Tumor Necrosis Factor and Interferon-γ Induce Distinct Patterns of Endothelial Activation and Associated Leukocyte Accumulation in Skin of *Papio Anubis*

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*Recombinant human interferon (IFN)-γ (2 × 10⁴ or 2 × 10⁵ U), tumor necrosis factor (TNF, 10⁴ or 10⁵ U), or both were injected intracutaneously into baboons (*Papio anubis*), and biopsies were examined at various intervals for evidence of altered endothelial cell antigen expression, endothelial morphology, and leukocyte infiltration. IFN-γ induced increased binding of anti-HLA-DP mAb by 24 hours and a mild-to-moderate accumulation of mononuclear cells. TNF induced increased binding of anti-endothelial leukocyte adhesion molecule (ELAM)-1 mAb by 2 hours that was associated with polymorphonuclear leukocyte accumulation, and increased binding of anti-intercellular adhesion molecule (ICAM)-1 mAb by 9 hours that was associated with the onset of progressive mononuclear leukocyte accumulation. TNF also caused endothelial cell hypertrophy and increased vascular permeability. The combination of IFN-γ and TNF induced a set of changes that qualitatively resemble those of a delayed hypersensitivity reaction to simian agent 8 envelope antigen. These findings are consistent with the concept that cytokine-activated endothelium plays an important role in the adhesion and subsequent extravasation of leukocytes during immune inflammation. (Am J Pathol 1989, 135: 121–133)*

During cell-mediated immune inflammation, endothelial cells of postcapillary venules synthesize new proteins normally absent from resting vascular beds[1-4] and develop a characteristically altered morphology.[5-7] These changes are manifestations of a process that has been termed endothelial activation,[5,8,9] reflecting alterations in endothelial cell functions, such as heightened leukocyte adhesion and emigration. The *in vivo* inflammatory endothelial cell changes are paralleled by *in vitro* phenotypic[10] and morphologic[11,12] alterations that can be induced by exposure to cytokines. Moreover, there is evidence that certain cytokine-induced surface molecules expressed on cultured endothelial cells mediate enhanced leukocyte adhesion.[13,14] Specifically, *de novo* expression of endothelial leukocyte adhesion molecule (ELAM)-1[14,15] and increased expression of intercellular adhesion molecule (ICAM)-1[15] are both induced on cultured endothelial cells by tumor necrosis factor (TNF) and interleukin (IL-)1. ICAM-1 (but not ELAM-1) can also be increased by interferon (IFN)-γ, albeit to a lesser extent than by TNF or IL-1.[15] ELAM-1 and ICAM-1 have been shown to mediate, at least in part, adhesion of polymorphonuclear leukocytes (PMN)[14] and lymphocytes,[16,17] respectively, although the importance of ICAM-1-dependent lymphocyte binding may be relatively minor. IFN-γ,[18,19] but not TNF or IL-1,[20-22] additionally induces endothelial HLA class II antigens, for which a role in the adhesion of T lymphocytes has been claimed.[23]

Based on these considerations, we postulated that during inflammation, local extravascular cells release cytokines that cause venular endothelial cells to become activated and to express new surface proteins or increased quantities of constitutive surface proteins that are involved in the adhesion of circulating leukocytes, a critical prelude to their emigration. Indeed, Dumonde et al[24] found that crude cytokine preparations injected into human skin induce endothelial morphologic changes similar to those occurring in immune reactions,[5-7] as well as in PMN and mononuclear cell accumulation. To test our hy-

pothesis, we intracutaneously injected human recombinant TNF and IFN-γ into baboons of the species *Papio anubis*. This model was selected because from preliminary observations we know that the human cytokines under study are biologically active on baboon cells and that monoclonal antibodies (mAbs) reactive with human endothelial and leukocyte antigens can be used to recognize homologous antigens in this species. Our initial studies focused on TNF and IFN-γ because these two cytokines are characteristically produced by those activated T lymphocytes that adoptively transfer cell-mediated immune responses.[25-27] Biopsies from injected sites were taken at set intervals, and specimens were examined by routine histology, immunohistochemistry, and electron microscopy. Here we report that these cytokines induced endothelial phenotypic changes and leukocyte accumulation. IFN-γ led to increased anti-HLA-DP binding by endothelial cells and modest mononuclear cell accumulation. TNF caused early endothelial binding of an antibody directed against ELAM-1 that correlated with PMN accumulation, and later increased endothelial binding of anti-ICAM-1, which correlated with mononuclear cell accumulation. The combination of TNF and IFN-γ induced mononuclear cell infiltration and changes in ELAM-1, ICAM-1, and HLA-DP expression that resembled those induced in a delayed hypersensitivity response to antigen.

Materials and Methods

Animals

Five healthy adult baboons (two males and three females) of the species *Papio anubis* housed at the New England Regional Primate Research Center were used; all injections and biopsies were performed under intramuscular ketamine anesthesia. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School, which approved the experimental protocol.

Antigen and Cytokines

Delayed hypersensitivity reactions were induced with 50 μg of simian agent 8 envelope antigen (provided by J. Hilliard, Southwest Foundation, San Antonio, TX) diluted in saline for injection (Lyphomed Inc., Rosemont, IL). This antigen is derived from a virus to which animals of the species *Papio anubis* have been exposed.[28] Cytokines used were purified preparations of carrier-free human recombinant IFN-γ at 2×10^4 or 2×10^5 U (3.35×10^7 U/mg protein, provided by Biogen Inc., Cambridge, MA) and carrier-free human recombinant TNF at 10^4 or 10^5 U (2.5

$\times 10^7$ U/mg protein, provided by W. Fiers, State University of Ghent, Belgium), diluted in saline as above. This TNF preparation showed no evidence of endotoxin activity as measured in *in vitro* endothelial cell assays, and did not show the *in vivo* actions described below when heat denatured before injection.

Injection and Biopsy Procedures

Skin to be injected was sheared and cleaned with iodine solution and 70% isopropyl alcohol. Antigen, cytokines, and saline controls were injected intracutaneously into the backs of animals in a volume of 0.1 ml using a 28-gauge insulin syringe (Becton Dickinson, Rochelle Park, NJ), and the sites were marked with a circle of indelible ink. When different cytokines were given simultaneously to different sites, these were injected at least 8 cm apart, whereas different injections of the same cytokine were sited at least 4 cm apart. Injections were given in batches to most animals, and further injections to the same individual animals were not given within 14 days of previous injection. Specimens were taken using a 1 cm diameter biopsy punch and bisected, with half being fixed in 10% buffered formalin and the other half mounted in OCT compound and snap frozen in isopentane/liquid nitrogen. Biopsies were taken at combinations of 0, 2, 6, 9, 12, 24, and 48 hours. One-to-six biopsy sites were examined for each time and test substance.

Routine Histology and Electron Microscopy

Formalin-fixed tissue was routinely processed in paraffin and sections were stained with hematoxylin and eosin (H&E). These were scored blindly (by two observers; J.M.M. and R.S.C.) for the intensity of extravascular cellular accumulation using set scales for PMN and mononuclear cells. The independently assessed scores were comparable, and one set (that of JMM) is shown in the results section. PMN accumulation was assessed separately in the upper two thirds of the dermis and the lower third of the dermis combined with the subcutaneous tissue. These areas were scored as follows: 0, less than 6 PMN present; 1, 6 to 40 PMN present; 2, more than 40 PMN present, with moderate numbers of focal collections and/or relatively few scattered; 3, more than 40 PMN present with extensive foci and relatively few PMN scattered or relatively few foci and moderate numbers scattered; and 4, extensive foci and marked numbers of scattered PMN. The scores for the two areas assessed were then added to provide a total score for the section (possible range 0 to 8). Mononuclear cell accumulation was also assessed separately in the two areas noted above, as

follows: 1, no increase in mononuclear cells; 2, slight increase; 3, moderate increase; 4, moderate-to-marked increase; and 5, marked increase. The scores for both areas were added to provide a score for the whole section, and 1 unit subtracted from this total (so that the baseline score for the few mononuclear cells present in control skin be set at 1 rather than 2, with a possible range, therefore, of 1 to 9). Blocks of selected biopsies were processed for electron microscopy after fixation by 2% paraformaldehyde/2.5% glutaraldehyde in cacodylate buffer, using standard techniques.

Immunohistochemistry

Cryostat sections (6 μ) were air dried, fixed in acetone for 5 minutes, and air dried again. Primary antibodies diluted in phosphate-buffered saline/1% fetal bovine serum/0.1% sodium azide were applied for 60 minutes, the sections washed twice in phosphate-buffered saline/0.2% gelatin, and exposed to peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako Corp., Santa Barbara, CA) in the same diluent as primary antibodies, including 1% baboon serum, for 60 minutes. The sections were then washed again, exposed to 0.25 mg/ml 3-amino-9-ethylcarbazole (Aldrich, Milwaukee, WI) in 2% N,N-dimethylformamide/0.1M acetate buffer (pH 5.2) with 0.03% H_2O_2 for 6 minutes, washed in water, and counterstained with hematoxylin. Preliminary studies indicated that the murine mAbs listed below recognize antigens in the tissue of *Papio anubis*.

The mAbs used were H4/18 (anti-ELAM-1); RR1/1 (anti-ICAM-1) provided by T. Springer (Dana-Farber Cancer Institute, Boston, MA); B7/21 (anti-HLA-DP) and Leu2a (anti-CD8) obtained from Becton Dickinson (Mountain View, CA); LB3.1 (anti-HLA-DR) provided by J. L. Strominger (Harvard University, Cambridge, MA); T11 (anti-CD 2), T4 (anti-CD4) and B1 (anti-CD20, B cells) obtained from Coulter (Hialeah, FL); anti-Tac provided by T. Waldmann (National Institutes of Health, Bethesda, MD); and EBM/11 (anti-monocyte/macrophage) obtained from Dako Corp. A control mAb of irrelevant specificity was also used. Initial studies revealed intense staining of normal cutaneous endothelial cells using anti-HLA-DR; basal anti-HLA-DP (B7/21) binding was weaker, and thus considered to be more appropriate for assessment of potential increased antigen expression. Initial studies also revealed basal staining of endothelium with anti-ICAM-I (RR1/1). Based on preliminary experiments, dilutions of B7/21 and RR1/1 that generally gave weak or no staining in control skin were selected, these dilutions allowing assessment of cytokine-mediated increases in antigen expression. For other mAbs, optimal concentrations for staining were used. Endothelial binding of H4/18, RR1/1,

and B7/21 was scored blindly (by J.M.M.) on the basis of extent and intensity of microvascular (excluding arteriolar) staining using set scales that ranged from 0 to 6. These sections were also scored blindly by a second observer (J.S.P.), and major differences in results resolved by conference. The following scoring system was used for H4/18 binding: 0, no binding; 1, 1-to-4 vessels positive; 2, 5-to-10 vessels positive; 3, 11-to-20 vessels positive, generally staining weakly; 4, 11-to-20 vessels positive, generally with moderate-to-strong staining; 5, more than 20 vessels positive, generally with weak staining; and 6, more than 20 vessels positive, generally with moderate-to-strong staining. Endothelial RR 1/1 binding was scored as follows: 0, no binding; 1, 1-to-5 vessels positive; 2, 6-to-10 vessels positive, generally with weak staining; 3, 6-to-10 vessels positive, generally with moderate-to-strong staining; 4, 11-to-20 vessels positive, generally with weak staining; 5, 11-to-20 vessels positive, generally with moderate-to-strong staining, or more than 20 vessels positive, generally with weak staining; and 6, more than 20 vessels positive, generally with moderate-to-strong staining. B7/21 binding was scored on the basis of the most strongly staining vessels: 0, negative; 2, weak staining; 4, moderate staining; and 6, strong staining.

Results

Normal Skin and Delayed-Hypersensitivity Reactions

Binding patterns of mAbs in the normal skin of *Papio anubis* were consistent with those expected in human skin. Anti-ELAM-1 did not bind to control endothelium (Figure 1A, Table 1) and, at the antibody concentrations used, the endothelium of most vessels was negative with anti-ICAM-1 (Figure 1C, Table 1), although rare vessels were faintly positive in some sections. Baseline endothelial anti-HLA-DP binding was variable among different animals, being undetectable in three of four animals with the standard techniques used (Figure 1E, Table 1), and diffusely present in venules in the fourth. H&E-stained sections revealed that uninjected skin contained a few mononuclear cells (typically perivascularly in the dermis) and no PMN (Table 1). Some of these resident leukocytes were CD2+, with both CD4+ and CD8+ subpopulations being represented. Rare cells stained weakly with anti-Tac. Scattered cells positive with EBM/11 (anti-macrophage) were also present in normal skin. No cutaneous CD20+ cells (B cells) were identified, although the antibody did stain B cells in baboon lymph node follicles. Injection of simian agent 8 envelope induced antigen by 24 to 48 hours endothelial binding of anti-ELAM-1, anti-ICAM-1, and anti-HLA-DP, endothelial hypertrophy, and a dense perivascu-
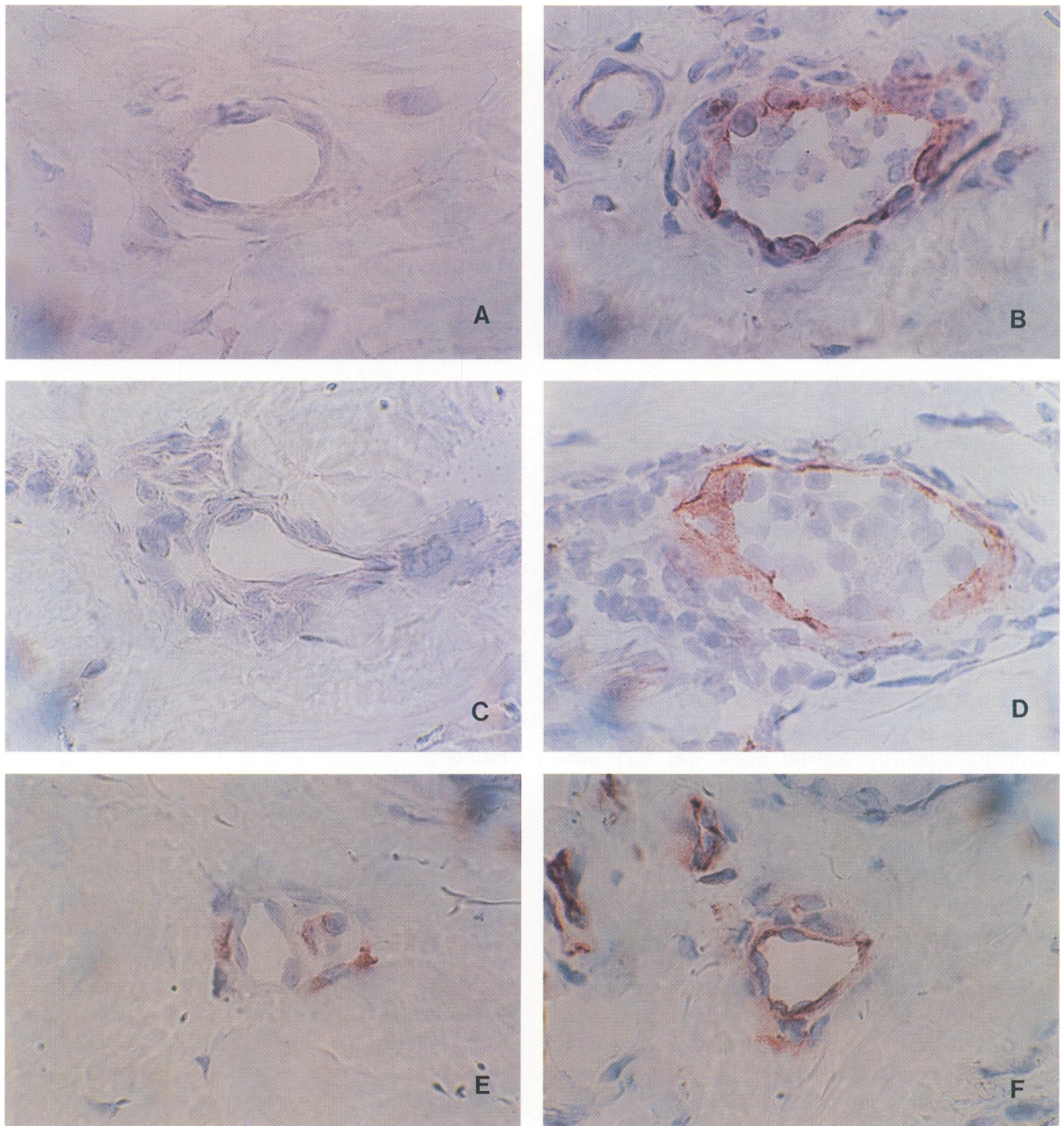
Figure 1. *Immunohistochemical assessment of endothelial antigens in cryostat sections of untreated and cytokine injected skin (each pair of micrographs is from specimens of the same animal) stained with antibodies to human: ELAM-1 (A and B); ICAM-1 (C and D); and HLA-DP (E and F).* **A:** *Control venule unstained after application of anti-ELAM-1.* **B:** *Two hours after injection of TNF (10⁴ U), anti-ELAM-1+ venule is now seen, with adherent intravascular PMN. An adjacent capillary (top left of panel) is negative.* **C:** *Venule in control skin without visible staining using anti-ICAM-1.* **D:** *24 hours after TNF (10⁵ U) there is marked anti-ICAM-1 binding to a venule that contains and is surrounded by mononuclear cells.* **E:** *Microvessel in control skin is negative with anti-HLA-DP.* **F:** *48 hours after injection of 2 × 10⁵ U IFN-γ, anti-HLA-DP binding is now seen (all counterstained with hematoxylin, ×650.)*

lar accumulation of mononuclear cells (Table 1), most which were CD2+.

Effects of TNF Injection

Preliminary experiments established that carrier-free human rTNF at 10^4 U per injection site was the minimum dose at which histologically evident reactions were routinely evoked. TNF (10^4 U) injection was followed by the onset of marked endothelial binding of anti-ELAM-1 by 2 hours and increased binding of anti-ICAM-1 between 6 and 9 hours (Figures 1B, 1D, and 2, Table 1). Anti-ELAM-1 binding was somewhat diminished by 24 hours, but not completely absent (Figure 2, Table 1). Venular endothelial cell hypertrophy (Figure 3C), visible in paraffin sections,

Table 1. *Semiquantitative Scores of Cutaneous Endothelial Antigen Expression and Cellular Accumulation in Baboons (Mean Score and Number of Biopsies Studied)*

| Injection	Time	Endothelial antigen expression			Cellular accumulation	
		ELAM-1	ICAM-1	HLA-DP*	PMN	Mononuclear
None (control)		0.3 (4)	0.8 (4)	0.0 (3)	0.0 (4)	1.3 (4)
Saline	24 hours	0.0 (5)	0.8 (5)	0.0 (4)	0.2 (6)	1.5 (6)
	48 hours	0.0 (2)	1.0 (2)	0.0 (1)	0.0 (2)	2.0 (2)
SA8	24 hours	3.0 (2)	3.5 (2)	4.0 (1)	2.5 (2)	6.5 (2)
antigen	48 hours	2.8 (4)	4.5 (4)	1.0 (2)	1.5 (4)	8.3 (4)
TNF (10⁴ U)	24 hours	2.7 (3)	4.0 (2)	0.0 (3)	2.8 (4)	3.5 (4)
TNF (10⁵ U)	24 hours	6.0 (2)	5.0 (2)	0.0 (1)	4.7 (3)	6.0 (3)
	48 hours	0.5 (2)	2.5 (2)	0.0 (1)	3.5 (2)	6.5 (2)
IFN-γ (2 × 10⁴ U)	24 hours	0.5 (2)	1.5 (2)	1.0 (2)	0.0 (3)	1.7 (3)
IFN-γ (2 × 10⁵ U)	24 hours	2.3 (3)	0.7 (3)	2.0 (2)	1.3 (4)	3.0 (4)
	48 hours	0.0 (3)	0.7 (3)	3.0 (2)	0.0 (3)	3.7 (3)
TNF (10⁴ U) and IFN-γ (2 × 10⁴ U)	24 hours	4.7 (3)	4.3 (3)	0.7 (3)	3.8 (4)	4.3 (4)
TNF (10⁵ U) and IFN-γ (2 × 10⁵ U)	24 hours	4.5 (2)	6.0 (2)	2.0 (1)	3.7 (3)	3.7 (3)
	48 hours	2.0 (2)	6.0 (2)	4.0 (1)	6.0 (3)	4.0 (2)

* HLA-DP results from one animal, in which strong basal expression was present, are not included. In this animal HLA-DP expression was increased further after administration of SA8 antigen, IFN-γ, and combined TNF and IFN-γ, but not after saline or TNF alone.

developed by 24 hours, particularly after 10⁵ U TNF. The onset of induced anti-ELAM-1 and anti-ICAM-1 binding appeared coincident with accumulation of PMN and mononuclear cells, respectively (Figure 2). By 2 hours postinjection, PMN were adherent within and extravasating from postcapillary venules (Figure 3A), which were dilated in the region of the subcutis. The PMN were mostly neutrophils with a smaller proportion of eosinophils. Mononuclear cells appeared to begin to accumulate by 9 hours in the animal studied at early time points. The mononuclear cell infiltrate was maximal at 24 to 48 hours (Figures 3B and 3C). The nature of the mononuclear cell infiltrates was deduced from marker studies, approximately half of them binding anti-CD2 (Figure 3D). CD4+ and CD8+ cells were both present, without a clear numerical predominance of either. Scattered cells staining with anti-Tac were also seen, but CD20+ cells were not. Monocytes/macrophages identified by EBM/11 appeared to account for the remainder of the mononuclear cell infiltrate. Accumulating leukocytes were generally seen around small vessels or adjacent to skin appendages, although subcutaneous PMN accumulation was generally more diffuse than that in the dermis. PMN also collected focally within skin appendages at late times.

Effects of IFN-γ Injection

Preliminary experiments established that carrier-free human rIFN-γ at 2 × 10⁴ U per injection site was the mini-

mum dose at which histologically evident reactions were routinely evoked. IFN-γ at 2 × 10⁴ or 2 × 10⁵ U induced an increase in endothelial binding of anti-HLA-DP by 24 to
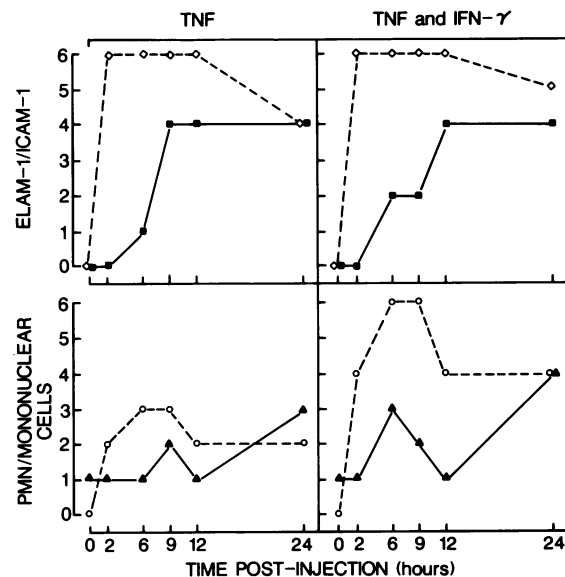
Figure 2. *Semiquantitative scores for cutaneous endothelial antigenic changes and associated leukocyte accumulation after injection of either TNF (10⁴ U) alone or TNF (10⁴ U) combined with IFN-γ (2 × 10⁴ U), all of which were injected simultaneously into the same animal. ◇----◇: Anti-ELAM-1 binding; ■——■: anti-ICAM-1 binding; ○----○: PMN accumulation; ▲——▲: mononuclear cell accumulation. Two additional time-course studies in other animals were performed with combined TNF (10⁵ U) and IFN-γ (2 × 10⁵ U) that gave similar results, ie, an early binding of anti-ELAM-1 and influx of PMN, and later increased binding of anti-ICAM-1 and mononuclear cell accumulation.*
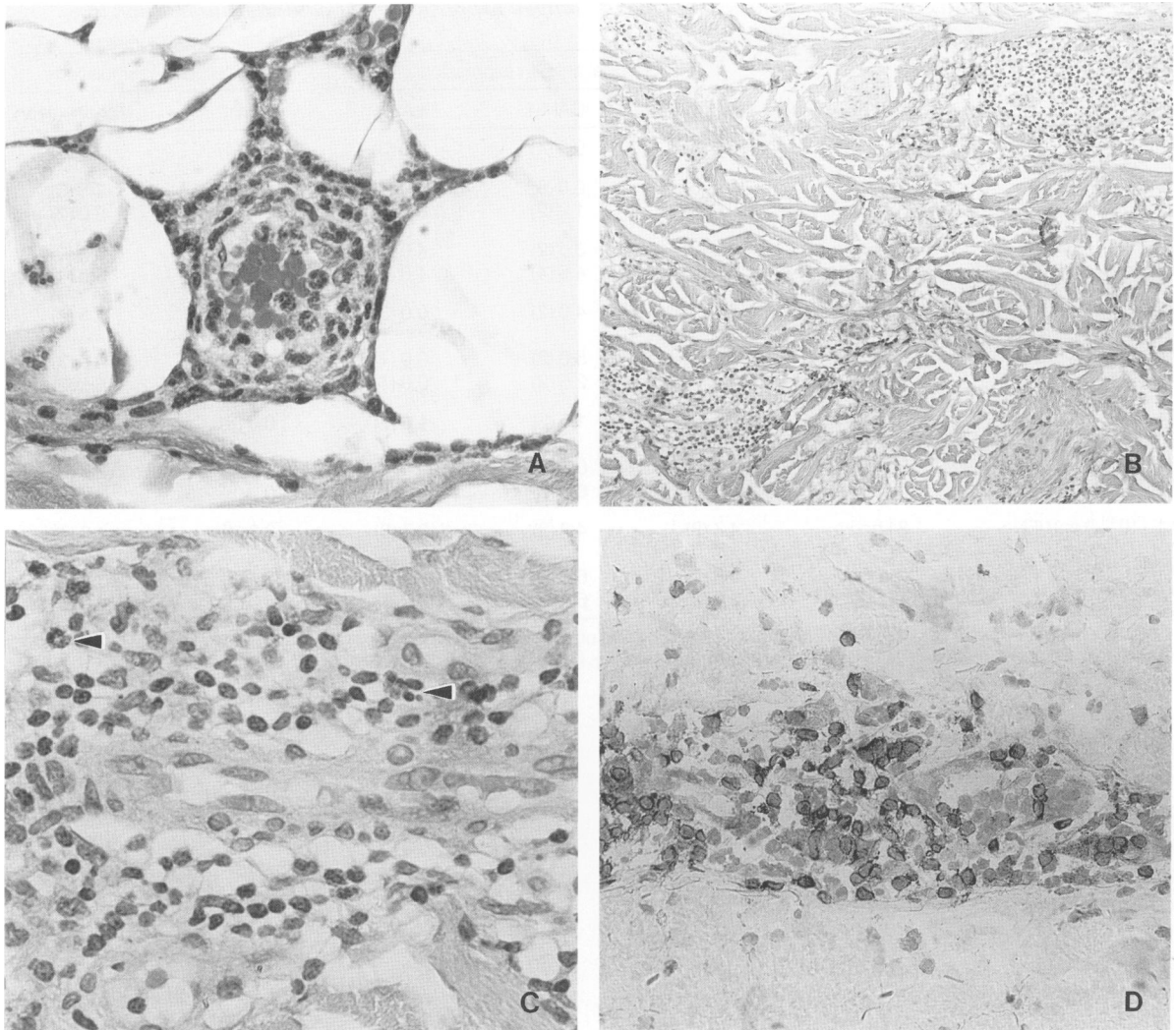
Figure 3. *Leukocyte accumulation in TNF-injected baboon skin as assessed by H&E stained paraffin sections, except D, which is an immunostained cryostat section. A: Subcutaneous venule with extravasating PMN 6 hours after treatment with TNF ($10^4 U$, ×300). B: Mononuclear cell accumulation 48 hours after TNF ($10^5 U$, ×100). C: High-power view of the same section seen in B. There is a central venule with hypertrophic endothelium. The leukocytes surrounding this vessel are preponderantly mononuclear cells, with occasional PMN (arrowheads, ×400). D: Section of skin injected 24 hours earlier with TNF ($10^5 U$) showing numerous perivascular CD2+ cells (hematoxylin counterstain, ×250).*

48 hours (Figure 1F, Table 1) but little anti-ELAM-1 binding and essentially no increased anti-ICAM-1 binding (Table 1). H&E-stained sections did demonstrate a modest accumulation of mononuclear cells (Table 1) by 12 hours but virtually no PMN accumulation. Although the changes induced by IFN-γ were mild and their onset difficult to establish with certainty, it appears that mononuclear cell infiltration precedes the onset of increased anti-HLA-DP binding. The mononuclear cells were mostly CD2+. CD4+ and CD8+ cells were present in roughly similar proportions, and occasional Tac+ cells were seen. CD20+ cells were not identified.

Effects of Combined TNF and IFN-γ Injection

Combined injection of TNF and IFN-γ, in general, produced results similar to those of TNF alone. Specifically,

after combined cytokines at higher or lower doses, there was, as was the case after injection of TNF alone, resultant endothelial binding of anti-ELAM-1 and increased binding of anti-ICAM-1 (Table 1). However, the intensity of the endothelial antigenic changes at 24 and 48 hours was more marked when TNF was given in combination with IFN-γ than after TNF alone (Table 1). In addition, in two of three cases, combinations of TNF and IFN-γ at both dose levels led to increased anti-ICAM-1 binding as early as 6 hours, rather than at 9 hours when the TNF was given alone. Endothelial cell hypertrophy was visible by 24 hours. PMN, mostly neutrophils, were seen emigrating from dilated subcutaneous and deep dermal venules 2 hours after injection. The accumulation of PMN was enhanced after TNF (10^4 U) and IFN-γ ($2 × 10^4$ U), in comparison with TNF (10^4 U) alone (Figure 2). Mononuclear

cells began to accumulate by 6 hours after injection. The number of mononuclear cells present 12 hours after administration of TNF (10^4 U), combined with IFN-γ, (2×10^4 U), was less than that at 9 hours after injection, as was the case after administration of TNF (10^4 U) alone (Figure 2). This suggests a biphasic response. However, at higher doses of combined cytokines, this pattern was not observed. The accumulating mononuclear cells were predominantly CD2+, with CD4+ and CD8+ cells present in similar proportions, and scattered Tac+ cells seen. Increased numbers of EBM/11+ mononuclear cells also accumulated, but no CD20+ cells were present. Detectable diffuse keratinocyte anti-ICAM-1 binding developed in animals treated with combined cytokines at both higher and lower doses. In contrast to endothelial staining, no keratinocyte anti-ICAM-1 binding was noted at sites injected with single cytokine. In both animals given the higher doses of combined cytokines, focal necrosis was seen in the epidermis and epithelium of appendages at 48 hours, in association with dense PMN accumulation at these sites.

Ultrastructural Studies

Electron microscopy was performed on selected skin blocks 2, 6, 9, 12, and 24 hours after injection with 10^4 U TNF or 10^4 U TNF and 2×10^4 U IFN-γ. The induced alterations were similar after TNF alone or combined cytokines, and will be described together. At 2 hours, postcapillary venules demonstrated neutrophils in various states of adhesion and emigration (Figure 4). At 9 to 12 hours lymphocytes and monocytes were seen adhering to and emigrating across endothelium (Figures 5A and 5B). There was focal cytoplasmic swelling of some endothelial cells. Although no endothelial gaps were clearly detected in the sections examined, evidence of increased permeability was apparent by the presence of amorphous, presumably proteinaceous, material in the vessel wall (Figure 5A). At 12 and 24 hours, fibrin was present in the interstitium (Figure 5A). By 24 hours, some venules had hypertrophied endothelial cells that bulged into the lumen and exhibited focally dilated endoplasmic reticulum and increased numbers of organelles (Figure 6).

Discussion

These studies indicate that recombinant cytokines can induce *in vivo* endothelial cell alterations and leukocytic accumulation that resemble those occurring during antigen-induced inflammation in sensitized animals. IFN-γ on its own caused increased expression of endothelial HLA-DP and accumulation of relatively few mononuclear cells.

Both *de novo* endothelial cell ELAM-1 expression and increased ICAM-1 expression were induced by TNF, which also led to the accumulation of large numbers of PMN and mononuclear cells. The addition of IFN-γ to TNF led to an earlier expression of ICAM-1 and somewhat enhanced endothelial antigen expression at later times (Figure 2, Table 1). Furthermore, the combination caused greater PMN accumulation than after TNF alone (Figure 2, Table 1). Mononuclear cell accumulation was, however, not consistently augmented with the combination (Table 1).

The cytokine-induced endothelial antigenic changes *in situ* were similar to those induced on cultured human endothelial cells.[15,18,20,22] Overall, the results of *in vivo* injections of cytokines were that early endothelial expression of ELAM-1 was associated with the onset of PMN accumulation, and later increased expression of ICAM-1 was associated with the onset of mononuclear cell accumulation (Figure 2, Table 1). This is consistent with the hypothesis that these molecules may be important in mediating the adhesion of the corresponding leukocyte populations. These findings are generally in keeping with *in vitro* data concerning leukocyte adhesion,[14,16] although there is evidence that endothelial ICAM-1 plays a role in the adhesion of neutrophils.[29] While it may indeed be the case that constitutively expressed levels of ICAM-1 play a part in the attachment of circulating neutrophils *in vivo,* we found that early PMN margination and emigration preceded the onset of increased ICAM-1 expression. It should also be noted that modest mononuclear cell accumulation occurred after IFN-γ injection in the absence of a demonstrable increase in ICAM-1 expression. At 24 and 48 hours, the endothelial antigenic profile and histologic appearances of skin injected with TNF, or particularly with combined TNF and IFN-γ, resembled in nature, if not always in degree, the delayed hypersensitivity reaction to simian agent 8 antigen. The results are thus in keeping with the hypothesis that *during immune inflammation TNF and IFN-γ produced by local extravascular cells are important in mediating initial, and potentially continued,[30] cellular accumulation through their action on endothelial cells.* In addition to endothelial antigenic changes, cytokine injection (TNF alone or TNF with IFN-γ) induced ultrastructural alterations that have previously been described in delayed hypersensitivity reactions. Specifically, aside from leukocyte emigration, there was evidence of increased vascular permeability and clear extravascular fibrin deposition and endothelial cell hypertrophy.[5,31] Some of the hypertrophic endothelial cell changes may reflect activation, early events in replication, or both.[5,32]

Other interpretations of our results should be considered. First, it is possible that the reaction to cytokines represented a true allergic phenomenon against foreign (human) protein, but this seems unlikely because early
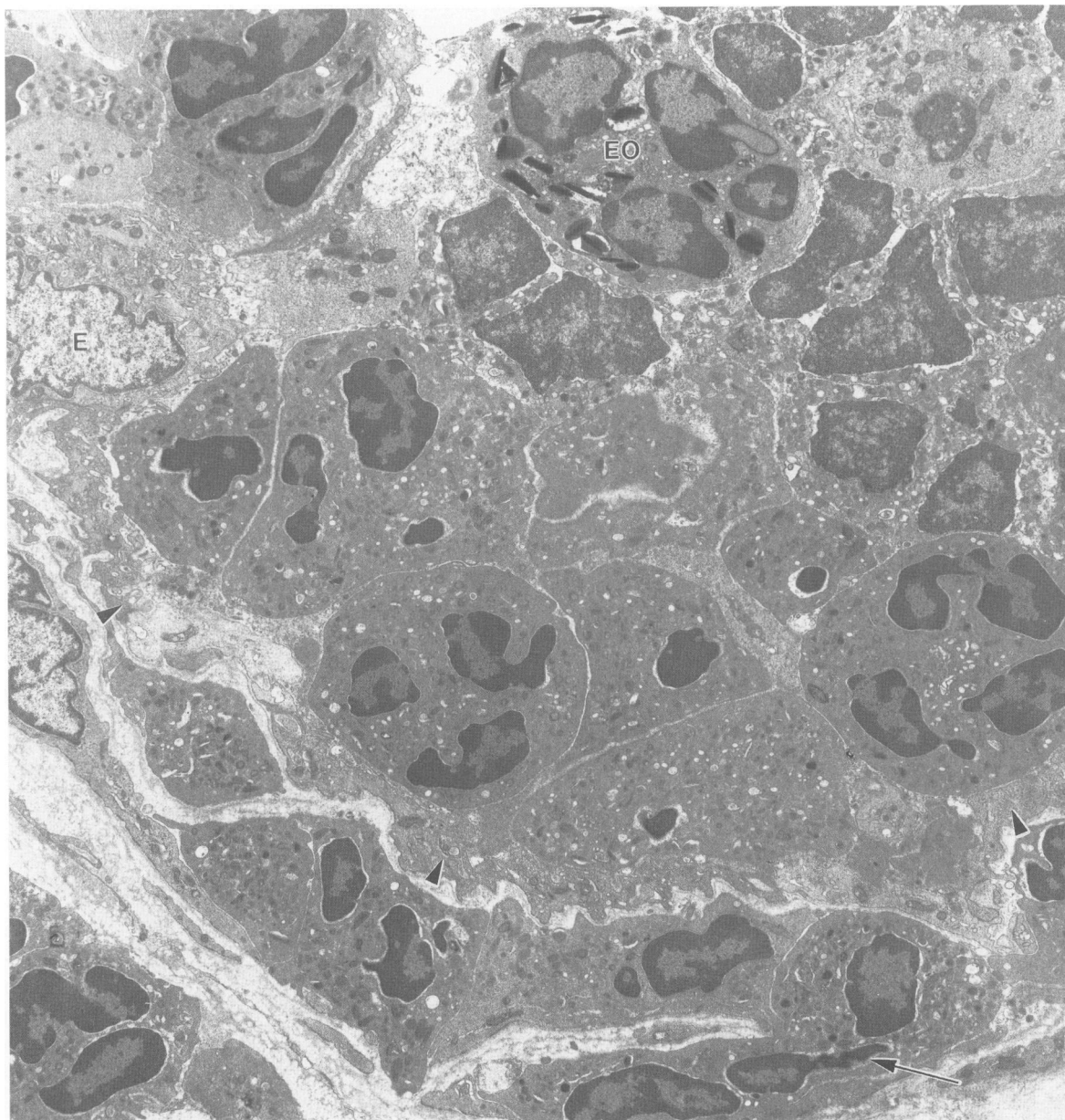
Figure 4. *Part of a postcapillary venule in baboon skin injected 2 hours earlier with TNF (10⁴ U). An endothelial cell nucleus (E) is visible, as is attenuated endothelial cytoplasm (arrowheads). Within the venular lumen (top right) are numerous adherent neutrophils, and an eosinophil (EO). An extravasating neutrophil is seen (arrow), as well as one at the bottom left of the micrograph that previously emigrated (Uranyl acetate and lead citrate, ×5600).*

changes occurred in animals that had not previously been exposed to the administered cytokines, and each cytokine had a characteristic effect on endothelial cells that was broadly comparable to that occurring *in vitro.* In addition, administration of heat-inactivated TNF (10⁴ or 10⁵ U) induced essentially no infiltrate (data not shown). These results argue against an immune reaction to this cytokine. Second, the endothelial cell changes may have occurred through mediators other than those injected, perhaps derived from accumulating leukocytes or resident tissue

cells. Although it is likely that emigrating leukocytes contribute to the evolution of the reaction, expression of adhesion molecules was coincident with the onset of leukocyte accumulation and, further, is generally consistent with the direct effect of these cytokines *in vitro.* Moreover, ELAM-1 and MHC class II antigen expression have been stimulated in explanted human skin by TNF and IFN-γ, respectively, in the absence of circulating leukocytes.[33,34] However, TNF-induced ELAM-1 expression *in vivo* appears to persist longer than expression in cultured cells or in ex-
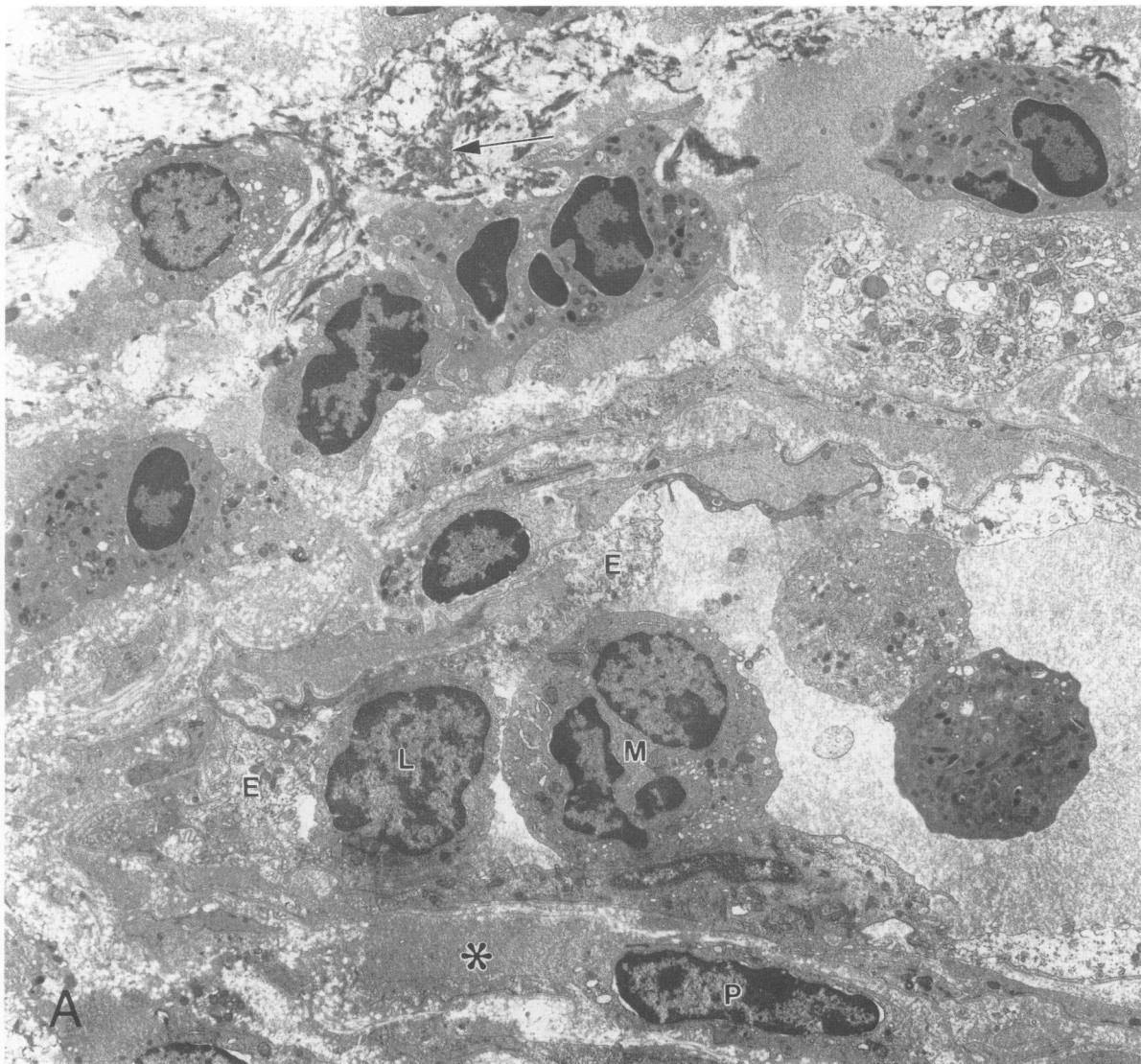
Figure 5. *Cutaneous venules 12 hours after TNF (10⁴ U) combined with IFN-γ (2× 10⁴ U) injection. **A**: Intraluminal lymphocyte (L) and monocyte (M) adherent to endothelium (E), adjacent to which is a pericyte (P). Extraluminal amorphous material, probably extravasated plasma protein, is visible (*), as is fibrin (arrow) (×4950). **B**: Lymphocyte traversing venular endothelium (Uranyl acetate and lead citrate, ×5000).*
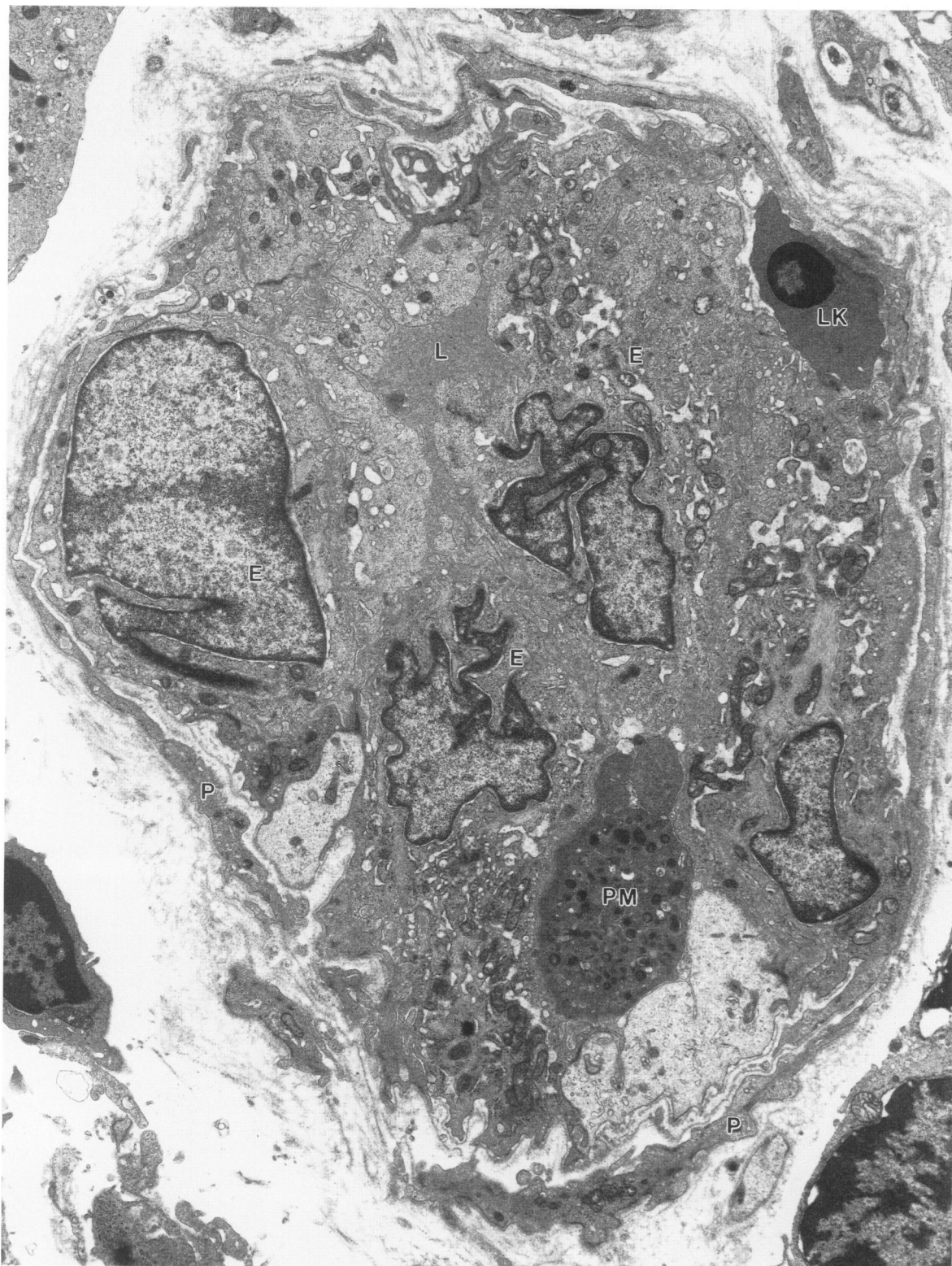
Figure 6. *Postcapillary venule 24 hours after administration of TNF (10^4 U). The endothelial cells (E) are hypertrophied, showing increased organelles, and focally dilated endoplasmic reticulum, and almost obliterate the lumen (L). Also seen are part of an intraluminal polymorphonuclear leukocyte (PM) and an emigrating leukocyte (LK). P, pericyte (Uranyl acetate and lead citrate, ×7500).*

planted skin; leukocyte-derived IL-1[35] may well contribute to sustained ELAM-1 expression. Third, the expression of epitopes of endothelial adhesion molecules could be a direct consequence of the leukocytic emigration induced by cytokines rather than the cause (without necessarily requiring secondary mediators). This seems unlikely because injections of leukotriene B$_4$, a leukocyte-directed inflammatory agent, induced extravascular PMN accumulation at early times (20 minutes) without detected induction of ELAM-1 (data not shown). Fourth, other (uncharacterized) endothelial molecules than the ones assessed in this study may also play a role in leukocyte adhesion. This possibility, in fact, seems likely because IFN-γ-induced mononuclear cell accumulation occurs without any clear ICAM-1 induction and probably precedes induced HLA-DP expression. Moreover, monocytes, which appear to accumulate concomitantly with lymphocytes in our study, have not been reported to recognize either ELAM-1 or ICAM-1 *in vitro*. Finally, this study does not exclude an additional effect of cytokines on leukocytes that would increase their adhesivity to endothelium. Indeed, in the case of TNF, *in vitro* studies suggest an early (10 minute) leukocyte-dependent increased adhesion related to the CD 11/18 complex, as well as a later (4 to 6 hour) endothelial-dependent increase in adhesion that is in part related to ELAM-1.[14,36]

Other authors have assessed the ability of injected cytokines to induce local PMN and mononuclear cell accumulation in various species, but their studies did not include simultaneous assessment of endothelial antigens considered relevant to leukocyte adhesion. PMN accumulation has been induced by IL-1, lymphotoxin, TNF, and other agents in rabbits[37-41] and by lymphotoxin in mice.[38] Human IL-1 and TNF additionally have been noted to induce some mononuclear cell accumulation after intravitreous administration to rabbits.[41] Issekutz, Stoltz, and van der Meide[42] found that injection of recombinant IFN-γ into rats was followed by accumulation of circulating radiolabeled lymphocyte populations in approximately one fourth of the number accumulating during a delayed hypersensitivity reaction. Another study involving injection of IFN-γ into the skin of humans suffering from leprosy showed that this led to accumulation of T lymphocytes and other cells,[43] although accumulation was stated to be not as great as that attained during a tuberculin reaction.[43,44] The present study, using initially normal skin of *Papio anubis* injected with recombinant human cytokines, demonstrates that, at the times and doses assessed, IFN-γ did not induce as much mononuclear cell accumulation as TNF alone or TNF combined with IFN-γ. TNF (with or without IFN-γ) additionally induced brisk PMN accumulation. Whether or not the concentrations of cytokines injected in these studies are physiologic (ie, whether or not local leukocytes produce levels of cytokine

concentrations equivalent to those of injections) should be questioned. This cannot be answered by present techniques. However, the extent of endothelial antigenic changes elicited by injected cytokines in our study was comparable to that produced in response to antigen.

In conclusion, endothelium is strategically placed to participate in cellular accumulation during inflammation by recognizing extravascularly produced mediators such as TNF and IFN-γ, undergoing activation, and thereby becoming adhesive for luminal leukocytes. The present study provides evidence that such a sequence of events could occur *in vivo*, and suggests that adhesion of circulating polymorphonuclear and mononuclear leukocytes may be related, in part, to *de novo* endothelial expression of ELAM-1 and to increased endothelial expression of ICAM-1 respectively.

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