# Complement-Induced Expression of Chemokine Genes in Endothelium: Regulation by IL-1-Dependent and -Independent Mechanisms[1]

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**Activation of complement in the vicinity of endothelium is thought to contribute to the tissue manifestations of inflammatory and immune responses. Endothelial cells contribute to these processes in part by the elaboration of chemokines that activate various leukocytes and direct their migration into tissues. We investigated the mechanisms by which activation of complement on endothelial cell surfaces might influence the expression of chemokine genes in endothelial cells. In a model for the immune reaction occurring in a xenograft, human serum, as a source of xenoreactive anti-endothelial Abs and complement, induced expression of the monocyte chemotactic protein-1 (MCP-1), IL-8, and RANTES genes. The MCP-1 and IL-8 genes were expressed within 3 h as a first phase and at >12 h as a second phase. The RANTES gene was expressed in porcine endothelial cells only 12 h after exposure to human serum. The expression of these genes required activation of complement and assembly of membrane attack complex, as it was inhibited by soluble CR1 and did not occur in the absence of C8. The early phase of MCP-1 and IL-8 gene expression did not require de novo protein synthesis. The late phase of MCP-1, IL-8, and RANTES gene expression predominantly required the production of IL-1$\alpha$ as an intermediate step. The results indicate that the expression of chemokine genes in endothelial cells occurs as a function of differential responses to complement and may in part be conditioned by the availability of IL-1$\alpha$.** *The Journal of Immunology,* **1998, 161: 4388–4395.**

Endothelial cells play a critical role in integrating leukocytes into immune and inflammatory lesions because of their strategic position at the interface between circulating cells and tissues (1). Endothelial control of leukocyte migration and activation is mediated in part by expression of adhesion molecules and proinflammatory cytokines, including chemokines (2, 3). Chemokines are members of a large family of structurally and functionally related cytokines that, tethered to endothelial cell surfaces, attract and activate leukocytes, facilitating emigration across endothelium (4–6). Endothelial cell production of chemokines is of special interest because chemokines secreted by the endothelium can appear immediately at the tissue-blood interface where they can exert their effects.

Endothelial cells produce chemokines in response to a variety of endogenous and exogenous stimuli, including IL-1 (7–10), TNF-$\alpha$ (7, 9, 11), low density lipoprotein (12), endotoxin (7), and complement (13). The expression of chemokines by endothelial cells differs depending on the stimulators applied, and this differential expression may contribute to the selectivity of cell recruitment in various types of inflammation, since only a limited set of immune cells is sensitive to each chemokine (14).

The present study examined the mechanisms and kinetics by which complement might induce the expression of chemokine genes in endothelial cells, thus influencing inflammatory reactions. To this end, we used cultured porcine aortic endothelial cells as a model for the donor, and human serum as a source of anti-endothelial cell Abs and complement that are known to be involved in xenograft rejection (15). Using such a model, it was earlier observed that complement stimulates endothelial cells with loss of barrier and anticoagulant functions (16–18). We studied the complement-mediated regulation of expression of chemokines MCP-1,[4] IL-8, and RANTES in cultured endothelial cells because of discrete as well as overlapping functional characteristics of these molecules. For example, IL-8 attracts and activates neutrophils (19, 20), while MCP-1 and RANTES attract and activate monocytes and subsets of T cells (21–25). We show here that complement differentially induces the expression of the MCP-1, IL-8, and RANTES genes as a direct consequence of its action on endothelial cells and through an intermediate step, the synthesis and elaboration of IL-1$\alpha$.

## Materials and Methods

### *Reagents*

Samples of human blood from healthy individuals were used as a source of anti-endothelial cell Abs and complement (26). Blood collected in sterile, pyrogen-free containers was allowed to clot at 4°C, and the serum fraction was separated and stored as aliquots at −80°C until used. The titer of Abs in serum was determined by ELISA, as described previously using cultured porcine endothelial cells as target (27). Human serum immunodepleted of C8 and purified human C8 were purchased from Quidel (San Diego, CA). Recombinant human IL-1$\alpha$ was obtained from Genzyme (Boston, MA). IL-1RA and anti-IL-1$\alpha$ neutralizing Abs were obtained from R & D Systems (Rockford, IL). Neutralizing anti-human IL-8 Ab was a gift from Dr. Nikolai N. Voitenok (Foundation for Fundamental Research, Minsk, Republic of Belarus). Anti-human MCP-1 and anti-human RANTES Abs (R & D Systems, Minneapolis, MN) were gifts from Dr. Terry O. Harville,

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[4] Abbreviations used in this paper: MCP-1, monocyte chemotactic protein-1; C8, complement component-8; IL-1RA, interleukin-1 receptor antagonist; sCR1, soluble complement receptor-1; MAC, membrane attack complex.



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Duke University (Durham, NC). Soluble complement receptor-1 (sCR1) was obtained from T-Cell Sciences (Cambridge, MA). Actinomycin D was purchased from Boehringer Mannheim (Indianapolis, IN). Cycloheximide and LPS (LPS, *Escherichia coli* serotype 0111:B4) were obtained from Sigma (St. Louis, MO). Porcine IL-8, IL-1$\alpha$, IL-1$\beta$, and TNF-$\alpha$ primers were provided by Dr. Robin S. Monroe (Duke University). Porcine IL-1$\alpha$ cDNA was obtained from Dr. Soheyla Saadi and Robert A. Holzknecht (Duke University). Human IL-8 cDNA was obtained from Drs. Joost J. Oppenheim and Kouji Matsushima (National Cancer Institute, National Institutes of Health, Frederick, MD). Human MCP-1 cDNA was obtained from Dr. Barrett Rollins (Dana-Farber Cancer Institute, Boston, MA). Human RANTES cDNA was obtained from Dr. Thomas J. Schall (DNAX Research Institute, Palo Alto, CA).

### *Endothelial cells*

Endothelial cells were explanted from porcine aortae and cultured in DMEM supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine, 50 U/ml penicillin, and 50 $\mu$g/ml streptomycin (Life Technologies, Grand Island, NY) as previously described (28, 29). The endothelial cells were characterized by their morphology, including a cobblestone appearance when confluent, and by the ability to take up acetylated low density lipoprotein (28). During our initial screening of a number of primary endothelial cell lines for their capacity to induce various cytokine genes in response to human serum, we identified an endothelial cell line that failed to express IL-1$\alpha$, IL-1$\beta$, and TNF-$\alpha$ in response to complement. These cells had normal morphologic characteristics and sensitivity to complement as measured by deposition of normal amounts of iC3b after incubation with human serum and by a normal level of complement-mediated cytotoxicity (data not shown) (30). Experiments were performed with cells at passages 4 to 10 that were grown to confluence in 60-mm tissue culture dishes (Corning, Corning, NY).

### *Stimulation of endothelial cells*

Confluent monolayers of endothelial cells were washed twice with DMEM and then incubated with human serum diluted in DMEM. Human serum that had been heated to 56°C for 30 min to inactivate complement was used as a control. Endothelial cells were also stimulated with LPS, IL-1$\alpha$, or TNF-$\alpha$ in DMEM containing heat-inactivated FBS, as discussed in the text.

### *Northern blot analysis*

Total RNA was obtained by the single step method, using acidic guanidinium thiocyanate (31). Samples of total RNA (10 $\mu$g) were electrophoresed, blotted by capillary transfer onto nylon membranes (Amersham, Arlington Heights, IL), and cross-linked to membranes by UV irradiation (CL 1000 Ultraviolet Crosslinker, UVP, San Gabriel, CA) as described previously (32). Blots were prehybridized at 68°C in QuikHyb (Stratagene, CA) containing 200 $\mu$g of herring sperm DNA and were hybridized for 2 h with $^{32}$P-labeled probes at 68°C in the same buffer. Blots were washed in 1× SSC/0.5% SDS at 23°C followed by high stringency washes in 0.1% SSC/0.1% SDS at 50°C and were exposed to Kodak BIOMAX film (Eastman Kodak, Rochester, NY). Blots were stripped by exposure to boiling 0.1% SDS before reprobing. The probes used were the RT-PCR fragment of porcine IL-8 (GAA TTC CTT CCA AAC TGG CTG TTG CC and AAG CTT CTG TAC AAC CTT CTG CAC CC) (33), actin (ATG TTT GAG ACC TTC AAC AC and CAC GTC ACA CTT CAT GAT GGA) (34), the PCR fragment of porcine IL-1$\alpha$ (GAA TTC CAA CAT ACA GCT TCC AGA GC and AAG CTT TGG GTG TCT CAG GCA GCT CC) (35), the PCR fragment of porcine IL-1$\beta$ (GAA TTC CCA TAG TAC CTG AAC CCG CC and AAG CTT TCA TCG GCT TCT CCA CTG CC) (36), and the PCR fragment of TNF-$\alpha$ (GAA TTC CGT TGT AGC CAA TGT CAA AGC C and AAG CTT CCA GGT AGA TGG GTT CGT ACC) (37). RNA loading was assessed by staining the blots for 5 min with 0.01% methylene blue in 0.3 M sodium acetate. Quantitative analysis of blots was accomplished using a Betascope (Betagen, Waltham, MA) or an Ultroscan XL laser densitometer (LKB, Gaithersburg, MD).

### *Metabolic labeling, immunoprecipitation, and ELISA*

Endothelial cells were labeled with [$^{35}$S]methionine and [$^{35}$S]cysteine (DuPont-New England Nuclear, Boston, MA) as described previously (32). Aliquots of labeled culture supernatants were immunoprecipitated using anti-human MCP-1, anti-human IL-8, and anti-human RANTES Abs. Samples containing immunoprecipitated proteins were electrophoresed in 15% discontinuous SDS-polyacrylamide gels according to the method of Laemmli (38). Proteins were visualized by fluorography (39) using Kodak BIOMAX film. The levels of MCP-1, IL-8, and RANTES proteins in the culture supernatant of stimulated endothelial cells were quantitated in duplicate using human chemokine ELISA kits obtained from Endogen (Woburn, MA).

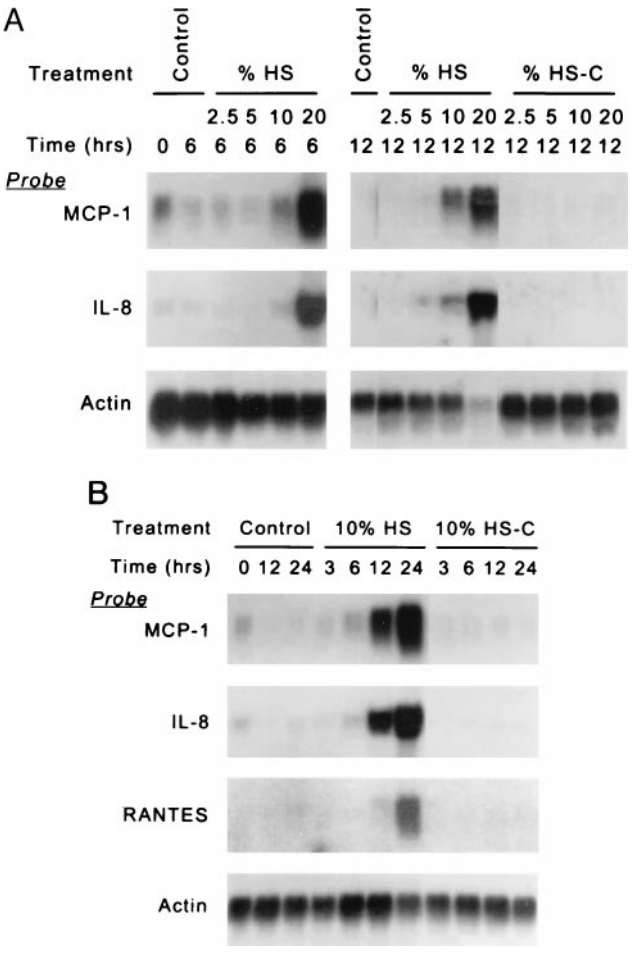


**FIGURE 1.** Induction of chemokine MCP-1, IL-8, and RANTES gene expression by complement in endothelial cells. *A*, Kinetics of chemokine gene expression induced by various concentrations of human serum containing natural xenoreactive Abs with complement (HS) or without complement (HS-C). *B*, Kinetics of chemokine gene expression induced by 10% HS with or without complement. Northern blots of total RNA samples were hybridized with $^{32}$P-labeled cDNA probes as indicated. The results shown are representative of five experiments.

## Results

### *Differential expression of MCP-1, IL-8, and RANTES genes in porcine endothelial cells stimulated with human Ab and complement*

Cultured porcine aortic endothelial cells expressed low levels of MCP-1, IL-8, and RANTES mRNAs under resting conditions (Fig. 1). To begin to determine how activation of complement might induce the expression of various chemokine genes in endothelial cells, the pattern of expression of MCP-1, IL-8, and RANTES genes in response to human serum containing xenoreactive natural Ab and complement was investigated. As shown in Figure 1 and Table I (columns on left), exposure of endothelial cells to human serum induced a notable increase in the levels of MCP-1, IL-8, and RANTES mRNAs. However, the rate of increase in IL-8 and MCP-1 mRNAs was different from the rate of induction of the RANTES gene in response to complement. Thus, IL-8 and MCP-1 mRNAs were increased as early as 6 h, while RANTES mRNA was not expressed until 12 h had elapsed. The rate of induction of MCP-1, IL-8, and RANTES mRNAs was a function of the amount

Table I. *Pattern of expression of chemokine genes in complement-stimulated porcine aortic endothelial cells*[a]

| Chemokine Genes | Complement-Mediated IL-1α Expressing Cells | | Complement-Mediated Non-IL-1α Expressing Cells | |
|---|---|---|---|---|
| | HS, 10% | HS, 20% | HS, 10% | HS, 20% |
| MCP-1 | Delayed (>12 h) | Immediate (<1 h) | No | Immediate (<1 h) |
| IL-8 | Delayed (>12 h) | Immediate (<1 h) | No | Immediate (<1 h) |
| RANTES | Delayed (>12 h) | Delayed (>6 h) | No | No |

[a] Human serum.

of complement applied to the endothelial cells, as MCP-1 and IL-8 mRNAs were not up-regulated until 12 h when cells were exposed to a 10% concentration of human serum, and lower concentrations of human serum induced no up-regulation of these transcripts.

*Role of complement in the induction of MCP-1 and IL-8 mRNAs in porcine endothelial cells stimulated with human Ab and complement*

We examined whether human serum-induced expression of MCP-1, IL-8, and RANTES genes was due to complement activation. Endothelial cells treated with varying concentrations of heat-inactivated human serum did not express increased levels of MCP-1, IL-8, and RANTES mRNAs (Fig. 1, *A* and *B*). To test whether the heat-labile component was complement, endothelial cells were treated with human serum in the presence or the absence of sCR1, which inhibits C3 convertase. As shown in Figure 2*A*, sCR1 abolished human serum-induced up-regulation of MCP-1, IL-8, and RANTES genes. It was previously demonstrated that complement activation and assembly of membrane attack complex were required for complement-induced up-regulation of the IL-1α gene (17). To investigate whether the assembly of membrane attack complex (MAC) induces the initial expression of MCP-1 and IL-8 mRNAs, the induction of these transcripts was examined in endothelial cells stimulated with a high concentration (20%) of C8-depleted human serum. As shown in Figure 2, *B* and *C*, human serum depleted of C8 did not induce MCP-1 and IL-8 mRNAs. However, the C8-depleted serum reconstituted with purified C8 induced IL-8 and MCP-1 mRNAs. Taken together, these results (Figs. 1 and 2) suggest that complement activation leading to assembly of MAC on endothelial cells causes expression of MCP-1, IL-8, and RANTES mRNAs.

*Secretion of chemokine proteins by porcine endothelial cells stimulated with human Ab and complement*

We next examined whether porcine endothelial cells that expressed high levels of mRNA for MCP-1, IL-8, and RANTES genes also secreted the corresponding proteins. Endothelial cells stimulated with human serum were labeled with [$^{35}$S]methionine and [$^{35}$S]cysteine for 2 h, and the chemokines secreted from the cells were immunoprecipitated. SDS-PAGE analysis of the immunoprecipitates revealed high levels of IL-8, MCP-1, and RANTES in the supernatants of the stimulated cells but very low levels in controls (Fig. 3). Consistent with the later induction of RANTES mRNA, culture medium was found to contain RANTES protein at 16 h, but not at 3 h, following stimulation (Fig. 3*C*). The sizes of these porcine chemokines, estimated by migration in polyacrylamide gels, were similar to those of human chemokines (40). The levels of MCP-1, IL-8, and RANTES proteins were quantitated by ELISA in the culture supernatant of human serum-stimulated

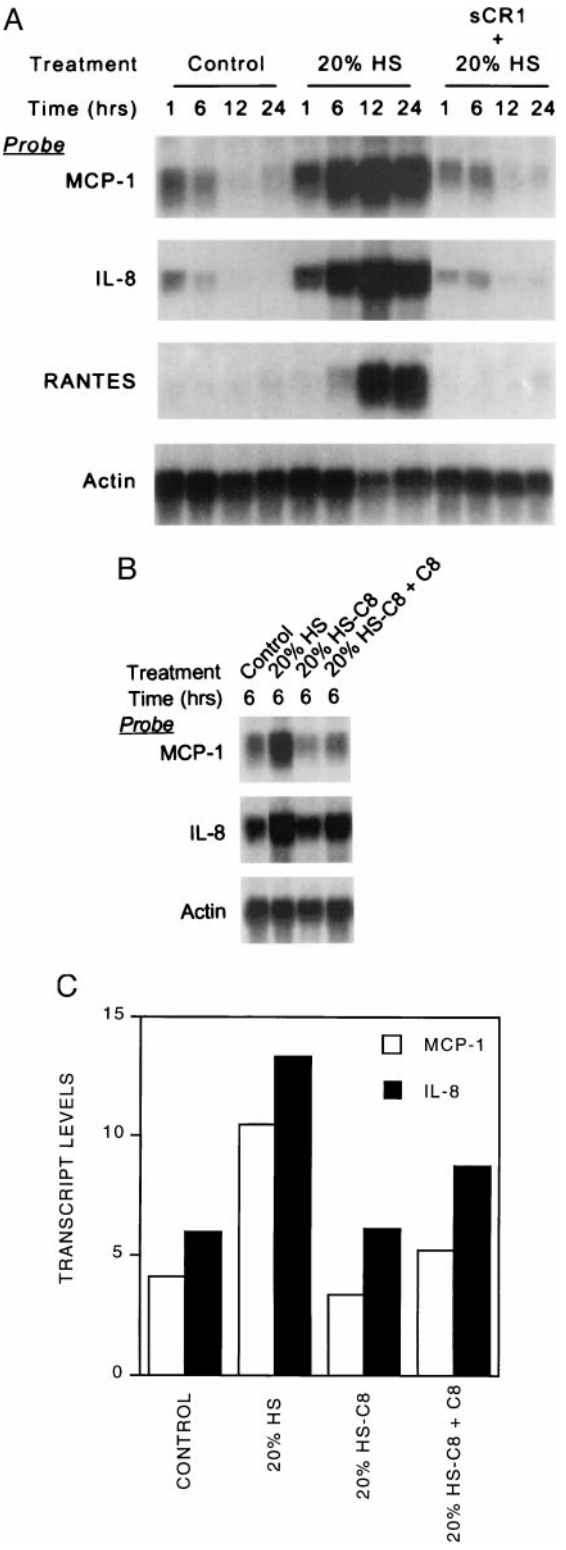


**FIGURE 2.** Role of complement activation in the expression of MCP-1, IL-8, and RANTES genes in endothelial cells. *A*, Cultured porcine aortic endothelial cells were stimulated with human serum containing xenoreactive Ab and complement (HS) in the presence or the absence of 6 μg/ml sCR1 as indicated. Northern blots of total RNA samples were hybridized with $^{32}$P-labeled cDNA probes as indicated. *B* and *C*, Cultured porcine aortic endothelial cells were stimulated with human serum, C8-depleted human serum, or C8-depleted human serum reconstituted with purified C8 (25 μg/ml). *B*, Northern blots of total RNA samples were hybridized with $^{32}$P-labeled cDNA probes as indicated. *C*, Hybridization signals on x-ray film were quantified using a laser densitometer. The values were normalized to the level of actin mRNA. The results shown are representative of three experiments.

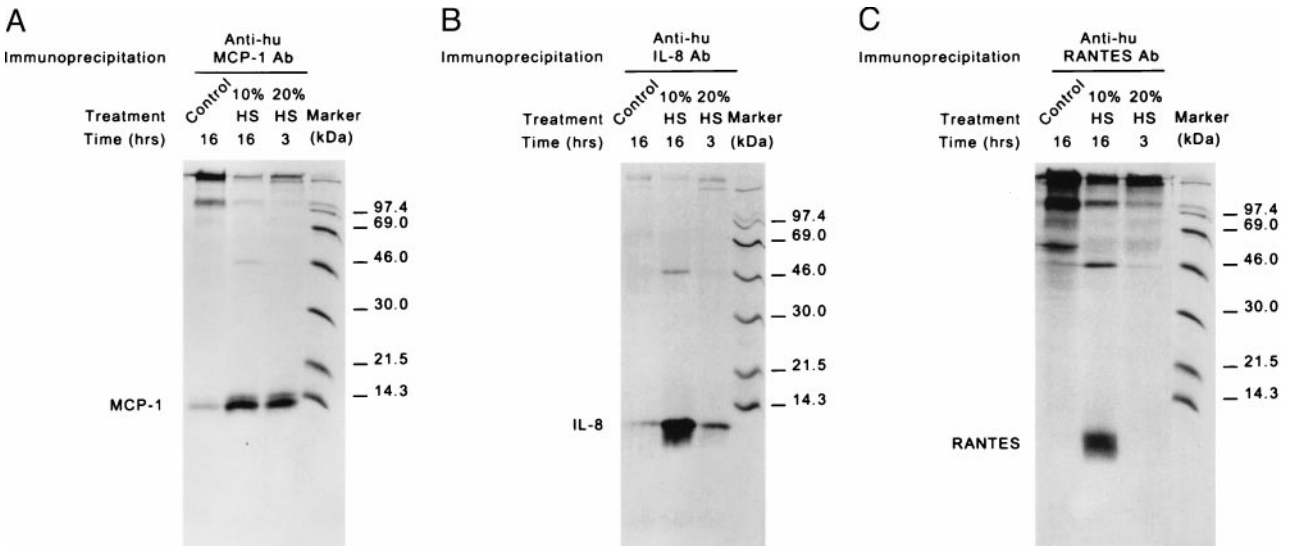


**FIGURE 3.** Secretion of MCP-1, IL-8, and RANTES by endothelial cells after stimulation with complement. Cultured porcine aortic endothelial cells were stimulated for 3 or 16 h with human serum containing xenoreactive Ab and complement (HS) as indicated and were metabolically labeled as described in *Materials and Methods*. mAbs specific for MCP-1 (*A*), IL-8 (*B*), or RANTES (*C*) were added to the supernatants, and immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. The results shown are representative of three experiments.

endothelial cells. Table II shows one experiment that is representative of four conducted. As was seen with immunoprecipitation analysis, high levels of IL-8, MCP-1, and RANTES were detected in the culture supernatants of the stimulated endothelial cells, while low levels of MCP-1 and undetectable levels of IL-8 and RANTES were seen in controls. A lower concentration (10%) of human serum induced MCP-1, IL-8, and RANTES at 16 h following stimulation; higher concentrations (20%) of human serum induced an early increase in MCP-1 and IL-8 but not RANTES. Although immunoprecipitation and ELISA have different levels of sensitivity, the results by ELISA were similar to those obtained with immunoprecipitation.

### *Different phases of MCP-1, IL-8, and RANTES mRNA induction by complement*

The early induction of MCP-1 and IL-8 mRNAs by an increased level of human serum may be a direct consequence of the action of complement. Conversely, the apparent delay in induction (>12 h) of MCP-1, IL-8, and RANTES mRNAs by human serum under some conditions suggested that these genes might be regulated by intermediary factors produced by the endothelial cells in response to primary stimuli. Previous work indicated that the physiology of endothelial cells was potentially regulated by endogenous IL-1$\alpha$ (41). Specifically, it was shown that the induction of cyclo-oxygenase in endothelial cells was mediated by synthesis of IL-1$\alpha$ and its action on endothelial cells. In addition, previous work from our laboratory indicated that following stimulation of porcine endothelial cells with complement, the induction of tissue factor was mediated by IL-1$\alpha$ produced by the endothelial cells in response to MAC (17). Our analysis using PCR and Northern blots revealed that endothelial cells express very little IL-1$\beta$ mRNA and undetectable levels of TNF-$\alpha$ mRNA in response to human serum (data not shown). Thus, we considered the possibility that IL-1$\alpha$ might be a potential factor regulating the late phase induction of chemokine genes. To directly test whether the expression of MCP-1, IL-8, and RANTES mRNAs is determined in part by expression of IL-1$\alpha$, porcine aortic endothelial cells were stimulated with human serum in the absence or the presence of neutralizing Ab against human IL-1$\alpha$ or in the presence of IL-1RA. As Figure 4 shows, the delayed induction of MCP-1 and IL-8 mRNAs by 10 and 20% human serum was strongly inhibited by anti-IL-1$\alpha$ Ab (91 and 80% inhibition, respectively). On the other hand, the early (at 3 h) up-regulation of MCP-1 and IL-8 mRNAs by 20% human serum was minimally inhibited by the anti-IL-1$\alpha$ Ab (25% inhibition). Similar levels of inhibitory response were observed with the inclusion of IL-1RA (Fig. 4). These results suggest that MCP-1 and IL-8 genes are induced by two mechanisms, one involving an initial phase that is IL-1$\alpha$ independent and another late phase that is dependent upon synthesis of IL-1$\alpha$ and its action on endothelial cells. In contrast, up-regulation of RANTES mRNA was always inhibited by anti-IL-1$\alpha$ Ab or IL-1RA, suggesting that RANTES gene induction by complement requires the synthesis and availability of IL-1$\alpha$.

Table II. *MCP-1, IL-8, and RANTES protein levels in the culture supernatant of complement-stimulated porcine aortic endothelial cells*[a]

| Treatment | MCP-1 (pg/ml) | IL-8 (pg/ml) | RANTES (pg/ml) |
|---|---|---|---|
| Control (3 h) | 5 | 0 | 0 |
| Human serum, 10% (3h) | 110 | 0 | 0 |
| Human serum, 20% (3h) | 155 | 2 | 0 |
| Control (16 h) | 20 | 0 | 0 |
| Human serum, 10% (16 h) | 370 | 38 | 95 |
| Human serum, 20% (16 h) | 200 | 8 | 140 |

[a] Values shown are means of duplicate samples tested by ELISA.

To demarcate IL-1-independent and IL-1-dependent mechanisms of induction of MCP-1, IL-8, and RANTES genes by complement, we studied a line of endothelial cells that do not express detectable amounts of IL-1$\alpha$, IL-1$\beta$, or TNF-$\alpha$ (Fig. 5*A* and data not shown) in response to complement. During routine culture, these cells constitutively expressed low levels of MCP-1 and IL-8 mRNAs but not IL-1$\alpha$ or RANTES mRNA (Fig. 5*A*). Stimulation of these cells with a high concentration (20%) of human serum induced heightened levels of MCP-1 and IL-8 mRNAs but negligible levels of IL-1$\alpha$ and RANTES mRNAs. This is in contrast to the expression of all three chemokine mRNAs in normal IL-1-expressing cells (compare Fig. 5, *A* with *B*; Table I). Following

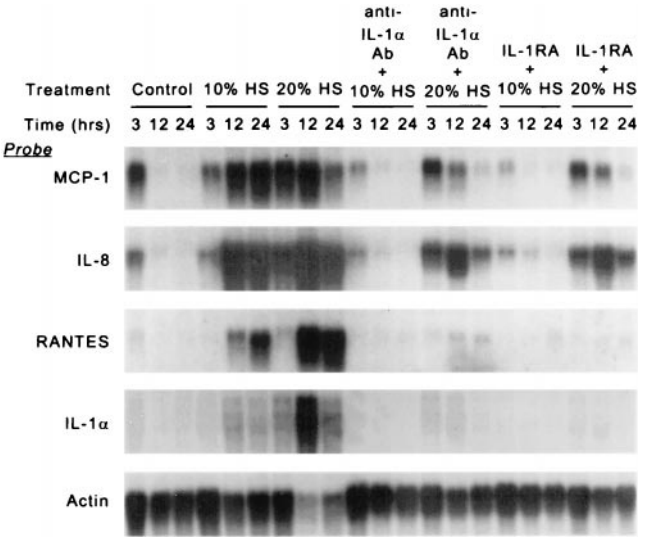


**FIGURE 4.** Role of endogenous IL-1$\alpha$ in the expression of MCP-1, IL-8, RANTES, and IL-1$\alpha$ genes to complement in endothelial cells stimulated with human serum. Cultured porcine aortic endothelial cells were stimulated with human serum containing xenoreactive Ab and complement (HS) in the presence or the absence of 10 $\mu$g/ml anti-IL-1$\alpha$ Ab or 12 ng/ml IL-1RA as indicated. The control Ab was noninhibitory to the induction of these cytokine genes in porcine aortic endothelial cells (17). Northern blots of total RNA samples were hybridized with $^{32}$P-labeled cDNA probes as indicated. The results shown are representative of three experiments.

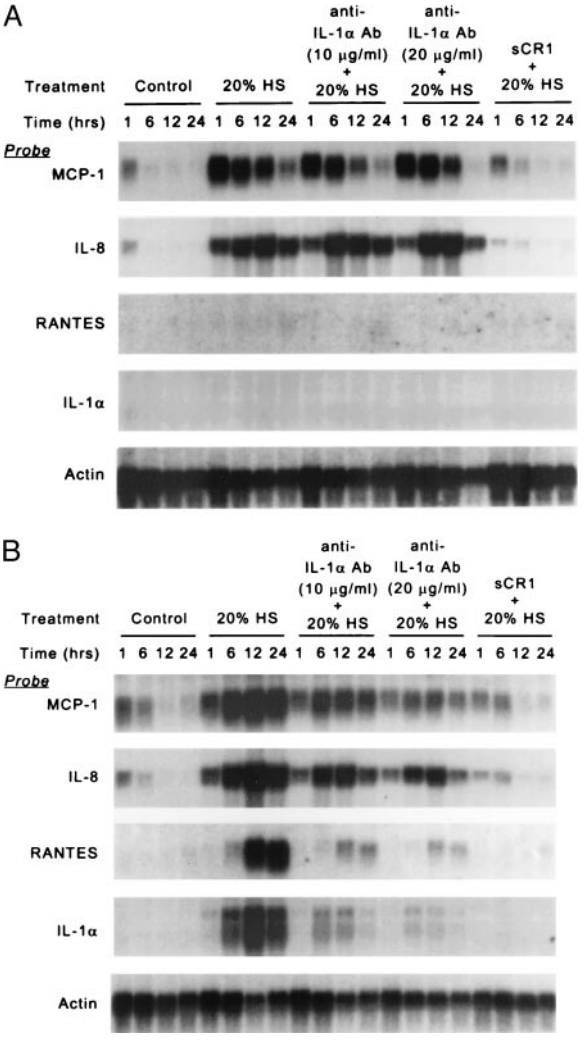


**FIGURE 5.** Differential induction of chemokine genes in endothelial cells that vary in the ability to synthesize IL-1$\alpha$. Porcine aortic endothelial cell cells that do not express IL-1$\alpha$ in response to complement (*A*) and normal porcine aortic endothelial cells that express IL-1$\alpha$ in response to complement (*B*) were stimulated with human serum containing xenoreactive Ab and complement (HS) and then tested for the induction of MCP-1, IL-8, and RANTES mRNAs in the presence or the absence of 10 $\mu$g/ml anti-IL-1$\alpha$ Ab, 20 $\mu$g/ml anti-IL-1$\alpha$ Ab, or 6 $\mu$g/ml sCR1 as indicated. Northern blots of total RNA samples were hybridized with $^{32}$P-labeled cDNA probes as indicated. The results shown are representative of three experiments.

exposure to lower concentrations (10%) of human serum, IL-1-nonexpressing cells did not express MCP-1, IL-8, or RANTES mRNA (data not shown), while IL-1-expressing cells did so (Fig. 4 and Table I). Furthermore, the anti-IL-1$\alpha$ Ab that significantly inhibited late induction of MCP-1, IL-8, and RANTES mRNAs in IL-1-expressing cells (Fig. 5*B*) did not alter the levels of MCP-1 and IL-8 mRNAs in IL-1-nonexpressing cells (Fig. 5*A*). IL-1-nonexpressing cells were able to respond normally to other types of stimulation, as treatment of the cells with rIL-1$\alpha$ (Fig. 6), LPS (Fig. 6), or rTNF-$\alpha$ (data not shown) induced RANTES as well as MCP-1 and IL-8 mRNAs. Taken together, these results demonstrate that RANTES gene induction by complement for the most part depends on the production of endogenous IL-1$\alpha$, while MCP-1 and IL-8 genes are induced directly by complement.

### *Effects of transcriptional and translational inhibitors on the initial up-regulation of MCP-1 and IL-8 mRNAs in porcine endothelial cells stimulated with human Ab and complement*

We investigated whether initial induction of MCP-1 and IL-8 mRNAs in endothelial cells by complement was the result of transcriptional or post-transcriptional mechanisms. Endothelial cells that did not express IL-1 in response to complement were stimulated with 15% human serum for 2 h and were further cultured for additional time with or without actinomycin D (Fig. 7). MCP-1 and IL-8 mRNAs decayed similarly in actinomycin D-treated and untreated cells. These experiments suggest that the human serum-induced increase in MCP-1 and IL-8 mRNA levels resulted from induction of MCP-1 and IL-8 gene transcription. We then investigated whether the initial expression of MCP-1 and IL-8 genes required de novo protein synthesis, since these genes are induced as a direct consequence of the action of complement. As shown in Figure 8, incubation with cycloheximide alone for 3 h led to an increased basal level of expression of MCP-1 and IL-8 mRNAs. Incubation with cycloheximide followed by stimulation with 15% human serum resulted in a further increase in the levels of MCP-1 and IL-8 mRNAs compared with those in endothelial cells treated with either cycloheximide or 15% human serum alone. These results demonstrate that de novo protein synthesis is not required for the initial induction of MCP-1 and IL-8 gene transcription in endothelial cells stimulated with a high concentration of human serum. Further, we extended our studies to check the effects of cycloheximide to ascertain the role of protein synthesis in the late phase (beyond 12 h) expression of MCP-1, IL-8, and RANTES mRNAs in 10% human serum-stimulated endothelial cells. Cycloheximide alone induced the expression of MCP-1, IL-8, and RANTES mRNAs similar to the levels up-regulated by human serum in the presence or the absence of cycloheximide (data not shown). These results suggest that protein synthesis, presumably the synthesis of endonucleases, is an important factor in degrading the mRNA synthesized during stimulation and determining the kinetics of these responses.

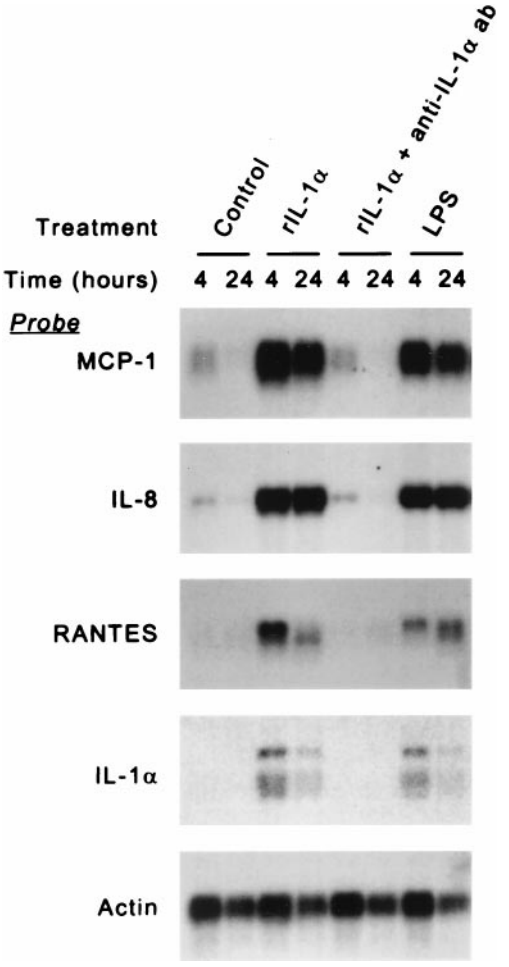


**FIGURE 6.** Complement-stimulated endothelial cells that do not produce IL-1$\alpha$ respond to exogenous IL-1$\alpha$ or LPS with the induction of IL-1$\alpha$, RANTES, IL-8, and MCP-1 mRNAs. Cultured porcine aortic endothelial cells that do not express IL-1$\alpha$ in response to complement were stimulated with rIL-1$\alpha$ (800 U/ml) in the presence or the absence of 10 $\mu$g/ml anti-IL-1$\alpha$ Ab or 20 $\mu$g/ml LPS as indicated. Northern blots of total RNA samples were hybridized with $^{32}$P-labeled cDNA probes as indicated. The results shown are representative of three experiments.

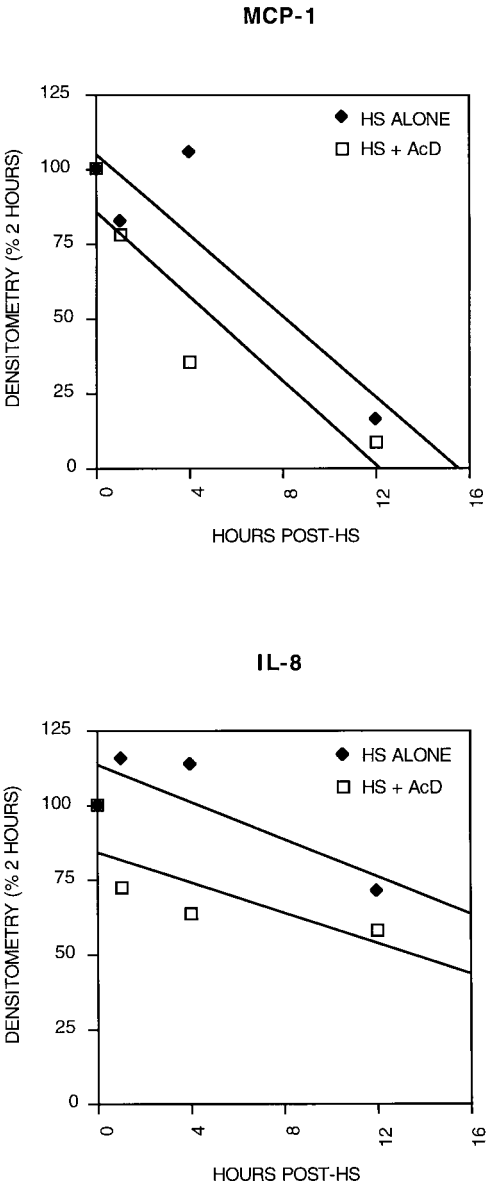


**FIGURE 7.** Effect of RNA synthesis inhibition on complement-induced MCP-1 and IL-8 transcript stability in endothelial cells. Cultured porcine aortic endothelial cells that do not express IL-1$\alpha$ in response to complement were stimulated for 2 h with 15% human serum containing xenoreactive Ab and complement (HS). Actinomycin D (AcD) was then added at 5 $\mu$g/ml (time zero), and cells were incubated for the indicated times. Northern blots of total RNA samples were hybridized with $^{32}$P-labeled MCP-1 or IL-8 cDNA probes. Hybridization signals on x-ray film were quantified using a laser densitometer. The values were normalized to 100 at time zero for comparison of transcript stability and were represented as symbols. The lines are the linear regression fitted to these points. The results shown are representative of two experiments.

## Discussion

It is increasingly evident that complement is involved either directly or indirectly in many facets of immune and inflammatory responses (42). A number of studies have shown that the deposition of complement components on leukocytes as well as tissue cells triggers cell activation and the release of inflammatory mediators (43, 44). Notably, in a vascular xenograft model using porcine endothelial cells and human serum, C5b-C9 (MAC) has been demonstrated to induce the expression of biologic response modifiers such as P-selectin and IL-1$\alpha$ (17, 45). Due to alterations of the physiologic status induced by complement, the endothelial cells loose barrier function and thereby may amplify inflammatory responses in part by directing the trafficking of inflammatory cells. The association of cellular infiltration with humoral immune responses involving activation of complement may well depend upon the production of a cascade of chemokines by endothelial cells. Thus, the pattern of expression of chemokine genes that occurs in response to complement or other inflammatory mediators may contribute to the kinetics and selectivity of cell recruitment, since a limited set of immune cells is sensitive to each chemokine (5, 14). Although MAC is thought to induce MCP-1 and IL-8 genes, the mechanisms leading to the induction of these genes and the pattern of chemokine gene expression that ensues are uncertain (13). The present report addresses the relationship between activation of complement and differential expression of chemokines MCP-1, IL-8, and RANTES in endothelial cells. Both MCP-1 and IL-8 responded early with a prolonged induction as a result of high level complement activation. RANTES expression, on the other hand, occurred late regardless of the quantity of complement components generated. The late phase induction of the MCP-1, IL-8,

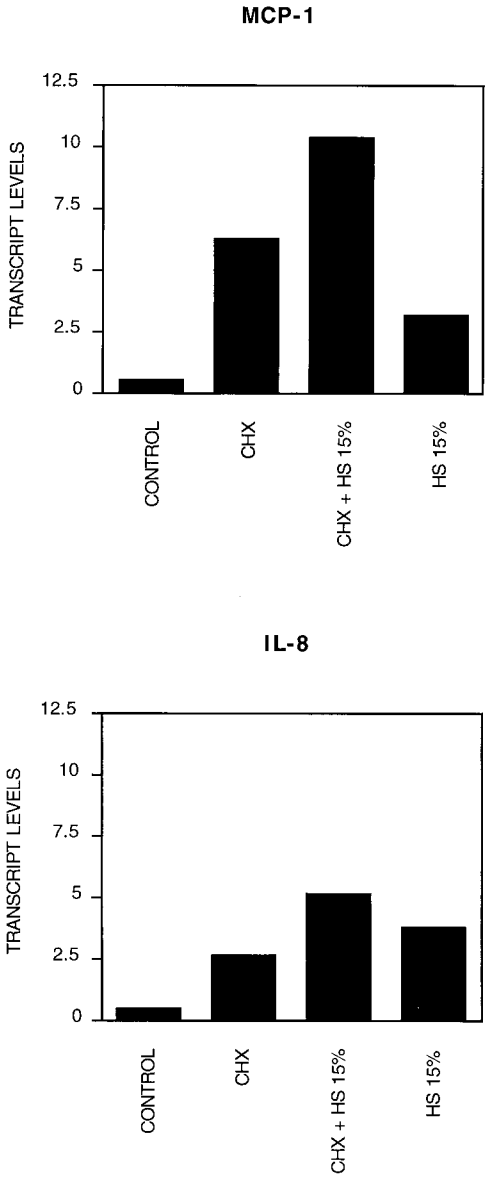


**FIGURE 8.** Effect of protein synthesis inhibition on early MCP-1 and IL-8 gene expression to complement in endothelial cells. Cultured porcine aortic endothelial cells that do not express IL-1$\alpha$ in response to complement were pretreated for 0.5 h with or without 10 $\mu$g/ml cycloheximide (CHX) and then stimulated with 15% human serum containing xenoreactive Ab and complement (HS) for 3 h. Northern blots of total RNA samples were hybridized with $^{32}$P-labeled MCP-1 or IL-8 cDNA probes. Hybridization signals on x-ray film were quantified using a laser densitometer. The values were normalized with the levels of total RNA. The results shown are representative of two experiments.

and RANTES genes indicates that the level of induction of intermediary factors by complement could regulate the expression of these chemokine genes. Two phases of chemokine gene induction are clearly separated by inhibition of IL-1$\alpha$. In the absence of any role for IL-1$\beta$ or TNF-$\alpha$, inclusion of anti-IL-1$\alpha$ Ab or IL-1RA only minimally inhibited the expression of MCP-1 and IL-8 genes during the early phase, but prevented later expression of MCP-1, IL-8, and RANTES genes. Based on this finding, we reasoned that the incomplete inhibition of expression of chemokines genes by anti-IL-1$\alpha$ Ab or IL-1RA during the early phase of stimulation reflects a primary response to complement. In other words, both complement and IL-1$\alpha$ rapidly synthesized by complement might have simultaneously influenced the expression of IL-8 and MCP-1 genes during the early phase in cells exposed to higher concentrations of human serum. IL-1$\alpha$-dependent late phase induction of these chemokine genes suggests that the IL-1$\alpha$ gene is more sensitive to complement-induced signaling pathways. Additionally, it may be due to IL-1 inducing its own biosynthesis (46). The dependence of MCP-1, IL-8, and RANTES expression on endogenous IL-1$\alpha$ activity suggests that factors governing local availability of IL-1, such as regional blood flow, could play an important role in inflammation. Consequently, the present study indicates that the expression of chemokines in endothelial cells occurs as a function of differential responses to complement. Consistent with these observations, it has been demonstrated that early induction of the IL-8 and MCP-1 genes and delayed induction of RANTES occur in a number of cell types, including endothelial cells, in response to stimulation by a variety of other agents (7–10, 47, 48).

The pattern of induction of chemokine genes would appear to correlate with in vivo cellular infiltration associated with acute vascular and cellular rejection. It has been well documented that IL-8 predominantly attracts and activates neutrophils, whereas MCP-1 attracts and activates monocytes and T cells (19–22, 25). Although RANTES is known to attract and activate monocytes, a number of studies indicate that RANTES is a potent attractant and activator of T cells (23, 24). Infiltration of neutrophils and macrophages into xenografts has been observed at early time points, consistent with potential involvement of IL-8 and MCP-1 (49). The notable accumulation of T cells during the 2 to 3 days following transplantation of xenogeneic organs may reflect the expression of RANTES brought about by complement activation (50). A slower pattern of expression of these genes and cellular infiltration was recently observed in vivo during skin allograft rejection (51), perhaps reflecting the relative absence of active complement products.

Using IL-1-nonexpressing cells, the present study allowed a separation of primary and secondary mechanisms of induction of MCP-1 and IL-8 genes by MAC. Our investigation with IL-1-nonexpressing cells revealed that early induction of MCP-1 and IL-8 mRNAs required no de novo protein synthesis and appeared to occur at the transcriptional level in primary response to activated complement components. It appears from these observations that MAC may modulate the activity of already existing transcriptional factors and thereby up-regulate the expression of MCP-1 and IL-8 genes.

The signaling pathway by which MAC specifically promotes the expression of MCP-1 and IL-8 genes in endothelial cells has yet to be determined. Previous studies have shown the ability of MAC to induce a number of different cell signaling pathways that could possibly regulate the expression of these genes. Studies from other laboratories demonstrated that the complement components C5b-8 and C5b-9 up-regulate intracellular levels of diacylglycerol and activate protein kinase C (52, 53). MAC may also influence cell function by interacting with guanine nucleotide-binding proteins (G proteins). It is possible that by these pathways deposition of the MAC may modulate the expression or activity of transcriptional factors, in turn leading to a selective up-regulation of MCP-1 and IL-8 genes. A recent study has demonstrated that the translocation of NF-$\kappa$B is necessary for endothelial activation with the induction of IL-8 and MCP-1 genes (54). Activation of NF-$\kappa$B has been shown to play a crucial role in the induction of many different genes by a number of stimuli in the endothelial cells (55). In this context, the IL-1-nonexpressing cells might constitute a unique system to understand the complement-mediated primary response of MCP-1 and IL-8 genes in endothelial cells. Additional studies are underway to understand the precise pathways by which MAC

modulates the expression of these chemokine genes in endothelial cells.

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## References

1. Bjerknes, M., H. Cheng, and C. A. Ottaway. 1986. Dynamics of lymphocyte-endothelial interactions in vitro. *Science 231:402.*
2. Huber, A. R., S. L. Kunkel, R. F. Todd III, and S. J. Weiss. 1991. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science 254: 99.*
3. Springer, T. A. 1995. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu. Rev. Physiol. 57:827.*
4. Baggiolini, M., B. Dewald, and B. Moser. 1994. Interleukin-8 and related chemotactic cytokines-CXC and CC chemokines. *Adv. Immunol. 55:97.*
5. Rot, A. 1992. Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. *Immunol. Today 13:291.*
6. Shimizu, Y., W. Newman, Y. Tanaka, and S. Shaw. 1992. Lymphocyte interactions with endothelial cells. *Immunol. Today 13:106.*
7. Strieter, R. M., S. L. Kunkel, H. J. Showell, D. G. Remick, S. H. Phan, P. A. Ward, and R. M. Marks. 1989. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-$\alpha$, LPS, and IL-1 beta. *Science 243:1467.*
8. Rollins, B. J., T. Yoshimura, E. J. Leonard, and J. S. Pober. 1990. Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE. *Am. J. Pathol. 136:1229.*
9. Sica, A., J. M. Wang, F. Colotta, E. Dejana, A. Mantovani, J. J. Oppenheim, C. G. Larsen, C. O. Zachariae, and K. Matsushima. 1990. Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. *J. Immunol. 144:3034.*
10. Sica, A., K. Matsushima, J. Van Damme, J. M. Wang, N. Polentarutti, E. Dejana, F. Colotta, and A. Mantovani. 1990. IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. *Immunology 69:548.*
11. Dixit, V. M., S. Green, V. Sarma, L. B. Holzman, F. W. Wolf, K. O'Rourke, P. A. Ward, E. V. Prochownik, and R. M. Marks. 1990. Tumor necrosis factor-$\alpha$ induction of novel gene products in human endothelial cells including a macrophage-specific chemotaxin. *J. Biol. Chem. 265:2973.*
12. Cushing, S. D., J. A. Berliner, A. J. Valente, M. C. Territo, M. Navab, F. Parhami, R. Gerrity, C. J. Schwartz, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl. Acad. Sci. USA 87:5134.*
13. Kilgore, K. S., C. M. Flory, B. F. Miller, V. M. Evans, and J. S. Warren. 1996. Membrane attack complex of complement induces interleukin-8 and monocyte chemoattractant protein-1 secretion from human umbilical vein endothelial cells. *Am. J. Pathol. 149:953.*
14. Miller, M. D., and M. S. Krangel. 1992. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. *Crit. Rev. Immunol. 12:17.*
15. Parker, W., S. Saadi, S. S. Lin, Z. E. Holzknecht, M. Bustos, and J. L. Platt. 1996. Transplantation of discordant xenografts: a challenge revisited. *Immunol. Today 17:373.*
16. Platt, J. L., G. M. Vercellotti, B. J. Lindman, T. R. Oegema, Jr., F. H. Bach, and A. P. Dalmasso. 1990. Release of heparan sulfate from endothelial cells: implications for pathogenesis of hyperacute rejection. *J. Exp. Med. 171:1363.*
17. Saadi, S., R. A. Holzkhecht, C. P. Patte, D. M. Stern, and J. L. Platt. 1995. Complement-mediated regulation of tissue factor activity in endothelium. *J. Exp. Med. 182:1807.*
18. Saadi, S., and J. L. Platt. 1995. Transient perturbation of endothelial integrity induced by antibodies and complement. *J. Exp. Med. 181:21.*
19. Yoshimura, T., K. Matsushima, S. Tanaka, E. A. Robinson, E. Appella, J. J. Oppenheim, and E. J. Leonard. 1987. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc. Natl. Acad. Sci. USA 84:9233.*
20. Colditz, I., R. Zwahlen, B. Dewald, and M. Baggiolini. 1989. In vivo inflammatory activity of neutrophil-activating factor, a novel chemotactic peptide derived from human monocytes. *Am. J. Pathol. 134:755.*
21. Rollins, B. J. 1991. JE/MCP-1: an early-response gene encodes a monocyte-specific cytokine. *Cancer Cells 3:517.*
22. Loetscher, P., M. Seitz, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1994. Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human $CD4^+$ and $CD8^+$ T lymphocytes. *FASEB J. 8:1055.*
23. Schall, T. J., K. Bacon, K. J. Toy, and D. V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature 347:669.*
24. Murphy, W. J., D. D. Taub, M. Anver, K. Conlon, J. J. Oppenheim, D. J. Kelvin, and D. L. Longo. 1994. Human RANTES induces the migration of human T lymphocytes into the peripheral tissues of mice with severe combined immune deficiency. *Eur. J. Immunol. 24:1823.*
25. Uguccioni, M., M. D'Apuzzo, M. Loetscher, B. Dewald, and M. Baggiolini. 1995. Actions of the chemotactic cytokines MCP-1, MCP-2, MCP-3, RANTES, MIP-1$\alpha$ and MIP-1$\beta$ on human monocytes. *Eur. J. Immunol. 25:64.*
26. Platt, J. L., B. J. Lindman, R. L. Geller, H. J. Noreen, J. L. Swanson, A. P. Dalmasso, and F. H. Bach. 1991. The role of natural antibodies in the activation of xenogenic endothelial cells. *Transplantation 52:1037.*
27. Platt, J. L., M. A. Turman, H. J. Noreen, R. J. Fischel, R. M. Bolman, and F. H. Bach. 1990. An ELISA assay for xenoreactive natural antibodies. *Transplantation 49:1000.*
28. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest. 52:2745.*
29. Ryan, U. S., and G. Maxwell. 1986. Isolation, culture and subculture of endothelial cells: mechanical methods. *J. Tissue Culture Methods 10:3.*
30. Holzknecht, Z. E., and J. L. Platt. 1995. Identification of porcine endothelial cell membrane antigens recognized by human xenoreactive antibodies. *J. Immunol. 154:4565.*
31. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem. 162: 156.*
32. Selvan, R. S., J. H. Butterfield, and M. S. Krangel. 1994. Expression of multiple chemokine genes by a human mast cell leukemia. *J. Biol. Chem. 269:13893.*
33. Lin, G., A. E. Pearson, R. W. Scamurra, Y. Zhou, M. J. Baarsch, D. J. Weiss, and M. P. Murtaugh. 1994. Regulation of interleukin-8 expression in porcine alveolar macrophages by bacterial lipopolysaccharide. *J. Biol. Chem. 269:77.*
34. Ponte, P., S. Y. Ng, J. Engel, P. Gunning, and L. Kedes. 1984. Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res. 12:1687.*
35. Maliszewski, C. R., B. R. Renshaw, M. A. Schoenborn, J. F. Urban, Jr., and P. E. Baker. 1990. Porcine IL-1$\alpha$ cDNA nucleotide sequence. *Nucleic Acids Res. 18:4282.*
36. Huether, M. J., G. Lin, D. M. Smith, M. P. Murtaugh, and T. W. Molitor. 1993. Cloning, sequencing and regulation of an mRNA encoding porcine interleukin-1$\beta$. *Gene 129:285.*
37. Kuhnert, P., C. Wuthrich, E. Peterhans, and U. Pauli. 1991. Porcine tumor necrosis factor-encoding genes: sequence and comparative analysis. *Gene 102:171.*
38. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227:680.*
39. Bonner, W. M., and R. A. Laskey. 1974. Film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem. 46: 83.*
40. Schall, T. J. 1991. Biology of the RANTES/SIS cytokine family. *Cytokine 3:165.*
41. Maier, J. A. M., P. Voulalas, D. Roeder, and T. Maciag. 1990. Extension of the life-span of human endothelial cells by an interleukin-1$\alpha$ antisense oligomer. *Science 249:1570.*
42. Morgan, B. P. 1995. Physiology and pathophysiology of complement: progress and trends. *Crit. Rev. Clin. Lab. Sci. 32:265.*
43. Rinder, C. S., H. M. Rinder, B. R. Smith, J. C. K. Fitch, M. J. Smith, J. B. Tracey, L. A. Matis, S. P. Squinto, and S. A. Rollins. 1995. Blockade of C5a and C5b-9 generation inhibits leukocyte and platelet activation during extracorporeal circulation. *J. Clin. Invest. 96:1564.*
44. Ward, P. A. 1996. Role of complement, chemokines, and regulatory cytokines in acute lung injury. *Ann. NY Acad. Sci. 796:104.*
45. Coughlan, A. F., M. C. Berndt, L. C. Dunlop, and W. W. Hancock. 1993. In vivo studies of P-selectin and platelet activating factor during endotoxemia, accelerated allograft rejection, and discordant xenograft rejection. *Transplant. Proc. 25:2930.*
46. Dinarello, C. A. 1994. Interleukin-1. *Adv. Pharmacol. 25:21.*
47. Stellato, C., L. A. Beck, G. A. Gorgone, D. Proud, T. J. Schall, S. J. Ono, L. M. Lichtenstein, and R. P. Schleimer. 1995. Expression of the chemokine RANTES by a human bronchial epithelial cell line: modulation by cytokines and glucocorticoids. *J. Immunol. 155:410.*
48. Kondo, T., Y. Watarai, A. C. Novick, H. Toma, and R. L. Fairchild. 1997. T cell-dependent acceleration of chemoattractant cytokine gene expression during secondary rejection of allogeneic skin grafts. *Transplantation 63:732.*
49. Mejia-Laguna, J. E., A. Martinez-Palomo, C. E. Biro, B. Chavez, F. Lopez-Soriano, and M. Garcia-Cornejo. 1972. Morphologic study of the participation of the complement system in hyperacute rejection of renal xenotransplantation. *Am. J. Pathol. 69:71.*
50. Leventhal, J. R., A. J. Matas, L. H. Sun, S. Reif, R. M. Bolman III, A. P. Dalmasso, and J. L. Platt. 1993. The immunopathology of cardiac xenograft rejection in the guinea pig to rat model. *Transplantation 56:1.*
51. Kondo, T., A. C. Novick, H. Toma, and R. L. Fairchild. 1996. Induction of chemokine gene expression during allogeneic skin graft rejection. *Transplantation 61:1750.*
52. Wiedmer, T., B. Ando, and P. J. Sims. 1987. Complement C5b-9-stimulated platelet secretion is associated with a $Ca2^+$-initiated activation of cellular protein kinases. *J. Biol. Chem. 262:13674.*
53. Carney, D. F., T. J. Lang, and M. L. Shin. 1990. Multiple signal messengers generated by terminal complement complexes and their role in terminal complement complex elimination. *J. Immunol. 145:623.*
54. Kilgore, K. S., E. Schmid, T. P. Shanley, C. M. Flory, V. Maheswari, N. L. Tramontini, H. Cohen, P. A. Ward, H. P. Friedl, and J. S. Warren. 1997. Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-$\kappa$B activation. *Am. J. Pathol. 150:2019.*
55. Read, M. A., M. Z. Whitley, A. J. Williams, and T. Collins. 1994. NF-$\kappa$B and I$\kappa$Ba: an inducible regulatory system in endothelial activation. *J. Exp. Med. 179: 503.*