Vol. 210, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 5, 1995 Pages 51-57

INTERFERON γ-INDUCIBLE PROTEIN 10 (IP-10), A MEMBER OF THE C-X-C CHEMOKINE FAMILY, IS AN INHIBITOR OF ANGIOGENESIS

R. M. Strieter[1], S. L. Kunkel*, D. A. Arenberg, M. D. Burdick, and P.J. Polverini†

Departments of Medicine and *Pathology, University of Michigan Medical School, Ann Arbor, Michigan

†Section of Oral Pathology, University of Michigan Dental School, Ann Arbor, Michigan

Received March 28, 1995

Angiogenesis is fundamental to a variety of physiological and pathological processes. While a number of factors have been identified that induce neovascularization, it is becoming increasingly apparent that endogenous angiostatic factors may play an important role in the regulation of angiogenesis during wound repair, chronic inflammation, and growth of solid tumors. In this study, we demonstrate the novel finding that IP-10, a member of the C-X-C chemokine family, is a potent inhibitor of both IL-8 and bFGF-induced angiogenic activity using *in vitro* and *in vivo* assays of angiogenesis. These findings support the contention that IP-10 may be a pivotal cytokine in the regulation of neovascularization.

Angiogenesis is an essential biological event encountered in a number of physiological and pathological processes including, embryonic development, formation of inflammatory granulation tissue during wound healing, chronic inflammation, and growth of malignant solid tumors (1,2). Among the most extensively studied of these angiogenesis-dependent physiological processes is wound repair (3). An important feature of wound-associated angiogenesis is that it is locally transient and tightly controlled. The abrupt termination of angiogenesis that accompanies the resolution of the wound response suggests two possible mechanisms of control, neither of which are mutually exclusive. First, there is probably a marked reduction in angiogenic mediators. Second, a simultaneous increase occurs in the level of angiostatic factors that inhibit new vessel growth (4). Although a number of investigations studying angiogenesis have focused on the identification and mechanism of action of angiogenic factors, recent evidence suggests that angiostatic factors may play an equally important role in the control of neovascularization (4-13).

Recently, interleukin-8 (IL-8), a member of the C-X-C chemokine family, has been found to be a potent mediator of angiogenesis (14-16). In contrast, platelet factor 4 (PF4), another member of the C-X-C chemokine family, has been shown to have angiostatic properties (17). The major difference between IL-8 and PF4 is the presence of the N-terminal ELR (Glu-Leu-Arg) motif

[1]Corresponding Author. FAX:(313) 764-4556.

0006-291X/95 $5.00

that precedes the first cysteine amino acid residue of IL-8, and is important in ligand/receptor interactions on neutrophils (18,19). We hypothesize that members of the C-X-C chemokine family that lack the ELR motif may function as inhibitors of angiogenesis, in a similar manner as PF4.

In this study, we demonstrate that interferon γ-inducible protein 10 (IP-10), another member of the C-X-C chemokine family that lacks the N-terminal ELR motif (20), is a potent inhibitor of angiogenesis. IP-10 was found in a dose-dependent manner to inhibit both IL-8 and basic fibroblast growth factor (bFGF)-induced endothelial chemotaxis *in vitro* and corneal neovascularization *in vivo*. These findings support the notion that C-X-C chemokines may play an important role in the regulation of angiogenesis by acting as either angiogenic or angiostatic factors.

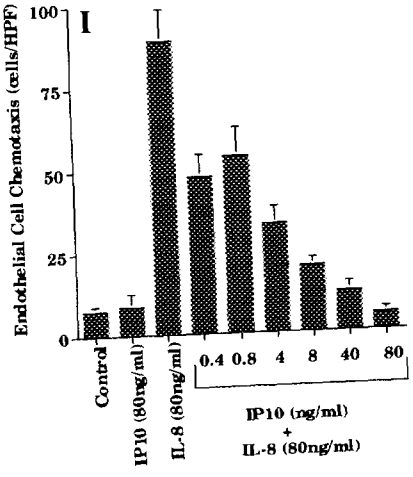
Materials and Methods

Reagents: Human recombinant IL-8 and IP-10 were purchased from Pepro Tech Inc. (Rocky Hill, NJ). bFGF was purchased from R&D Systems Inc. (Minneapolis, MN).
Endothelial cell chemotaxis: Endothelial cell chemotaxis was performed in 48-well chemotaxis chambers (Nucleopore Corp.) as previously described (14,16). Briefly, bovine adrenal gland capillary endothelial cells (BCE) were suspended at a concentration of 10^6 cells/ml in DME with 0.1% BSA and placed into each of the bottom wells (25μl). Nucleopore chemotaxis membranes (5 micron pore size) were coated with 0.1 mg/ml gelatin. The membranes were placed over the wells, chambers sealed, inverted, and incubated for 2 hours to allow cells to adhere to the membrane. The chambers were then reinverted, 50 μl test media was dispensed into the top wells and reincubated for an additional 2 hours. Membranes were then fixed and stained with Diff-Quick staining kit (American Scientific Products) and cells which had migrated through the membrane were counted in ten high power fields (HPF; 400X). Results were expressed as the number of endothelial cells that migrated per HPF. Each sample was assessed in triplicate. Experiments were repeated at least three times.
Corneal micropocket model of angiogenesis: In vivo angiogenic activity was assayed in the avascular cornea of Long Evans rat eyes, as previously described (14-16). Briefly, cytokines were combined with sterile Hydron (Interferon Sciences Inc.) casting solution, and 5 μl aliquots were air-dried on the surface of polypropylene tubes. Prior to implantation, pellets were rehydrated with normal saline. Animals were anesthetized i.p. with ketamine (150mg/kg) and atropine (250ug/kg). Rat corneas were anesthetized with 0.5% proparacaine hydrochloride ophthalmic solution followed by implantation of the Hydron pellet into an intracorneal pocket (1 to 2 mm from the limbus). Six days after implantation, animals were pretreated i.p. with 1000 Units of heparin, anesthetized with ketamine (150mg/Kg), and perfused with 10 mls of colloidal carbon via the left ventricle. Corneas were harvested and photographed. Positive neovascularization responses were recorded only if sustained directional ingrowth of capillary sprouts and hairpin loops towards the implant were observed. Negative responses were recorded when either no growth was observed or when only an occasional sprout or hairpin loop displaying no evidence of sustained growth was detected.
Statistical analysis: Data are expressed as means±SEM. Data that appeared to be statistically significant were compared by Student's *t*- test and considered significant if $p<0.05$.

Results

IP-10 inhibits IL-8-induced angiogenic activity. To assess the affect of IP-10 on angiogenic activity, we began our evaluation by utilizing endothelial cell chemotaxis in the presence or absence of IL-8 (80ng/ml) with or without varying concentrations of IP-10 (0.4ng/ml to 80ng/ml) (Figure 1I). IL-8 alone (89±10 cells per HPF) resulted in maximal endothelial chemotaxis, as compared to either media (control; 7.5±1.5 cells per HPF) or IP-10 (8±4 cells per HPF) alone. However,

when IL-8 (80ng/ml) was combined in the presence of varying concentrations of IP-10 (0.4ng/ml to 80ng/ml), IP-10 was found to significantly attenuate IL-8 induced chemotactic activity by 46% to 93%, respectively. The inhibitory potency of IP-10 for IL-8-induced endothelial chemotaxis was similar to that of PF4 (data not shown). To determine the affect of IP-10 on



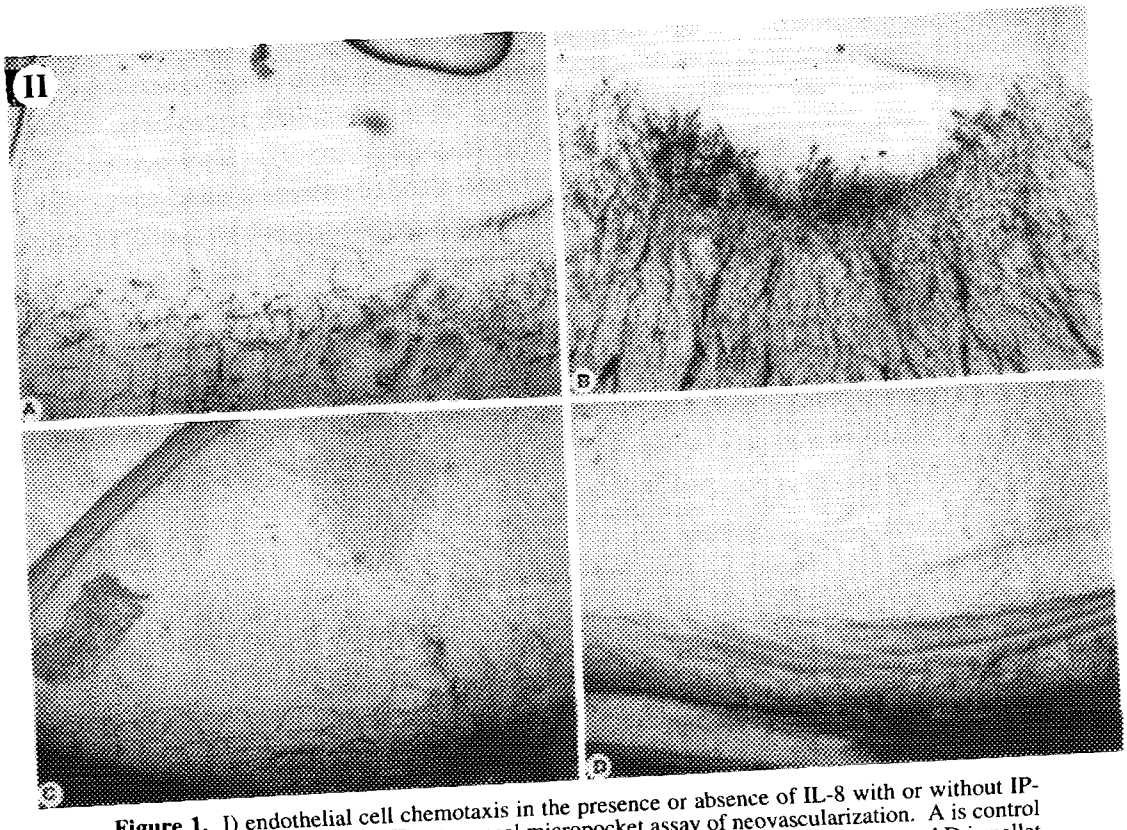


Figure 1. I) endothelial cell chemotaxis in the presence or absence of IL-8 with or without IP-10. Control is media alone. II) rat corneal micropocket assay of neovascularization. A is control pellet alone, B is pellet containing IL-8 (80ng), C is pellet containing IP-10 (80ng), and D is pellet containing combined IL-8 (80ng) and IP-10 (80ng). All photomicrographs are at 50X magnification.

neovascularization *in vivo*, we next evaluated the affect of this chemokine on IL-8-induced angiogenesis utilizing the previously well characterized rat corneal micropocket model (14-16). As shown in Figure 1II, Hydron pellets alone or incorporated with either IP-10 (80ng), IL-8 (80ng), or IL-8 (80ng) combined with IP-10 (80ng) were embedded into the normally avascular rat cornea. IL-8 induced positive corneal angiogenic responses in >90% of the corneas (n=6) without evidence for neutrophilic infiltration. In contrast, Hydron pellets alone (n=6) or incorporated with IP-10 (n=6) resulted in a positive neovascular response in <17% of the corneas. Moreover, when IP-10 was added to Hydron pellets incorporated with IL-8, IP-10 significantly abrogated IL-8-induced angiogenic activity in >83% of the corneas (n=6). These findings support the notion that members of the C-X-C chemokine family may be acting as either angiogenic or angiostatic factors, and the net biological balance of these C-X-C chemokines may play an important role in modulating neovascularization.

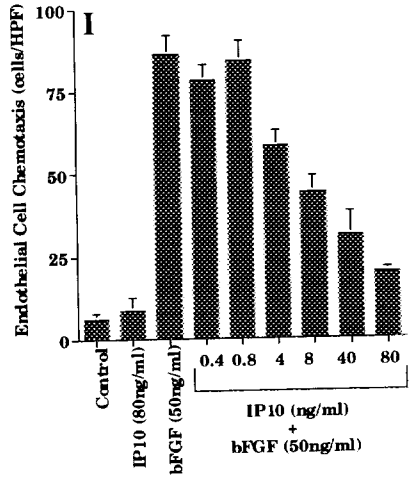
IP-10 inhibits bFGF-induced angiogenic activity. While the above findings provided evidence that IP-10 could inhibit the angiogenic activity of IL-8 in both *in vitro* and *in vivo* assays of angiogenesis, we next determined whether IP-10 could attenuate the angiogenic activity of bFGF, a well studied angiogenic factor and a non-chemokine (1-4). Using endothelial cell chemotaxis in the presence or absence of bFGF (50ng/ml) with or without varying concentrations of IP-10 (0.4ng/ml to 80ng/ml) (Figure 2I). bFGF alone induced maximal endothelial chemotaxis (85±5 cells per HPF), as compared to either media (control; 6±2 cells per HPF) or IP-10 (8±4 cells per HPF) alone. However, IP-10 in a dose-dependent manner (4ng/ml to 80ng/ml) was found to significantly inhibit bFGF-induced endothelial chemotaxis by 33% to 76%, respectively. These findings were similar to the affect of IP-10 on IL-8 angiogenic activity. Since our findings demonstrated that IP-10 inhibited bFGF-induced endothelial cell chemotaxis *in vitro*, we extended our studies to assess the affect of IP-10 on bFGF-induced neovascularization *in vivo*. Hydron pellets incorporated with either IP-10 (80ng), bFGF (50ng), or bFGF (50ng) combined with IP-10 (80ng) were embedded into rat corneas (Figure 2II). bFGF induced positive corneal angiogenic responses in 100% of the corneas (n=6). In contrast, the addition of IP-10 into Hydron pellets incorporated with bFGF (n=6) resulted in a significant reduction of bFGF-induced neovascularization within the corneas, <20% of the corneas were positive. These results demonstrate that IP-10 may play an important role in regulating neovascularization induced by either a C-X-C chemokine or non-chemokine angiogenic factor.

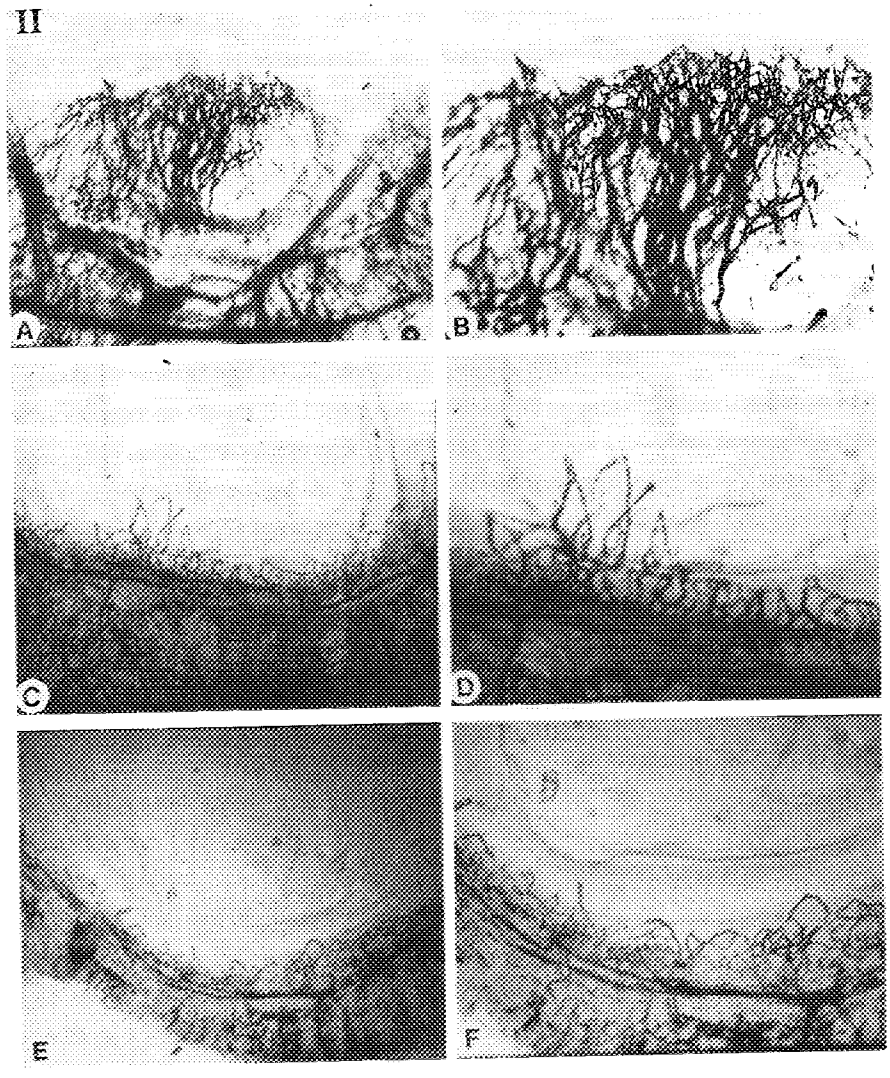
Discussion

The C-X-C chemokine family of cytokines appear to have proinflammatory and reparative activities (21-24). These cytokines are basic heparin-binding proteins less than 10kD, and have four highly conserved cysteine amino acid residues with the first two cysteines separated by one

---

Figure 2. I) endothelial cell chemotaxis in the presence or absence of bFGF with or without IP-10. Control is media alone. II) rat corneal micropocket assay of neovascularization. A and B are the same pellet containing IL-8 (80ng) at 25X and 50X magnification, respectively. C and D are the same pellet containing IP-10 (80ng) at 25X and 50X magnification, respectively. E and F are the same pellet containing combined IL-8 (80ng) and IP-10 (80ng) at 25X and 50X magnification, respectively.


I
Endothelial Cell Chemotaxis (cells/HPF)
100
75
50
25
0
Control
IP10 (80ng/ml)
bFGF (50ng/ml)
0.4 0.8 4 8 40 80
IP10 (ng/ml)
+
bFGF (50ng/ml)


II
A
B
C
D
E
F

non-conserved amino acid residue. These chemokines are all clustered on human chromosome 4, and exhibit between 20% to 50% homology on the amino acid level (21-24). Twelve different C-X-C chemokines have been identified and include PF4, NH_2-terminal truncated forms of platelet basic protein [PBP; connective tissue activating protein-III (CTAP-III), beta-thromboglobulin (βTG), and neutrophil activating protein-2 (NAP-2)], IL-8, growth-related oncogene (GROα), GROβ, GROγ, IP-10, monokine induced by gamma-interferon (MIG), epithelial neutrophil activating protein-78 (ENA-78), and granulocyte chemotactic protein-2 (GCP-2) (21-27). PF4, the first member of the C-X-C chemokine family to be described, was originally identified for its ability to bind to heparin, leading to inactivation of heparin's anticoagulation function. Both IP-10 and MIG are interferon-inducible C-X-C chemokines (20,25,28). While IFNγ induces the production of IP-10 and MIG, it attenuates the expression of IL-8 (29).

Our laboratory and others have found that IL-8 is a potent angiogenic factor (14-16). Recombinant IL-8 mediates both endothelial cell chemotactic and proliferative activity *in vitro* and angiogenic activity *in vivo* (14). In contrast, PF4 has been shown to have angiostatic properties (17), and attenuates growth of tumors *in vivo* (30). While the angiostatic activity of PF4 was initially felt to be due to its heparin binding domain (COOH-terminus) (17,30), recent studies have shown that a PF4 mutant lacking both the heparin-binding domain and functional heparin binding, is equipotent to native PF4 for the attenuation of tumor growth (31). Although it remains unclear whether the COOH-terminus of these chemokines dictates their biological role in angiogenesis, the difference in C-X-C chemokine function could also be explained by other structural domains. A salient amino acid sequence has been identified in the primary structure of the C-X-C chemokine family that appears, in part, to account for the ability of these chemokines to bind and activate neutrophils. These amino acids are Glu-Leu-Arg, the ELR motif, which is absent in certain members of the C-X-C chemokine family (PF4, IP-10, and MIG) that display markedly reduced ability to bind and activate neutrophils. In addition, when the ELR motif was introduced into PF4, this chemokine gained 1000-fold potency in mediating neutrophil chemotaxis (19). Thus, these structural differences, in part, may explain the disparity of angiogenic activity of the C-X-C chemokine family and support the hypothesis that the expression of angiogenic and angiostatic C-X-C chemokines may play a significant role in the regulation of neovascularization.

In this study, we demonstrated a novel finding that IP-10, a member of the C-X-C chemokine family of cytokines, behaves as an angiostatic regulator of neovascularization. IP-10 was found in a dose-dependent manner to attenuate the *in vitro* and *in vivo* angiogenic activity of both IL-8 and bFGF in bioassays of angiogenesis. These findings support the contention that the members of the C-X-C chemokine family may exert disparate effects in mediating angiogenesis for primarily four reasons. First, members of the C-X-C chemokine family that display binding and activation of neutrophils share the same N-terminal ELR motif homology that immediately precedes the first cysteine amino acid residue, whereas, PF4, IP-10, and MIG lack this motif (18,19). Second, IL-8 (contains ELR motif) is angiogenic, while PF4 and IP-10 that lack the ELR motif are angiostatic (14-17). Third, the interferons (IFNα, IFNβ, and IFNγ) are known inhibitors of angiogenesis (32-35). While the interferons down-regulate the expression of the angiogenic factor, IL-8 from monocytes (33), they induce the expression of IP-10 from a number of cells, including keratinocytes, fibroblasts, endothelial cells, and mononuclear phagocytes (20,28). The

IP-10 produced by these cells may act in an autocrine and paracrine manner as a pivotal angiostatic factor that regulates angiogenesis during wound repair, chronic inflammation, and neovascularization associated with tumorigenesis. Thus, the balance in expression of C-X-C chemokines under conditions of neovascularization may be important in regulating net angiogenesis.

Acknowledgments: This work was supported, in part, by NIH grants CA66180, HL50057, and 1P50HL46487 (R.M.S.), HL39926 (P.J.P.), and HL31693 and HL35276 (S.L.K.).

References

1. Folkman, J., and Cotran, R. (1976) International Rev. Exp. Pathol. 16, 207-248.
2. Folkman, J., and Klagsbrun, M. (1987) Science 235, 442-447.
3. Leibovich, S. J., and Weisman, D. M. (1988) Prog. Clin. Biol. Res. 266, 131-145.
4. Bouck, N. (1990) Cancer Cells 2, 179-185.
5. Eisenstein, R., Kuettner, K. E., Neopolitan, C., Sobel, L. W., and Sorgente, N. (1975) Am. J. Pathol. 81, 337-347.
6. Sorgente, N., Kuettner, K. E., Soble, L. W., and Eisenstein, R. (1975) Lab Invest 32, 217-222.
7. Brem, H., and Folkman, J. (1975) J. Exp. Med. 141, 427-439.
8. Lee, A., and Langer, R. (1983) Science 221, 1185-1187.
9. Langer, R., Conn, H., Vacanti, J., Haudenschild, C. C., and Folkman, J. (1980) Proc. Natl. Acad. Sci. USA. 77, 4331-4335.
10. Brem, S., Preis, I., Langer, R., Brem, H., Folkman, J., and Patz, A. (1977) Am. J. Ophthalmol. 84, 323-328.
11. Lutty, G. A., Thompson, D. C., Gallup, J. Y., Mello, R. J., and Fenselau, A. (1983) Inv. Opthalmol. Vis. Sci. 24, 52-56.
12. Madri, J. A., Pratt, B. M., and Tucker, A. M. (1988) J. Cell Biol. 106, 1375-1384.
13. Ingber, D. E., Madri, J. A., and Folkman, J. (1986) Endocrinology 119, 1768-1775.
14. Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G., and Strieter, R. M. (1992) Science 258, 1798-1801.
15. Strieter, R. M., Kunkel, S. L., Elner, V. M., Martonyl, C. L., Koch, A. E., Polverini, P. J., and Elner, S. G. (1992) Am J. Pathol. 141, 1279-1284.
16. Smith, D. R., Polverini, P. J., Kunkel, S. L., Orringer, M. B., Whyte, R. I., Burdick, M. D., Wilke, C. A., and Strieter, R. M. (1994) J. Exp. Med. 179, 1409-1415.
17. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer S. L., Carson, H. F., and Sharpe R. J. (1990) Science 247, 77-79.
18. Hébert, C. A., Vitangcol, R. V., and Baker, J. B. (1991) J. Biol. Chem. 266, 18989-18994.
19. Clark-Lewis, I., Dewald, B., Geiser, T., Moser, B., and Baggiolini M. (1993) Proc. Natl. Acad. Sci USA 90, 3574-3577.
20. Luster, A. D., Unkeless, J. C., and Ravetch, J. V. (1985) Nature 315, 672-676.
21. Baggiolini, M., Walz, A., and Kunkel, S. L. (1989) J. Clin. Invest. 84, 1045-1049.
22. Matsushima, K., and Oppenheim, J. J. (1989) Cytokine 1, 2-13.
23. Oppenheim, J. J., Zachariae, O. C., Mukaida, N., and Matsushima, K. (1991) Annu. Rev. Immunol. 9, 617-648.
24. Miller, M. D., and Krangel, M. S. (1992) Crit. Rev. Immunol. 12, 17-46.
25. Farber, J. M. (1993) Biochem. Biophys. Res. Comm. 192, 223-320.
26. Proost, P., De Wolf-Peeters, C., Conings, R., Opdenakker, G., Billiau, A., and Van Damme, J. (1993) J. Immunol. 150, 1000-1010.
27. Walz, A., Burgener, R., Car, B., Baggiolini, M. Kunkel, S. L., and Strieter, R. M. (1991) J. Exp. Med. 174, 1355-1362.
28. Kaplan, G., Luster, A. D., Hancock, G., and Cohn, Z. (1987) J. Exp. Med. 166, 1098-1108.
29. Gusella, G. L., Musso, T., Bosco, M. C., Espinoza-Delgado, I., Matsushima, K., and Varesio, L. (1993) J. Immunol. 151, 2725-2732.
30. Sharpe, R. J., Byers, H. R., Scott, C. F., Bauer, S. I., and Maione, T. E. (1990) J. Natl. Cancer Inst. 82, 848-853.
31. Maione, T. E., Gray, G. S., Hunt, A. J., et. al. (1991) Cancer Res. 51, 2077-2083.
32. Sidky, Y. A., and Borden, E. C. (1987) Cancer Res. 47, 5155-5161.
33. Zetter, B. R. (1988) Chest 93, 159S-166S.
34. Pober, J. S, and Cotran, R. S. (1990) Pathol. Rev. 70, 427-451.
35. Stout, A. J., Gresser, I., and Thompson, D. (1993) Int. J. Exp. Path. 74, 79-85.