Effect of immune complexes in serum from patients with rheumatoid vasculitis on the expression of cell adhesion molecules on polymorphonuclear cells

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Abstract

Objective

Immune complexes (IC) are frequently detected in patients with rheumatoid vasculitis (RV). To explore the pathogenic role of IC in the development of vasculitis among patients with rheumatoid arthritis (RA), we examined the effect of IC on the expression of cell adhesion molecules (CAM) on polymorphonuclear cells (PMN).

Methods

PMN from healthy volunteers were incubated with the sera from 26 patients with RA including 9 patients with RV, and the expression of CAM on the PMN was assessed by flow cytometry.

Results

We found that 67% (6/9) of the serum samples from RV patients and 18% (3/17) of the samples from RA patients without RV revealed up-regulated CD11b expression. On the other hand, 89% (8/9) of the samples from RV patients and 12% (2/17) of the samples from RA patients without RV revealed up-regulated CD18 expression. However, the expression of CD11a was not affected. Up-regulation of CD11b and CD18 on PMN was also induced by the immunoglobulin G (IgG) fraction of the sera of RV patients. Moreover, L-selectin expression on PMN was down-regulated by the sera or IgG of some patients with RV. These changes in CAM expression on PMN induced by IgG of RV patients were not observed when PMN were incubated with the IgG of RV patients from which the IC formed by IgG had been removed.

Conclusion

These results suggest that IC formed by IgG in patients with RA are involved in the development of vasculitis by affecting the expression of CAM on PMN.

Key words

L-selectin, CD11b, CD18, immune complexes, rheumatoid vasculitis.

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Introduction

Polymorphonuclear cells (PMN) play an important role in the development of inflammatory diseases such as rheumatoid arthritis (RA). RA is a chronic inflammatory disease characterized by infiltration and accumulation of leukocytes in the inflamed joints. PMN migrate into the synovial fluid, generate toxic oxygen metabolites, and release granular protease, resulting in destruction of cartilage and bone (1). This process is initiated by adherence of circulating PMN to activated vascular endothelial cells, mediated by cell adhesion molecules (CAM) such as L-selectin (CD62L) and integrins including CD11/CD18 (2, 3) on PMN. Activation of PMN by numerous physiologic stimuli results in shedding of L-selectin and up-regulation of CD11b/CD18 expression. Upon stimulation, the level of CD11a/CD18 expression is unaltered; instead, the CD11a/CD18 molecules undergo a conformational change, resulting in activation of these molecules (4). In fact, the PMN in the inflamed joints express a lower level of L-selectin and a higher level of CD11b on their surface than PMN in peripheral circulation of RA patients (5, 6). Moreover, PMN on which the expression of CD11b and CD18 is up-regulated compared to that on the PMN of healthy subjects, are also present in the circulation of some patients with RA (7).

The active phase of RA is known to be associated with an increase of immune complexes (IC) formed by rheumatoid factor(s) (RF) in the sera of RA patients. Although the exact pathogenic role of RF has not yet been elucidated, RF participates in the pathogenesis of RA through the formation of IC. In the synovial fluid of the inflamed joints, transmigrated plasma cells synthesize RF, especially IgG-RF, which is released into the synovial fluid, resulting in the formation of large IC (8). These IC in the synovial fluid activate NADPH oxidase on neutrophils of the inflamed joint and induce the release of myeloperoxidase (MPO) from the neutrophils (9). However, IC are more often observed in the sera of RA patients with extra-articular disease manifestation such as vasculitis, than in those with the disease confined to the joints (10). Recently, it was reported that cross-linking of the Fc receptor affects the expression of CAM on PMN (11). These reports led us to hypothesize that IC affect the level of CAM expression on PMN in peripheral circulation and might be involved in the development of vasculitis among RA patients. To verify this hypothesis, we investigated the expression of CAM on PMN after incubation with the sera or IgG from patients with RA. Since PMN release IL-8 (12) which affects the expression of CAM on PMN (2, 3, 13), we also determined the expression of IL-8 in PMN which had been incubated with the IC from patients with RV to evaluate whether changes in the expression of CAM on PMN are related to IL-8 release.

Materials and methods

Patients and controls

The sera of 26 patients with RA, 16 patients with systemic lupus erythematosus (SLE), and 14 normal subjects were evaluated. The clinical characteristics of the patients enrolled in this study are summarized in Table I. RA patients (26 subjects) were diagnosed according to the 1987 Revised Criteria of the American College of Rheumatology (ACR, ref. 14). Nine of the 26 patients exhibited one or more of the following symptoms of rheumatoid vasculitis (RV, Table I). Biopsy was performed in these 9 cases, and if it unequivocally demonstrated the presence of leukocytoclasis or fibrinoid necrosis of the vascular wall, the patient was classified as having histologically proven RV (15, 16). Sixteen patients fulfilled the ACR criteria for systemic lupus erythematosus (SLE) (17). The control group consisted of 14 normal subjects without any medical or known inflammatory conditions. Since complements affect the expression of CAM on PMN (3), each serum sample was incubated at 56°C for 30 min to inactivate the complements.

Preparation of PMN

PMN were isolated from heparinized venous blood from randomly selected healthy volunteers with Mono Poly-

Table I. Patients with rheumatoid vasculitis: Clinical manifestations, laboratory data and treatments.

Case	Sex	Age	Disease duration (years)	No. of painful joints	No. of swollen joints	CRP < 0.3 mg/dl	RF < 20 IU/dl	IgG-RF < 2.0	CIC < 4.2 mg/ml	Extra-articular symptoms and signs	Treatments for vasculitis
1	F	57	6	8	2	4.3	1044	5.3	4.4	Digital ulcers, polyneuritis multiplex, episcleritis, nodules	Prednisolone 30 mg/d, alprostadil (LipoPGE1) 10 mg/d, div, sarpogrelate hydrochloride 300 mg/d
2	F	63	9	10	8	9.9	1060	6.4	28.6	Skin ulcers, nodules, episcleritis, pneumonitis	Prednisolone 15 mg/d, azatiopurine 50 mg/d, alprostadil (LipoPGE1) 10 mg/d,div, sarpogrelate hydrochloride 300 mg/d
3	F	71	15	15	6	14.5	86	3.6	8.4	Episcleritis, pericarditis, mononeurits multiplex	Methylprednisolone 1g/d for 3 days, beraprost sodium 120 mg/d
4	M	63	4	6	4	7.5	241	3.7	3.7	Mononeurits multiplex, pleuritis, pneumonitis, digital ulcers, nodules, necrotizing glomerulonephritis	Methylprednisolone 1g/d for 3 days, PP*, beraprost sodium 120 mg/d
5	M	63	24	10	4	7.6	174	0.9	8.3	Digital ulcers and gangrene, polyneuritis, pneumonitis	Methylprednisolone 500 mg/d for 3 days, PP, dipyridamole 75 mg/d, limaprost alfadex 30 mg/d
6	M	72	10	12	5	14.2	1067	6.2	10.5	Mononeurits multiplex, pleuritis, nodules	Prednisolone 40 mg/d, PP, ticlopidine hydrochloride 300 mg/d
7	M	49	6	10	10	6.4	102	5.5	5.5	Episcleritis, digital gangrene, leg ulcers, nodules, pneumonitis	Prednisolone 40 mg/d, methylprednisolone 500 mg/d for 3 days, cyclophosphamide 50 mg/d, dipyridamole 75 mg/d
8	F	61	18	8	6	17.6	531	3.1	6.3	Leg ulcers, episcleritis, pneumonitis, polyneuropathy	Prednisolone 30 mg/d, PP, ticlopidine hydrochloride 300 mg/d, alprostadil (LipoPGE1) 10 mg/d,div
9	F	51	30	6	4	19.9	304	7.5	4.8	Digital ulcers and gangrene, mononeurits multiplex	Prednisolone 30 mg/d, alprostadil (LipoPGE1) 10 mg/d,div, **PP, cilostazol 200 mg/d, ethyl icosapentate 1800 mg/d**

Case	Sex	Age	Disease duration (yrs.)	No. of painful joints	No. of swollen joints	CRP <0.3 mg/dl	RF < 20 IU/dl	IgG-RF <2.0	CIC <4.2 mg/ml
Mean ± SD positive (%)	M/F = 4/5	61.1 ± 7.9⁺	13.6 ± 9.0	9.4 ± 2.9⁺	5.4 ± 2.4⁺	11.3 ± 5.4⁺ 100	512 ± 429⁺ 100	4.7 ± 2.0⁺ 88.9⁺	8.9 ± 7.7⁺ 88.9⁺
RA (n = 17) positive (%)	M/F = 2/15	49.8 ± 16.2	8.4 ± 8.4	4.5 ± 3.9	2.3 ± 2.5	5.3 ± 5.4 82.4	166 ± 177 88.2	1.7 ± 1.7 41.2	2.9 ± 1.0 17.6
SLE (n = 16) positive (%)	M/F = 1/15	38.6 ± 9.82	NE	NE	NE	NE	36.9 ± 56.8⁺ 25.0⁺	1.4 ± 1.0 6.0	3.6 ± 1.9# 50.0

*PP; plasmapheresis (double filtration method), ⁺p<0.05 as compared to RA, #p<0.05 as compared with RV, NE: not examined.

Resolving Medium (Flow Laboratories Inc., McLean, USA), according to the manufacturer's instructions. After isolation, the PMN were washed with phosphate-buffered saline (PBS), suspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), and kept at 4°C until use.

Reagents and antibodies (Ab)

TNF was purchased from Genzyme Corp (Cambridge, USA). Lipopolysaccharide (LPS) (from *E. coli*, B0111) was purchased from Sigma Chemical Co. (St. Louis, USA). Anti-human L-selectin monoclonal antibody (mAb) was purchased from Chemicon International, Inc. (Temecula, USA). Anti-human CD11a mAb (25.3, mouse IgG1), anti-human CD11b mAb (Bear 1, IgG1) and anti-human CD18 mAb (7E4, mouse IgG1) were purchased from Immunotech (Marseille, France).

Flow cytometry

PMN (5 x 10^5 cells/100 ml) suspended in RPMI 1640 medium containing 10% FBS were incubated with sera, IgG or TNF (10 ng/ml) in a CO_2 incubator for the specified length of time. The cells were then washed twice with ice-cold RPMI 1640 medium containing 5% FBS (washing solution), and maintained on ice during subsequent preparation of the cells for flow cytometry. The cells were stained for 30 min with a saturating concentration of the primary mAb diluted with washing solution, followed by reaction with phycoerythrin (PE)-conjugated goat anti-mouse IgG (Immunotech) for 30 min. After fixation with 1% paraformaldehyde in PBS, the cells (10,000/group) were analyzed by flow cytometry using EPICS ELITE (Colter, Hialeah, USA). Data were expressed as follows: % expression of CAM = Mean fluorescence intensity (MFI) of treated PMN / MFI of untreated PMN x 100.

Purification of IgG from sera

IgG was purified from sera using the MAbTrapG II kit (Pharmacia, Uppsala, Sweden). Briefly, 1 ml of serum diluted with PBS at 1:10 was centrifuged at 8,000 g for 10 min, and the supernatant was filtered with a 0.45 m filter. After this solution had been loaded on a protein G Sepharose column (1 ml), the column was washed with 2 ml of the Binding Buffer. Then, IgG was eluted with the Elution Buffer and neutralized with the Neutralizing Buffer. Fractions of 500 l were collected. After purification, the solution containing IgG was condensed using Microcon 30 (Amicon Inc., Beverly, USA) and diluted with PBS at a concentration of 10 mg/ml. The solution of IgG from each RA and normal subject was kept at room temperature for 30 min. The IgG solution was centrifuged at 8,000g for 10 min, and the sediment and the supernatant were defined as self-aggregated IC and soluble IgG (IgG not included in IC) in RA, respectively. The self-aggregated IC was used after resuspension in PBS. The purity of IgG was assessed by standard SDS-PAGE, and the final protein concentration of IgG was evaluated using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, USA).

Quantitation of soluble L-selectin (sL-selectin) and IL-8

PMN (1 x 10^6 cells/100 ml) suspended in RPMI 1640 medium containing 10% FBS were treated with IgG (200 g/ml), TNF (10 ng/ml) or LPS (1 g/ml) for 2 hr in a CO_2 incubator, and then the supernatant was collected. The level of sL-selectin and IL-8 in the supernatant was determined using the sL-selectin ELISA kit (Bender MedSystem, Vienna, Austria) and Predicta IL-8 ELISA kit (Genzyme), respectively, according to the manufacturer's instructions. The sensitivity of these assays is 0.3 ng/ml for sL-selectin and 1 pg/ml for IL-8.

Determination of mRNA level in IL-8

After incubation with IgG (200 g/ml) or LPS (1 g/ml) for 2 hr, the PMN (1 x 10^6 cells) were lysed in 0.2 ml of RNA zole B (Tel-test Inc., Friendswood, USA). Total RNA was extracted with chloroform and precipitated with isopropanol. The RNA pellet was washed with ethanol, resuspended in 30 l of diethylpyrocarbonate-treated (DEPC) water, and reverse-transcribed by adding 0.5 g of oligo-dT primer (Promega, Madson, USA). The RNA/ primer mixture was incubated at 70°C for 10 min, and then immediately cooled on ice, followed by incubation at 42°C for 45 min. The reverse transcription (RT) reactions were terminated by incubating the samples at 95°C for 5 min. Next, polymerase chain reaction (PCR) amplification of the target gene sequence was carried out in a reaction mixture which contained 1 l cDNA, 5 l PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3, Boehringer Mannheim,Mannheim,Germany), 1 l of 10 mM dNTPs (PCR Nucleotide Mix, Boehringer Mannheim), 0.5 pmol of the respective 5' primer (IL-8: 5'-ATGA CTTCCAAGCTGG-CCGTGGCT-3'; and -actin: 5'-GCCCAGAGCAAGAGAAGGTAT-3') and 3' primer (IL-8: 5'-TCTCAGCC-CTCTTCAAAAACT TCTC-3'; and -actin: 5'-GGCCATCT CTTGCTC-GAAGT-3') and 0.5 units of Taq polymerase (Boehringer Mannheim) to a total volume of 50 l water. The PCR mixture was overlaid with mineral oil, and then amplified in a thermal cycler. The amplification profile was as follows: initial denaturation at 94°C for 5 min, then denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min for 35 cycles, and a final step at 72°C for 5 min.

Rheumatoid factors and other laboratory parameters

RF was determined with the latex turbidimetric immunoassay (TIA). The normal range was less than 20 IU/ml. IgG-RF was determined by ELISA (Eitest IgG-RF, Eisai, Tokyo, Japan) and the value was expressed as index (normal range: less than 2.0). C-reactive protein (CRP) were determined with TIA (normal range: less than 0.3 mg/dl). IC was determined utilizing the CICmRF method (Nissui Ltd., Tokyo, Japan) and normal range was less than 4.2 g/ml. Sera at the onset of vasculitis were selected and used for analyses.

Statistical analysis

The means of all groups were analyzed for overall significance of difference by Kruskal-Wallis analysis and differences between groups were tested by

Dann's test or Mann-Whitney's U test. P values < 0.05 were considered to be statistically significant.

Results

Expression of CAM on PMN upon incubation with sera from RA, SLE, and normal subjects

Incubation of PMN with the sera (1:50) of RA, SLE and normal subjects for 2 hrs resulted in down-regulation of L-selectin expression and up-regulation of CD11b and CD18 expression in all cases. When the cut-off value of normal down-regulation of L-selectin expression was set as the mean - 2 SD of the level of L-selectin expression of the Normal group, lower expression of L-selectin on PMN than the cut-off values were observed after incubation of PMN with the sera of 4 of the 9 (45%) RV patients and 6 of the 17 (35%) RA patients without vasculitis. When the cut-off value of normal up-regulation of CD11b and CD18 was set as the mean + 2 SD of the level of expression of the Normal group, higher expression of CD11b on PMN than cut-off values were observed after incubation of PMN with the sera of 6 of the 9 (67%) RV patients and 3 of the 17 (18%) RA patients without vasculitis. Moreover, the level of CD18 expression on PMN was higher than the cut-off value after the incubation of PMN with the sera of 8 of the 9 (89%) RV patients and 2 of the 17 (12%) RA patients without vasculitis (Fig. 1).

The significant differences between RV patients and RA patients without vasculitis was observed in the case of CD18 expression (Kruskal-Wallis analysis following Dann's test; $p < 0.05$). When RA patients including RV patients were divided into negative and positive groups for CAM expression by the cut-off values, there were significant differences between the groups with respect to IgG-RF (CD11b, C18) and CIC (CD18, Table II). On the other hand, the level of expression of CD11a did not change upon incubation with any of the serum samples used in this study. The sera of SLE patients did not induce significant changes in the level of expression of L-selectin, CD11a, CD11b or CD18 on PMN.

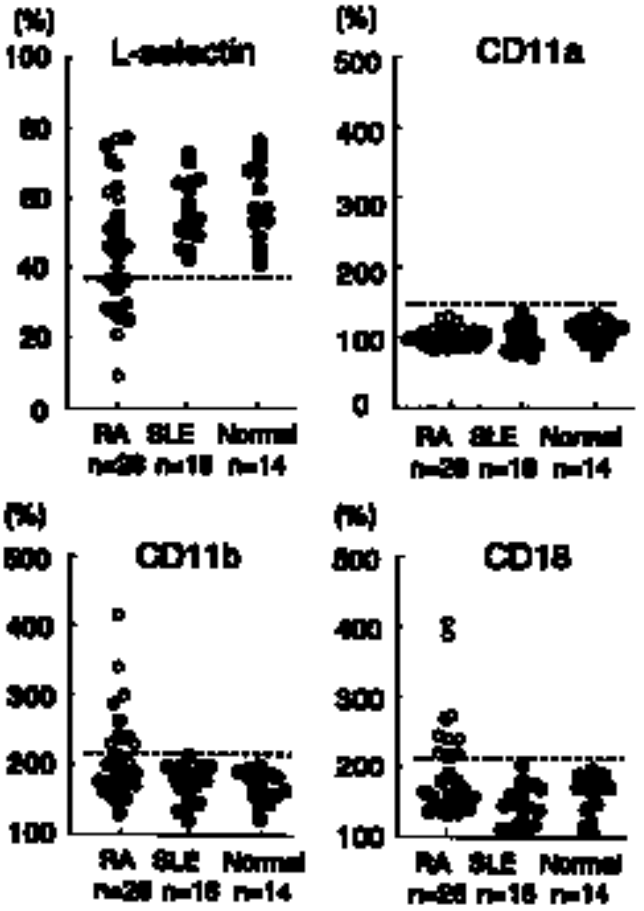


Fig. 1. Expression of CAM on PMN upon incubation with the sera of RA patients, SLE patients and normal subjects. After incubation of PMN with the serum of a subject (1:50) for 2 hrs,the expression of L-selectin,CD11a,CD11b and CD18 on the PMN was analyzed by flow cytometry as described in the *Materials and Methods.* The cut-off values (mean + 2 SD of the Normal group for CD11a,CD11b and CD18; mean - 2 SD of the Normal group for L-selectin) are indicated by the broken line. : serum of patients with rheumatoid vasculitis (RV).

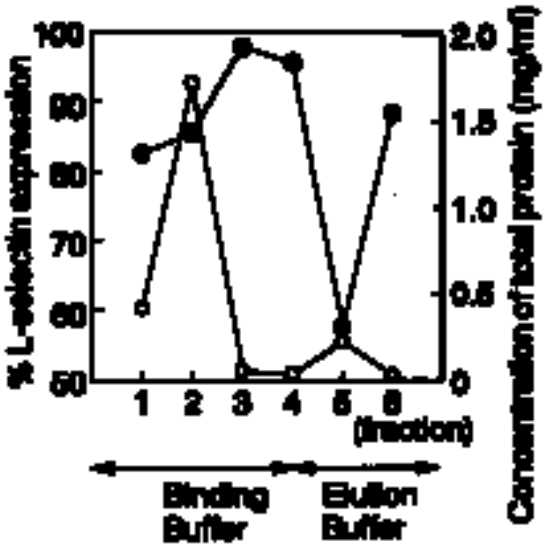
Table II. Laboratory indices of RA patients:positive and negative groups for expression of cell adhesion molecules.

		No. of patients	CRP (mg/dl)	RF (IU/dl)	IgG-RF	CIC (g/ml)
L-selectin	Positive	10	7.8 ± 6.4	417 ± 407	3.2 ± 2.4	4.6 ± 2.8
	Negative	16	6.6 ± 5.9	228 ± 264	2.5 ± 2.2	5.3 ± 6.5
CD11b	Positive	9	9.5 ± 6.1	458 ± 459	4.0 ± 2.4*	7.4 ± 8.4
	Negative	17	6.2 ± 6.4	195 ± 167	2.1 ± 2.1	3.7 ± 2.2
CD18	Positive	10	8.8 ± 5.9	435 ± 436	4.3 ± 2.3*	8.1 ± 7.6*
	Negative	16	6.5 ± 6.2	192 ± 200	1.8 ± 1.7	3.0 ± 1.4

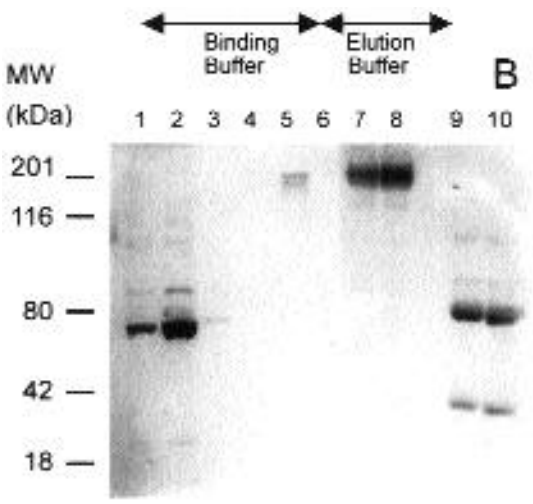
Laboratory indices in RA patients including RV patients represented as mean ± SD.
*$p < 0.05$ as compared with negative group.

Expression of CAM on PMN upon incubation with IgG purified from the serum of RV patients

To determine the factor(s) in the sera of RV patients which down-regulate L-selectin expression and up-regulate CD11b and CD18 expression on PMN, we examined the expression of CAM on PMN after incubation with IgG purified from the serum of RV patients. Figure 2A shows a representative elution profile of the protein G Sepharose column for purification of IgG from the



(A)



(B)

Fig. 2. Elution profile (**A**) and SDS-PAGE analysis (**B**) of IgG which was purified from the serum of a patient with RV. (**A**) Elution profile. 10 l of each fraction was incubated with PMN for 2 hrs, and the level of L-selectin expression was evaluated by flow cytometry as described in the *Materials and Methods*. indicates the expression of L-selectin on PMN. indicates the concentration of total protein in each fraction.
(**B**) SDS-PAGE analysis. Fractions 1-6 diluted with PBS at 1:10 (Lanes 1-6) from A, soluble IgG (Lanes 7 and 9; 1 g/lane) and self-aggregated IC (Lanes 8 and 10; 1 g/lane) were resolved on 5-20% gradient SDS gel under non-reducing conditions (Lanes 1-8) or reducing conditions (Lanes 9 and 10). The proteins were visualized by Coomassie Blue R-250.

serum of a RV patient. The down-regulation of L-selectin expression on PMN was observed after incubation with the fraction including IgG (Fraction 5 in Fig. 2A).

Upon incubation of PMN with the IgG fraction from the serum of RV patients for various lengths of time, the level of expression of L-selectin on PMN changed in a time-dependent manner (Fig. 3). Incubating PMN with TNF (10 ng/ml) down-regulated L-selectin expression on PMN within 15 min. However, incubation of PMN with the IgG fraction (200 g/ml) from the sera of RV patients, resulted in a gradual decrease of L-selectin expression over 2 hrs (Fig. 3).

Upon incubation of PMN with various concentrations of IgG from RV patients and normal subjects for 2 hrs, a concentration-dependent decrease in L-selectin expression and a concentration-dependent increase in CD11b and CD18 expression were observed (Fig. 4). Representative flow cytometric results upon incubation of PMN with the IgG fraction from a RV patient and that of a normal subject, are shown in Figure 5. Namely, L-selectin expression was down-regulated and the expression of CD11b and CD18 was up-

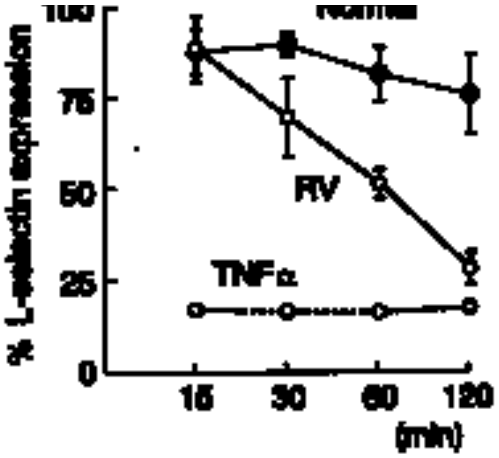


Fig. 3. Kinetics of down-regulation of L-selectin expression on PMN by IgG. PMN were incubated with the fraction which contained IgG from the sera of RV patients (n = 3) or normal subjects (n = 3),or TNF (10 ng/ml) for the indicated lengths of time. The expression of L-selectin on PMN was analyzed by flow cytometry as described in the *Materials and Methods* section. Data are expressed as the mean ± SEM (n = 3).

regulated by 200 g/ml of IgG from patients with RV. Similar changes in the expression of CAM on PMN were observed by treatment with TNF (10 ng/ml). On the other hand, IgG from normal subjects did not affect the expression of CAM on PMN.

Expression of CAM on PMN upon incubation with self-aggregated IC from the sera of patients with RV

Incubation of PMN with the IgG of 8 RV patients whose sera up-regulated the expression of CD18 from which IC had been removed (soluble IgG from RV in Table III), induced smaller changes in the expression of CAM on PMN. On the other hand, self-aggregated IgG (200 g/ml) isolated from the IgG fraction of these 8 RV patients induced not only significant up-regulation of CD 11b and CD18 expression but also significant down-regulation of L-selectin expression on PMN (Table III). However, there were no changes in composition between soluble IgG and self-aggregated IgG (Fig. 2B). Moreover, $F(ab')_2$ fragments of IgG purified from the sera of RV patients did not affect the expression of CAM on PMN (data not shown).

The down-regulation of L-selectin expression resulting from shedding of L-selectin was confirmed by observation

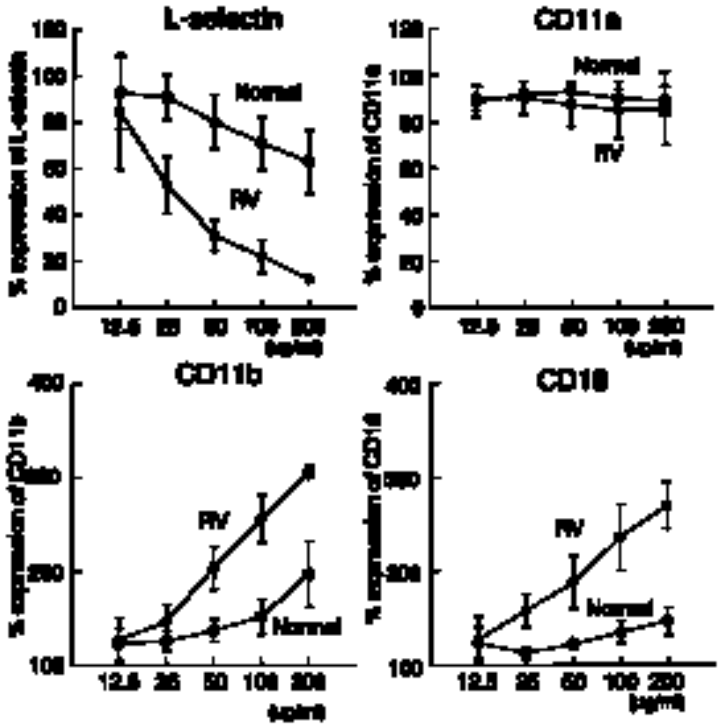


Fig. 4. Dose-dependent effect of IgG on the expression of CAM on PMN. PMN were incubated with various concentrations of IgG isolated from the serum of RV patients (n = 3) and normal subjects (n = 3) for 2 hrs, and analyzed by flow cytometry as described in the *Materials and Methods.* Data are expressed as mean ± SEM (n = 3).

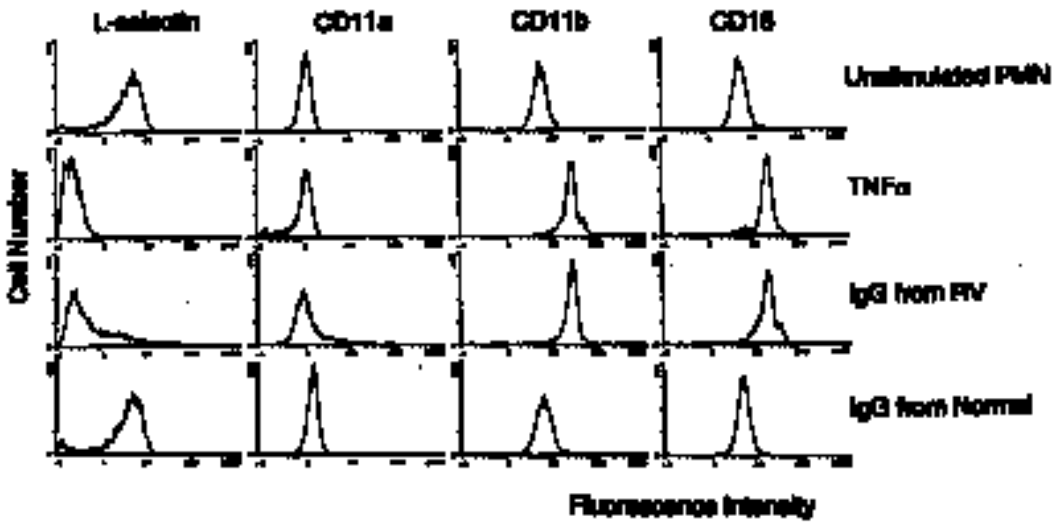


Fig. 5. Representative results of flow cytometric analysis of the expression of CAM on PMN. PMN were incubated with IgG (200 g/ml) of a RV patients, normal subject, or TNF (10 ng/ml) for 2 hrs, and then were analyzed with flow cytometry as described in the *Materials and Methods.*

Table III. Effects of IgG on the expression of cell adhesion molecules.

	L-selectin	CD11a	CD11b	CD18
RV				
Self-aggregated IC (n = 8)	27.7 ± 2.8*	95.7 ± 1.4	193 ± 11*	221 ± 19*
Soluble IgG (n = 8)	84.3 ± 7.4	101.0 ± 1.2	144 ± 7	182 ± 8
Normal				
Soluble IgG (n = 5)	92.0 ± 2.8	95.8 ± 2.8	144 ± 7	164 ± 8

Expression of cell adhesion molecules (%) were determined by flow cytometry as described in *Materials and Methods,* and represents the mean ± SEM.
*p < 0.05 as compared with soluble IgG from normal subjects (Normal).

that the level of sL-selectin in the supernatant of PMN after incubation with self-aggregated IgG (200 g/ml) was significantly higher than that in the supernatant after incubation with the IgG fraction of normal subjects. However, the level of sL-selectin in the supernatant after incubation of PMN with soluble IgG from patients with RV (200 g/ml) did not differ significantly (Fig. 6).

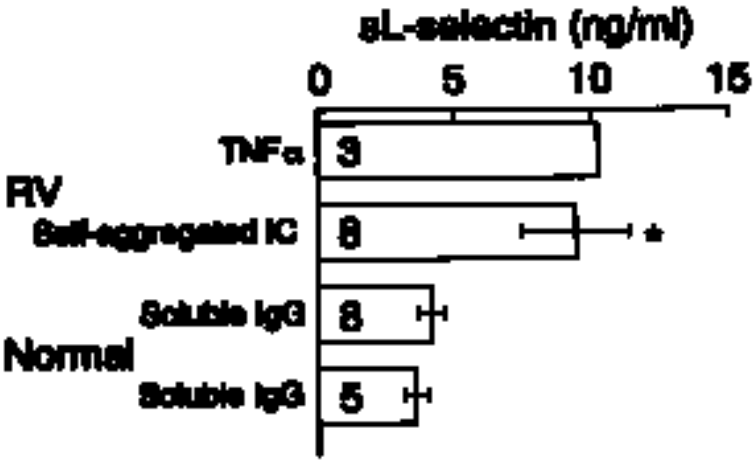


Fig. 6. Level of sL-selectin in the supernatant of PMN. After incubation with self-aggregated IC (200 g/ml) or soluble IgG (200 g/ml) from RV patients,or IgG (200 g/ml) from normal subjects for 2 hrs,the level of sL-selectin in the supernatant of PMN was determined with ELISA. Data are expressed as mean ± SEM. The number in each column indicates the number of samples examined.
*$p < 0.05$ compared with the level of sL-selectin upon incubation with IgG from normal subjects.

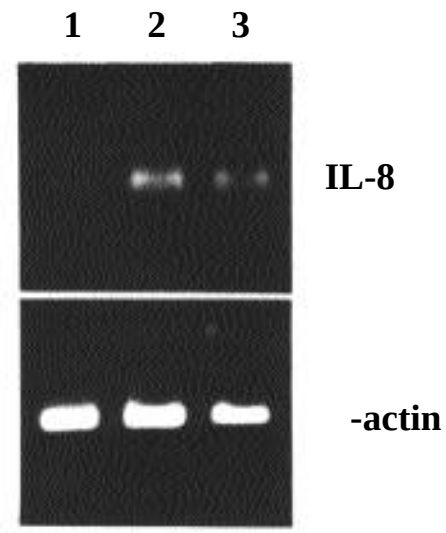


Fig. 7. Expression of mRNA of IL-8 and -actin in PMN. After incubation of PMN with self-aggregated IC or LPS (1 g/ml), the level of mRNA was determined with RT-PCR as described in the Materials and Methods. Lane 1, unstimulated PMN; Lane 2,PMN incubated with LPS; Lane 3, PMN incubated with IC from RV.

Expression of IL-8

To evaluate the involvement of IL-8 in the change in expression of L-selectin, CD11b and CD18 on PMN by self-aggregated IgG, we examined the mRNA level of IL-8 in PMN upon incubation with self-aggregated IC from RV patients. IL-8 mRNA was detected by RT-PCR in PMN which had been incubated for 2 hrs in medium (unstimulated PMN) as well as with LPS (1 g/ml) or self-aggregated IC (200 g /ml). The level of IL-8 mRNA in PMN which had been incubated with self-aggregated IC or LPS was increased in comparison with that in unstimulated PMN (Fig 7). In fact, IL-8 (35.3 ± 9.7 pg/ml, mean ± SEM, n = 5) was released from unstimulated PMN over 2 hrs. Moreover, the level of IL-8 in the supernatant of PMN was significantly higher after incubation with self-aggregated IC (71.3 ± 8.5 pg/ml, mean ± SEM, n = 5).

Discussion

We have shown in this study that the sera of some patients with RA induced shedding of L-selectin and up-regulation of CD11b and CD18 expression on the PMN. Moreover, similar changes in the expression of CAM on PMN were observed when PMN were incubated with IgG purified from these serum samples. During the purification of IgG, the IgG of patients with RV aggregated and sedimented after neutralization of the solution eluted from the protein G Sepharose column, indicating that IgG-RF formed IC *in vitro*. In fact, the level of IgG-RF was significantly higher in patients whose sera induced the up-regulation of CD11b and CD18 (Table II). Aggregation of IgG did not occur during the purification of IgG from the sera of normal subjects. Incubation of PMN with IgG solutions from which IC had been removed (soluble IgG in RA, Table III), did not change the expression of CAM on PMN. These results suggest that IC formed by IgG-RF affect the expression of CAM on PMN. Dimeric IgG-RF in the peripheral circulation normally binds with two IgG molecules. Although the pathogenicity of these complexes is not fully understood (17), our results suggest that IC formed by IgG-RF in the peripheral circulation may be involved in the development of vasculitis in RA.

Although anti-neutrophil cytoplasmic antibodies (ANCA) have been detected in the sera of some patients with RA (19, 20), ANCA were not detected in the serum samples used in this study. Moreover, the F $(ab')_2$ fragment of IgG purified from the sera of RV patients did not affect the expression of CAM on PMN (data not shown). These results indicate that the Fc portion of IgG is required to induce changes in the expression of CAM on PMN. This suggests that IC formed by RF affect the expression of CAM through the Fc receptor on PMN. There are two structurally and functionally distinct Fc receptors on the surface of unstimulated PMN. Homotypic or heterotypic cross-linking of Fc receptor induces up-regulation of the expression of CD11b and CD18. Although the cross-linking of Fc RIIa induces shedding of L-selectin, cross-linking of Fc RIIIb does not result in a change in the level of L-selectin expression (11).

In this study, the expression of CD11b and CD18 on PMN upon incubation with the sera of RV patients was significantly higher than the expression of CD11b and CD18 on PMN incubated with the sera of normal subjects. However, the level of L-selectin expression on the surface of PMN incubated with the sera of RV patients and that of PMN incubated with the sera of normal

subjects did not differ significantly. IC formed by bovine serum albumin (BSA) and anti-BSA increased the expression of CD11b and CD18 on PMN while it did not affect L-selectin expression (21). There is a ten-fold greater number of Fc RIIIb than Fc RIIa on the surface of PMN (22). IC formed by IgG-RF mainly bind to Fc RIIIb, thereby activating PMN in the circulation. However, the IgG fraction from the sera of RV patients, as well as the self-aggregated IC, may have cross-linked not only to Fc RIIIb but also to Fc RIIa in this study. Self-aggregated IC may induce shedding of L-selectin, whereas the sera may not affect the expression of L-selectin on PMN (Fig. 1).

Autoantibodies against vascular endothelial cells in patients with WG and scleroderma stimulate the production of IL-1 in vascular endothelial cells. IL-1 released from endothelial cells stimulates the expression of CAM on endothelial cells (23, 24). Although these changes in the expression of CAM on PMN induced by the IgG of RV patients were indistinguishable from those induced by TNF , down-regulation of L-selectin expression on PMN treated with IgG from RV patients occurred gradually than that on PMN treated with TNF (Fig. 3). Moreover, the mRNA of IL-1 and TNF was not detected by RT-PCR in PMN which had been stimulated with self-aggregated IC for 2 hrs (data not shown). Although the expression of mRNA for IL-8 in PMN and the level of IL-8 in the medium were increased by incubation with self-aggregated IC, the amount of IL-8 in the medium (71.3 ± 8.5 pg/ml) did not affect the expression of CAM on PMN after incubation of PMN with self-aggregated IC for 2 hrs (data not shown). These findings suggest that IgG in the sera of RA patients directly induces changes in the expression of CAM on PMN, but not via cytokine production.

IC have been detected in the sera of patients with SLE as well as RA (25). The level of CD11b/CD18 expression on the PMN of SLE patients is directly correlated with disease activity, although there was no relationship between the level of L-selectin expression on PMN and disease activity (26). However, the sera of SLE patients did not affect the expression of CAM on PMN in this study. The level of CIC was lower in SLE patients than that of RV patients (Table I). Agalactosyl IgG-IC was present in the serum from RA patients, but absent in the sera from SLE patients (27). Then, there is a possibility of quantitative and/or qualitative differences of IC that may affect the expression of CAM.

PMN can seriously compromise the function of the vasculature by injuring endothelial cells. Activated PMN release protease and superoxide, leading to vasculitis (28). The binding of CD11b/CD18 to intercellular adhesion molecules-1 leads to the production of superoxide in PMN (29). Many cytokines are present in the circulation of patients with RA (1). Then, IC formed by RF in combination with these cytokines may result in the development of vasculitis in RA.

References

1. EDWARDS SW, HALLETT MB: Seeing the wood for the trees: The forgotten role of neutrophils in rheumatoid arthritis. *Immunol Today* 1997; 18: 320-4.
2. CRONSTEIN BN, WEISSMANN G: The adhesion molecules of inflammation. *Arthritis Rheum* 1993; 36: 147-57.
3. SPRINGER TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: The multi-step paradigm. *Cell* 1994; 76: 301-14.
4. LUB M, VAN KOOYK Y, FIGDOR CG: Ins and outs of LFA-1. *Immunol Today* 1995; 16: 479-83.
5. HUMBRÍA A, DÍAZ-GONZÁLEZ F, CAMPANERO MR *et al*.: Expression of L-selectin, CD43, and CD44 in synovial fluid neutrophils from patients with inflammatory joint diseases. *Arthritis Rheum* 1994; 37: 342-8.
6. CROCKARD AD, THOMPSON JM, MCBRIDE SJ, EDGAR JD, MCNEILL TA,BELL AL: Markers of inflammatory activation: Upregulation of complement receptors CR1 and CR3 on synovial fluid neutrophils from patients with inflammatory joint disease. *Clin Immunol Immunopathol* 1992; 65: 135-42.
7. MCCARTHY DA, BERNHAGEN J, TAYLORMJ *et al*.: Morphological evidence that activated polymorphs circulate in the peripheral blood of patients with rheumatoid arthritis. *Ann Rheum Dis* 1992; 51: 13-8.
8. MANNIK M, NARDELLA FA, SASSO EH: Rheumatoid factors in immune complexes of patients with rheumatoid arthritis. *Springer Semin Immunopathol* 1988; 10: 215-30.
9. ROBINSON JJ, WATSON F, PHELAN M, BUCKNALL RC, EDWARDS SW: Activation of neutrophils by soluble and insoluble immunoglobulin aggregates from synovial fluid of patients with rheumatoid arthritis. *Ann Rheum Dis* 1993; 52: 347-53.
10. REYNOLDS WJ, YOON SJ, EMIN M, CHAPMAN KR, KLEIN MH: Circulating immune complexes in rheumatoid arthritis: A prospective study using five immunoassay. *J Rheumatol* 1986; 13: 700-6.
11. KOCHER M, SIEGEL ME, EDBERG JC, KIMBERLY RP: Cross-linking of Fc receptor IIa and Fc receptor IIIb induces different pro-adhesive phenotypes on human neutrophils. *J Immunol* 1997; 159: 3940-8.
12. LLOYD AR, OPPENHEIM JJ: Poly's lament: The neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. *Immunol Today* 1992; 13: 169-72.
13. DETMERS PA,POWELL DE, WALZ A,CLARK-LEWIS I, BAGGIOLINI M, COHN ZA: Differential effects of neutrophil-activating peptide 1/IL-8 and its homologues on leukocyte adhesion and phagocytosis. *J Immunol* 1991; 147: 4211-7.
14. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
15. VOSKUYL AE,ZWINDERMAN AH,WESTEDT ML, VANDENBROUCKE JP, BREEDVELD, FC, HAZES JMW: The mortality of rheumatoid vasculitis compared with rheumatoid arthritis. *Arthritis Rheum* 1996; 39: 266-71.
16. HASHIMOTO H, ABE T, NOBUNAGA M, YOSHIZAWA H, KYOGOKU M, MISHIMA Y: Proposal of revised criteria for malignant rheumatoid arthritis. *Ryumachi* 1989; 29: 268-76.
17. AN EM,COHEN AS, FRIESJF *et al*.: The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; 25: 1271-7.
18. MANNIK M: Rheumatoid factors in the pathogenesis of rheumatoid arthritis. *J Rheumatol* 1992; 19 (Suppl. 32): 46-9.
19. MULDER AHL,HORST G, VAN LEEUWEN MA, LIMBURG PC,KALLENBERG CGM: Anti-neutrophil cytoplasmic antibodies in rheumatoid arthritis. *Arthritis Rheum* 1993; 36: 1054-60.
20. CAMBRIDGE G, WILLIAMS M, LEAKER B, CORBETT M, SMITH CR: Anti-myeloperoxidase antibodies in patients with rheumatoid arthritis: Prevalence, clinical correlates, and IgG subclass. *Ann Rheum Dis* 1994; 53:24-9.
21. MOLAD Y, HAINES KA, ANDERSON DC, BUYON JP, CRONSTEIN BN: Immunocomplexes stimulate different signaling events to chemoattractants in the neutrophil and regulate L-selectin and $_2$-integrin expression differently. *Biochem J* 1994; 299: 881-7.
22. RICHARD S, SHAW AS, SHOWELL HJ, CONNELLY PA: The role of individual Fc receptors in aggregated IgG-stimulated protein tyrosine phosphorylation in the human neutrophil. *Biochem Biophys Res Com* 1994; 199: 653-61.
23. PAPA ND, GUIDALI L,SIRONI M *et al*.:Anti-endothelial cell IgG antibodies from patients with Wegener's granulomatosis bind to

human endothelial cells *in vitro* and induce adhesion molecule expression and cytokine secretion. *Arthritis Rheum* 1996; 39: 758-66.

24. CARVALHO D, SAVAGE COS, BLACK CM, PEARSON JD: IgG antiendothelial cell autoantibodies from scleroderma patients induce leukocyte adhesion to human vascular endothelial cells *in vitro*. *J Clin Invest* 1996; 97: 111-9.
25. THEOFILOPOULOS AN, DIXONFJ: The biology and detection of immune complexes. *Adv Immunol* 1979; 28: 89-220.
26. MOLAD Y, BUYON J, ANDERSON DC, ABRAMSON SB, CRONSTEINBN: Intravascular neutrophil activation in systemic lupus erythematosus (SLE): Dissociation between increased expression of CD11b/CD18 and diminished expression of L-selectin on neutrophils from patients with active SLE. *Clin Immunol Immunopathol* 1994; 71: 281-6.
27. BOND A, KERR MA HAY FC: Distinct oligosaccharide content of rheumatoid arthritis-derived immune complexes. *Arthritis Rheum* 1995; 38: 744-9.
28. GREENWALD RA: Oxygen radical, inflammation,and arthritis:Pathophysiological considerations and implications for treatment. *Semin Arthritis Rheum* 1991; 20: 219-40.
29. SHAPPEL SB, TOMAN C, ANDERSON DC, TAYLOR AA, ENTMAN NL, SMITH CW: Mac 1 (CD11b/CD18) mediates adherence-dependent hydrogen peroxide production by human and canine neutrophil. *J Immunol*