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Immunological studies on Kawasaki disease. I. Appearance of Hanganutziu–Deicher antibodies

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SUMMARY

Sera of patients with Kawasaki disease were studied for heterophile antibodies by means of enzyme immunoassay (EIA) with enzyme conjugated antisera to human IgM, IgG, IgA and IgE. Antibodies of IgM (43%), IgG (3%), IgA (11%) and IgE (49%) classes were demonstrated that combined with high molecular weight glycoprotein (HMWGP) of bovine red blood cells (BRBC) one of the antigenic preparations of the Hanganutziu–Deicher (H-D) heterophile system. Studies on sequential sera of the patients revealed that HMWGP antibodies of IgM and IgE classes began to appear in the second week, reached their peaks in the third week of the disease and declined gradually thereafter. Absorption studies on the positive sera showed that the HMWGP antibody activities were abolished by BRBC, sheep red blood cells and guinea-pig kidney tissues, confirming H-D specificity of these antibodies. EIA inhibition studies showed that the antibody activity was inhibited by HMWGP and partially by asialo-HMWGP and NGNA ganglioside rich preparation of BRBC but not by purified Paul–Bunnell or Forssman antigens. These results indicate that the H-D antibodies under investigation consist of antibodies of two different specificities; one directed against asialo-HMWGP and the other NGNA ganglioside of BRBC. Circulating immune complexes (IC) were demonstrated in 23% of the patients by means of anti-antibody inhibition test. Evidence was presented that IC in the sera of five patients were composed of H-D (HMWGP) antigen and its corresponding antibodies.

Keywords Kawasaki disease H-D antibody immune complexes

INTRODUCTION

Since the first report on Kawasaki disease (Kawasaki, 1967) or acute febrile mucocutaneous lymph node syndrome, many immunological studies have been carried out (see the review by Yanagihara & Todd, 1980). Elevated serum levels of IgM, IgG, IgA and IgE have been described (Lauer *et al.*, 1976; Bergeson & Schoenike, 1977; Siegel & Wenner, 1976; Goldsmith, Gribetz & Straus, 1976; Kusakawa & Heiner, 1976). Recently the polyclonal B cell activation seen at the acute phase of the disease was shown to be due to abnormalities of regulatory T cell subsets; increase in numbers of helper T cells and decrease in activities of suppressor T cells (Geha, Rosen & Merler, 1973; Leung *et al.*, 1983). Circulating immune complexes (IC) (Corbeel *et al.*, 1977; Yata, Nakagawa & Sawa, 1977;

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Milgrom *et al.*, 1980) as well as deposition of IgG in walls of coronary arteries or in cardiac myofibriles were also reported (Hamashima, Tasaka & Hirose, 1976).

In the present study sequential sera of patients with Kawasaki disease were studied for the presence of heterophile antibodies especially those of Hanganutziu–Deicher (H-D) specificity, since H-D antibodies have been demonstrated in various diseases including those of unknown aetiology (Kasukawa *et al.*, 1976; Nishimaki, Kano & Milgrom, 1979; Kano *et al.*, 1981; Morito, Kano & Milgrom, 1982; Tamura *et al.*, 1983, see also review, Kano & Milgrom, 1977). Attempts were also made to demonstrate circulating IC by means of anti-antibody inhibition test (Kano *et al.*, 1978; Kano, Nishimaki & Milgrom, 1979; Milgrom & Kano, 1978, see also review, Kano & Milgrom, 1980) and to identify the antigen(s) of the IC by dissociation experiments using purified soluble antigens of known specificities (Nishimaki *et al.*, 1978; Wozniczko-Orlowska & Milgrom, 1981).

MATERIALS AND METHODS

Human sera. Sera of 56 patients with Kawasaki disease, 6 months to 3 years of age, were obtained at the Pediatric Clinics of the Oofuna Kyosai Hospital, the Kanagawa Medical Center for Children, Kanagawa, Japan, and the Second Hospital of the Tokyo Women's Medical College, Tokyo, Japan. The diagnosis was made on the basis of characteristic clinical manifestations of the disease (Kawasaki, 1967; Yanagihara & Todd, 1980) and laboratory findings of leukocytosis with nuclear left-ward shift, increased erythrocyte sedimentation rate and positive CRP test (Yanagihara & Todd, 1980). Majority of the sera were obtained between the second and the fourth week of the illness (stage II). Sequential sera were also obtained weekly from some patients, between the first week and the sixth month after the onset of the disease. Age matched control sera were obtained from 35 healthy children who visited the Hospitals for vaccination, or yearly physical examination.

Sera of patients with infectious mononucleosis (IM) and various other diseases were from the collections at the Department of Microbiology, State University of New York at Buffalo, Buffalo, New York, USA. These sera shown to contain heterophile antibodies of Paul–Bunnell (P-B), and/or H-D specificity in the previous studies (Kasukawa *et al.*, 1976; Nishimaki *et al.*, 1979; Kano *et al.*, 1981; Morito *et al.*, 1982; Tamura *et al.*, 1983) were used as positive controls throughout this study. The sera were stored at $-20°C$ and were heat-inactivated at 56°C for 30 min before use.

Heterophile antigens. Purified P-B antigens and H-D antigens (high molecular weight glycoprotein [HMWGP] and a fraction rich in NGNA ganglioside) were prepared from bovine red blood cell (BRBC) stromata (Merrick *et al.*, 1977; Merrick, Zadarlik & Milgrom, 1978; Higashi *et al.*, 1977; Nowak, Jain & Merrick, 1982). Forssman antigen was obtained from guinea-pig kidney (GPK) tissues (Laine *et al.*, 1974). All these antigens were kindly supplied by Dr J. M. Merrick of the Department of Microbiology, State University of New York at Buffalo.

Haemagglutination tests. The tests with trypsinized BRBC and sheep red blood cells (SRBC) were performed according to our routine procedures used in our previous studies (Kasukawa *et al.*, 1976; Nishimaki *et al.*, 1979; Kano *et al.*, 1981; Morito *et al.*, 1982; Tamura *et al.*, 1983).

Enzyme immunoassay (EIA). EIA with H-D (HMWGP) and P-B antigens were performed following the procedure established previously (Morito *et al.*, 1982; Tamura *et al.*, 1983; Palosuo & Milgrom, 1981). Briefly, a solution of HMWGP at a concentration of 5 μg/ml or a solution of P-B antigen at a concentration of 1 μg/ml were prepared in 0·05 M carbonate–bicarbonate buffer, pH 9·6. To wells of microELISA plates (Terumo, Inc., Tokyo, Japan) was added 200 μl of the antigen solution and the plates were incubated at 4°C for 16 h. The antigen coated wells were washed three times with phosphate-buffered saline, pH 7·2 containing 0·05% Tween 20 (PBS-Tween) and then dilutions of tested serum were added to the wells. The plates were incubated at 20°C for 2 h and the wells were washed three times with PBS-Tween. Thereafter 200 μl of alkaline phosphatase conjugated goat anti-human IgM, IgG, IgA or IgE (Tago Inc., Burlingame, California, USA) at predetermined optimal dilutions (Morito *et al.*, 1982; Tamura *et al.*, 1983) were added to the wells and the plates were incubated at 20°C for 2 h. After the wells were washed three times with PBS-Tween, 200 μl of a 1 mg/ml solution of *p*-nitrophenyl phosphate disodium (Sigma Chemical Co., St Louis, Missouri, USA) were added to each well, the plates were incubated at 37°C for 1 h and

the enzyme reaction was stopped by adding 150 μl of 1·0 N NaOH. The intensity of colour was estimated by a spectrophotometer (Beckman Inc., Anaheim, California) at 405 nm. All tests were performed in duplicate and mean OD value was calculated for each specimen.

EIA inhibition test. Patients' sera at 1:50 or 1:100 dilutions giving OD values of 0·5–1·0 against HMWGP with the anti-IgM, anti-IgA and anti-IgE conjugates were selected. To the diluted serum was added an equal volume of an H-D antigen preparation: purified HMWGP or HMWGP treated with neuraminidase (Nowak *et al.*, 1982) or a fraction rich in NGNA ganglioside, all prepared from BRBC (Merrick *et al.*, 1978; Nowak *et al.*, 1982) or otherwise an equal volume of P-B or F antigen. All these antigens were added at various concentrations; the mixtures were incubated at 4°C for 16 h and then 200 μl of each mixture were transferred to the HMWGP coated wells and EIA was performed as described above. The decrease in OD values of the sera was expressed as % inhibition following the previously established formula (Morito *et al.*, 1982; Tamura *et al.*, 1983; Palosuo & Milgrom, 1981).

Absorption of pathologic sera. Selected sera were subjected to absorption experiments according to the routine procedure employed previously (Kasukawa *et al.*, 1976; Nishimaki *et al.*, 1979; Kano *et al.*, 1981; Morito *et al.*, 1982; Tamura *et al.*, 1983); they were absorbed three times with sediments of homogenized GPK tissues, homogenized normal human kidney tissues, SRBC or BRBC and tested by EIA with HMWGP and/or agglutination test with trypsinized BRBC.

Antiantibody (AA) inhibition test. This test was performed following the procedure established previously (Kano *et al.*, 1978) and employed in the previous studies (Kano *et al.*, 1978, 1979; Milgrom & Kano, 1978). AA is a serum factor resembling rheumatoid factor, but showing much higher specificity of reaction with IC than the rheumatoid factor; the bibliography on this topic was reviewed by Kano *et al.*, 1978; Milgrom & Kano, 1978. Briefly, 50 μl of tested serum were mixed with an equal volume of an AA containing serum at a dilution corresponding to 4 agglutinating units and incubated at 20°C for 1 h. To each mixture 50 μl of a 1% suspension of human group O Rh positive erythrocytes sensitized by an anti-CD serum Ripley (Kano *et al.*, 1978, 1979; Milgrom & Kano, 1978) were added and the tubes were incubated at 20°C for 1 h. Thereafter the tubes were centrifuged at 200 r/min for 2 min and agglutination was examined macroscopically after shaking the tubes gently.

Dissociation of IC by soluble antigens. This procedure has been employed to identify the antigens of IC of various pathological sera in the previous studies (Nishimaki *et al.*, 1978; Wozniczko-Orlowska & Milgrom, 1981). For this test sera with AA inhibition titres of 8 or more were selected. To each serum at various dilutions were added equal volumes of purified heterophile antigens at 0·5 mg/ml concentration. For controls, solutions of bovine and human γ globulins at the same or higher concentrations were included. The mixtures were incubated at 37°C for 3 h. Thereafter AA inhibition tests were performed on these specimens as outlined above.

RESULTS

Sera of 56 patients with Kawasaki disease, which were obtained 2–4 weeks after the onset of the disease along with sera of age matched control subjects were at first examined by agglutination tests with trypsinized BRBC and SRBC. A significant number of the patients' sera gave BRBC agglutinin titres of 160–1,280 which were higher than those of the controls. Preliminary experiments on the positive sera showed that antibody activity of the patients' sera against trypsinized BRBC was abolished by absorption with SRBC, BRBC and GPK, indicating H-D nature of the antibodies (see the review, Kano & Milgrom, 1977).

The same 56 Kawasaki disease sera were then studied by EIA against H-D (HMWGP) and P-B antigens using enzyme conjugated antisera to human IgM, IgG, IgA or IgE. For these experiments 1:100 dilutions of the sera were employed for IgM, IgG and IgA and 1:50 dilutions for IgE antibodies. It was found that mean OD value of the patients' sera using the anti-IgM conjugate was $0{\cdot}507 \pm 0{\cdot}457$ and using the anti-IgE conjugate it was $0{\cdot}233 \pm 0{\cdot}332$. These values were significantly higher than those of the controls, $0{\cdot}271 \pm 0{\cdot}201$ and $0{\cdot}011 \pm 0{\cdot}027$, respectively. The mean value with the anti-IgA conjugate was slightly higher for Kawasaki disease than that for the control sera and

Table 1. Enzyme immunoassay with HMWGP* and sera† of 35 patients with Kawasaki disease

	Mean OD at 405 nm ± sd values	
	Anti-IgM conjugate	Anti-IgE conjugate
Sera obtained at		
1st week	0·339 ± 0·220	0·026 ± 0·071
2nd week	0·507 ± 0·459	0·164 ± 0·477
3rd week	0·579 ± 0·491	0·292 ± 0·601
4th week	0·420 ± 0·281	0·233 ± 0·332
5th week	0·425 ± 0·291	0·155 ± 0·243
Normal control sera	0·271 ± 0·201	0·011 ± 0·027

* High molecular weight glycoprotein of bovine erythrocytes.
† Serum dilutions of 1:100 for IgM and 1:50 for IgE were used.

the values with the anti-IgG conjugate did not differ between pathological and control sera. Patients' sera that had OD values against HMWGP exceeding the mean values of the controls by 2 s.d. were tentatively considered positive. The incidence of such cases at the height of the disease (2–4 weeks) was 43% for IgM, 3% for IgG, 11% for IgA and 49% for IgE antibodies. OD values of the patients' sera against P-B antigen using the anti-IgM and anti-IgE conjugates did not differ significantly from those of the controls.

Subsequently sequential sera obtained at weekly intervals from 35 patients were studied by EIA with HMWGP for IgM and IgE antibodies. As shown in Table 1, mean OD values for both classes began to increase in the second week of the illness, reached their maximum in the third week and decreased gradually thereafter. In inspecting the HMWGP antibody responses of individual patients it was noted that 12 patients had HMWGP antibodies of both IgM and IgE classes, three had IgM antibodies alone, the other five patients had IgE antibodies alone and the remaining had neither of them.

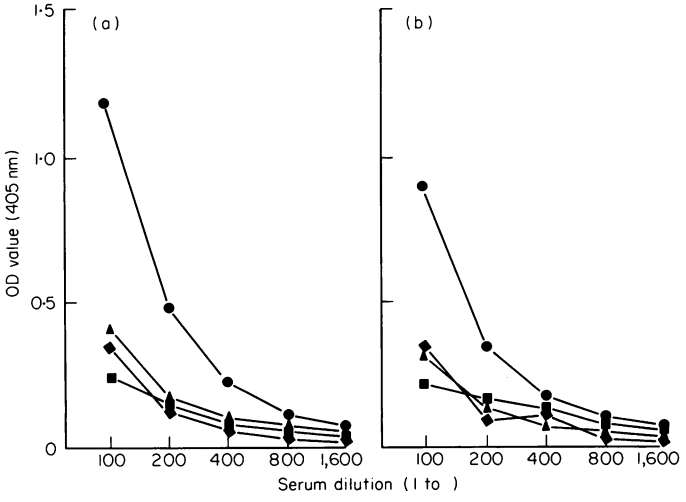

Fig. 1. Enzyme immunoassay with HMWGP and two Kawasaki disease sera (aK73 and b=K34) using anti-IgM conjugate. OD values of unabsorbed sera (●——●) and sera absorbed with SRBC (▲——▲), BRBC (♦——♦) and GPK (■——■).

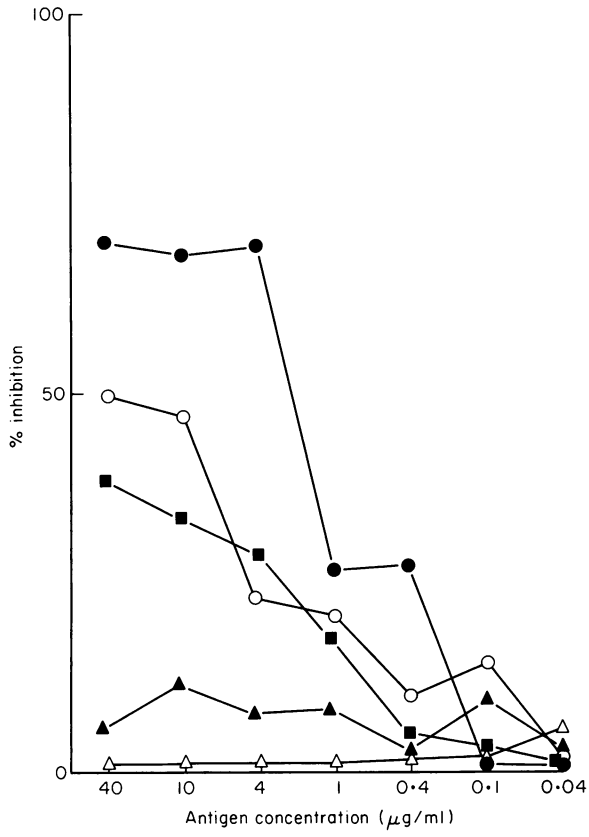

Fig. 2. Enzyme immunoassay with HMWGP and Kawasaki disease serum using anti-IgM conjugate. Inhibition of reaction by HMWGP (●——●), neuraminidase treated HMWGP (○——○), NGNA ganglioside (■——■), P-B antigen (▲——▲) and F antigen (△——△).

Specificity of the HMWGP antibodies under investigation was at first studied by absorption experiments in EIA with the anti-IgM conjugate. Representative results of these experiments are shown in Fig. 1. As seen in this figure, absorption of two sera with SRBC, BRBC and GPK tissues resulted in significant reduction in the OD values against HMWGP; from 1·3 to less than 0·4 in the patient K73 and from 0·9 to less than 0·35 in the patient K34. There were no significant reductions when these sera were absorbed with human erythrocytes or apparently normal human kidney tissues (the results of these control experiments are not shown in the figure).

EIA inhibition tests were performed on selected sera with HMWGP antibodies of IgM, IgA and IgE classes. As shown in Fig. 2, HMWGP at a concentration of 40 μg/ml produced 70% inhibition of the IgM antibodies, whereas inhibition by the P-B or the F antigen at the same concentration was less than 10%. Neuraminidase treated HMWGP showed 50% inhibition and a fraction rich in NGNA ganglioside of BRBC stromata 38% inhibition. Similar results were obtained in inhibition studies on IgA and IgE antibodies.

Subsequently sequential sera of the 35 patients were studied by the AA inhibition test for the presence of circulating IC. Sera of eight patients (23%) gave positive results: AA inhibition titres of the positive sera varied from 4 to 64. IC were detectable in sera of six patients in 3–6 weeks after the onset of the disease. Serum of one patient was positive already in the second week of the disease and serum of one other patient became positive as late as 6 months after the onset of the disease.

In comparing AA inhibition titres and H-D antibody activities of the sequential sera, inverse relationships between titres of the IC on the one hand and the IgM and IgE H-D antibody activities

Table 2. Dissociation of IC in Kawasaki disease serum K128 by various soluble antigens

	Inhibition of AA by serum K128 in the presence of						
	heterophile antigens (0·5 mg/ml)*					γ globulins (5 mg/ml)	
Dilution of serum (1 to)	Saline	HMWGP	NGNA	P-B	F	Bovine	Human
4	−	++	−	−	−	−	−
8	−	++	−	−	−	−	−
16	−	++	−	−	−	−	−
32	−	++	−	−	−	−	−
64	−	++	+	−	−	−	−
128	++	++	++	++	++	++	++

* HMWGP = High molecular weight glycoprotein of bovine erythrocytes; NGNA = NGNA ganglioside rich fraction of bovine erythrocytes; P-B = Paul–Bunnell antigen of bovine erythrocytes; F = Forssman antigen of guinea-pig tissues.

on the other hand were noted. This was rather surprising since AA detects exclusively IC formed by IgG antibodies (Kano *et al.*, 1978, 1979; Milgrom & Kano, 1978; Kano & Milgrom, 1980) and only a few Kawasaki disease sera contained H-D antibodies of IgG class. Therefore, we did not anticipate that IC found by us would be composed of H-D antibodies and their corresponding antigens. It was, however, observed in dissociation experiments with purified heterophile antigens that some IC positive sera lost their AA inhibitory activities upon incubation with HMWGP. Representative results of such experiments are shown in Table 2. As seen in the table, the patient's serum lost its AA inhibitory activity upon incubation with the HMWGP at a concentration of 0·5 mg/ml whereas NGNA ganglioside, P-B and F antigens at the same concentration did not affect its activity. Of seven other AA inhibitory sera examined four lost their inhibitory activity by preincubation with HMWGP at the same concentration, whereas incubation with NGNA ganglioside, P-B and F antigens at the same concentrations did not affect their activity. Sera of the remaining three patients were not affected by incubation with any of these antigens. For additional controls, SLE sera and IC prepared by tetanus toxoid and human anti-toxoid antibodies were included: The HMWGP as well as other antigens did not affect AA inhibitory activity of these specimens. These results indicated that the IC of the 'dissociation positive' sera was probably composed of the H-D antigen and its corresponding antibodies of the IgG class.

DISCUSSION

The aetiology of Kawasaki disease is still uncertain in spite of the extensive investigation conducted for the past decade (Yanagihara & Todd, 1980). The most serious features of the disease are the pathological changes in the cardiovascular system such as panvasculitis, aneurysma and occlusion of coronary arteries resulting in a rather high mortality (Yanagihara & Todd, 1980). As mentioned in the Introduction, the polyclonal B cell activation observed at the acute phase of the disease (Lauer *et al.*, 1976; Bergeson & Schoenike, 1977; Siegel & Wenner, 1976; Goldsmith *et al.*, 1976; Kusakawa & Heiner, 1976; Geha *et al.*, 1973; Leung *et al.*, 1983) may contribute to the formation of circulating IC and, consequently, to the deposition of IC in capillary walls (Hamashima *et al.*, 1976).

Human heterophile antibodies, especially those of H-D group (see the review, Kano & Milgrom, 1977) have been demonstrated in a variety of diseases including systemic lupus erythematosus, rheumatoid arthritis, Chediak–Higashi syndrome, various infections as well as some malignancies (Kasukawa *et al.*, 1976; Nishimaki *et al.*, 1979; Kano *et al.*, 1981; Morito *et al.*, 1982; Tamura *et al.*, 1983). In the present study, H-D antibodies of IgM and IgE classes were demonstrated in sera of

patients at the height of the Kawasaki disease. Studies on the specificity of these antibodies suggested that the antibodies detectable by EIA with HMWGP were directed against two distinct antigens; asialo-HMWGP and NGNA ganglioside, since addition to the positive sera of either one of these antigens resulted in 40–50% inhibition of their antibody activities. The fact that these antibodies were absorbable by GPK tissues puts them into the 'GPK positive' group of human heterophile antibodies (Kano, Merrick & Milgrom, 1983)

The definite explanation for the mode of formation of the heterophile antibodies in the Kawasaki disease cannot be offered at the present time. However, similar to the postulated explanation for formation of heterophile antibodies in other diseases (Kasukawa *et al.*, 1976; Nishimaki *et al.*, 1979; Kano *et al.*, 1981; Morito *et al.*, 1982; Tamura *et al.*, 1983), it may be proposed that H-D antigenic determinants might have been produced due to the alteration of self components as a result of the morbid processes in the Kawasaki disease.

In agreement with the studies by other investigators (Corbeel *et al.*, 1977; Yata *et al.*, 1977; Milgrom *et al.*, 1980), IC were demonstrated in the present study in the sera of patients (23%), mostly at later stages of the Kawasaki disease by the AA inhibition test. The dissociation of IC by soluble antigens has been successfully employed to identify the antigens of circulating IC (Nishimaki *et al.*, 1978; Wozniczko-Orlowska & Milgrom, 1981) as well as IC deposited in pathological tissues (Penner *et al.*, 1982; Sugisaki *et al.*, 1982). As mentioned in the Results, the conversion of the AA inhibiting sera into noninhibiting sera by addition of H-D (HMWGP) antigen was not expected in view of the fact that only a few sera tested had H-D antibodies of IgG class and AA detects only IC formed by IgG antibodies (Kano *et al.*, 1978, 1979; Milgrom & Kano, 1978; see also the review, Kano & Milgrom, 1980). Still the presented results brought evidence that the circulating IC demonstrated in the sera of some patients are formed by the H-D antigen and its corresponding antibodies of the IgG class. It would appear, therefore, that the IgG antibodies appear in the patients's circulation more frequently in the form of an IC than in a free state.

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