

Classical pathway complement activation in Kawasaki syndrome

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In this study the complement breakdown products C3d, C4d, Bb and membrane attack complex were measured in plasma of patients with Kawasaki syndrome. The results suggested strong activation of the classical activation pathway. However, there was no significant decrease in hemolytic titer or in the concentrations of the intact proteins C3, C4, and B. The relationship between the serum concentrations of cytokines and complement components was examined; increased interleukin-6 concentration on the fifth day after the onset of fever was found to correlate well with the C3 and B concentrations in serum obtained 5 days later. We conclude that complement activation occurred in Kawasaki syndrome via the classical pathway but that the inflammatory reaction was accompanied by increased production of complement components. As a result, there was increased formation of activation products without changes in the serum complement levels. (J ALLERGY CLIN IMMUNOL 1994;93:520-5.)

Key words: Kawasaki syndrome, complement, cytokine, C4d, C3d, membrane attack complex, Bb, IL-6, classical pathway

Several agents have been speculated to induce Kawasaki syndrome (KS), but there are also contradictory findings regarding the roles of Epstein-Barr virus¹ and retrovirus.² The etiologic agents are still unknown, but the mechanism of augmentation of inflammatory response is gradually being elucidated. Some symptoms, including fever and vasculitis, arise as a result of immunologic reactions, which involve the action of cytokines, especially interleukin-2 (IL-2) receptor,³ IL-6,⁴ and tumor necrosis factor- α .⁵ Many factors have been identified as the causative or enhancing agents in vasculitis, including anti-endothelial cells,⁶ anti-cardiolipin,⁷ anti-neutrophil cytoplasm,⁸ and anti-actin⁹ antibodies. The positive immune complex results that have been reported in KS include IgG immune complex measured by C1q, Raji cells, polyethylene glycol,¹⁰ and platelet agglutination methods^{11, 12} and IgA immune complex measured by the anti-C3 method.¹³ Complement activation has not been considered to be accelerated in KS because the reported serum complement levels

Abbreviations used

EDTA: Ethylenediaminetetraacetic acid

IL: Interleukin

KS: Kawasaki syndrome

MAC: Membrane attack complex

are elevated.¹⁴ However, the serum levels of C5a and C3a that have an anaphylatoxin-like activity and C3 breakdown products have recently been reported to be elevated.¹⁵ The mechanism of induction of the complement augmentation through immunologic reactions in KS remains obscure.

In this study we investigated the immunologic changes, especially in factors producing the inflammatory reaction, in patients with KS. The complement breakdown products C4d, C3d, Bb, and membrane attack complex (MAC), were detected in the ethylenediaminetetraacetic acid (EDTA) plasma of patients with this syndrome, and the levels of C4d and MAC were particularly high. As in some other inflammatory states, serum levels of complement components are increased because of cytokine stimulation of liver production of acute-phase reactants.¹⁶ Therefore the levels of serum complement component might not be decreased even when there is prominent complement activation. This possibility is also discussed in connection with cytokine effects.

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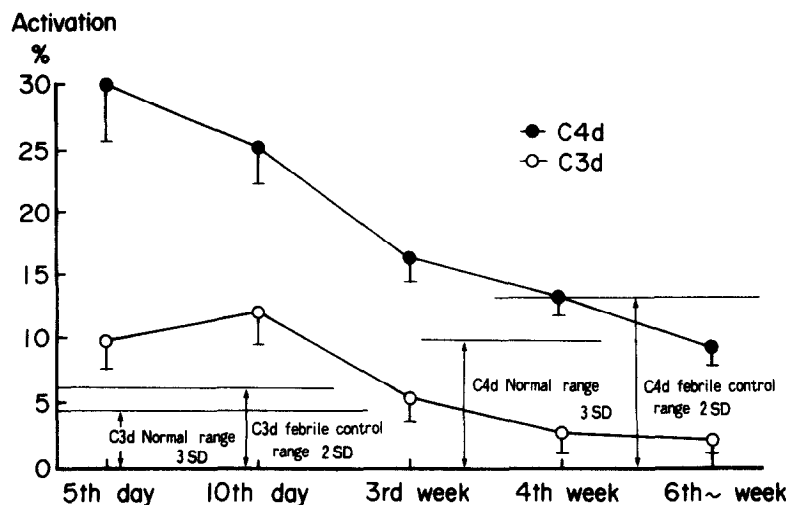


FIG. 1. The plasma concentrations of the complement breakdown products C3d and C4d in Kawasaki syndrome at various intervals. The normal range for C3d concentration in EDTA plasma is below 6%. The mean value in the healthy control group was $1.83\% \pm 1.32\%$, and the mean value in the febrile control group was $2.42\% \pm 1.70\%$. C3d activity does not increase after separation of serum. The production of C4d at up to 10% activation occurs spontaneously in serum at 37°C for 4 hours. The activation in the healthy control subjects was $4.63\% \pm 2.14\%$ and that in the febrile control subjects was $7.15\% \pm 3.23\%$. In patients with KS the C4d activity on the fifth and tenth days was significantly increased compared with that during week 6 (both, $p < 0.001$) and with those in healthy and febrile control subjects (all, $p < 0.001$).

METHODS

Patients and sample selection

The 40 patients with KS were all inpatients at hospitals in the Kanto area of Japan. All had clinical features that fulfilled at least five major diagnostic criteria for KS. The selected patients, whose ages ranged from 6 months to 5 years (mean age \pm SEM, 1.95 ± 1.23 years), had not received γ -globulin therapy and were treated with salicylates and/or anticoagulants. Ten millimoles per liter EDTA was added to heparinized plasma, which was stored at -80°C . Serial samples were obtained in 26 patients at the following intervals: the fifth day (5.2 ± 1.2 days) after onset of fever; tenth day (10.8 ± 1.9 days); week 3 (20.1 ± 2.4 days); week 4 (28.5 ± 3.5 days); and week 6 (52.8 ± 7.8 days). In all 40 patients the levels of complement breakdown products were measured at least twice before the third week after onset of fever.

The healthy control subjects were 32 patients scheduled to undergo minor surgery for hernia or cleft palate. Their ages ranged from 6 months to 5 years (2.15 ± 1.23 years). The febrile control subjects consisted of 32 patients with respiratory disease who manifested more than 38°C fever, which continued for 4 days or more. Their ages ranged from 8 months to 5 years (2.98 ± 2.12 years). These subjects exhibited no exanthema or other vasculitis symptoms, and their clinical condition, other than upper respiratory symptoms, returned to normal within 1 week. Informed consent for use of a portion of the patients' plasma was obtained from the parents.

The method of complement breakdown products and cytokine assay

C4d and C3d were assayed by the two-directional rocket method, the details of which have been reported elsewhere.¹⁶ The underlying principle of this method is that there is faster movement of C4d and C3d than of C4 and C3 during electrophoresis. The precipitation lines of C4d and C3d are formed in the alpha area, whereas those of C4 and C3 appear in the beta area.

Zymosan-treated plasma (10 mg/ml incubated at 37°C on 3 consecutive days) was used as the sample to show 100% activation of C4. Trypsin-treated purified C3 (incubated for 3 minutes, followed by addition of Trasylol to stop the reaction) was prepared as the sample for that of C3d standard. The standard sample for 0% activation was fresh plasma to which 10 mmol/L EDTA was added and compared with standard samples made at known concentrations of C3 (80 mg/dl) and C4 (30 mg/dl).

To assess Bb and MAC levels, individual monoclonal mouse antibody attached to beads was added with EDTA phosphate-buffered saline-diluted plasma ($20 \times$ to $100 \times$). After washing, the beads were incubated with horseradish peroxidase-conjugated antibodies to B or neoantigen, respectively. The beads were washed again and assayed with peroxidase-diammonium reaction method (produced by the Quidel Co., San Diego, Calif.).¹⁷ C3 and C4 were measured by laser nephelometry (supplied by Kyouwa Medex, Tokyo, Japan), and IL-6 was evaluated by the ELISA method with two different monoclonal antibodies (Fujirebio Inc., Tokyo, Japan).¹⁸

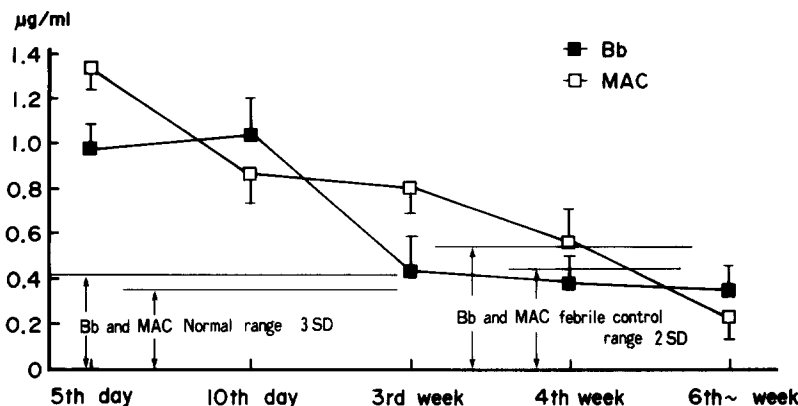


FIG. 2. The time course of the MAC and Bb plasma concentrations in patients with KS. The level of MAC during week 3 was significantly increased compared with that during week 6 and with the control range (healthy control group, 0.182 ± 0.062 $\mu\text{g/ml}$; febrile control group, 0.231 ± 0.110 $\mu\text{g/ml}$) (all, $p < 0.001$). The level of Bb on the tenth day was significantly higher than the normal range (healthy control group, 0.215 ± 0.071 $\mu\text{g/ml}$; febrile control group, 0.292 ± 0.138 $\mu\text{g/ml}$) (both, $p < 0.01$).

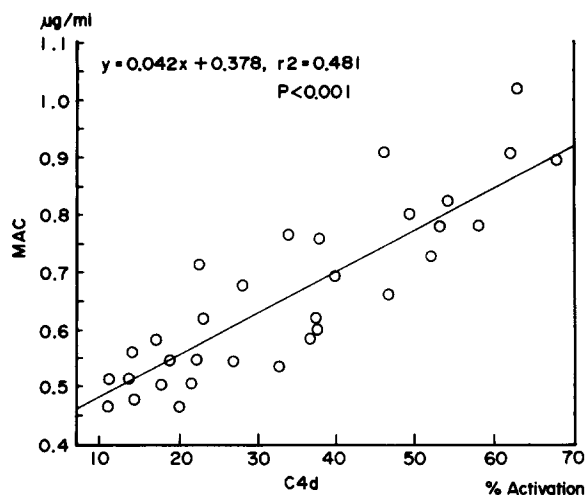


FIG. 3. The relationship between the plasma concentration of C4d and that of MAC. In the fluid phase, classical activation occurred and was strong enough to activate the terminal components ($r = 0.694$, $p < 0.001$).

Data analysis

Data are expressed as means \pm SEM, unless otherwise indicated. Statistical analysis of variance and Pearson's correlation coefficients were analyzed with the software Statview II and Super ANOVA Macintosh (Abacus Concepts, Berkeley, Calif.). Significance of intra- and intergroup differences was determined with two-tailed Student's t test and Pearson's correlation test. A p value of less than 0.05 was considered significant.

RESULTS

Chronologic changes in the plasma concentration of C3d and C4d

In the patients with KS, the concentrations of both the complement breakdown products C4d

and C3d exceeded the normal range (Fig. 1). C4d concentration was high during the fifth ($29.8\% \pm 4.2\%$ of maximal activation) and the tenth ($25.5\% \pm 2.7\%$) days after onset of fever but was within the normal range during week 6 ($p < 0.01$). The C4d value was also significantly higher than those in the healthy ($4.63\% \pm 2.14\%$) and febrile ($7.15 \pm 3.23\%$) control groups (both, $p < 0.001$). The elevation of the concentration of C3d in patients with KS from the fifth ($9.8\% \pm 1.3\%$) to the tenth days ($12.3\% \pm 1.2\%$) was less marked than that of the C4d concentration.

The time course of Bb and MAC concentrations in KS plasma

The MAC concentration was elevated on the fifth (1.32 ± 0.12 $\mu\text{g/ml}$) and tenth days (0.88 ± 0.17 $\mu\text{g/ml}$) and returned to the normal range (healthy control range, below 0.37 $\mu\text{g/ml}$) during week 6. The concentration of Bb was also increased on the fifth (0.98 ± 0.14 $\mu\text{g/ml}$) and tenth days (1.23 ± 0.24 $\mu\text{g/ml}$). However, the degree of activation of factor B was less marked, returning to the normal range (healthy control range, below 0.43 $\mu\text{g/ml}$) by week 3 (Fig. 2).

The relationship between C4d and MAC plasma concentrations

There was a significant correlation between C4d and MAC concentrations in KS plasma (Fig. 3), suggesting that the classical pathway via C4 was activated as far as the terminal components. The levels of Bb and C3d were not significantly correlated with that of MAC.

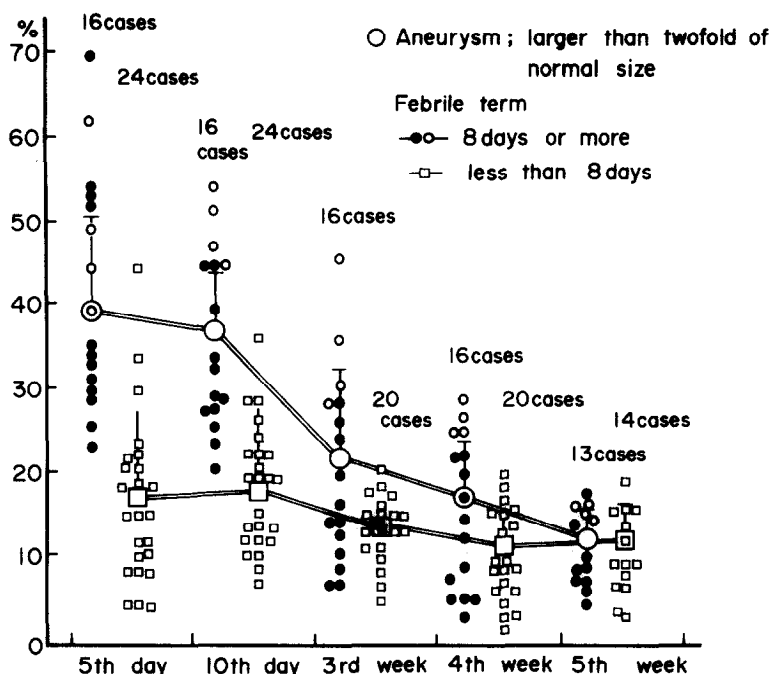


FIG. 4. Chronologic change of plasma C4d concentration according to the duration of febrile term. Patients with fever that continued for 8 days or more showed persistently high C4d concentrations, especially on the fifth and tenth days. There was a significant difference between this group and the other group with shorter febrile terms in C4d concentration during the first 4 weeks ($p < 0.001$).

C4d plasma concentration in patients with KS classified by duration of febrile period

Patients with fever that persisted for 8 days or more showed significantly higher concentrations of C4d on the fifth and tenth days than did those with fever of shorter duration (Fig. 4). In patients with more severe heart complications, markedly high C4d concentrations persisted throughout the active phase of illness.

The relationship of IL-6 concentration on the fifth day to plasma complement concentrations on the fifth and tenth days

Although the serum concentration of IL-6 on the fifth day was not correlated with either C3 or B levels on the fifth day, it was significantly correlated with both C3 and B concentrations on the tenth day (C3: $r = 0.56$, $p < 0.01$; B: $r = 0.603$, $p < 0.01$) (Fig. 5).

DISCUSSION

In KS the serum complement levels are known to be elevated,¹⁴ as are those of other inflammatory proteins. However, complement activation cannot be determined on the basis of protein concentration or hemolytic titer, as it can in some types of nephritis and systemic lupus erythemato-

sis, in which complement activation leads to decreased serum complement values. Recent studies of complement production have indicated that the levels of serum complements do not directly reflect complement activation.¹⁹ Blood concentrations of complements express the difference between production and consumption. Consequently, when the production of complements is stimulated by cytokines, decrease of protein concentrations or of hemolytic activity is unlikely to ensue, even if there is enhanced complement activation. In fact, the complement cascade is activated in other types of vasculitis, such as allergic purpura²⁰ and juvenile rheumatoid arthritis,²¹ but neither protein concentrations nor titers of complements have been reported to decrease except in rare cases. However, an elevated C3d concentration has been reported in these diseases. For this reason, the release of cytokines as a result of inflammation linked with increased levels of inflammatory proteins has been cited.²² Indeed, in our investigation of the relationship between serum concentration of IL-6 and concentrations of C3 and B, no correlation was observed in the serum samples taken on the same day, whereas the IL-6 concentration on the fifth day of illness was found to be well correlated with con-

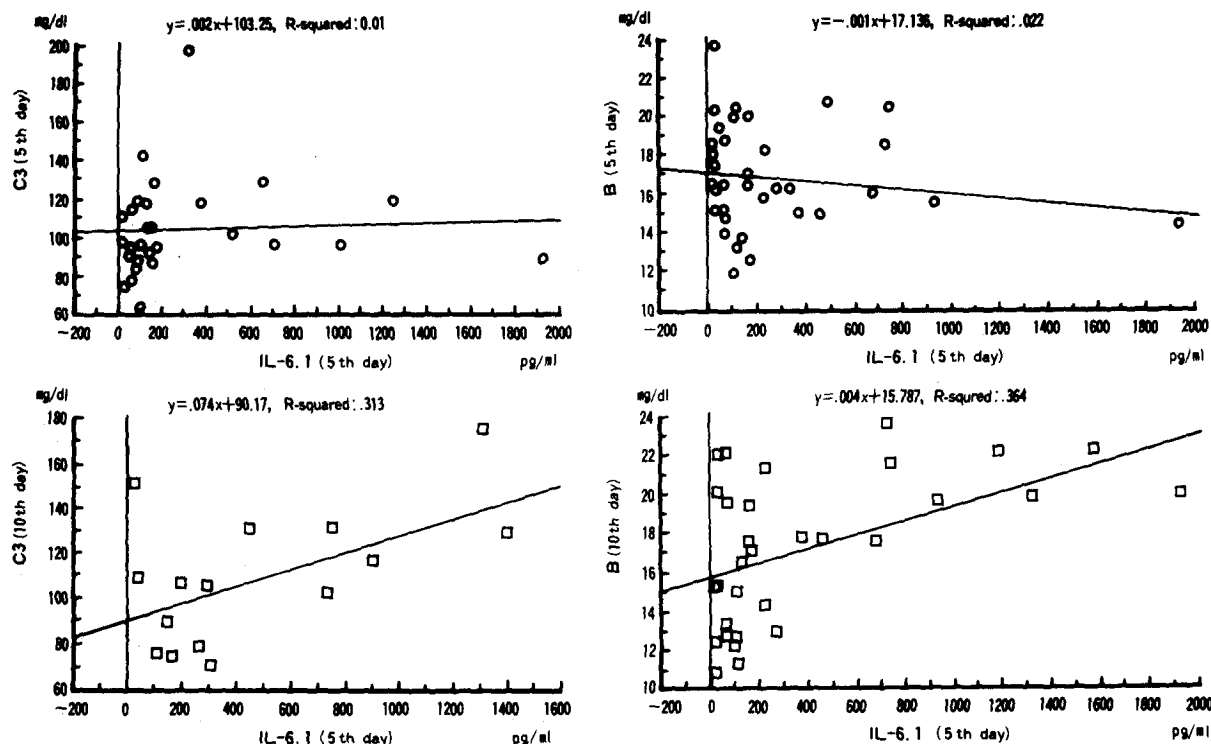


FIG. 5. The relationship of IL-6 concentration in KS plasma on the fifth day to C3 and B concentrations on the fifth and tenth days. The concentration of IL-6 on the fifth day was not correlated with that of C3 on the same day. However, it was significantly ($p < 0.001$) correlated with C3 level on the tenth day (5 days later).

centrations of C3 and B 5 days later. These findings raise the questions of whether production of inflammatory proteins is potentiated by IL-6 and whether the early phase IL-6 concentration is important in determining the inflammatory reaction. The possibility of substances other than IL-6 accelerating hepatic production of inflammatory proteins cannot be excluded. Between the fifth and tenth days, some autoantibodies and immune complexes are also detected in KS serum.^{6-8, 11-13} They activate or injure endothelial and immunologic cells, resulting in production of other cytokines and chemical mediators, which affect hepatic protein synthesis.

The role of these complement breakdown products in KS, other than the roles as anaphylatoxin (C3a, C5a) and chemotaxis (C5a), has not been studied. When IgM autoantibody²³ or immune complex attaches on the vascular wall, complement activation and macrophage phagocytosis occur at once, and cytokine production and tissue damage follows. Such complement products function as causative agents of inflammation.

Recently, complement breakdown products have been reported to play a role in the regulation

of cytokine production. Human recombinant C5a was found to mediate the release of IL-6 in peripheral blood-derived mononuclear cells²⁴ and Bb to enhance the IL-1 production of macrophages stimulated with lipopolysaccharide, although this latter effect was diminished after the procedure to ultrapurify Bb without contamination.²⁵ Furthermore, C3d or C3 synthetic peptides not only support the growth of human blastoid B-cell lines in serum-free media but also stimulate the proliferative response of CR2-positive cells.²⁶ These findings would account for the high cytokine levels and the polyclonal activation of B cells in KS.

Accordingly, complement activation affects the prognosis of patients with KS through these immunologic augmentation mechanisms. The level of C4d in KS plasma in the early stage is high and correlates well with that of MAC, which suggests the production of breakdown products such as C3a, C5a, and Bb. The duration of the febrile period in patients with KS is thought to reflect the severity of associated heart disease.²⁷ Patients with fever lasting for 8 days or more were found to have significantly higher incidence of subse-

quent cardiac involvement than those whose febrile period was shorter than 7 days.²⁸ Clinically, our patients with high C4d levels had longer febrile terms than did those in whom C4d levels were low, and the elevated C4d values in these patients may be associated with more severe heart complications; in these patients, abnormally high C4d values persisted throughout the active phase of illness.

Although the pathogenic factor remains unknown in KS, these results indicate that the production of immune complex, as well as that of cell or virus fragments, primes the activation of both complement and macrophages and that the complement breakdown products enhance cytokine production and B-cell activation. The interaction between these fluid-phase reactions and cellular immunity may increase inflammatory reactions in KS.

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