JCI The Journal of Clinical Investigation

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J Clin Invest. 1987;79(2):468-472. https://doi.org/10.1172/JCI112835.

Research Article

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Reversal of Lymphocyte Activation In Vivo in the Kawasaki Syndrome by Intravenous Gammaglobulin

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Abstract

The effect of intravenous gammaglobulin (IVGG) on the immunoregulatory abnormalities found during acute Kawasaki syndrome (KS) was studied in a randomized trial of IVGG plus aspirin (ASA) versus ASA alone. Before therapy, patients in each treatment group had increased numbers of circulating HLA-DR-bearing Leu 3+ helper T cells, a deficiency of Leu 2+ suppressor/cytotoxic T cells, and increased levels of spontaneous IgG and IgM synthesis by peripheral blood mononuclear cells. There were no significant differences ($P > 0.1$) between immunologic parameters measured on day 1 and day 4 in the ASA-treated group. In contrast, patients treated with ASA plus IVGG had by day 4 a highly significant decrease in HLA-Dr+ Leu 3+ helper T cells ($P < 0.001$), an increase in Leu 2+ suppressor/cytotoxic T cells ($P < 0.01$), and a decrease in spontaneous IgG ($P < 0.01$) and IgM synthesis ($P < 0.001$). These changes were associated with a reduction in the secretion of T cell–derived B cell helper factors ($P < 0.001$). These findings indicate that treatment with IVGG suppresses the marked T and B cell activation found in patients with acute KS.

Introduction

Kawasaki syndrome (KS)[1] is an acute febrile childhood disease of unknown etiology that is associated with the development of coronary artery aneurysms in 15–20% of the cases (1, 2). Children with KS are usually placed on daily aspirin (ASA) as an anti-inflammatory agent and to prevent platelet aggregation in blood vessels. There is however no definitive evidence that ASA therapy deminishes the frequency of coronary artery lesions in KS. Recently, it has been reported that administration of intravenous gammaglobulin (IVGG) in high doses during the acute phase of KS reduces the frequency of coronary artery aneurysms (3, 4).

We have previously demonstrated that acute KS is characterized by immunoregulatory abnormalities of circulating lymphocytes. These abnormalities include a deficiency of suppressor/cytotoxic T cells, increased numbers of activated helper T cells, and increased B cell activation (5, 6) that is reflected by high levels of spontaneous immunoglobulin synthesis. Although multiple factors are likely to be involved in the pathogenesis of KS, the effect of high dose IVGG on the immunoregulatory imbalance observed during acute KS was of interest to us in attempting to unravel mechanisms by which IVGG prevents the development of coronary artery aneurysms in this disease. During an ongoing multicenter trial in the United States to study the effects of ASA alone versus ASA plus IVGG on the clinical outcome of KS, we have examined the effect of these agents on the immunoregulatory abnormalities in acute KS. The results of this study suggest that IVGG reverses the activation of T and B cells in vivo in acute KS.

Methods

Subjects. The study population consisted of 29 patients with acute KS who were seen at Children's Hospital between January, 1984 and March, 1985. Eligibility for this study required that the patient meet the diagnostic criteria for KS and that they enter the study within 10 d of onset of fever. The diagnostic criteria for KS were five or all six of the following signs: (*a*) fever of unknown etiology lasting for 5 d or more; (*b*) bilateral conjunctival injection; (*c*) changes of lips and oropharynx including (*i*) dryness, redness, and fissuring of the lips, (*ii*) diffuse redness of the oropharynx, (*iii*) protuberance of tongue papillae, i.e. "strawberry tongue;" (*d*) changes in the peripheral extremities including: (*i*) reddening of palms and soles (initial stage), (*ii*) indurative edema (initial stage), and (*iii*) periungal desquamation (convalescent stage); (*e*) polymorphous erythematous rash of trunk; and (*f*) acute nonpurulent cervical lymphadenopathy with lymph nodes measuring 1.5 cm in diameter. Antistreptococcal titers and throat cultures for group A beta hemolytic streptococci were negative on each enrolled study subject. A control group consisting of 15 age- and sex-matched asymptomatic children was also studied. None of the patients or controls had ever received steroids or other immunosuppressive agents before their lymphocyte analysis. Informed consent was obtained from each subject and/or their parents before entry into the study.

Study design. Eligible patients were randomized using sequences of random numbers to one of two therapies. The first group received only ASA: 100 mg/kg per d in four divided doses until the 14th day, after the onset of fever, followed by daily ASA at a dose of 3–5 mg/kg per d. The second group received the same course of ASA as the first group and, in addition, received an infusion of intravenous gammaglobulin (Immuno-AG Co., Vienna, Austria) as a 5% solution at a dose of 400 mg/kg daily, for four consecutive days. Heparinized blood samples for lymphocyte studies were obtained on day 1 before the first IVGG infusion and on day 4, 2–3 h after the last IVGG infusion.

Cell separation. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia Fine Chemicals, Piscataway, NJ) of heparinized venous blood. T and B cells were separated by rosetting PBMC with neuraminidase-treated sheep red cells as previously described (6).

Monoclonal antibodies. The specificities of the monoclonal antibodies used in this study are presented in Table I. All monoclonal reagents were purchased from the Becton-Dickinson Monoclonal Center, Inc. (Mountain View, CA). The monoclonal antibodies were conjugated with either

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Received for publication 18 July 1986 and in revised form 16 September 1986.

1. *Abbreviations used in this paper:* ASA, aspirin; FITC, fluorescein isocyanate conjugate; IVGG, intravenous gammaglobulin; KS, Kawasaki syndrome; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; Th, T helper cells; Ts, T suppressor/cytotoxic cells.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/87/02/0468/05 $1.00
Volume 79, February 1987, 468–472

Table I. Specificity of Monoclonal Antibodies

Monoclonal antibody	Specificity
Anti-Leu 2a	Cytotoxic/suppressor T cell
	Subset of natural killer cells
Anti-Leu 3	Helper/inducer T cells
Anti-Leu 4	All T cells
Anti-HLA-DR	Activated T cells
	B cells
	Monocyte/macrophages

fluorescein isocyanate (FITC) or phycoerythrin (PE). Since anti-Leu 2a and anti-HLA-DR react with both T cells and non-T cells (see Table I), two-color immunofluorescence studies were used. To enumerate the percent of suppressor/cytotoxic T cells, PBMC were incubated with a combination of PE anti-Leu 2a and FITC anti-Leu 4 and the cells analyzed on the FACS IV cell sorter for dual staining. Similarly, activated helper T cells were enumerated by analyzing for cells that simultaneously reacted with PE anti-Leu 3 and FITC anti-HLA-DR.

For the remainder of this communication, we will define PBMC reacting with: (*a*) anti-Leu 4 antibody as "T cells," (*b*) anti-Leu 3 antibody as "T helper cells (Th)," (*c*) anti-Leu 3 and anti-HLA-DR antibodies as "activated Th," and (*d*) anti-Leu 2 and anti-Leu 4 antibodies as "T suppressor/cytotoxic cells (Ts)."

Two-color flow cytometry of peripheral blood lymphocytes. PBMC (1×10^6) in 0.1 ml RPMI 1640 medium with 2½% fetal calf serum (FCS) and 0.1% sodium azide (wash medium) were incubated with 1 μg (10 μl) of FITC-conjugated antibody and 1 μg of PE-conjugated antibody at 4°C for 30 min. The stained cells were then washed three times with wash medium and resuspended in 2 ml of phosphate-buffered saline (PBS) before flow cytometry analysis. Controls consisted of cells stained with FITC- or PE-conjugated, isotype-matched nonimmune myeloma protein (Becton-Dickinson & Co., Mountain View, CA).

The two-color immunofluorescence experiments were analyzed with a fluorescence-activated cell sorter (FACS IV; Becton-Dickinson & Co.) as previously described (7) using 488-nm light (400 mW, argon laser) to excite both fluorochromes. Data were collected in a Consort-40 PDP/11 computer system (Becton-Dickinson & Co.) for analysis of both single-color and two-color data. A total of 50,000 cells were analyzed in each experiment.

Measurement of spontaneous immunoglobulin (Ig) production. Spontaneous IgG and IgM synthesis was measured by culturing 1×10^6 PBMC/ml in RPMI 1640 medium containing 10% FCS (complete medium in the presence or absence of 100 μg/ml cycloheximide. After 6 d at 37°C in a 5% Co_2 incubator, culture supernatants were assayed for IgG and IgM by a standard enzyme-linked immunosorbent assay (ELISA) method as previously described (8). De novo IgG synthesis was calculated by determining the difference in IgG concentration between cycloheximide-treated cultures and cultures incubated in the absence of the protein synthesis inhibitor.

Assay of T cell supernatants for helper activity. T cell supernatants were generated by culturing T cells at 1×10^7/ml for 24 h in complete medium. Supernatants were then collected, filtered through a 0.45-μm Millipore filter, and frozen at − 20°C until tested. T cell supernatants were added at a 1:1 dilution to cultures of normal B cells (1×10^6 cells/ml) in complete medium in the presence or absence of 100 μg/ml cycloheximide. After 6 d of culture, the de novo IgG content of B cell culture supernatant was determined with a standard ELISA method as previously described (8).

Statistical evaluation. The two treatment groups were compared with regard to parameters at enrollment as well as to the change in these parameters between day 1 and day 4 of each treatment protocol using the Wilcoxon Rank Sum Test. We regarded a P value of: (*a*) < 0.05, as significant, (*b*) < 0.01, as highly significant, and (*c*) > 0.05 as not significant.

Results

Characteristics of patient study groups. Of the 29 patients with KS enrolled in this study, 14 were randomized to the ASA treatment group and 15 to the ASA plus IVGG treatment group. As shown in Table II, treatment groups were similar with respect to age distribution, sex, and duration of illness before entry into the study. Furthermore, patients enrolled into the two treatment groups did not differ significantly ($P > 0.1$) with regard to temperature or erythrocyte sedimentation rate.

Effects of IVGG on T cell subsets in KS. The T cell subset distribution in patients with KS at the time of enrollment into the study is also summarized in Table II. There were no significant differences in the percentage of circulating Leu 4+ T, Leu 3+ Th, activated HLA-DR+ Leu 3+ Th or Leu 2+ Ts between the two treatment groups. PBMC from both groups demonstrated a significant decrease in the percentages of T cells in comparison with age-matched controls. This decrease in total T cells represented an absolute T cell lymphopenia because the mean value for the number of lymphocytes in the peripheral blood of patients with acute KS was lower than that obtained for the group of age-matched controls (Table II). The mean lymphocyte counts in patients at enrollment was similar in the two treatment groups. The decrease of circulating T cells in acute KS reflected a significant decrease in both Leu 2+ Ts and Leu 3+ Th. The decrease in Ts, however, was proportionately greater than that observed with Leu 3+ Th. As a result, KS patients entered into this study had an abnormally increased ratio of circulating Leu 3+ Th to Leu 2+ Ts. This ratio was 6.6±5.2 in the ASA-treated group and 8.0±3.3 in the IVGG group, compared with 2.4±0.4 in the control group ($P < 0.01$). More importantly, both groups had a highly significant elevation in the percentages of circulating activated HLA-DR+ Leu 3+ Th.

Table II. Clinical and Laboratory Features of KS Patients at Time of Enrollment into Trial

	Study population		
Study parameter	ASA ($n = 14$)	ASA plus IVGG ($n = 15$)	Normals ($n = 15$)
Sex			
Male	7	8	7
Female	7	7	8
Mean age (*yr*)	2.1±0.4[§]	2.6±0.6	2.8±0.7
Time of entry into trial (days after onset of fever)	5.5±0.3	6.2±0.5	—
Body temperature (°C)	38.5	38.7	37*
ESR (wintrobe)	51±6	54±9	—
Total lymphocyte count	3,613±1,147	3,169±2,045	4,300±1,550
% PBMC reactive with monoclonal antibody:			
Leu 4+	54±4[¶]	48±4[¶]	62±5
Leu 3a+	36±3[¶]	36±3[¶]	43±5
Leu 2a+/Leu 4+*	8±4[‖]	7±4[‖]	18±2
Leu 3a+/HLA-DR+‡	21±5[‖]	32±7[‖]	2±2
Ratio of Leu 2+ to Leu 3+ T cells	6.6±5.2[‖]	8.0±3.3[‖]	2.4±0.4

* % Ts were expressed as the % PBMC co-staining with anti-Leu 2-PE and anti-Leu 4-FITC.
‡ % Activated Th were calculated by determining the % PBMC co-staining with anti-Leu 3-PE and anti-HLA-DR FITC.
§ Values are expressed as mean±SEM.
‖ $P < 0.01$ when compared with normal controls.
¶ $P < 0.05$ compared with normal controls.

Fig. 1 summarizes the T cell subset distribution of PBMC from both treatment groups on day 1 and day 4 of the study. After 4 d of treatment, the IVGG plus ASA group underwent a greater increase in the percentage of Leu 4+ T cells, Leu 3+ Th, and Leu 2+ Ts (Fig. 1, *A–C*) than the ASA-treated group; these differences were not statistically significant ($P = 0.1$) for changes in Leu 4+ T cells and Leu 3+ Th. Patients treated with IVGG plus ASA did, however, have a significantly greater increase in their percentages of Leu 2+ Ts ($P < 0.01$) after the 4-d treatment period than did patients treated exclusively with ASA. The most dramatic change in T cell phenotype involved the percentage of circulating HLA-DR+ Leu 3+ Th before versus after treatment with IVGG plus ASA. Between the first and fourth treatment day, the percentage of activated HLA-DR+ Th in patients treated with IVGG plus ASA fell an average of 25.2%, as compared with a rise of 1.5% ($P < 0.001$) in children who received only aspirin (Fig. 1 *D*).

Effect of IVGG on the production of T cell–derived B cell helper factors. Activated Th from patients with acute KS have been previously demonstrated to produce soluble helper factors that induce B cells to secrete IgG (6). To determine whether the decrease in HLA-DR+ Th was associated with a functional change of helper cells, we examined the capacity of the patient's T cells to spontaneously release B cell helper factors on day 1 and day 4 of each treatment. Supernatants from 24-h cultures of freshly isolated unstimulated T cells derived from four patients in the ASA-treated group, five patients in the ASA plus IVGG–treated group and five normal controls were tested for their capacity to induce IgG production in cultures of purified B cells from a single normal donor. Fig. 2 shows that T cell supernatants derived from the KS patients before treatment induced significantly more ($P < 0.01$) IgG synthesis than supernatants of normal T cells. There was no significant difference between either treatment group in the capacity of their T cell supernatants to induce IgG production on day 1 of the protocol. After 4 d of treatment with IVGG plus ASA, T cell supernatants from the five KS patients under this regimen had a marked reduction in their ability to induce IgG production in the same B cell donor. Between day 1 and day 4 of the study, the decline in capacity of T cells to secrete B cell helper factors was significantly greater in the IVGG plus ASA–treated group than in the ASA-treated group ($P < 0.001$).

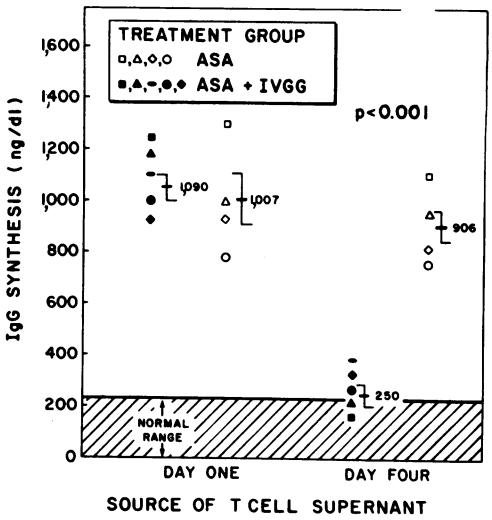

Figure 2. Effect of intravenous gammaglobulin plus aspirin vs ASA treatment alone on the generation of B cell helper factors by T cells. Data points indicate the mean IgG production of triplicate cultures induced by T cell supernatants from individual KS patients. The cross-hatched area represent the mean±2 SD of IgG production induced by T cell supernatants from five normal donors. The mean±SEM is shown for each group. Between enrollment and protocol day 4, T cells from patients treated with IVGG + ASA had a significantly greater decrease in their capacity to secrete B cell helper factor ($P < 0.001$) than T cells from patients treated exclusively with ASA.

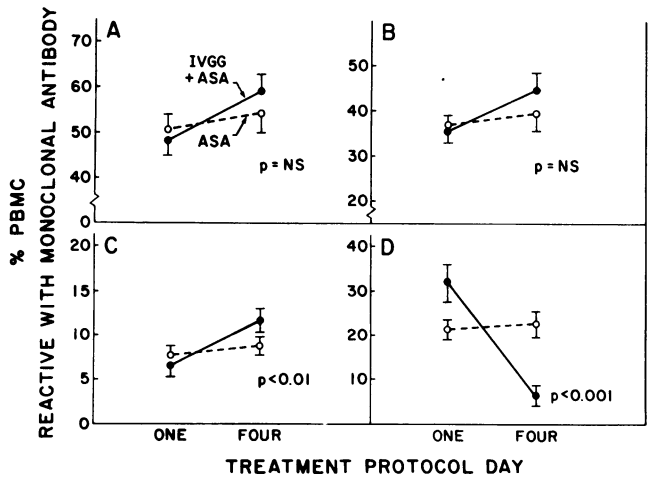

Figure 1. (*A*) Leu 4+ T cells; (*B*) Leu 3+ T cells; (*C*) Leu 2+ T cells; (*D*) DR+ Leu 3+ T cells. T cell populations in 14 KS patients treated with ASA and 15 KS patients treated with ASA plus intravenous gammaglobulin (ASA + IVGG). Values are expressed as mean±SEM. *P* values comparing the changes in monoclonal antibody binding between day 1 and day 4 of each treatment protocol are shown. Specificities of each monoclonal antibody are defined in Table I.

After treatment with ASA plus IVGG there was a highly significant decrease in spontaneous immunoglobulin synthesis as compared with the group receiving ASA treatment alone. Between enrollment and treatment day 4, PBMC from patients treated with ASA plus IVGG had a mean decrease in IgG synthesis of 853±142 ng/ml ($P < 0.001$), and a mean decrease in IgM synthesis of 554±56 ng/ml ($P < 0.001$). In contrast, after 4 d of treatment with ASA alone, PBMC secreted slightly higher levels of IgG (mean increase, 85±78 ng/ml) and IgM (mean increase, 28±24 ng/ml) than PBMC obtained at enrollment.

Effect of IVGG on spontaneous immunoglobulin synthesis. In this series of experiments, we examined the level of spontaneous IgG and IgM production by PBMC obtained on day 1 and day 4 of each treatment protocol. Before treatment, spontaneous IgG and IgM synthesis in acute KS patients was significantly elevated (Fig. 3). There was no significant difference between the pretreatment spontaneous IgG and IgM synthesis of the acute KS patients assigned to the ASA regimen versus those assigned to the ASA plus IVGG regimen.

Discussion

In the present study we found that high dose IVGG suppresses the marked activation of T and B cells found in acute KS. After four consecutive days of 400 mg/kg per d of IVGG plus ASA there was a significant decrease in the numbers 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

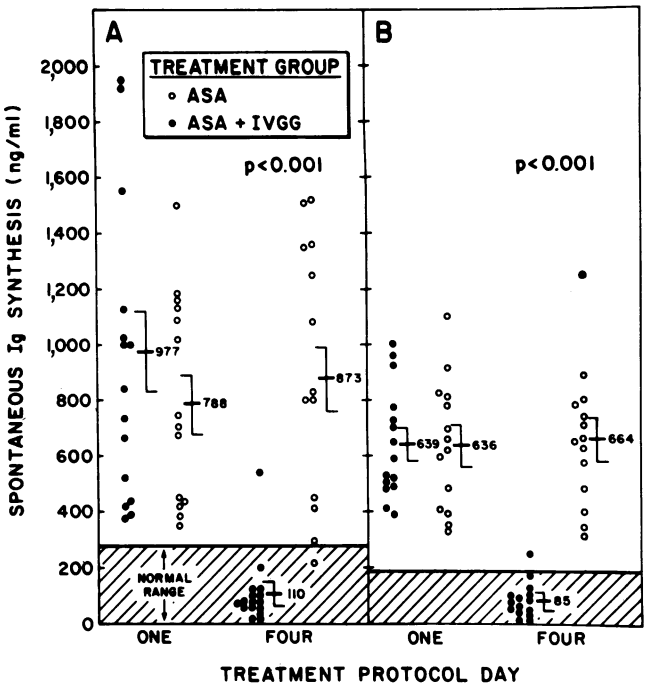

Figure 3. (*A*) IgG synthesis; (*B*) IgM synthesis. Effect of intravenous gammaglobulin plus ASA versus ASA alone on spontaneous IgG and IgM production. Values are expressed as the mean±SEM. *P* values comparing the decline in antibody production after treatment with 4 d of IVGG + ASA versus ASA alone are shown. Treatment with IVGG + ASA resulted in a significantly greater fall in Ig synthesis ($P < 0.001$) than treatment with ASA alone.

B cell helper factors, and a decrease in spontaneous IgG and IgM production by PBMC. In contrast, treatment with ASA for 4 d did not cause any significant changes in the abnormal T cell distribution or B cell activation found in active KS.

The current investigation also documented an absolute T lymphopenia during the first 10 d after onset of KS. The T lymphopenia was reflected in a loss of helper T cells and a disproportionately greater loss of suppressor/cytotoxic T cells. Infusion of high dose IVGG increased the total T cell numbers and helper T cell numbers to normal levels. Although the percent suppressor/cytotoxic T cells after infusion of IVGG increased significantly when compared with the ASA-treated group, they remained lower than those of age-matched controls.

The mechanisms by which IVGG reverses the immunoregulatory abnormalities in KS are unknown. Potential mechanisms include: (*a*) feedback inhibition of antibody-producing system; (*b*) increased antigen clearance of the infectious agent or toxin mediating KS; and (*c*) nonspecific immunologic blockade via the infusion of anti-HLA-DR antibodies in pooled IVGG or binding of intact IgG to Fc receptors of the lymphoid and/or reticuloendothelial cell system.

The optimal production of immunoglobulin requires a favorable balance between helper T cells and suppressor T cells. Increased numbers of suppressor T cells accompanied by the loss of activated helper T cells could account for the marked decrease in spontaneous IgG and IgM production by PBMC in acute KS after IVGG treatment. Durandy et al. (9) has reported that lymphocytes obtained from nonimmunodeficient children treated with intramuscular preparations of gammaglobulin have a reduced capacity to proliferate and to mature into IgG and IgM secreting plasma cells in vitro after stimulation with pokeweed mitogen. There was evidence for both activation of suppressor T cells and for direct inhibition of B lymphocytes by IgG aggregates. Small amounts of IgG aggregates present in the relatively high doses of infused IVGG and possibly aggregates formed between the infused IgG and circulating antigen may result in the activation of suppressor cells and inhibition of B cell function in KS patients. More recently Stohl has reported that monomeric IgG in commercially available preparations of IVGG can inhibit in vitro pokeweed mitogen–induced B cell differentiation (10).

The vasculitic process of KS that results in the development of coronary artery aneurysms is believed by many investigators to be immunologically mediated. This premise is supported by the following observations: (*a*) an increased incidence of HLA-Bw22 in Japanese children with KS (11) and of HLA-Bw51 in caucasian children (12); (*b*) the marked T cell imbalance and polyclonal B cell activation during acute KS (5, 6); (*c*) tissue biopsies of small and medium-sized arteries, including coronary arteries, reveal a panvasculitis and perivasculitis with marked mononuclear cell infiltration (13, 14). Some investigators have also reported immunoglobulin and fibrin deposits in the tunica media of coronary arteries in autopsy specimens of KS (15); and (*d*) circulating immune complexes have been detected in KS primarily between 2 and 4 weeks after the onset of fever corresponding to the time in the course of the disease during which arterial aneurysms are most likely to appear (16, 17).

Activated helper T cells such as those present in acute KS secrete high levels of gamma interferon (18, 19). Gamma interferon induces the in vitro synthesis and surface expression of major histocompatibility class I and class II antigens, as well as several other proteins on vascular endothelial cells (20–22). Groenewegen and co-workers (23) have recently demonstrated that the expression of major histocompatibility complex (MHC) class II antigen on vascular endothelial cells in vivo was dependent on the secretion of lymphokines from circulating T cells. We recently found that sera from patients with acute KS contained antibodies that cause complement-mediated lysis of gamma interferon–treated endothelial cells, but not of untreated endothelial cells (24). These findings suggest that T cell activation in acute KS could be a predisposing factor to vasculitis.

Based on the above observations, we postulate that IVGG may prevent the development of coronary artery aneurysms in KS by interrupting the immunologic cascade that results in blood vessel injury at several sites. First, by reducing the level of T cell activation and the secretion of lymphokines, IVGG would cause a reduction in the expression of new surface antigens on vascular endothelium. Second, by inhibiting antibody production, IVGG would inhibit the formation of antibodies to neoantigens on endothelial cells. Finally, by binding to Fc receptors of reticuloendothelial cells, IVGG would reduce the binding of immune complexes to such cells. These possibilities are currently being investigated in our laboratory.

Acknowledgments

The authors wish to thank Mrs. Nancy Hunt, Mr. Nizar Maksad, and Mr. Alan Flint for technical assistance, and Mrs. Adrienne B. Sisco for preparation of this manuscript. We thank the Immuno-AG Co. for supplying the intravenous gammaglobulin used in this study.

This paper was supported by U. S. Public Health Service grants AM-31925-01, AI-21163, AI-20373-01, HL-34545, and RR-02172, and by a grant from the National Foundation, March of Dimes. Dr. Leung is

the recipient of New Investigator Research Award 5R23 HL30082, and Dr. Geha is the recipient of Allergic Diseases Academic Award K07 AI-0440.

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