

Case report

Presence of C5a-potentiating activity in the plasma of a patient with Kimura's disease

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To determine whether the response to the active complement fragment C5a in polymorphonuclear leukocytes (PMN) obtained from a patient with Kimura's disease is similar to that in normal subjects, we evaluated superoxide anion (O_2^-) generation after stimulation with C5a. The patient's PMN produced more O_2^- than did those from healthy controls after stimulation with human C5a *in vitro*. The response returned to normal with the improvement in clinical indicators after initiation of steroid administration. We conducted the following experiments with the plasma of this patient during the active disease stage to establish the presence of a humoral factor that potentiated the C5a-response of PMN. Incubation of PMN from normal controls having the same blood type as the patient at 37°C for 2 h with the patient's plasma at the active disease stage revealed that the cells generated abundant O_2^- after stimulation with C5a. Incubation of normal PMN with the patient's plasma during the inactive disease stage showed no potentiated response to C5a. This activity was lost after incubation at 56°C for 30 min. Coincubation of PMN with methylprednisolone up to 100 µg/ml did not suppress this activity. Although the plasma concentration of C5a at the active stage was mildly elevated (13 ng/ml), it was below the limit of detection (< 10 ng/ml) at the inactive stage. These results suggest the presence of a heat-labile, humoral factor in the patient's plasma that upregulated the response of PMN to C5a and that was not suppressed by *in vitro* treatment with a steroid. This factor may influence the acute inflammatory reaction in this disorder.

M. Abe¹, K. Tanaka¹, J. Kudo²,
Y. Fukuoka³, N. Hara¹

¹Research Institute for Diseases of the Chest and ²The First Department of Internal Medicine, Kyushu University Faculty of Medicine;

³Department of Immunology, Research Institute for Tuberculosis and Cancer, Tohoku University, Japan

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Masayoshi Abe, MD
Research Institute for Diseases of the Chest
Faculty of Medicine, Kyushu University, B-30
3-1-1 Maidashi, Higashi-ku
Fukuoka 812
Japan

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While investigating the influence of complement activation on polymorphonuclear leukocytes (PMN) from patients with various inflammatory disorders, we found that the PMN obtained from a patient with Kimura's disease generated quantities of O_2^- after C5a stimulation. This rare disease is characterized histologically by the presence of granulomas consisting of lymphoid follicles and massive eosinophilic infiltrates in the subcutaneous tissues and lymph nodes (8, 10, 14). It is often characterized by painless soft masses behind the ear associated with such laboratory abnormalities as marked eosinophilia, elevated IgE (9), high expression of the IL-5 gene in T cells (7), and chronic glomerulonephritis (11, 18).

We will demonstrate that the PMN obtained from this patient with Kimura's disease showed hyperresponsiveness to human C5a *in vitro*, and present evi-

dence for a humoral factor in his plasma that can upregulate the C5a-response of PMN from normal subjects.

Case report

A 48-year-old elementary schoolteacher was in good health until July 1985 when he noticed subcutaneous tumors on the right lateral chest and bilateral posterior auricular skin. There had been no exposure to drugs or known toxins. He then developed allergies to various foods such as meat, beans, milk, egg, shrimp, and fish, and frequent episodes of generalized urticaria. His physician detected eosinophilia and treated him for food allergies with a restricted diet and antihistamines. Seven years later (December 1991), the persistence of the subcutaneous tu-

mors and food allergies led to his admission to the Kyushu University Hospital for detailed examination and treatment. He exhibited generalized skin pigmentation caused by chronic urticaria. Elastic hard subcutaneous masses were present around his ears and on the right side of the chest. His white cell count was $13\,300/\text{mm}^3$ with $7400/\text{mm}^3$ eosinophils. Tests for fecal parasites were negative, and the results of Ouchterlony's double-diffusion tests against antigens of 10 helminthic parasites were negative. His serum IgE level was as high as 11 500 IU/ml. A tumor specimen obtained at biopsy revealed eosinophilic lymphoid granuloma, and Kimura's disease was diagnosed.

The patient was treated alternatively with nonsteroidal antipyretics, eicosapentaenoic acid, or a corticosteroid. Four weeks later, on prednisolone 40 mg daily, he was improved. The urticaria had disappeared, and there was a marked reduction in the subcutaneous masses and eosinophilia. Blood eosinophils and serum IgE had decreased to $300/\text{mm}^3$ and 6900 IU/ml, respectively. He was discharged from the hospital on prednisolone 20 mg daily, and is now in good condition.

Material and methods

The following agents were used: LSM (lymphocyte separation medium; Organon Teknika, NC, USA), L-glutamine (Wako Chem, Kyoto, Japan), cytochrome C (Sigma, SL), cytochalasin E (Sigma), and phorbol 12-myristate 13-acetate (Sigma). Purified human C5a was generously donated by Dr T. E. Hugli (Scripps Research Institute, La Jolla, CA, USA). Recombinant C3a of human anaphylatoxin was obtained as previously reported (4). Water was distilled and filtered with Puric-R (Organo, Kyoto, Japan).

PMN preparations

Heparinized venous blood was drawn from the patient and from healthy subjects into heparinized syringes and exposed to dextran sedimentation for 30 min. The leukocyte-rich supernatant was then collected and layered on LSM. After centrifugation for 30 min at 400 g, the pellet was washed with HEPES buffer. The cells were exposed to distilled water for 20 s to lyse the erythrocytes and an equal amount of 1.8% saline was added. After centrifugation, the PMN recovered in the pellet were resuspended in HEPES buffer. The number of cells was counted, and their viability was determined to be at least 90% in the presence of 0.1% trypan blue.

Incubation of PMN. Plasma samples were obtained from the patient before and after the administration

of the steroid. PMN (1×10^6) from normal subjects were incubated with 0.3–0.4 ml of the patient's plasma at 37°C in a CO₂ incubator for 2 h. Each aliquot was then centrifuged at 200 g for 10 min. PMNs recovered in the pellet were resuspended in HEPES buffer for the assay of superoxide anion (O_2^-) production.

Superoxide anion production by PMN. O_2^- was measured by the superoxide dismutase inhibitable ferricytochrome C reduction method with PMA (0.6 µg/ml), recombinant human C3a (100 ng/ml), and purified human C5a (10 ng/ml) as the triggers. In brief, the reaction mixtures (total volume = 1 ml) containing 4×10^5 PMN and 100 µM ferricytochrome C (type VI, Sigma) with or without drugs in the HEPES buffer were preincubated at 37°C for 7 min. Each stimulant was then added to the reaction mixture. The change in absorbancy at 550 nm referenced to that of 540 nm was followed on a recorder attached to a double-beam spectrophotometer (Hitachi 556, Tokyo, Japan). The release of O_2^- was determined by the molar absorption coefficient for reduced-minus-oxidized ferricytochrome C of $19.1 \times 10^3 \text{ M}^{-1} \times \text{cm}^{-1}$. The maximum rate of O_2^- production was expressed as nmol cytochrome C reduced/ 4×10^5 PMN per min by the initial reaction rate.

Results

Time course of the outcome

After steroid treatment was initiated, the patient's serum IgE levels decreased transiently, and the eosinophil count fell markedly (Fig. 1). The levels of C3, C4, and CH50 before initiation of the steroid were 70 mg/dl (38–96), 21 mg/dl (11–46 mg/dl), and 42 (30–50), respectively, all within the normal range. Fragments of complement proteins, C3a, C4a, and C5a des Arg were mildly elevated; i.e., 854 (50–200), 362 (50–250), and 13 ng/ml (<10 ng/ml), respectively, before treatment. However, after the initiation of steroid treatment, the C5a returned to normal (less than 10 ng/ml), while C3a and C4a were persistently elevated to 848 and 429 ng/ml, respectively.

Time course of O_2^- generation from PMN by stimulation with C5a or PMN

PMNs obtained from this patient generated large amounts of O_2^- , 5.03 and 5.86 nmol/ 4×10^5 cells/min, after stimulation with C5a 10 ng/ml and PMA 600 ng/ml, respectively, before steroid treatment. In contrast, PMN from normal subjects generated O_2^- amounts of 0.42 and 0.71 in response to the respective stimuli. After the beginning of steroid treatment, the patient's PMN generated less O_2^- ,

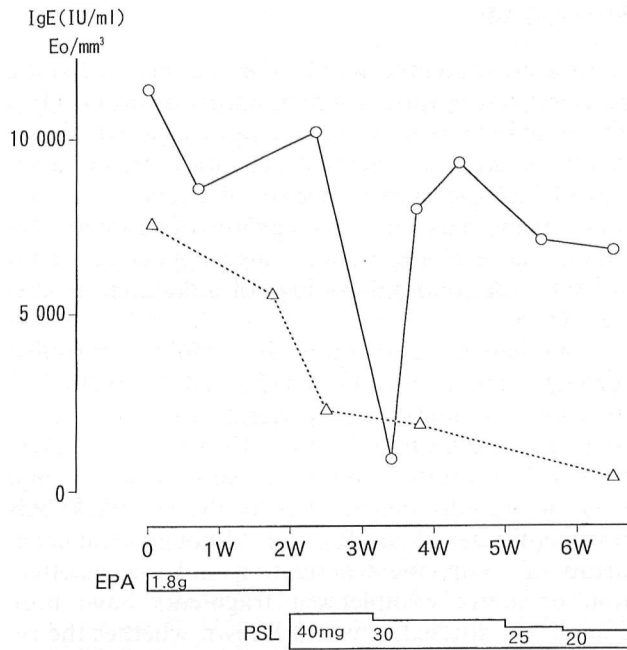


Fig. 1. Time-course study of blood eosinophils, E_o (Δ --- Δ) and IgE (○ — ○). Eosinophil counts and IgE levels are expressed as absolute values per microliter and as IU per microliter, respectively. Oral eicosapentaenoic acid (EPA) was prescribed at dose of 1.8 g/d. Oral prednisolone was initiated at 40 mg/d, and then tapered to 20 mg/d.

0.45 and 1.5 nmol/ 4×10^5 cells/min by stimulation with C5a and PMA, respectively (Fig. 2).

Potentiation of O_2^- generation after stimulation with C5a after incubation with patient's plasma

PMNs were obtained from normal controls with the same blood type (Type A) as the patient. We com-

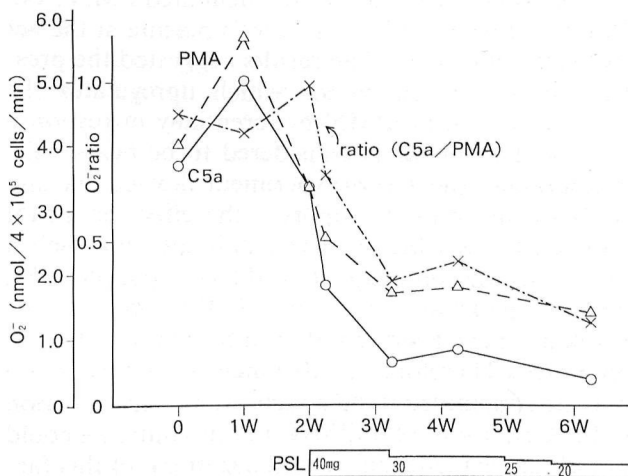


Fig. 2. Time-course study of O_2^- generation from PMN after stimulation with human C5a (○ — ○), PMA (Δ --- Δ), and their ratio (C5a/PMA) (× ··· ×).

pared O_2^- generation after stimulation with PMA with that obtained with C5a, after incubation at 37°C in a CO_2 incubator at a density of 1×10^6 cells per 0.3–0.4 ml plasma of the patient during active disease stage (active plasma) or inactive stage (inactive plasma). As shown in Fig. 3, the amount of O_2^- after stimulation with PMA was the same or lower after 2-h incubation with the patient's active plasma. In contrast, while the amount of O_2^- increased after stimulation with C5a and incubation for the same period, incubation of PMN with inactive plasma did not potentiate O_2^- generation after stimulation with C5a. Consequently, the ratio of O_2^- production by PMN after stimulation with C5a to that after PMA stimulation showed a higher value, 3.1, under the former condition than the 1.6 obtained under the latter one. The same experiment was repeated with PMN obtained from three healthy controls with type A blood. As summarized in Table 1, incubation of PMN with either the active or inactive plasma generated similar amounts of O_2^- after stimulation with PMA, whereas after stimulation with C5a, the PMN

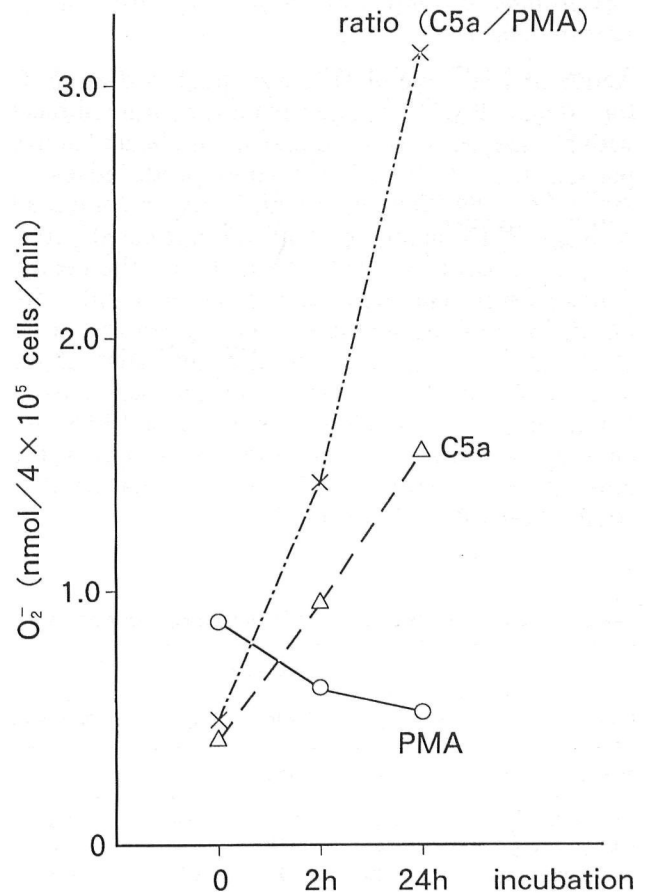


Fig. 3. Changes in O_2^- generation by PMN after incubation with plasma from patient with active Kimura's disease. Y-axis denotes O_2^- generation (nmol/ 4×10^5 cells/min) from PMN after stimulation with C5a and PMA, and their ratio (C5a/PMA). Symbols as in Fig. 2 legend.

Table 1. Amounts of O_2^- produced by PMN after stimulation with C5a, PMA, and C3a and 2-h incubation with patient's plasma during active or inactive stage

Plasma	Donor	O_2^- amount			Ratio* of C5a to PMA
		C5a*	PMA	C3a	
Active stage	Case 1	1.57	1.73	0.16	0.91
	Case 2	0.93	1.63	0.08	0.57
	Case 3	0.84	1.36	0.06	0.62
Inactive stage	Case 1	0.76	1.80	0.04	0.42
	Case 2	0.50	1.73	0.04	0.29
	Case 3	0.24	1.26	0.03	0.19

Each tube containing 4×10^5 PMN was suspended in patient's plasma obtained at active or inactive disease stage and incubated at 37°C in CO_2 incubator for 2 h. Each tube was centrifuged at 200 g for 10 min. Cell pellet was resuspended in HEPES buffer. Values for O_2^- production after stimulation with C5a, C3a, and PMA were expressed as nmol cytochrome c reduced/ 4×10^5 PMN per min. Asterisk indicates significant difference ($P < 0.05$) between active and inactive plasma by statistical analysis with paired T test.

incubated with the active plasma consistently produced greater amounts of O_2^- than the PMN with the inactive plasma.

Deactivation of C5a-activating factor in plasma by heat treatment

Active and inactive plasma was incubated at 56°C for 30 min. PMN obtained from a healthy subject with blood type A were incubated with heated active plasma at 37°C for 2 h, and they produced 84 or 89% of O_2^- by stimulation with PMA, as compared with the PMN incubated with the untreated active plasma. In contrast, PMN treated with the heated active plasma generated, after stimulation with C5a, 44 or 56% of the amount of O_2^- generated from PMN after incubation with the untreated active plasma. The presence of methylprednisolone, 10 or 100 $\mu\text{g}/\text{ml}$, in the incubation mixture of PMN, including the active plasma, did not suppress the amount of O_2^- (109 or 102% of the control) after stimulation with C5a (Table 2).

Table 2. Effects of steroid hormone or heat on C5a-potentiating factor in plasma

Plasma	O_2^- amounts			Ratio of C5a to PMA
	C5a	PMA	C3a	
Active plasma	0.43	0.85	0.08	0.51
Heat (56°C , 30 min)	0.19	0.71	0.04	0.27
Steroid (10 $\mu\text{g}/\text{ml}$)	0.47	1.10	0.09	0.43
Steroid (100 $\mu\text{g}/\text{ml}$)	0.44	1.00	0.09	0.44
Inactive plasma	0.10	0.81	0.00	0.12
Heat (56°C , 30 min)	0.12	0.69	0.04	0.17

Plasma obtained at active and inactive disease stage was heated at 56°C for 30 min. Methylprednisolone was added to incubation mixture containing PMN and active plasma at concentrations of 10 or 100 $\mu\text{g}/\text{ml}$ and incubated at 37°C for 2 h. Other experimental conditions were same as in Table 1.

Discussion

Activation of complement has been implicated in the pathogenesis of various inflammatory disorders (16). The anaphylatoxins C3a, C4a, and C5a, which are produced after complement activation, trigger a series of biologic events: release of chemical mediators, chemotaxis, and aggregation of granulocytes (5, 6). These biologic effects also appear to be involved in the pathophysiology of inflammatory disease (15).

Complement activation is tightly controlled through various receptors (15) and by regulating proteins including decay-accelerating factor (12), homologous restriction factor (13, 19), and others (3, 17). The resulting anaphylatoxins, C3a, C4a, and C5a, are rapidly deactivated by the enzymes, carboxypeptidases N and R (1, 2). Although the mechanisms of complement activation and the deactivation of active complement fragments have been extensively studied, it is not known whether the response to C5a in the PMN obtained from patients with various diseases is similar to that in normal subjects. The PMN are one of the most important target cells for the anaphylatoxins, C3a and C5a (5).

After stimulation with C5a, the PMN generate O_2^- , a generation that may be important in the defense against infection and in the progression of inflammation. Such effects of C5a are believed to be mediated through the C5a receptors on PMN. We found that the PMN from a patient with Kimura's disease generated much greater amounts of O_2^- after stimulation with C5a than did PMN from healthy controls. The hyperresponsiveness of PMN to C5a gradually returned to near normal values after the initiation of steroid treatment.

We speculated that the PMN from this patient may have been upregulated by some humoral factor. To confirm this hypothesis, we incubated PMN from healthy subjects with the patient's plasma at the active stage of disease. The results suggested the presence of a humoral factor which upregulates the O_2^- generation by PMN preferentially in response to C5a. This factor is considered to be heat-labile. Additionally, the *in vitro* experiment showed that steroids do not directly suppress the effect on PMN induced by this factor. The candidates for such a factor include complement products, especially C5a, and interleukins 3, 5, 6, and 8. C5a and various cytokines have been reported to be heat-resistant at 56°C (5). Therefore, a substance other than these may be considered to be a factor in the upregulation of the C5a response of PMN. In this study, we could not obtain evidence of the cellular origin of this factor.

Since the anaphylatoxin C5a is believed to play an important role in various inflammatory disorders,

the C5a-potentiating factor in this patient's plasma may have been important in regulating the cellular response after complement activation. Further study is needed to identify the factor that upregulates the C5a response of PMN.

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