Switch Moiety in Agonist/Antagonist Dual Effect of S19 Ribosomal Protein Dimer on Leukocyte Chemotactic C5a Receptor

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The cross-linked homodimer of S19 ribosomal protein (RP S19) induces monocyte predominant infiltration due to a dual effect on the C5a receptor in leukocyte chemotaxis, agonistic to monocytes and antagonistic to polymorphonuclear leukocytes (PMN) (H. Nishiura, Y. Shibuya, T. Yamamoto, Lab Invest 1998, 78:1615–1623). The agonistic ligand moiety was recently determined to be -Leu131-Asp132-Arg133- (Y. Shibuya et al, Am J Pathol 2001, 159:2293–2301). In this study we determined the moiety responsible for the antagonistic function. A C-terminal analogue peptide of RP S19, with 18 residues containing the agonistic ligand moiety as a part, reproduced the dual function in the leukocyte chemotaxis. A C5a analogue peptide attracted PMN as well as monocytes. When C-terminal 12 residues of RP S19 after the agonistic moiety, IAGQVAAANKKH, were connected to the C5a peptide, the chimeric peptide newly obtained the dual function, indicating that the C-terminal portion of RP S19 functions as a converter from the agonist to the antagonist. C-terminal truncation analyses indicated that the C-terminal His was not essential but the next Lys was necessary for the converter function. The homodimer of a mutant RP S19 that was truncated for the C-terminal 4 residues lost the monocyte selectivity in the leukocyte infiltration *in vivo* as in the case of the leukocyte chemotaxis *in vitro*. These results indicated that the conversion of the RP S19 dimer from agonist to antagonist of C5a receptor is attributed to the IAGQVAAANKK moiety between Ile134 and Lys144. (Am J Pathol 2003, 162:1381–1388)

To elucidate the molecular mechanism that underlies the monocyte/macrophage predominant infiltration in the chronic inflammation, we have identified the major monocyte chemotactic factor in extracts of rheumatoid arthritis

synovial lesion. The monocyte chemotactic factor was a cross-linked homodimer of S19 ribosomal protein (RP S19) that is a component of the protein producing machinery, ie, the ribosome.^{1,2} RP S19, consisting of 145 amino acid residues, is dimerized by a transglutaminase-catalyzed reaction and released extracellularly during apoptotic process.^{3,4}

The RP S19 dimer attracts monocytes by binding to the receptor of C5a.⁵ C5a, composed of 74 amino acid residues with leukocyte chemotactic activity, is proteolytically liberated from the α -chain of complement component 5. The C5a receptor, composed of 340 amino acid residues, is a member of the G protein-coupled receptor family and has the structural motif of seven hydrophobic transmembrane α -helices linked by extra- and intracellular hydrophilic loops.⁶ The RP S19 dimer, as well as C5a, activates the C5a receptor by a two-step binding mechanism.^{7,8} The first binding substantiates the high-affinity ligand-receptor interaction, and the second binding triggers the intracellular signal transduction in the cell migration. The first ligand site of C5a is a three-dimensionally formed basic cluster composed of His15, Arg46, and Lys49, and that of the RP S19 dimer is a basic cluster region containing the Lys41-His42-Lys43 tandem.⁸ The second binding moieties of C5a and the RP S19 dimer are -Leu72-Gly73-Arg74-COOH^{7,9} and -Leu131-Asp132-Arg133-⁸, respectively. The β -carboxyl group of Asp132 of the latter functions equivalently to the α -carboxyl group of the COOH-terminal (C-terminal) Arg74 of the former.⁸ A peptide analogue (Ac-QQRDLDRAGQVAAANKK) that contains the second ligand moiety of the RP S19 dimer is capable of inducing chemotaxis, although about a two-magnitude higher concentration is needed than the RP S19 bearing the first ligand moiety.⁸ The RP S19 dimer and C5a, therefore, share the chemotactic receptor due to the local structural similarity at the receptor binding moieties between them, although the overall homology of the amino acid sequence between them is only 4%.

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Interestingly enough, the RP S19 dimer but not C5a behaves as an antagonist to the C5a receptor of polymorphonuclear leukocytes (PMN). The dual function, agonistic to C5a receptor of monocytes but antagonistic to that of PMN, causes the monocyte predominant infiltration *in vivo*.⁵ To explain the dual function of the RP S19 dimer, one can assume the presence of a moiety that turns the agonist to the antagonist. This moiety, which is a sort of molecular switch, should not be present in the C5a molecule, and it should convert C5a to an antagonist of C5a receptor on PMN when the moiety was chemically connected to the C5a molecule.

As described above, the peptide analogue of RP S19 composed of the second binding moiety and of the following C-terminal 11 amino acid residues exhibited the monocyte chemotaxis.⁸ Fortunately enough, this analogue did not attract PMN as in the case of the RP S19 dimer in our preliminary experiment, indicating the presence of the element responsible for the conversion from the agonist to the antagonist in the 18 amino acid residues of this peptide. Based on this observation, we currently determined the switch moiety of the RP S19 dimer responsible for the dual function in the leukocyte chemotaxis.

Materials and Methods

Reagents and Others

Hanks' balanced salt solution (HBSS) and RPMI 1640 medium were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) was a product of GIBCO BRC (Paisley, Scotland). Mono-Poly Resolving medium was a product of Flow Laboratory (Herts, UK). Bovine serum albumin (BSA) and formyl-Met-Leu-Phe were purchased from Sigma Chemical (St. Louis, MO). A multi-well chamber for chemotaxis assay was obtained from Neuro Probe (Bethesda, MD). Nuclepore filters were purchased from Nuclepore (Pleasant, CA). Restriction endonucleases (*Nde*I and *Bam*H I), Plasmid Pure Prep, TaKaRa RNA PCR kit, Microcon & Micropure, and *Escherichia coli* (*E. coli*) strain JM109-competent cells were purchased from TaKaRa Biomedicals (Otsu, Japan). Native Pfu DNA polymerase was purchased from STRATAGENE (La Jolla, CA). JETSORB gel extraction kit was purchased from GENOMED GmbH (Bad Oeynhausen, Germany). SeaKem GTG agarose and NuSieve GTG agarose were purchased from FMC (Rockland, ME). *E. coli* strain BL21 (DE3)-competent cells and plasmid pET11a were obtained from Novagen, Inc. (Madison, WI). All other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) or from Wako Pure Chemicals (Osaka, Japan) unless otherwise specified.

Preparation of Analogue Peptides

The peptides were synthesized by a conventional solid-phase method with fluorenylmethoxycarbonyl (Fmoc) amino acid-resins. After the synthesis, the peptides were separated from the resin with a trifluoroacetic acid-con-

taining solvent. After extraction with water, the peptides were purified by preparative reverse-phase high-performance liquid chromatography (HPLC) with a YMC C18 column (Φ 10 $\times$ 250 mm, Yamamura, Kyoto, Japan). The peptides thus prepared demonstrated single peaks with the absorbance at 220 nm in analytical reverse-phase HPLC with a Wako C18 column (Φ 4.6 $\times$ 150 mm). After freezing and drying, the peptides were dissolved into sterile phosphate-buffered saline (PBS, pH 7.4) containing 2 mg/ml BSA and used in the chemotaxis assay.

Preparation of Wild Type and Mutant Recombinant RP S19

Two types of recombinant RP S19, the wild type and a truncated mutant (Asn142-His145 deletion) were prepared. A cDNA for the wild-type RP S19 was prepared as described previously.⁵ The truncated mutant was prepared by the polymerase chain reaction (PCR) using a sense primer, 5'AGGCCGCCATATGCCTGGAGTTACTGTAAAAGA3', an antisense primer, 5'AGCCGGATCCTCTAGGCAGCTGCCACCT3', Pfu DNA polymerase, and the wild-type cDNA as the initial template. The sense primer oligonucleotides were prepared as described previously.⁸ The antisense primer was made to order by TaKaRa Biomedicals. The wild-type RP S19 cDNA and the mutant were extracted from agarose gel using the Microcon & Micropure (TaKaRa Biomedicals) after the agarose gel electrophoresis subcloned into the pET11a plasmid vector using the *Nde*I and *Bam*H I cloning sites and transformed into the cloning host *E. coli* JM109-competent cells by the heat shock method. PET11a, bearing an insert of RP S19 cDNA (recombinant plasmid), was purified from a positive colony culture, and analyzed for the DNA sequence using *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Perkin Elmer, Foster City, CA) for performing fluorescence-based dideoxysequencing reactions according to the method of Prober et al¹⁰ to confirm the mutagenesis. These constructs of the wild-type RP S19 recombinant plasmid and the mutant were transformed to the expression host *E. coli* BL21 (DE3)-competent cells.

RP S19 molecules of the wild type and the mutant were respectively extracted from the periplasmic fraction of the *E. coli*, and were purified by HPLC using a SP-5PW column and a HiTrap heparin column (Pharmacia), in this order, as described previously.⁵ The preparations of the wild-type RP S19 and the mutant thus obtained demonstrated single bands with an apparent molecular size of 15.5 kd in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), respectively (data not shown). Judging from the SDS-PAGE patterns, their purity was more than 95%.

Preparation of Dimers of Recombinant RP S19

The purified recombinant RP S19 molecules were respectively treated with factor XIIIa (final concentration 1 units/ml) in the presence of heparin (1 units/ml) and 5 mmol/L CaCl₂ for 60 minutes at 37°C as previously de-

scribed.² The cross-linked dimers of the recombinant proteins were then purified by immuno-affinity column chromatography with an anti-isopeptide bond monoclonal antibody column (Coval Ab, Lyon, France), and by HPLC with a Hitachi 5C4-300 column (Nacalai Tesque) as described previously.² Each cross-linked recombinant RP S19 dimer enriched in a HPLC fraction was evaporated with a vacuum centrifuge concentrator (Savant), dissolved into sterilized PBS, diluted with PBS containing 2 mg/ml of BSA, and used in the chemotaxis assay. When the quality of the preparations was examined by SDS-PAGE, it was always more than 90%, and the contaminant in each preparation was the monomer (data not shown). The protein concentration of these dimers thus prepared was determined by the absorbance at 280 nm under the assumption that an absorbance unit 1.0 was equivalent to 1 mg/ml.

Leukocyte Chemotaxis Assay

Mononuclear cells and PMN were isolated from heparinized human venous blood of healthy donors according to the method of Fernandez et al¹¹ as described previously. The monocytes and PMN were respectively suspended at a cell density of 1×10^6 cells/ml in RPMI 1640 containing 10% FBS, and in HBSS containing 0.5% BSA for the multi-well chamber assay. The multi-well chamber was used according to the method of Falk et al¹² using a Nuclepore filter with a pore size of 5 μ m for monocytes and of 3 μ m for PMN. After incubation for 90 minutes, each membrane was separated, fixed with methanol, and stained with Giemsa solution. The total number of monocytes or of PMN migrated beyond the lower surface of the membrane (the cells adhered to the bottom of the membrane) was counted in five microscopic high-power fields. The results are expressed as the number of migrated monocytes or PMN.

Histological Examination

Samples were injected intradermally into two guinea pigs with 27-gauge needles. The animals were exsanguinated under ether anesthesia 12 hours after the injection. The skin lesions were immediately resected and fixed in 10% formalin. Paraffin sections were prepared at 4- μ m thickness and stained with hematoxylin and eosin in the usual way. In a morphometric analysis on the infiltrated monocytes and PMN, the number of each cell type of the leukocytes was respectively counted at a microscopic high-power field (ocular lens $\times 10$, objective lens $\times 40$, and zoom lens $\times 2$) in three different areas just above the panniculus carnosus muscle. The infiltrated leukocyte numbers were compared between monocytes and PMN as mean $\pm$ SD per high-power field. The animal experiments were performed under the control of the Animal Experiment Committee, Kumamoto University School of Medicine (Kumamoto, Japan).

Results and Discussion

Selective Chemotactic Effect of Peptide Analogue of RP S19

Reproduction of the leukocyte chemotactic capacity of C5a by a decapeptide analogue of C5a, YSFKDMQLGR, was reported.¹³ The Leu-Gly-Arg-COOH moiety of this peptide is the ligand site to the C5a receptor with the receptor activation capacity as in the case of C5a. We have previously reported that a peptide analogue of RP S19, Ac-GQRDLDRAGQVAAANKK, which contains the second ligand moiety (Leu-Asp-Arg) of the RP S19 dimer to C5a receptor, reproduced the chemotactic effect of the RP S19 dimer to monocytes.⁸

In the current study, the chemotactic capacities to monocytes and to PMN of the analogue peptides of C5a and RP S19 were comparatively examined. As shown in Figure 1, the C5a analogue peptide attracted both monocytes and PMN. In contrast to this, the RP S19 analogue peptide lacked the chemotactic capacity to PMN as in the case of the RP S19 dimer.⁵

These different behaviors between the C5a analogue peptide and the RP S19 analogue peptide are identical to those between C5a and the RP S19 dimer.⁵ This suggests that the RP S19 analogue peptide would have the antagonistic effect to the C5a receptor of PMN as in the case of the RP S19 dimer. This possibility was next examined.

Antagonist Effect of RP S19 Analogue Peptide on C5a Receptor of PMN

As shown in Figure 2, the analogue peptide of RP S19 inhibited the chemotactic effect of the C5a analogue peptide to PMN in a dose-dependent manner. The inhibitory effect was not observed in the chemotactic response of PMN to formyl-Met-Leu-Phe, which is a formyl peptide receptor-mediated PMN response to bacterial metabolites.¹⁴ The receptor-specific inhibitory effect of the RP S19 analogue peptide indicated that the analogue peptide is an antagonist of the C5a receptor of PMN.

These results strongly suggested that the analogue peptide contains the moiety converting the agonist effect to the antagonist one in PMN chemotaxis. We assumed that the C-terminal region with 12 amino acid residues following to the agonist moiety, Leu-Asp-Arg, would possess the converting capacity. We initially examined this possibility by preparing a chimeric peptide that possessed the C5a ligand sequence followed by the C-terminal sequence of RP S19.

Selective Chemotactic Effect of Chimeric Peptide Bearing Ligand Moiety of C5a and C-Terminal Sequence of RP S19

As described above, the Leu-Gly-Arg-COOH moiety in the C5a analogue peptide is essential for its chemotactic capacity.¹³ In addition to this, we have reported that the α -carboxyl group of the C-terminal Arg, which is essential for the ligand function, could be substituted by the β -car-

Figure 1. Selective chemotactic effect of peptide analogue of S19 ribosomal protein (RP S19). A C5a analogue peptide (YSFKDMLGR, closed squares) and a RP S19 analogue peptide (Ac-GQRDLDRAGQVAAANKK, open circles), both of which induce C5a receptor-mediated monocyte chemotaxis. The chemotactic capacities of these analogue peptides to PMN were comparatively examined. Leukocyte chemotaxis assay used was the multi-well chamber assay using a Nuclepore filter with a pore size of 5 or of 3 μ m for monocytes or for PMN, respectively. After a 90-minute incubation, the membrane was stained with Giemsa solution and the total number of monocytes (a) or PMN (b) migrated beyond the membrane was counted in five microscopic high-power fields. In the negative control experiment, PBS containing 0.1 mg/ml BSA was used as the attractant (the open columns). The results of a representative examination in at least three different examinations are shown. The experiments were carried out with triplicate samples. Values are expressed as mean $\pm$ SD.

Figure 2. Antagonist effect of RP S19 analogue peptide on C5a receptor of PMN. PMN chemotaxis assay was performed using the C5a analogue peptide (final concentration 10^{-7} M, closed squares) or formyl-Met-Leu-Phe (f-MLF, final concentration 5×10^{-8} M, open circles) as the attractant in the presence of various concentrations of the RP S19 analogue peptide. The results of a representative examination in at least three different examinations are shown. The experiments were carried out with triplicate samples. Number of migrated PMN is expressed as mean $\pm$ SD.

boxyl group of an Asp residue, when the Arg is not at the C-terminal and the Asp positions just before the Arg such as the -Leu-Asp-Arg-Ile- sequence in RP S19.⁸ Concerning this information, we designed the chimeric peptide that bore the C-terminal 12 amino acid residues of RP S19 following the C5a deca-peptide in which Gly9 was substituted by an Asp residue.

The chimeric peptide was subjected to chemotaxis assay using monocytes and PMN. In contrast to the simple C5a analogue peptide, the chimeric peptide lost the chemotactic activity to PMN, although it still maintained the activity to monocytes as much as the C5a analogue peptide (Figure 3).

Antagonist Effect of Chemotactic Peptide on C5a Receptor of PMN

As shown in Figure 4, the chimeric peptide inhibited the chemotactic response of PMN to the C5a analogue peptide in a dose-dependent manner. This result joins the result shown in Figure 2 to indicate that the C-terminal portion of RP S19 is responsible for the conversion from the agonist to the antagonist in the C5a receptor-mediated PMN chemotaxis.

This then raised the question as to whether the switching moiety should form a single molecule with the agonistic ligand moiety of the C5a analogue peptide, or is it also effective in a form separated from the analogue peptide. As shown in Figure 4, the C-terminal peptide of RP S19 in the free form did not inhibit the chemotactic response of PMN to the C5a analogue peptide at con-

Figure 3. Selective chemotactic effect of chimeric peptide between C5a analogue peptide and C-terminal moiety of RP S19. The C5a analogue peptide (**open circles**) and its chimera with a C-terminal moiety of RP S19, in which Gly9 was substituted by Asp9 (YSFKDMQLDRIAGQVAAANKKH, **closed circles**) were subjected to the monocyte (**a**) and PMN (**b**) chemotaxis assays, respectively. The **open columns** denote the negative control with PBS-BSA. The results of a representative examination in at least three different examinations are shown. The experiments were carried out with triplicate samples. Values are expressed as mean $\pm$ SD.

Figure 4. Antagonist effect of chimeric peptide on C5a receptor of PMN. The PMN chemotaxis assay was performed using the C5a analogue peptide (final concentration 10^{-7} M) as the attractant in the presence of various concentrations of the chimeric peptide (YSFKDMQLDRIAGQVAAANKKH, **closed circles**) or of a free form C-terminal moiety of RP S19 (IAGQVAAANKKH, **open circles**). The results of a representative examination in at least three different examinations are shown. The experiments were carried out with triplicate samples. Number of migrated PMN is expressed as mean $\pm$ SD.

centrations from 10^{-10} M to 10^{-6} M. This may indicate that the switch element must be directly connected to the agonistic ligand of the C5a receptor to evoke the inhibitory function, or that the element alone might have a very low binding affinity to PMN despite possessing the inhibitory function even in the free form.

Truncation Analysis to Select Unessential Amino Acid Residues at C-Terminal Portion of Chimeric Peptide in Terms of Antagonist Effect on C5a Receptor of PMN

To briefly examine essential amino acid residues in the C-terminal moiety of RP S19 for the antagonist function to the PMN C5a receptor, the sequential truncation analysis of C-terminal residues was carried out using the chimeric peptide. As shown in Figure 5, the cell type selectivity of the chimeric peptide was lost when the C-terminal 2 residues were truncated. These results indicated that the moiety with IAGQVAAANKKH is enough, namely, the C-terminal His residue of RP S19 is not necessary, for the antagonist effect on the PMN C5a receptor.

Influence of C-Terminal Truncation to Cyto-Selectivity of RP S19 Dimer

To confirm the responsibility of the C-terminal moiety to the switching effect in the natural ligand, the cross-linked homodimer of a mutant RP S19 with truncation of the

Figure 5. Truncation analysis of C-terminal portion of chimeric peptide in terms of the antagonist effect on C5a receptor of PMN. The native form and two truncated forms of the chimeric peptide were subjected to the leukocyte chemotaxis assays. The **closed circles** denote the chimeric peptide, and the **closed squares** and the **open circles** denote the chimeric peptides truncated with the C-terminal His, and with Lys-His, respectively. The **open columns** denote the negative control with PBS-BSA. The results of a representative examination in at least three different examinations are shown. The experiments were carried out with triplicate samples. Values are expressed as mean $\pm$ SD.

Figure 6. Influence of C-terminal truncation to cyto-selectivity of RP S19 dimer in leukocyte chemotaxis. The dimer (**closed squares**) and monomer (**open squares**) of a RP S19 mutant truncated with 4 amino acid residues at the C-terminal were subjected to the monocyte and PMN chemotaxis assays, respectively. The **hatched columns** with slash and the **open columns** denote the activities of the dimer of the wild-type RP S19 (the final concentration 10^{-9} M) and PBS-BSA as the negative control, respectively. The results of a representative examination in at least three different examinations are shown. The experiments were carried out with triplicate samples. Values are expressed as mean $\pm$ SD.

Figure 7. Induction of PMN-predominant infiltration by intradermal injection of the C-terminal-truncated RP S19 mutant dimer. The C-terminal-truncated RP S19 mutant dimer (**a**) as well as the wild-type RP S19 dimer (**b**) was intradermally injected into a guinea pig at the final concentrations of 10^{-6} M ($100 \mu\text{l}$). The skin lesions at 12 hours after the injection were histologically examined with hematoxylin and eosin staining. The results of a morphometric analysis on infiltrated monocytes (**open columns**) and PMN (**slashed columns**) are also shown (**c**). Three microscopic high-power fields of each histological slide were analyzed. Values are expressed as mean $\pm$ SD.

C-terminal 4 amino acid residues was prepared and its chemotactic capacities to monocytes and to PMN were measured. As shown in Figure 6, the dimer of the truncated RP S19 attracted not only monocytes but also PMN, while the wild-type RP S19 dimer attracted only monocytes. This result indicated again the responsibility of the C-terminal moiety of RP S19 in the switch effect on the C5a receptor-mediated leukocyte chemoattraction of the RP S19 dimer.

Loss of the Monocyte Predominance in the RP S19 Dimer Induced Leukocyte Infiltration by the C-Terminal Truncation of RP S19

We finally examined the leukocyte infiltration pattern induced by the intradermal injection of the C-terminal-truncated RP S19 mutant dimer in guinea pigs. Typical histological pictures at 12 hours after the intradermal injection of the RP S19 mutant dimer as well as the wild type at the concentration of 10^{-6} M are shown in Figure 7, a and b. We performed a morphometric analysis on the infiltrated leukocytes comparing the numbers between monocytes and PMN using the hematoxylin and eosin-stained sections (Figure 7c). The leukocyte infiltration pattern induced by the RP S19 mutant dimer injection is a mixed population type between monocytes and PMN, whereas the wild-type RP S19 dimer injection caused the monocyte predominant infiltration. This result confirmed the role of the C-terminal portion of the RP S19 dimer in the induction of the monocyte selective leukocyte infiltration as seen in the chronic inflammation.

In the present study, it was clarified that the conversion from the chemotactic agonist to the antagonist is attributed to the C-terminal moiety of RP S19 with an IAGQVAAANKK sequence. In the series of our study on the RP S19 dimer as the monocyte chemotactic factor, several functional moieties of the RP S19 molecule have been identified. They are the major glycosaminoglycan-binding site,² the first and second ligand sites to C5a receptor,⁸ residues mainly used for the intermolecular

cross-linking by transglutaminases,^{2,4} and the antagonist switch moiety as illustrated in Figure 8.

The conversion from the agonist to the antagonist in the RP S19 dimer-induced and C5a receptor-mediated chemotaxis is only effective to PMN but not to monocytes. This suggests the presence of a kind of cellular machinery on/in PMN responding to the C-terminal moiety of RP

Figure 8. Functional moieties of RP S19 or its homodimer. The underlined moiety with the notation "GAG-binding" is the major glycosaminoglycan-binding site. The moieties indicated with **white** letters and the notation "first ligand," and with the **bold** letters and the notation "second ligand" are the first and second ligand sites to C5a receptor, respectively. **White** letters **K** and **Q** with the notation "iso peptide" are the Lys and Gln residues intermolecularly cross-linked by transglutaminases. The underlined moiety with the notation "antagonist" is the moiety identified in the present study.

S19. This machinery would generate an intracellular signal that cancels the migration signal raised by the activated C5a receptor. It is very likely that the inhibitory machinery contains a molecule that directly interacts with the C-terminal moiety of RP S19. This molecule should not be present on/in monocytes. Apparently, the C-terminal moiety of RP S19 should be molecularly connected to the receptor ligand moiety to effectively exhibit the inhibitory function to the C5a receptor-mediated PMN chemotaxis. This strongly suggested that the acceptor molecule to the C-terminal moiety of RP S19 would closely associate to the C5a receptor of PMN. On the other hand, the C5a receptor gene is a single gene, and a single cDNA of the C5a receptor has been cloned so far.^{6,15} The alternative splicing is not known in the C5a receptor mRNA. These findings suggest that the acceptor molecule is not a moiety of the C5a receptor itself of PMN. Therefore, the acceptor molecule would be a sort of co-receptor molecule of the C5a receptor present on/in PMN. This molecule should be identified in the near future.

In terms of the cell type selection mechanism in the leukocyte infiltration *in vivo*, we have reported the inhibitory effect of C4a (complement component 4-derived anaphylatoxin) on monocytes.¹⁶ C4a inhibits monocyte chemotaxis in an indirect manner as promoting monocytes to release an autocrine inhibitory cytokine. This cytokine, which has not yet been identified, did not inhibit PMN chemotaxis.¹⁷ C4a is rich in rheumatoid arthritis synovial fluid where the PMN predominant infiltration is observed despite the chronic nature of the inflammatory disease.¹⁶ Thus, C4a and the RP S19 dimer would play key roles in causing the distinct leukocyte infiltration patterns in rheumatoid arthritis, the PMN predominant infiltration in the synovial fluid, and the monocyte-predominant one in the synovial granulomatous lesion.¹⁸

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