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Effects of Intravenous Immune Globulin on the Peripheral Lymphocyte Phenotypes in Kawasaki Disease

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The effect of intravenous immune globulin (IVIG) on the lymphocyte phenotypes in acute Kawasaki disease (KD) was studied in a random trial of IVIG-and-aspirin versus aspirin-alone. Before therapy, patients in each treatment group had an increased percentage of B cells, and a decreased percentage of T cells, CD4 T cells, CD8 T cells and CD5+ B cells. There was no significant difference in immunologic parameters between the two groups measured before therapy. Patients treated with IVIG-and-aspirin had by the fourth day developed a highly-significant increase in T cells, CD4 T cells and CD8 T cells and a decrease in B cells. Despite the decrease of B cells, there were significant increases in CD5+ B cells in both treatment groups. However, the degree of increase in the IVIG-and-aspirin treated group was significantly more noticeable than that in the aspirin-alone treated group. These findings indicate that treatment with IVIG restores the T- and B- cell abnormalities, especially CD5+ B-cell abnormalities found in patients with acute KD.

Key Words: Kawasaki disease, intravenous immune globulin, T cell, B cell, CD5+ B cell

Kawasaki disease (KD) is an acute systemic vasculitis which predominantly affects infants and young children. The major clinical features are prolonged fever of more than 5 days-duration, bilateral conjunctival injection, enlarged cervical lymph nodes, erythematous induration of hands and feet, inflammation of lips and tongue and polymorphous skin rashes. Even though the epidemiologic features of this disease suggest that it is related to an infectious agent, the etiology is still unknown (Kawasaki, 1967).

However, KD is an immunologically interesting disease because during its course immunological abnormalities, including activation of immunocompetent cells, can be observed (Leung, 1989). It is also reported that during the acute phase of KD, some autoantibodies such as anti type III collagen antibody (Kobayashi *et al.* 1992), antineutrophil cytosolic antibody (Guzman *et al.* 1994), IgM autoantibody to vascular endothelial cells activated by interferon-γ (Leung *et al.* 1986), and anti-heat shock protein 65 antibody (Yokoda, 1991) were found. One of the most remarkable immunologic findings in KD is probably the marked increase in circulating B cells which actively produce immunoglobulins (Leung, 1989). In a previous study, we observed that CD5+ B cells decreased during the acute phase of KD (Noh and Kim, 1995).

The major breakthrough in the treatment of KD was the introduction of high-dose intravenous immune globulin (IVIG). A high dose of IVIG is very effective in reducing the systemic symptoms and the prevalence of cor-

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onary artery abnormalities, which is the major complication of KD during the convalescent phase (Furusho *et al.* 1984). The mechanisms of action of IVIG in KD is unknown.

In this report, we have examined the effect of IVIG on the immunoregulatory cells in KD and speculated on the mechanisms of IVIG in KD.

MATERIALS AND METHODS

Patients

Venous blood was obtained from 29 patients (15 males) with KD, who were followed at the Severance Hospital, Yonsei University College of Medicine and Sowha Children's Hospital, Seoul, Korea. All patients satisfied at least five of the six criteria for the diagnosis of KD (Kawasaki, 1967). Atypical KD was excluded. Samples were obtained from 10 age-matched children with acute febrile disease (3 herpangina, 7 acute tonsillopharyngitis) and 10 children who were having routine blood work done before elective surgery. Samples of acute febrile disease were collected from the patients before any medication was given. Informed consent was obtained from the parents of children included in this study.

Study design

Eligible patients were separated into two groups by using sequences of random numbers to one of two therapies. The first group received only aspirin, 100 mg/kg per day in four divided doses until the 14th day after the onset of fever, followed by daily aspirin at a dose of 3~5 mg/kg per day. The second group received the same dosage of aspirin as the first group, but in addition they received an infusion of intravenous immune globulin (IVIG) at a dose of 2 g/kg. Heparinized blood samples for lymphocyte studies were obtained on the day before the initiation of IVIG infusion and 2~3 days after IVIG infusion.

Analysis of immunophenotypes of peripheral lymphocytes

To determine the phenotype of the peripheral lymphocytes in KD, the cell surface antigens were stained using fluorescein-conjugated (FITC) and/or phycoerythrin (PE)-conjugated anti-Leu-3a (CD4), anti-Leu-1 (CD5), anti-Leu-2a (CD8), anti-Leu-12 (CD19) monoclonal antibodies, which were purchased from Becton Dickinson Monoclonal Center (San Jose, CA, USA) by the whole-blood method. Briefly, 100 microliters of the heparinized venous blood was placed in each of 4 labeled tubes. One hundred microliters of monoclonal antibodies and PBS as negative controls were added. The cells were incubated in the dark at 4℃ for 30 min. The red blood cells in each tube were then lysed using 1 mL of lysing reagent. After incubation in the lysing reagent for 10 min, the cells were immediately centrifuged at 200×g for 5 min. The supernatants were removed, leaving approximately 50 microliters of fluid. The pellets were mixed gently with a vortex after adding 3 mL of 0.01 M PBS with 2% bovine serum albumin. After centrifugation at 200×g for 5 min, the supernatant was discarded, leaving approximately 50 microliters of fluid. The pellets were resuspended with 0.3 mL of 1% paraformal-dehyde in PBS and were analysed using a FACStar flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA).

Statistical analysis

The Students't test and ANOVA (analysis of variance) were used to analyze the significance of differences between the experimental groups and the normal controls.

RESULTS

Characteristics of patient study groups

Of the 29 KD patients, 14 were randomly selected to the aspirin treatment group and 15 to the aspirin plus IVIG treatment group. As shown in Table 1, treatment groups were similar with respect to age distribution, sex and duration of illness before entry into the study.

Effects of IVIG on T cell subsets in KD

The T-cell-subset distribution in patients

Table 1. Clinical and laboratory features of KD patients at time of enrollment into trial

Study parametet	Study population			
	IVIG+aspirin (n=15)	Aspirin only (n=14)	Acute febrile disease (n=10)	Normals (n=10)
Sex				
Male	8	7	5	5
Female	7	7	5	5
Mean age(yr)	2.5± 0.6	2.3± 0.5	2.7± 0.7	2.8± 0.8
Total lymphocyte count	3,523±1,045*	3,324±2,126*	4,312±10,55	4,331±1,521
%PBMC reactive with monoclonal antibody				
CD3	47.2±3.6*	50.1±5.4*	56.8±3.3	62.9 ±5.1
CD4	33.7±2.6*	35.0±3.9*	42.7±3.0	43.1 ±5.2
CD8	10.5±2.4*	11.7±2.3*	19.5±2.1	18.12±2.5
CD19	25.4±3.1*	32.1±5.4*	15.7±2.1	15.1 ±3.5
CD5+CD19+ in CD19+	15.2±1.0*	17.1±1.6*	38.3±2.2	40.1 ±5.1

*: $p<0.05$ compared with normal controls

+: $p<0.01$ when compared with normal controls

with KD at the time of enrollment into the study is also summerized in Table I. There were no significant differences in the percentage of circulating CD3 T cells, CD4 T cells and CD8 T cells between the two treatment groups. PBMC from both groups demonstrated a significant decrease in the percentages of T cells in comparison with the age-matched controls ($p<0.05$). This decrease in total T cells represented T-cell lymphopenia because the mean value for the number of lymphocytes in the peripheral blood of patients with acute KD was lower than that obtained from the group of age-matched controls ($p<0.05$)(Table 1). The mean lymphocyte counts in patients at enrollment was similar in the two treatment groups. The decrease of circulating T cells in acute KD reflected a significant decrease in both CD4 T cells ($p<0.05$) and CD8 T cells ($p<0.01$). However, the decrease in CD8 T cells was proportionately greater than that observed with CD4 T cells. As a result, KD had an abnormally increased ratio of circulatuing CD4 T cells to CD8 T cells.

Fig. 1 and 2 summerizes the T-cell-subset distribution of PBMC from both treatment groups on day 1 and day 4 of the study. Pa-

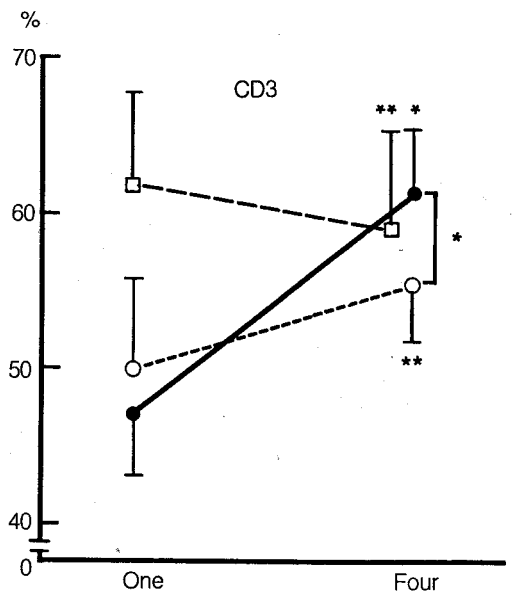

Fig. 1. *T cell populations in 14 KD patients treated with aspirin (○---○), 15 KD patients treated with aspirin and IVIG (●——●) and 10 acute febrile disease patients treated with aspirin and IVIG (□--□) on admission (One) and 4 days after admission (Four). Values are expressed as mean ±SEM. *: $p<0.05$, **: p=not significant.*

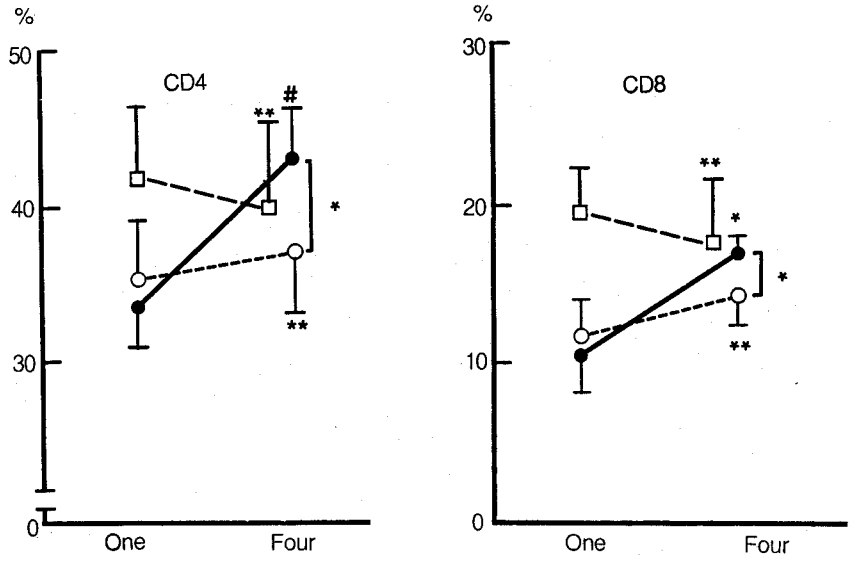

Fig. 2. *CD4 (left) and CD8 (right) T cell populations in 14 KD patients treated with aspirin (○----○), 15 KD patients treated with aspirin and IVIG (●—●) and 10 acute febrile disease patients treated with aspirin and IVIG (□—□) on admission (One) and 4 days after admission (Four). Values are expresed as mean±SEM. *: p< 0.05, #: p<0.005, **: p=not significant.*

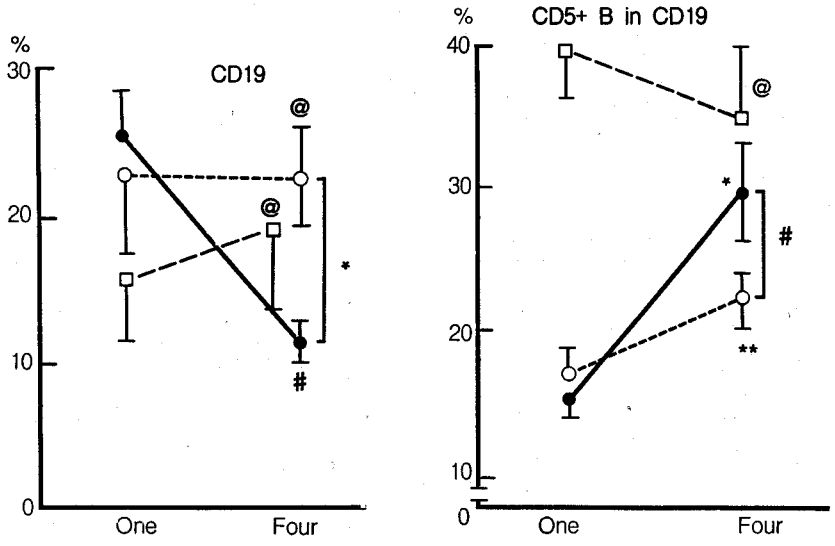

Fig. 3. *CD19+ conventional B cell (left) and CD5+ B cell (right) populations in 14 KD patients treated with aspirin (○----○), 15 KD patients treated with aspirin and IVIG (●—●) and 10 acute febrile disease patients treated with aspirin and IVIG (□—□) on admission (One) and 4 days after admission (Four). Values are expressed as mean±SEM. *: p<0.01, **: p: <0.05, # p: <0.001, @: p=not significant.*

tients treated with IVIG and aspirin had a significantly-greater increase in their percentages of T cells ($p<0.05$)(Fig. 1), CD4 T cells ($p<0.005$) and CD8 T cells ($p<0.05$) (Fig. 2) after the 4 days of treatment than did patients treated with aspirin only ($p>0.05$).

Effects of IVIG on B cell subsets in KD

The B-cell-subset distribution in patients with KD at the time of enrollment into the study is also summerized in Table 1. There were no significant differences in the percentage of circulating B cells and CD5+ B cells between the two treatment groups. PBMC from both groups demonstrated a significant increase in the percentages of B cells in comparison with age-matched controls ($p<0.01$). This increase in the total B cells represented B-cell lymphocytosis, which is probably the most remarkable immunologic finding in this disease. However, as we reported previously (Noh and Kim, 1995), the percentages of CD5 + B cells from both treatment groups demonstrated a significant decrease in comparison with age-matched controls ($p<0.01$).

Fig. 3 summerizes the B-cell-subset distribution of PBMC from both treatment groups on day 1 and day 4 of the study. Patients treated with IVIG and aspirin have a significantly-greater decrease in their percentages of B cells ($p<0.005$) after the 4 days of treatment than did patients treated with aspirin only ($p>0.05$).

Interestingly, there were significant increases in the percentages of CD5+ B cells in both groups: the group treated with aspirin only ($p<0.05$) and the group treated with IVIG and aspirin ($p<0.01$). However, the degree of increase in the IVIG-and-aspirin-treated group was significantly more noticeable than that in the aspirin-only-treated group ($p<0.001$).

In contrast to these results, the effects of IVIG on lymphocytes subsets in patients with acute febrile diseases showed the opposite results to those in patients with KD. However, these opposite findings were found to be not statistically significant (Fig. 1~3).

DISCUSSION

In the present study, we found that high-dose IVIG had an effect on the restoration of the immunological alterations found in acute KD. After IVIG infusion, there was a significant increase in T cells, CD4 T cells and CD8 T cells and a decrease in B cells. In contrast, treatment with aspirin only did not cause any significant changes in the abnormal T-cell or B-cell distribution found in acute KD.

IVIG has been used for over 10 years in various diseases, such as immunodeficiency diseases, autoimmune diseases, viral and bacterial infections, malignant diseases, sepsis, and epilepsy (ASHP Commission on therapeutics, Phelps *et al.* 1992). However, the exact action mechanisms of IVIG are still unknown except in immunodeficiency disease. There have been many reports on the action mechanisms of IVIG, such as the modulation of T-cell proliferation and suppressor T cell immune response (Luzi and Ferrara, 1993; Orvieto *et al.* 1993), modulation of cytokines and their receptors (Andersson *et al*, 1993; Skanssen-Saphir *et al.* 1994; Toyoda *et al.* 1994). Also IVIG influences on the cellular-compositional changes of the immune system were reported as the induction of an increase in the number of polymorphonuclear cells in the peripheral blood, and a decrease in the number of B cells and CD56+ cells (Hall, 1993). IVIG also contains autoantibodies to cytokines and CD5 molecules (Vassilve *et al.* 1993). However, these mechanisms cannot be applied in the treatment of KD. There were several possible mechanisms of IVIG action in KD: feedback inhibition of antibody synthesis, clearance of microbial agent or toxin, nonspecific immunologic Fc blockage, induction of suppressor T cells, and inhibition of autoantibodies by anti-idiotypic antibodies (Leung, 1989).

However, there are some controversies in IVIG effect on T cell changes (Leung *et al.* 1987; Nonoyama, 1991; Toyoda *et al.* 1994). Leung *et al.* (1987) observed that after IVIG therapy there was a significant decrease in the number of circulating HLA-DR+ helper T

cells, a significant increase in suppressor/cytotoxic T cells, a decrease in the capacity of T cells to release B-cell-helper factors, and a decrease in spontaneous IgG and IgM production by PBMC. However, on the contrary, Nonoyama (1991) reported that there was no significant change in CD8+ T cells.

In this study, after IVIG treatment there was also a significant increase in CD8 T cells. We also observed a statistically-significant increase in T cells and CD4 T cells, but which were found not to be statistically-significant in Leung's study (1987).

These different findings may result from a different regimen of infusion with IVIG. Our regimen used a continuous infusion of IVIG at a dose of 2 g/kg. In contrast, Leung's study showed an infusion of IVIG at a dose of 400 mg/kg per day for four consecutive days, which is not used these days. From these results, the continuous infusion of IVIG which was used in our study produces better results in immunological restoration than did Leung's method.

We did not focus this study on the spontaneous secretion of immunoglobulins from B cells. However, we observed the changes of the percentage of B cells in patients with KD. It is well known that there is a marked increase in circulating B cells in this disease. Like Nonoyama's result (1991), our study showed a significant decrease of B cell percentages after IVIG treatment. Durandy *et al.* (1981) and Stohl (1985) also reported that there was evidence for direct inhibition of B lymphocytes by gammaglobulin.

In a previous study, we observed that conventional B cells (CD5-, CD19+) increased, whereas CD5+ CD19+ B cells decreased during the acute phase of KD (Noh and Kim, 1995). Interestingly, there was a significant increase of CD5+ B cells without IVIG infusion. The percentage of CD5+ B cells increased with IVIG treatment much more than without IVIG treatment. With these results, it can be speculated that the initial action to correct immunological abnormalities in acute KD is the restoration of CD5+ B cells. Therefore, the major effect of IVIG in the treatment of KD is the restoration of CD5+ B cells.

The exact mechanisms of IVIG in the restoration of CD5+ B cells is not known yet; however, there has been a report that IVIG stimulated IgM production (Kondo *et al.* 1991). It is well known that CD5+ B cells produce mainly IgM (Schroeder and Dighiero, 1994). From these facts, the stimulatory effect of IVIG on IgM production may be from the stimulatory effect of IVIG on CD5+ B cells. However, further study is needed.

As mentioned, it is well documented that KD is characterized by marked immune activation and increased cytokine production (Leung, 1989). This could contribute to the endothelial damage that is observed in this disease. Clinically, IVIG appears to reduce the prevalence of coronary artery abnormalities in this disease by reducing the tissue inflammation that underlies the vasculitis (Furusho *et al.* 1984). Based on our results, we postulate that IVIG may prevent the development of coronary artery aneurysm by interrupting the immunologic abnormalities that result in blood-vessel injury. To resolve this issue, we need to reach a better understanding of the immunologic mechanisms through which IVIG works in KD.

For the control study, patients with acute febrile disease were treated with IVIG 2 g/kg, and then the changes of immunophenotypes of peripheral lymphocytes were tested. Interestingly, the changes of CD3, CD4, CD8, CD19, and CD5+ CD19+ cells were not statistically significant. However, the trend of changes in these cells after IVIG infusion was completely opposite to those in KD. It can be explained by the fact that the action mechanisms in the patients with KD are immunologically different from those in the normal physiologic controls.

In conclusion, IVIG infusion in acute KD has an effect on the restoration of altered compositions of immune cells such as CD3+, CD4+, CD8+ T cells, B cells and CD5+ B cells to normal levels, especially through the restoration of CD5+ B cells. These effects may also prevent blood-vessel injury in the development of coronary-artery aneurysm.

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