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Hiroo Hashimoto*

Masahiro Yamamura†

Koji Nishiya‡

Zensuke Ota**

*Okayama University,

†Okayama University,

‡Kochi Medical School,

**Okayama University,

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Hiroo Hashimoto, Masahiro Yamamura, Koji Nishiya, and Zensuke Ota

Abstract

The accumulation of polymorphonuclear leukocytes (PMN) in synovial fluid is a common feature of rheumatoid arthritis (RA). We studied the chemotactic response of PMN obtained from the synovial fluid and from the peripheral blood of patients with RA using a modified Boyden's method, in which interleukin-8 (IL-8) or N-formyl-methionyl-leucyl-phenylalanine (FMLP) was used as a chemotactic agent. The IL-8-induced response of peripheral blood PMN from 15 patients with RA did not differ from that of 15 healthy controls. A decreased chemotactic response to IL-8 was, however, observed in PMN from the synovial fluid of 12 patients with RA compared with peripheral blood cells of the same individual. This defective chemotactic ability of PMN was inversely correlated with the number of infiltrating cells in the synovial fluid. We also obtained similar results with FMLP. These results indicate that the chemotactic ability of PMN may be reduced after migrating to the synovial fluid.

KEYWORDS: Interleukin-8, chemotaxis, rheumatoid arthritis, synovial fluid PMN

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Impaired Interleukin-8-Dependent Chemotaxis by Synovial Fluid Polymorphonuclear Leukocytes in Rheumatoid Arthritis

Hiroo Hashimoto*, Masahiro Yamamura, Koji Nishiya^a and Zensuke Ota

Third Department of Internal Medicine, Okayama University Medical School, Okayama 700, Japan and ^aDepartment of Laboratory Medicine, Section of Blood Transfusion, Kochi Medical School, Nankoku-shi, Kochi 783, Japan

The accumulation of polymorphonuclear leukocytes (PMN) in synovial fluid is a common feature of rheumatoid arthritis (RA). We studied the chemotactic response of PMN obtained from the synovial fluid and from the peripheral blood of patients with RA using a modified Boyden's method, in which interleukin-8 (IL-8) or N-formyl-methionyl-leucyl-phenylalanine (FMLP) was used as a chemotactic agent. The IL-8-induced response of peripheral blood PMN from 15 patients with RA did not differ from that of 15 healthy controls. A decreased chemotactic response to IL-8 was, however, observed in PMN from the synovial fluid of 12 patients with RA compared with peripheral blood cells of the same individual. This defective chemotactic ability of PMN was inversely correlated with the number of infiltrating cells in the synovial fluid. We also obtained similar results with FMLP. These results indicate that the chemotactic ability of PMN may be reduced after migrating to the synovial fluid.

Key words: Interleukin-8, chemotaxis, rheumatoid arthritis, synovial fluid PMN

Rheumatoid arthritis (RA) is a chronic inflammatory disease which mainly affects synovial tissues, but its etiology is not well understood. The accumulation of a large number of polymorphonuclear leukocytes (PMN) within the joint spaces is a common feature of this disease. These infiltrating PMN are believed to contribute to the inflammatory condition of the disease through the release of degradative enzymes, oxygen-derived free radicals, and inflammatory cytokines (1, 2). It has been shown that both the chemotactic response and phagocytic capacity of synovial fluid PMN are defective in patients with

RA (3-6). The migration of PMN is mediated by a number of molecules with chemotactic activity, including small peptides of bacterial origin, complement component C5a leukotriene B₄, platelet-activating factor, and inflammatory cytokines. Because interleukin-8 (IL-8) is a potent chemotactic and activating factor for PMN, it is also termed neutrophil-activating peptide-1 (NAP-1) (7-9). IL-8 is now known to be produced by several cell types, including mononuclear cells, fibroblasts, endothelial cells and keratinocytes. Using a specific enzyme linked immunosorbent assay (ELISA) method, the presence of significant levels of IL-8 in synovial fluid has been demonstrated in the RA joints and there is a strong correlation between the concentration of IL-8 and both serum C-reactive protein and the number of infiltrating cells in the synovial fluid (10). IL-8 mRNA has been shown to be spontaneously produced not only by macrophages infiltrating into RA synovial tissue, but also by the synovial fluid mononuclear cells (11-13). These results suggest that this cytokine may also be involved in the accumulation of PMN in RA synovial fluid. Therefore, it is of interest to determine IL-8-induced chemotaxis in peripheral blood and synovial fluid PMN to characterize the role of PMN in RA. In the present study, we examined the IL-8-dependent chemotactic response in peripheral blood PMN from patients with RA and from healthy controls using a modified Boyden's chamber, and compared the chemotactic response of synovial fluid and peripheral blood PMN in patients with RA.

Materials and Methods

Subjects. All patients included in this study satisfied the American Rheumatism Association criteria for RA (14). The chemotactic response of peripheral

*To whom correspondence should be addressed.

Table 1 Clinical features of patients with rheumatoid arthritis

Case	Sex	Stage	Class	Age (years)	Disease duration (years)	ESR (mm/h)	CRP (mg/dl)	Titer of RF (IU/ml)	Number of cells in SF ($\times 10^6$ cells/ml)	Drug therapy
1 SY	(F)	I	II	50	14.0	52	2.7	4.9	6.5	GST
2 SI	(F)	III	II	61	3.7	49	2.2	50.7	7.3	D-Pc
3 HM	(F)	II	I	68	1.0	35	6.0	201.0	2.8	GST
4 MY	(M)	II	II	52	10.0	98	9.5	62.9	N. D.	GST, MTX
5 TF	(F)	III	II	50	2.6	32	3.3	47.5	1.5	GST, PSL
6 AK	(F)	IV	II	83	15.0	51	5.5	17.5	8.0	GST, MTX
7 TM	(F)	III	III	52	18.0	28	8.2	362.0	N. D.	BUC, PSL
8 SN	(F)	IV	IV	67	21.0	15	1.0	17.5	10.0	PSL
9 MI	(F)	II	II	55	5.5	56	7.9	104.7	2.6	TIO, PSL
10 TT	(F)	III	II	49	10.0	105	8.7	1560.0	7.9	NSAID only
11 SM	(M)	II	I	41	5.0	93	10.1	121.0	16.0	BUC, MIZ, PSL
12 FO	(F)	III	III	69	13.0	23	4.6	234.0	21.0	BUC, PSL
Mean \pm SE				58 \pm 3.4	9.9 \pm 1.9	53 \pm 8.7	5.8 \pm 0.9	232.0 \pm 124.6	8.4 \pm 1.9	

Abbreviations: ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; M, male; F, female; GST, gold salt thiomalate; D-Pc, *D*-penicillamine; MTX, methotrexate; TIO, tiopronin; BUC, bucillamine; MIZ, mizoribine; NSAID, non-steroidal anti-inflammatory drug; PSL, prednisolone; SF, synovial fluid; N. D., not done.

blood PMN from patients was compared to that of healthy controls on the same day in order to minimize the variation of assay to assay. These patients consisted of 12 women and 3 men, with a mean age of 47.0 years and a range of 26 to 62 years. All patients received non-steroidal anti-inflammatory drugs, and additional medication included gold salt (2 patients), *D*-penicillamine or bucillamine (10 patients), methotrexate (2 patients), and desferrioxamine (1 patient). Ten patients received low-dose steroids (prednisolone 10 mg/day or less). The healthy controls consisted of 12 women and 3 men with a mean age of 44.0 years and a range of 25 to 60 years. In separate experiments, both synovial fluid and peripheral blood samples were simultaneously obtained from 12 patients with RA and assayed for chemotaxis of PMN. Their clinical features are summarized in detail in Table 1.

Materials. Recombinant human monocyte-derived IL-8 (rIL-8) and N-formyl-methionyl-leucyl-phenylalanine (FMLP) were purchased from Genzyme Co., Cambridge, MA, USA and Sigma Chemical Co., St. Louis, MO, USA, respectively.

Preparation of PMN. The synovial fluid was obtained from patients with RA by arthrocentesis of knee joints and 100 u/ml heparin (Upjohn, Tokyo, Japan) and 20 u/ml hyaluronidase (Mochida Pharmaceutical Co.,

Tokyo, Japan) were immediately added to the samples. Heparinized venous blood was mixed with 1:3 (v/v) with 6% dextran saline solution, and PMN enriched plasma was collected after allowing the red cells to sediment for 30 min at 37°C. PMN were isolated from these peripheral blood and synovial fluid samples by collecting sedimented cells after centrifugation over Ficoll-Hypaque gradients. The cells were washed in medium and resuspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (GIBCO). Next, 1×10^5 cells were cytocentrifuged and the content of PMN was determined after May-Giemsa staining.

Assay for PMN chemotaxis. PMN chemotaxis was measured by a modified Boyden's method (15). PMN were placed on the upper compartment of a chamber separated by a cellulose nitrate filter with 5.0 μ m pores (Sartorius AG, Göttingen, Germany) and a medium in the lower compartment contained a chemotactic agent, rIL-8 or FMLP. After incubation at 37°C in a humidified 5% CO₂ atmosphere, the filter membrane was removed and nonmigrated cells were wiped away from the upper surface very gently. The filter membrane was then fixed with 3.5% formaldehyde and cells were stained with Papanicolaou's hematoxylin solution (Merck, Darmstadt,

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Germany). The number of migrated PMN on the lower surface of the membrane was counted microscopically in each of 10 randomly selected fields at a magnification of 200. The results were represented as the average count per high power field (HPF).

Statistical analysis. The statistical analysis between the values in two groups was performed by Student's *t*-test.

Results

IL-8-induced PMN chemotaxis. The PMN preparation consistently contained over 90 % PMN when

morphologically scored by May-Giemsa staining, as shown in Fig. 1a. The cells were settled on the upper side of the membrane which separated a Boyden's chamber into two compartments, and were incubated with IL-8 placed on the other side. PMN chemotaxis was estimated by counting the number of cells reaching the lower surface of the membrane, as shown in Fig. 1b. The results in Fig. 1c show the number of migrated PMN towards the medium containing different concentrations of IL-8 ranged from 1 to 500 ng/ml. The number of PMN increased in a dose-dependent manner although relatively less at 500 ng/ml which might be due to the presence of an excessively high concentration of IL-8. This indicates direc-

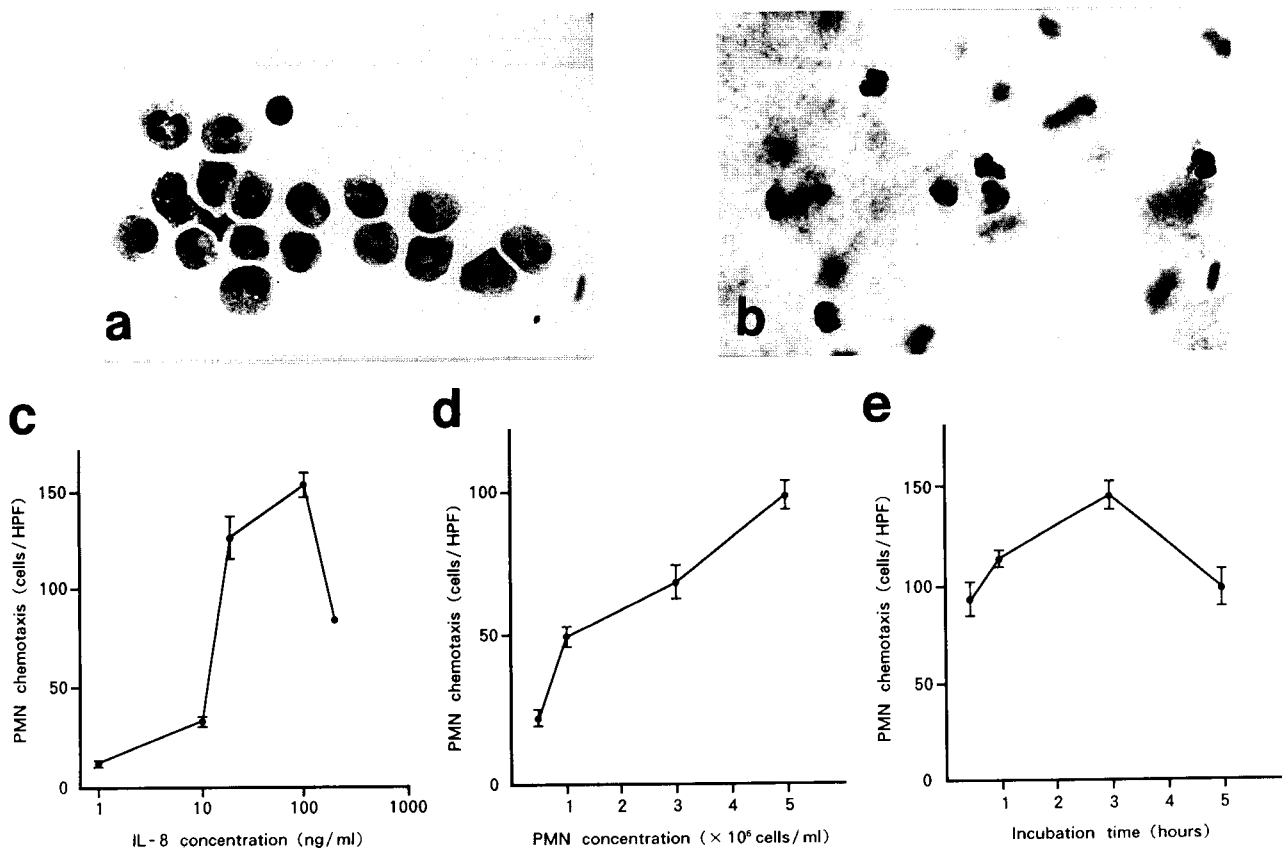


Fig. 1 Chemotactic response of polymorphonuclear leukocytes (PMN) to IL-8. **a:** Cytosmear of PMN preparation with May-Giemsa staining ($\times 1,000$). The PMN-enriched fraction was separated over Ficoll-Hypaque gradients after dextran sedimentation of red blood cells as described in Materials and Methods. **b:** PMN reaching the lower surface of the membrane were stained with hematoxylin ($\times 1,000$). **c:** PMN chemotactic response to varied IL-8 concentrations. PMN were incubated at 3×10^6 cells/ml for 1 h in the presence of different concentrations of IL-8. The number of migrated cells was counted. Results are expressed as the mean \pm SE. **d:** IL-8 induced chemotactic response by different concentrations of PMN in a Boyden's chamber. PMN at different concentrations were incubated for 1 h at 50 ng/ml of IL-8. **e:** Kinetics of PMN chemotactic response to IL-8. PMN were incubated at 3×10^6 cells/ml for 30 min through 5 h at 50 ng/ml of IL-8.

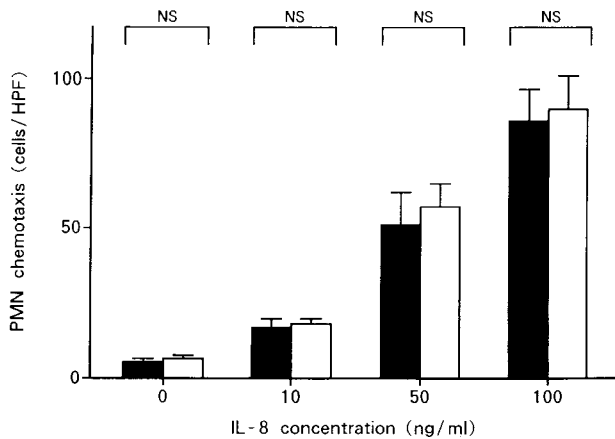


Fig. 2 Chemotactic response to IL-8 in peripheral blood polymorphonuclear leukocytes (PMN) from patients with rheumatoid arthritis and healthy controls. PMN were incubated at 3×10^6 cells/ml for 1 h at different concentrations of IL-8 and the number of migrated cells was counted. Results are expressed as the mean \pm SE. Open and closed columns represent healthy controls and patients with rheumatoid arthritis, respectively.

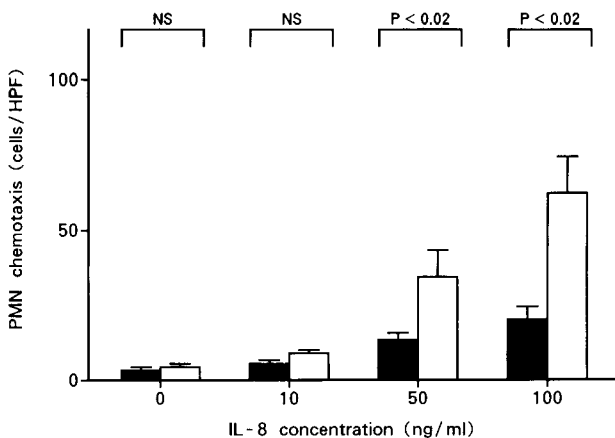


Fig. 3 Chemotactic response to IL-8 in peripheral blood and synovial fluid polymorphonuclear leukocytes (PMN) of patients with rheumatoid arthritis. PMN were incubated at 3×10^6 cells/ml for 1 h at different concentrations of IL-8 and the number of migrated cells was counted. Results are expressed as the mean \pm SE. Open and closed columns represent peripheral blood and synovial fluid PMN, respectively.

tional movement of cells in response to the concentration gradients of IL-8. The number of migrated PMN was also dependent upon the cell concentration in the upper

compartment, as shown in Fig. 1d. The kinetic study, given in Fig. 1e, showed that a 1-h incubation was sufficient to determine IL-8-induced chemotaxis by PMN. From these results, in our comparative study, PMN were incubated for 1 h at a concentration of 3×10^6 cells/ml in a Boyden's chamber, and IL-8 was used at concentrations of 10, 50, and 100 ng/ml.

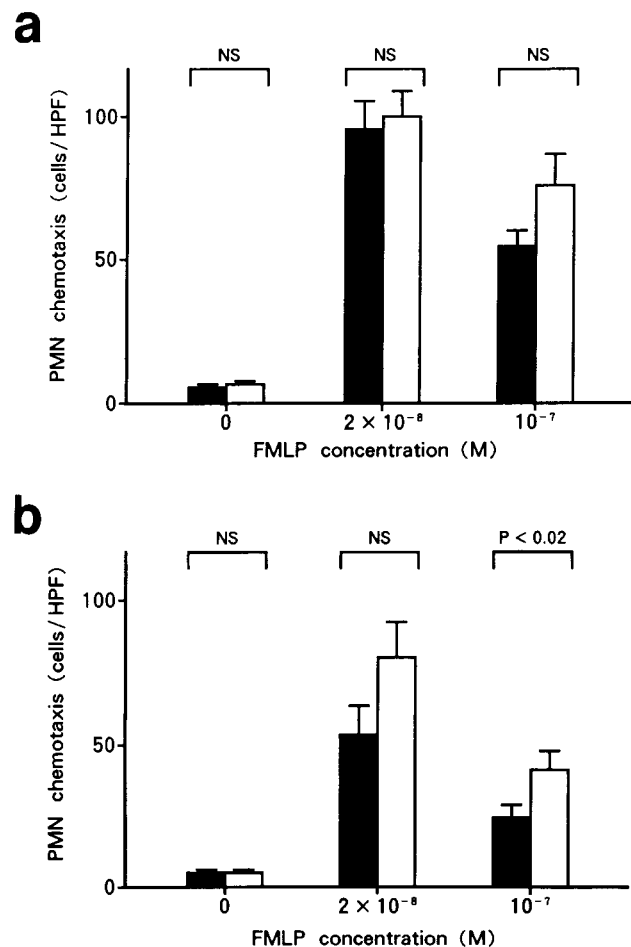


Fig. 4 Polymorphonuclear leukocyte (PMN) chemotactic response to N-formyl-methionyl-leucyl-phenylalanine (FMLP). PMN were incubated at 3×10^6 cells/ml for 1 h at different concentrations of FMLP and the number of migrated cells was counted. Results are expressed as the mean \pm SE. **a:** Chemotactic response to FMLP in peripheral blood PMN from patients with rheumatoid arthritis and healthy controls. Open and closed columns represent healthy controls and patients with rheumatoid arthritis, respectively. **b:** Chemotactic response to FMLP in peripheral blood and synovial fluid PMN from patients with rheumatoid arthritis. Open and closed columns represent peripheral blood and synovial fluid PMN, respectively.

Chemotaxis in RA peripheral blood PMN.

Fig. 2 shows IL-8-induced chemotactic response of peripheral blood PMN from 15 patients with RA and 15 age- and sex-matched healthy controls. Although the mean level of PMN response in patients with RA was slightly lower than that observed in controls, there was no significant difference between the two groups (RA patients *vs* healthy controls: mean \pm SE cells/HPF, 6.2 ± 0.9 *vs* 7.0 ± 1.3 without IL-8, 17.0 ± 2.9 *vs* 17.8 ± 2.3 at 10 ng/ml, 52.1 ± 10.4 *vs* 57.4 ± 8.0 at 50 ng/ml, and 86.0 ± 11.2 *vs* 90.2 ± 10.6 at 100 ng/ml).

Chemotaxis in RA synovial fluid PMN.

The chemotactic response of PMN prepared from the synovial fluid of 12 patients with RA was compared to that of peripheral blood cells of the same individual. As shown in Fig. 3, the response of synovial fluid PMN was decreased compared with peripheral blood cells (synovial fluid PMN *vs* peripheral blood PMN: 4.5 ± 0.5 *vs* 4.9 ± 0.8 without IL-8, 6.6 ± 0.8 *vs* 9.2 ± 1.7 at 10 ng/ml, 13.4 ± 2.9 *vs* 34.3 ± 7.9 at 50 ng/ml, and 20.0 ± 4.7 *vs* 61.8 ± 13.8 at 100 ng/ml). The decrease in chemotaxis of synovial fluid PMN was statistically significant when IL-8 was present at higher concentrations (50 and 100 ng/ml).

FMLP-induced PMN chemotaxis. To determine whether the reduced chemotactic ability of PMN observed in RA synovial fluid is due to a specific defect in IL-8, we compared the PMN chemotactic response to FMLP in the peripheral blood from 15 patients with RA and 15 healthy controls (Fig. 4a), and compared the peripheral blood and synovial fluid of 12 patients with RA (Fig. 4b). No difference in the response of peripheral blood PMN from patients with RA and controls was observed at the concentrations of 2×10^{-8} M (patients with RA *vs* healthy controls; 95.3 ± 10.3 *vs* 99.5 ± 9.1) and 10^{-7} M (54.0 ± 6.3 *vs* 75.3 ± 12.0). In contrast, compared with peripheral blood cells, the response of PMN from the synovial fluid of patients with RA was significantly decreased at a concentration of 10^{-7} M (synovial fluid *vs* peripheral blood: 53.3 ± 10.2 *vs* 78.6 ± 13.4 at 2×10^{-8} M; 24.1 ± 4.9 *vs* 40.8 ± 7.2 at 10^{-7} M).

Correlation between PMN chemotaxis and the number of cells in RA synovial fluid.

The PMN chemotactic response induced by 100 ng/ml IL-8 was inversely correlated with the number of cells infiltrating into synovial fluid as shown in Fig. 5. However, the response of synovial fluid PMN was not correlated with the disease activity assessed by the eryth-

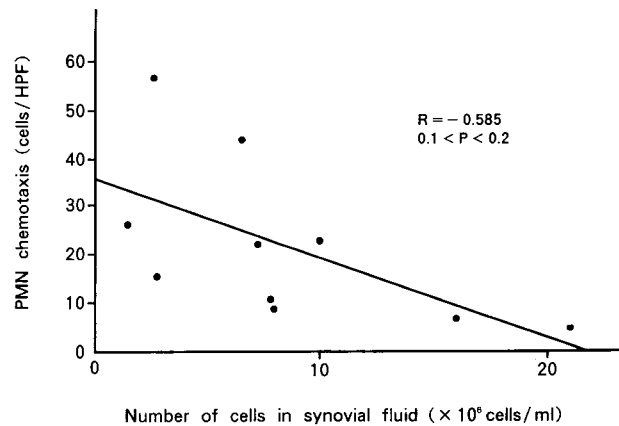


Fig. 5 Correlation of polymorphonuclear leukocyte (PMN) chemotactic response to IL-8 with the number of infiltrating cells in synovial fluid of patients with rheumatoid arthritis. The number of migrating cells was determined after 1 h of incubation at 100 ng/ml of IL-8.

rocyte sedimentation rate, serum C-reactive protein level, and titer of rheumatoid factor (data not shown). Furthermore, no significant correlation was noted between synovial fluid PMN response and drug therapy (with and without steroids) employed for individual patient (data not shown).

Discussion

Hollander *et al.* initially focused on the pathogenic role of PMN in the articular inflammation associated with RA (1, 2). They have observed PMN containing cytoplasmic granules in RA synovial fluid, and these cells have been termed "RA cells". Such cells would likely be attracted by chemotactic stimuli as a result of the formation of complexes of gamma-globulin with rheumatoid factor and phagocytose the deposit of these immune complexes. These cells actively release lysosomal enzymes, contributing to chronic inflammation and tissue destruction.

The mechanism of PMN accumulation in inflammatory tissue has been clarified in recent years, accompanied by the characterization of molecules with chemotactic activity. IL-8 was first identified and characterized as a polypeptide with chemotactic activity for PMN (7, 9). This cytokine also has been shown to have chemotactic activities for T cells and basophils, and to activate PMN. It is produced

by a variety of cell types including monocytes. IL-8, as with other inflammatory cytokines, has been shown to be produced by both RA synovial tissue and synovial fluid cells, and its protein is detected in RA synovial fluid at significant levels (10-13). These findings indicate that PMN migrating to RA synovial fluid may be exposed to IL-8 in addition to other mediators such as C5a, leukotriene B₄, and platelet-activating factor.

Our data showed that IL-8-induced chemotaxis of peripheral blood PMN from patients with RA was not different from that of healthy controls, corresponding with most of previously reported studies where zymosan-activated plasma and casein were used as chemotactic stimuli (16, 17). The chemotactic response of PMN in the synovial fluid of RA, however, was significantly decreased as compared to that of circulating cells. This also agrees with the previous results by Kemp *et al.* (3). They have found a markedly reduced chemotactic response to casein in synovial fluid PMN. We also obtained similar results with FMLP, a small chemotactic peptide of bacterial origin.

It has been recently reported that expression of IL-8 receptors is rapidly down-regulated via its internalization after binding to IL-8 (18). Since IL-8 has been detected in RA synovial fluid at much higher levels than in peripheral blood (10), the reduction in IL-8-dependent chemotaxis could be attributable to decreased receptor expression on synovial fluid PMN. We examined whether synovial fluid PMN could restore the response following incubation for 2 h at 37°C in medium without IL-8, which could allow reexpression of the receptor. However, this treatment only partially enhanced PMN responsiveness to IL-8 (data not shown), indicating that the impairment in IL-8-induced chemotaxis found in RA synovial fluid PMN could not be fully explained by this mechanism.

Peichl *et al.* have demonstrated the presence of anti-IL-8 antibody in the peripheral blood of RA, and a strong correlation between the antibody level and the disease activity (19). Although it remains unclear whether the cytokine antibody is actually operating *in vivo* as an inhibitor or merely a carrier, this implies that the natural antibody may be responsible for the suppression of IL-8-mediated PMN chemotaxis. However, we could not find a chemotactic defect in the circulating PMN. Moreover, PMN chemotaxis induced by FMLP, which is not thought to be present in the synovial fluid, was also impaired in RA synovial fluid. These results suggest that the chemotactic ability of PMN, but not the response to

IL-8, may be reduced after migrating to the synovial fluid.

There are a number of PMN infiltrating into RA synovial fluid. It is believed that PMN activation may occur in the synovial fluid or prior to their arrival. Previous studies have demonstrated that PMN from the synovial fluid (4-6) of patients with RA exhibit a defect in phagocytic capacity as well as in chemotactic ability (3). These findings suggest that PMN which accumulate in RA synovial fluid may become functionally impaired through the activation of PMN during migrating events. Our results lend more support to this idea.

The mechanisms responsible for the activation of PMN in the joint of RA are very complex. It has been reported that incubation of normal PMN with serum from patients with RA impairs their capacity to migrate chemotactically, presumably, in part, as a result of prior endocytosis of immune complexes containing rheumatoid factor (20-22). Indeed, most of PMN found in RA synovial fluid are characterized by the presence of phagocytosed rheumatoid factor complexes (2). These suggest that immune complexes could be a potent activator for PMN in RA joint. We found an inverse relationship between PMN chemotactic response to IL-8 and the number of cells in RA synovial fluid. The previous study has shown that the levels of IL-8 in synovial fluid are related to the number of infiltrating cells (10). It is therefore conceivable that IL-8, as well as immune complexes, strongly induces the chemotaxis and activation of PMN in the RA joint, and consequently reduces their chemotactic activity.

We demonstrated the impaired chemotactic response of PMN from the synovial fluid of patients with RA although a chemotactic defect was not found in their peripheral blood cells. Our results imply that synovial fluid PMN may become desensitized to chemotactic stimuli through the inflammatory process within the RA joint, but that this may not be due to an intrinsic defect in PMN function of patients with RA. However, the mechanisms of chemotactic desensitization of PMN at inflammatory sites remain to be elucidated.

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