesion to stimulated EC compared with the % adhesion to unstimulated EC.

[d] *p* values for the % adhesion to EC stimulated with a combination of cytokines compared with the % adhesion to EC stimulated with the more effective of the two agents alone.

[e] Not significant.

TABLE II

Increase in T cell adhesion to EC stimulated with TNF, IL-4, IFN-γ, TNF + IL-4, or TNF + IFN-γ[a]

Expt. No.	Δ % Adhesion[b]				
	TNF	IL-4	IFN-γ	TNF + IL-4	TNF + IFN-γ
1	25.20	14.65	14.61	42.91	38.11
2	32.44	15.32	14.96	51.24	41.20
3	32.57	15.78	15.41	51.68	38.70

[a] EC were stimulated for 18 h with PM, TNF (320 U/ml), IL-4 (200 U/ml), IFN-γ (250 U/ml), or combinations of TNF and IL-4 or TNF and IFN-γ and the adhesion of T cells to EC quantified in a 60-min adhesion assay.

[b] Results were expressed as Δ % adhesion (i.e., % adhesion to stimulated EC − % adhesion to unstimulated EC). Results are shown for three separate experiments.

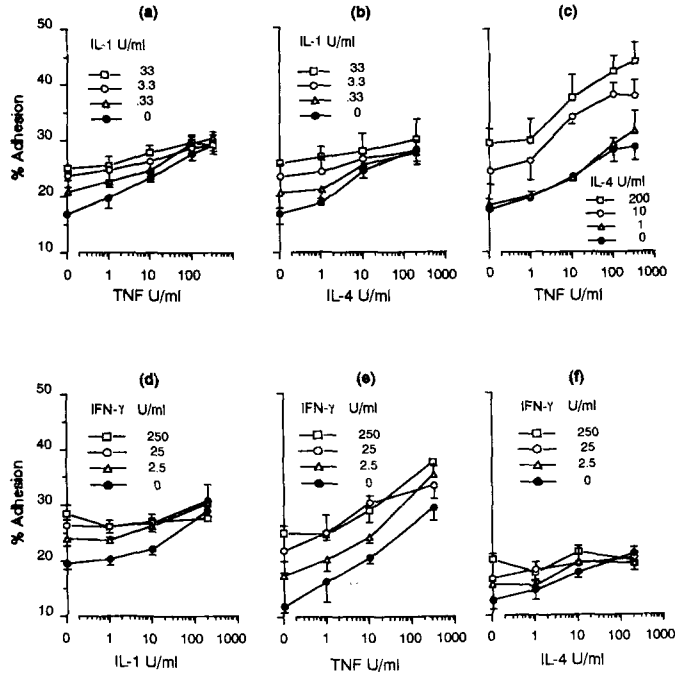

Figure 1. EC were stimulated for 18 h with different concentrations of cytokines in the following combinations: *a*) IL-1 and TNF, *b*) IL-1 and IL-4, *c*) TNF and IL-4, *d*) IL-1 and IFN-γ, *e*) TNF and IFN-γ, *f*) IL-4 and IFN-γ, then washed and used in an adhesion assay with T cells. Values represent the mean % adhesion ± SD of triplicates.

the effect of the single agents. However, in each case the dose response lines converged when the optimal concentration of each cytokine was used. In contrast, when EC were stimulated with combinations of TNF + IL-4 (Fig. 1*c*), their dose-response curves ran approximately parallel, and did not converge even at optimal concentrations, indicating that the effect of these two agents was additive at all concentrations tested. For TNF + IFN-γ-stimulated EC (Fig. 1*e*) the dose response pattern was similar to that of TNF + IL-4 although at optimal concentrations their combined effect was less than additive.

Effect of IL-4 or IFN-γ on TNF-stimulated EC adhesiveness for PMN. We have previously shown that the kinetics of cytokine-enhanced EC adhesiveness for PMN is different to that for T cells. Increased adhesiveness for PMN, induced by IL-1 or TNF peaks after 4 to 6 h stimulation, is starting to fall after 8 h stimulation and is significantly reduced after 24 h stimulation (15). In contrast, the increased adhesiveness for T cells peaks later and is maintained up to 72 h. In order to compare the effects of cytokine combinations on EC adhesiveness for PMN and T cells (Table III), it was therefore necessary to stimulate EC with cytokines for 6 h. At this time point PMN binding is optimal, although T cell adhesion is less than at 18 h. Whether used singly or in combination, all of the cytokines induced a significant increase in EC adhesiveness for T cells ($p < 0.002$ in each case). However, endothelial cell adhesiveness for PMN, although significantly increased by stimulation with TNF ($p < 0.003$) or IL-1 ($p < 0.001$), was not significantly increased by stimulation with IL-4, IFN-γ, or a combination of IL-4 + IFN-γ. Furthermore, although the combination of TNF with either IL-4 or IFN-γ resulted in a significant further enhancement of EC adhesiveness for T cells over and above that produced by TNF alone (TNF + IL-4, $p < 0.01$; TNF + IFN-γ, $p < 0.05$), there was no such further enhancement in adhesiveness for PMN. Indeed, combination of IL-4 with TNF or IL-1 partially inhibited the increase in PMN binding induced by TNF or IL-1 alone. However, out of three similar experiments, the inhibitory effect of IL-4 on IL-1-stimulated PMN-EC adhesion only reached statistical significance in the one experiment shown and the weak inhibitory effect of IL-4 on TNF-stimulated adhesion, although consistent in the three experiments performed, did not reach statistical significance. When the effects of TNF and IL-4 on adhesion of T cells and PMN are viewed together, it can be seen that although TNF alone led to approximately equal proportions of T cells (23.49%) and PMN (22.04%) adhering to EC, the addition of IL-4 led to nearly double the proportion of T cells adhering (29.92%) compared with PMN (15.59%).

The effect of anti-CD18 and anti-VCAM-1 mAb on T cell binding to EC stimulated with TNF + IL-4 or TNF + IFN-γ in combination. Preliminary experiments with the anti-VCAM-1 mAb 1.4C3 indicated that it did not inhibit unstimulated, IL-4-stimulated, or TNF-stimulated T-EC adhesion. However, mAb 1G11, also generated in our laboratory, was found to have inhibitory effects. Because both mAb showed an identical profile of binding to cytokine-stimulated EC, as determined by ELISA (data not shown), the possibility was raised that 1G11 might also recognize VCAM-1. In order to pursue this possibility further, the Ag recognized by mAb 1.4C3 was isolated from a [^{35}S]methionine-labeled lysate of TNF-stimulated EC, using mAb 1.4C3 affinity chromatography. The solubilized 1.4C3 Ag eluted from the column was then immunoprecipitated with mAb 1.4C3 or mAb 1G11, and analyzed by SDS-7.5% PAGE under reducing conditions. As can be seen in Figure 2, both mAb immunoprecipitated the same major band of approximately 105 kDa, together with a minor band of approximately 95 kDa. No significant material was immunoprecipitated from the mAb 1.4C3-Sepharose column eluate by mAb 6.5B5 (anti-ICAM-1). No additional band was identified when mAb 1G11 was used to immunoprecipitate Ag from a TNF-stimulated EC lysate before passage over the 1.4C3 mAb affinity column (not shown). Furthermore, by using mAb 1G11 it was possible to preclear from a lysate of TNF-stimulated EC the Ag recognized by mAb 1.4C3, and vice versa (Table IV).

Having established that the same molecule, VCAM-1, was recognized by mAb 1.4C3 and 1G11, we next investigated the role of VCAM-1 and CD11/CD18 in the increased EC adhesiveness for T cells induced by different cytokines and cytokine combinations. EC were optimally stimulated for 18 h with PM, TNF, IL-4, IFN-γ, TNF + IL-4, or TNF + IFN-γ and then treated with saturating

TABLE III

% Adhesion of PMN and T cells to EC stimulated for 6 h with different cytokine combinations[a]

Stimulus	PMN			T cells		
	Mean ± SD	p[b]	p[c]	Mean ± SD	p[b]	p[c]
PM	4.81 ± 1.38			8.94 ± 0.60		
TNF	22.04 ± 4.24	<0.003		23.49 ± 0.72	<0.001	
IL-1	20.85 ± 2.61	<0.001		22.56 ± 0.97	<0.001	
IL-4	6.39 ± 0.85	ns[d]		15.13 ± 1.02	<0.001	
IFN-γ	7.17 ± 2.46	ns		15.15 ± 1.11	<0.001	
TNF + IL-4	15.59 ± 0.99	<0.001	ns	29.92 ± 1.92	<0.001	<0.01
TNF + IFN-γ	21.04 ± 3.23	<0.001	ns	30.38 ± 3.68	<0.001	<0.05
IL-1 + TNF	20.92 ± 2.83	<0.001	ns	23.72 ± 3.69	<0.002	ns
IL-1 + IL-4	14.97 ± 0.05	<0.001	<0.02	22.93 ± 2.16	<0.001	ns
IL-1 + IFN-γ	24.75 ± 3.95	<0.001	ns	24.16 ± 2.67	<0.001	ns
IL-4 + IFN-γ	5.22 ± 1.87	ns	ns	16.21 ± 0.87	<0.001	ns

[a] EC were stimulated for 6 h with PM, IL-1 (20 U/ml), TNF (320 U/ml), IFN-γ (250 U/ml), IL-4 (200 U/ml), or combinations of any two cytokines and the % adhesion of PMN or T cells to EC quantified in a 30-min adhesion assay. The mean % adhesion ± SD of triplicates is shown for one experiment out of three similar experiments.

[b] p values for the % adhesion to stimulated EC compared with the % adhesion to unstimulated EC.

[c] p values for the % adhesion to EC stimulated with a combination of cytokines compared with the % adhesion to EC stimulated with the more effective of the two agents alone.

[d] Not significant.

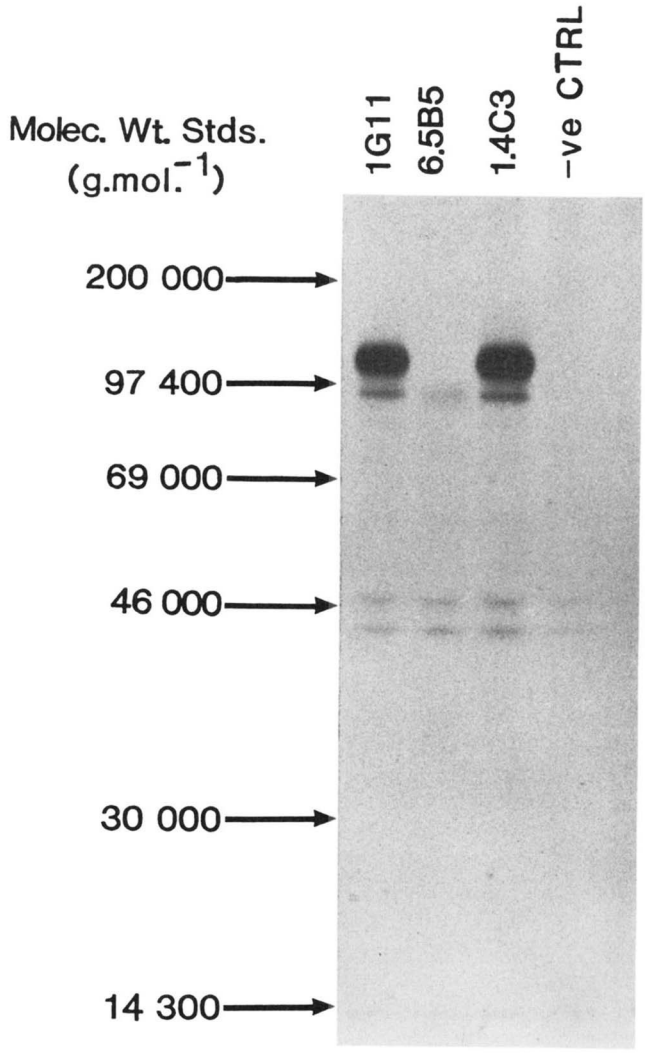

Figure 2. Autoradiograph of SDS-7.5% PAGE of proteins immunoprecipitated from the eluate of a mAb 1.4C3 affinity column. ^{35}S-labeled Ag was eluted from a mAb 1.4C3-Sepharose affinity column and immunoprecipitated with mAb 1G11, mAb 6.5B5 (anti-ICAM-1), or mAb 1.4C3, or control without mAb (−ve CTRL). Immunoprecipitates were then analyzed by SDS-7.5% PAGE and autoradiography. It can be seen that the same major band of approximately 105 kDa was immunoprecipitated by mAb 1.4C3 and 1G11 but not by mAb 6.5B5. Both mAb also immunoprecipitated a minor band of approximately 95 kDa.

TABLE IV

Preclearing of mAb 1.4C3 Ag with mAb 1G11[a]

mAb	Band	Specific Band Activity (cpm)		
		No preclearance	Precleared with mAb 1G11	Precleared with mAb 1.4C3
1.4C3	105 kDa	948	92	0
1G11	105 kDa	649	3	0
6.5B5	90 kDa	879	1319	801

[a] A lysate was made from EC stimulated with TNF (10 ng/ml) for 18 h in the presence of [^{35}S]methionine. The lysate was then precleared with either mAb 1.4C3 or mAb 1G11 before immunoprecipitation with mAb 1.4C3, mAb 1G11, or control mAb 6.5B5 (anti-ICAM-1). After SDS-7.5% PAGE and autoradiography, the visualized bands were cut from the gel and the radioactivity was counted (see *Materials and Methods*).

concentrations of the noninhibitory anti-VCAM-1 mAb 1.4C3, as control, or the inhibitory anti-VCAM-1 mAb 1G11 before use in adhesion assays. T cells were likewise pretreated with PM control or anti-CD18 mAb TS1/18. Compared with control mAb 1.4C3, 1G11 significantly inhibited T cell binding to TNF ($p < 0.01$), IL-4 ($p < 0.001$), TNF + IL-4 ($p < 0.005$), and TNF + IFN-γ ($p < 0.01$) stimulated EC but not to unstimulated or IFN-γ-stimulated EC (Fig. 3). Thus, mAb 1G11 abolished the increase in binding induced by IL-4 stimulation of EC, partially inhibited the increase in binding attributable to TNF stimulation, and had no effect on the increase in adhesion produced by IFN-γ stimulation whether in the presence or absence of anti-CD18 mAb. The effect of mAb 1G11 on T cell adhesion to IL-4 + TNF-stimulated EC was to inhibit the increase in adhesion down to the same level as the mAb inhibited the increase in adhesion induced by TNF alone (Fig. 3*a*). Although the degree of blocking by a single mAb is unlikely to be a simple measure of the proportion of adhesion attributable to a particular ligand, these data suggest that the extra increase in adhesion attributable to stimulation with IL-4 + TNF in combination may be entirely mediated by VCAM-1. In contrast, the effect of mAb 1G11 on T cell

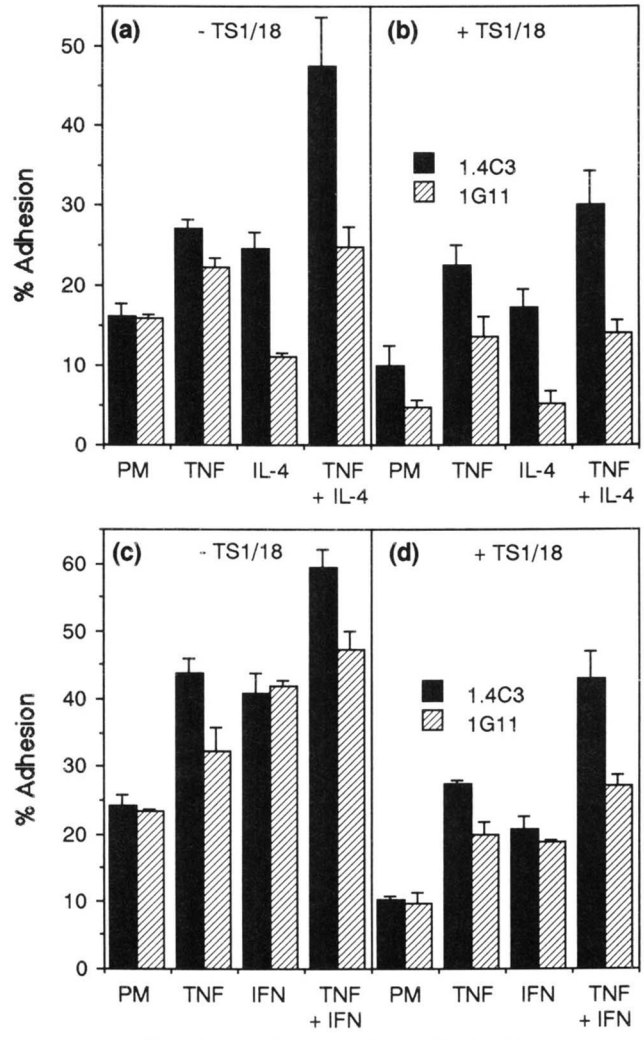

Figure 3. Effect of anti-VCAM-1 and anti-LFA-1 mAb on adhesion of T cells to EC stimulated with cytokine combinations. In one experiment (*a* and *b*), EC were stimulated for 18 h with PM control, TNF (320 U/ml), IL-4 (200 U/ml), or a combination of TNF and IL-4, and in another experiment (*c* and *d*) for 18 h with PM, TNF (320 U/ml), IFN-γ (250 U/ml) or a combination of TNF and IFN-γ. The EC were then washed and incubated for 30 min at 37°C with mAb 1.4C3 (control) or anti-VCAM-1 mAb 1G11 and used in adhesion assays with T cells preincubated for 30 min at 21°C with PM, (*a* and *c*), or anti-CD18 mAb TS1/18, (*b* and *d*). All mAb were used at 10 times the saturating dilution as determined by flow microfluorometry for T cells or ELISA for EC and were present during the adhesion assay as well as the preincubation step. Results are expressed as mean % adhesion ± SD of triplicates and are representative of four similar experiments for TNF and IL-4-stimulated EC and three for IFN-γ-stimulated EC.

adhesion to IFN-γ + TNF-stimulated EC was to inhibit the increase in adhesion by the same decrement as it inhibited the increase in adhesion caused by TNF alone, with the extra increase in binding attributable to stimulation with the combination remaining unaffected (Fig. 3c). Anti-CD18 mAb inhibited all levels of binding but had little effect on the increase in adhesion attributable to stimulation with individual or combinations of cytokines (Fig. 3, *b* and *d*). Finally, even in the presence of TS1/18 and 1G11 mAb together, the extra increase in adhesion induced by combinations of TNF and IFN-γ was not completely inhibited.

DISCUSSION

Previous studies on the role of cytokines in the control of T cell-EC adhesion have shown that the four cytokines studied here fall broadly into two pairs (3, 4, 13–15). The first pair consists of IL-1 and TNF, which have optimal actions 8 to 10 h after stimulation of EC. In contrast, the lymphokines IFN-γ and IL-4 have a delayed effect, with optimal actions at 18 to 24 h. The experiments reported in this paper support this classification by showing that TNF has additive effects on EC adhesiveness for T cells in conjunction with either IL-4 or IFN-γ, but that at optimal concentrations IL-1 + TNF or IL-4 + IFN-γ are no more effective than the more potent of each pair alone in enhancing EC adhesiveness for T cells.

Cytokine-inducible endothelial determinants responsible for T cell adhesion in this model have now been identified and cloned. Both ICAM-1 (CD54) (18, 33, 34) and VCAM-1 (or inducible cell adhesion molecule-110) (21, 22, 35) are members of the Ig supergene family and bind, respectively, to LFA-1 (CD11a/CD18) (24) and very late activation Ag-4 (CD49d/CD29) (36) on T cells. Although both ICAM-1 and VCAM-1 are induced or up-regulated by IL-1 or TNF (19, 21, 35, 37), only ICAM-1 is responsive to IFN-γ (18, 19), and only VCAM-1 is induced by IL-4 (20). Furthermore, we have recently reported that the effect of IL-4 is additive with that of TNF or IL-1 for the induction of VCAM-1, but that IL-4 partially inhibits TNF-, IL-1-, or IFN-γ-stimulated ICAM-1 expression (20).

In our original characterization of the 1.4C3 Ag we reported a molecular mass of approximately 95 kDa (19). Since this work was published we have become aware that the m.w. of ^{14}C-methylated phosphorylase b, which was used in our SDS-PAGE experiments as a marker, has been revised by the manufacturers from 92.5 kDa to 97.4 kDa. The adjusted molecular mass for the 1.4C3 Ag is therefore approximately 105 kDa. The size of the major band immunoprecipitated from the eluate of the 1.4C3 mAb affinity column in the present study is therefore consistent with the previous result. The identification of the 95 kDa minor band in the present study is likely to be related to the purity of the material eluted from the mAb affinity column and the resulting capacity to distinguish the band from background on examination of the autoradiograph. The molecular masses of the major and minor bands are therefore similar to those previously published for VCAM-1 (35).

We have described in this paper a novel anti-VCAM-1 mAb 1G11, which, unlike mAb 1.4C3, is capable of fully inhibiting enhanced T cell adhesion to IL-4-stimulated EC and partially inhibiting enhanced T cell adhesion to TNF-stimulated EC. With this mAb, together with the anti-CD18 mAb TS1/18, we have been able to dissect the molecular basis of the response to combinations of cytokines that additively enhance EC adhesiveness for T cells. We have observed that T cell binding to IL-4 + TNF-stimulated EC, in the presence of mAb 1G11, is not greater than to EC stimulated with TNF alone, suggesting that the additive effect of this combination may be attributable to augmented VCAM-1 induction. In contrast, T cell adhesion to IFN-γ + TNF-stimulated EC was significantly greater than to TNF-stimulated EC either in the presence or absence of anti-VCAM-1 and/or anti-CD18 mAb, suggesting the involvement of another as yet unidentified cytokine-inducible T cell ligand that is regulated by IFN-γ and perhaps also by TNF.

Until this study, the observed effects of IL-1 in enhancing EC adhesiveness for T cells have been similar to those of TNF. Thus, Cavender et al. observed that TNF + IL-1 were not additive at optimal concentrations, suggesting that they may stimulate the same intracellular activation pathway (3). Furthermore, cross-panning experiments have shown that the same subset of T cells binds to EC stimulated by either cytokine (38). We have found, however, that the effects of IL-1 and TNF are not identical because, in contrast to TNF, IL-1 was unable to cause additive effects with either IL-4 or IFN-γ. At present, the reason IL-1 and IL-4 do not additively enhance EC adhesiveness for T cells is unclear, but may relate to IL-1 being a relatively weak stimulus compared with TNF for VCAM-1 induction, particularly at 18 h or longer after stimulation (19, 20). The explanation for the lack of additive effect of IL-1 and IFN-γ will await the identification of the putative LFA-1 and VCAM-1-independent EC adhesion molecule induced by IFN-γ. We are currently investigating whether this is HLA-DR, as suggested by a previous publication (39).

The data in this paper show that although both IL-4 and IFN-γ each selectively enhance EC adhesiveness for T cells without enhancing EC adhesiveness for PMN, the two lymphokines do so by different mechanisms. Effects of IFN-γ and IL-4 on other EC functions are also dissimilar, with IFN-γ but not IL-4 being capable of up-regulating HLA class I and inducing HLA class II expression on EC (19, 40). Furthermore IL-4 but not IFN-γ is able to down-regulate EC expression of ICAM-1 and ELAM-1 (19). It was therefore perhaps surprising that the effects of optimal concentrations of IFN-γ and IL-4 on EC adhesiveness for T cells were not additive. As with the combination of IL-1 + IFN-γ, the explanation for this observation awaits the further identification of the adhesion molecules involved.

Just as IL-4 and IFN-γ do not enhance the adhesiveness of EC for PMN, they failed to increase the numbers of PMN binding to TNF- or IL-1-stimulated EC. Indeed, there was a tendency for IL-4 to inhibit TNF- or IL-1-stimulated PMN-EC adhesion, although on the whole these results were not statistically significant. However, although approximately equal proportions of T cells and PMN-bound TNF stimulated EC, nearly double the proportion of T cells compared with PMN bound EC stimulated with both TNF and IL-4. This phenomenon is likely to be caused not only by the positive effects of IL-4 and TNF on T cell-EC adhesion discussed above, but also by the ability of IL-4 to partially inhibit TNF- or IL-1-stimulated ELAM-1 or ICAM-1 induction (19).

The outcome of these studies investigating the effects of cytokine interactions on EC adhesiveness for T cells and PMN is the suggestion that the combination of IL-4, and to a lesser extent IFN-γ, with TNF may be important in causing an alteration in the EC membrane in response to TNF away from one suited to the adhesion of PMN toward one more suited to the binding of T cells. The controlling influence of the lymphokines IL-4 and IFN-γ on the action of TNF on EC may therefore be a major contributing factor to the development of a lymphocytic infiltrate at sites of immune mediated inflammation.

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