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Detection of Serum Antibody by the Antimitogen Assay against Streptococcal Erythrogenic Toxins. Age Distribution in Children and the Relation to Kawasaki Disease

YOSHIAKI ABE, SHOKO NAKANO, TAKAKO NAKAHARA, YUKIO KAMEZAWA, IWAO KATO, HIROHARU USHIJIMA, KAZUYA YOSHINO, SHIGERU ITO, SEIJI NOMA, SHOKO OKITSU, AND MASAKO TAJIMA

Departments of Pathology [Y.A.,S.N.], Pediatrics [H. U., K.Y., S I., S.N., S.O.], and Central Clinical Laboratory [M.T.], Teikyo University School of Medicine, Tokyo Department of Microbiology [T.N., Y.K.], Saitama College of Health, Urawa; and Department of Microbiology [I.K.], Faculty of Medicine, Chiba University, Chiba, Japan

ABSTRACT. We describe a new method to measure human serum antibody against streptococcal erythrogenic toxins that uses inhibition of lymphocyte mitogenicity of the toxins as the indicator. Sera from 53% of 53 Kawasaki disease patients contained specific inhibitory activity against A toxin, whereas only 15% had serum inhibitory activity against B toxin. The specific anti-A toxin serum inhibitor was found in 10% of 118 age-matched control patients suffering from various infections and allergic diseases ($p = 0.001$, compared to Kawasaki disease patients). Serum inhibitory activity was detected in a small number of patients with β-hemolytic streptococcal infection (3/19) and in none of the age-matched healthy children (0/17). However, four of seven cord blood sera samples and five of 13 sera samples from healthy neonates contained the inhibitor, a result suggesting passive transfer from mothers. Most of the antimitogen-positive sera were also positive by ELISA of IgG antibody against A toxin, and IgG fractions of the positive sera remained positive in both assays. Thus, it is possible that the specific serum inhibitor detected by the antimitogen assay represents anti-A toxin antibody. The role of toxin-producing bacteria in the pathogenesis of Kawasaki disease remains to be investigated. (*Pediatr Res* 27: 11–15, 1990)

Abbreviations

KD, Kawasaki disease
SET, streptococcal erythrogenic toxins
TdR, thymidine
Con A, concanavalin A
AMA, antimotogen assay
OD, outer diamer

KD, or mucocutaneous lymph node syndrome, is an acute febrile infantile disease that has become known as a new clinical entity since Kawasaki's first article was published in Japanese about 20 y ago (1). Kawasaki *et al.* (2) later gave an outline of clinical and epidemiologic aspects of the disease in English (2). KD has not only been extensively encountered in Japan, but has been reported also in Korea, the United States, Europe, and Australia (3–7). Pathology of the disease is characterized by systemic vasculitis simulating or identical to infantile periarteritis nodosa (8–13). Coronary arteries are most severely affected. Rare but sudden death is caused by myocardial infarction due to coronary thrombosis or by rupture of a coronary aneurysm. The etiology remains unknown, although the involvement of an infectious agent is suspected on clinical and epidemiologic grounds. Many attempts have been made to find the causative microorganism among viruses, bacteria, spirochetes, and fungi, but no definite evidence has been obtained (14). Suspicion that KD may be a toxin-related disease comes from several reports indicating similarities in symptoms between toxic shock syndrome and KD (15–18). Similarities to scarlet fever have been noted in skin and oral eruptions of KD, but KD affects much younger children than scarlet fever, and isolation of β-hemolytic streptococci is infrequent in patients with KD (2). However, some symptoms of KD are similar to those known to result from SET, which play a pathogenetic role in scarlet fever. These biologic properties include pyrogenicity, erythrogenic activity, and lymphocyte mitogenicity (19, 20). In our report, we describe a new method to measure the anti-SET antibody by using the lymphocyte mitogenicity of the toxins as the indicator. We have found a significantly higher frequency of serum inhibitory activity against erythrogenic toxin A mitogenicity in KD patients when compared with age-matched control children. The antitoxin inhibitory activity detected in sera of KD patients suggests the possibility that toxin-producing bacteria may contribute to pathogenesis of this disease.

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Correspondence Yoshiaki Abe, M.D., Department of Pathology, Teikyo University School of Medicine, Kaga 2-11-1, Itabashi, Tokyo 173, Japan.
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MATERIALS AND METHODS

R9eagents. 2-2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), BSA, carbonyl iron powder, and N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid were purchased from Sigma Chemical Co., St. Louis, MO.^{3}H-TdR, sp act 78 Ci/mM, was purchased from Amersham Japan Co., Tokyo, Japan. Other chemicals and reagents were Dulbecco's modified Eagle medium (GIBCO, Grand Island, NY), Vectastain ABC kit (Vector Laboratories, through Funakoshi Pharmaceutical Co., Tokyo, Japan), goat antihuman IgG serum (Tago Inc., Burlingame, CA), Con A, Miles-Yeda Ltd., Rehovot, Israel), protein A Sepharose (Pharmacia Japan Co., Tokyo, Japan), Tween 20 (Wako Pure Chemical Industries, Tokyo, Japan), polyvinyl alcohol (polymerization degree 2000, Koso Chemical Co. Tokyo, Japan), penicillin and streptomycin (Meiji, Tokyo, Japan). PBS, pH 7.2, 0.15

M, carbonate buffer, pH 9.6, 0.05 M, citrate buffer, pH 4.0, 0.1 M, and glycine-HCl buffer, pH 3.0, 0.1 M, were prepared as described (21)

SET preparations. Purified erythrogenic toxins A and B were obtained from culture supernatant of NY5 strain *Streptococcus pyogenes* from the stock of the Institute of Medical Science, the University of Tokyo, Tokyo, Japan. Ammonium sulfate precipitate at 3/4 saturation was used as crude toxin. Further purification was carried out as described (22). Physicochemical and biologic characteristics of the purified A toxin are briefly as follows. It produced a single band on SDS-PAGE, showing M_r 28 000, and had isoelectric points of 5.0 (major) and 5.2 (minor). HPLC revealed one sharp symmetric peak, showing a high degree of homogeneity. The result of amino acid analysis of the toxin was in accordance with Gerlach *et al.* (23) and with Weeks and Ferretti (24). An antiserum raised in a rabbit immunized with Freund's complete adjuvant against the purified A toxin produced a single precipitation line in gel diffusion, specifically abolished erythematous skin reaction, and inhibited the lymphocyte mitogenicity in a dose-dependent fashion. B toxin had a isoelectric point of 7.9. Specific antisera to inhibit biologic activities of the B toxin have not been obtained. The isoelectric points of the A and B toxins are similar to those of streptococcal pyrogenic exotoxins A and B, respectively (25, 26). We use the term erythrogenic toxin because we have routinely assayed biologic activity of the toxins by skin reaction in rabbits. Erythrogenic and mitogenic activities of the toxins have not until now been separable. Doses of the toxin preparations were shown in terms of protein concentration.

Animals. Adult JW-NIBS strain rabbits (The Nippon Institute for Biological Sciences, Tokyo, Japan) of either sex were used.

Rabbit lymphocyte culture. A modification of the previous methods (27, 28) was used. Peripheral blood from ear artery was defibrinated by glass beads in a flask, mixed with an equal volume of 1% polyvinyl alcohol in PBS containing carbonyl iron powder (20 mg/mL) after separation of serum, and incubated at 37°C for 30 min in a water bath. Leukocyte-rich supernatant was passed once through a magnet when necessary to remove neutrophils that had phagocytized iron. Purity of the lymphocytes was 85% or more, and the yield was approximately 1 x 10^6/per mL of blood. Lymphocytes thus obtained were washed three times in PBS, suspended at the cell density of 1 x 10^6/mL in Dulbecco's modified Eagle medium containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, penicillin (100 U/mL), streptomycin (100 μg/mL), and 20% inactivated autologous or homologous serum, distributed in 0.2 mL vol in each well of 96-well plastic plate, and cultured for 4 d in a CO_2 incubator. Mitogens were added at the start of culture. The purified A toxin was used at the concentration of 0.1 ng/mL of culture, B toxin and crude toxin at 1.0 ng/mL, and Con A at 5 μg/mL. ^{3}H-TdR was added at the concentration of 1 μCi/mL for the last 17 h-period of culture. Cells were harvested on Whatman GF/A filter after culture (Whatman Inc., Clifton, NJ). Radioactivity was determined by a liquid scintillation counter. Assays were done in triplicate or quadriplicate.

AMA. A total of 10 μL of 20-fold dilutions with PBS of human sera was added in each well at the start of culture. Percent inhibition of the mitogenic activity was calculated by ^{3}H -TdR incorporation as follows:

$$\left(1 - \frac{\text{cpm}_{\text{mitogen + test serum}} - \text{cpm}_{\text{no mitogen}}}{\text{cpm}_{\text{mitogen}} - \text{cpm}_{\text{no mitogen}}}\right) \times 100$$

Inhibition was assessed as positive when the difference of cpm between cultures containing both mitogen and test serum and those containing mitogen alone was statistically significant by *t* test in two or more separate experiments. The minimum significant inhibition was 13%.

ELISA. The purified A toxin preparation used as the antigen was diluted in carbonate buffer at the concentration of 2 μg/mL, distributed in 0.1 mL vol in each well of 96-well plastic plate, sealed and kept at 4°C overnight. Following washing, procedure was carried out three times at the end of each step by PBS containing 0.05% Tween 20. BSA in PBS (1 mg/mL) for recoating was distributed in 0.1 mL vol in each well and placed at room temperature for 15 min. Twenty μL of 20-fold dilutions of human sera, or fractions separated by protein A Sepharose column, were then added, and incubated for another 30 min. After washing, BSA in PBS was refilled, incubated for 15 min, and 10 μL of a goat antihuman IgG serum were added for another 30 min. The antiserum was used at 1000-fold dilution in PBS. Thereafter, each one drop (approximately 50 μL) of the reagents included in Vectastain ABC kit was successively distributed and incubated as above. They were normal rabbit serum, biotin-labeled anti-goat IgG rabbit IgG, avidin-biotin complex, and biotin-conjugated peroxidase. Finally, 0.1 mL of the substrate solution containing 0.04% H_2O_2 and 0.2 mM 2–2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) dissolved in the 10-fold diluted citrate buffer was distributed and incubated at room temperature for 30 min or more. The OD was measured at 405 nm. Wells without addition of human serum and the goat antihuman IgG serum served as blanks. Each test was carried out in duplicate. Mean OD of 0.20 or more was taken as positive. The cut-off value was determined by preliminary experiments using serial dilutions of high-titered human sera.

Fractionation of human sera by protein A Sepharose column. A total of 50 to 300 μL of human serum or Ig fraction prepared by ammmonium sulfate precipitation at 50% saturation was diluted to 1 mL with PBS, applied to 2.5 mL of swollen protein A Sepharose packed in a 5-mL syringe, and passed through the column by addition of PBS under monitoring by UV absorption. IgG was eluted by glycine-HCl buffer. Both the effluent and the eluate fractions were concentrated by use of PM10 filter (Amicon, Lexington, MA), diluted with PBS when necessary, and brought to the same volume to 20-fold dilution of the starting specimen of serum. One serum specimen in the amount of 50 μl was diluted to 46-fold volume. The IgG binding capacity of the swollen gel, 25 mg/mL, was found to be sufficient to bind total amount of anti-SET IgG antibody because no positive results of ELISA were seen in the effluent fractions.

Human serum specimens. The following serum specimens were tested: 169 specimens from 53 patients with KD hospitalized in Teikyo University Hospital and Tokyo Kosei-Nenkin Hospital between March 1984 and February 1987, 214 specimens from 160 hospitalized patients and outpatients with various infectious and allergic diseases in the above two hospitals during the same period, 23 specimens from 19 patients with β-hemolytic streptococcal infection, all confirmed by positive bacterial culture, in Tokyo Kosei-Nenkin Hospital and Oji Co-op Hospital, and 39 specimens from 24 healthy children from several institutions including the aforementioned hospitals. All the hospitals are located in the Tokyo metropolitan area, and the patients came from this area or from the suburbs. No patient was included in this study who had received intravenous human γ-globulin infusion.

RESULTS

AMA and ELISA in KD patients. Table 1 shows a representative experiment of AMA of sera from four patients with KD. All the sera tested inhibited the mitogenicity of A toxin but not those of B toxin and Con A. A slight degree of enhancement may be noteworthy in the response to B toxin and to Con A. Such slight and variable enhancement was occasionally seen in sera with negative AMA throughout the experiments. Weaker inhibition of crude toxin mitogenicity was seen in each of the four specimens when compared with that of A toxin. Among a total of 169 serum specimens tested from KD patients, 52 were considered positive in AMA against A toxin, *i.e.* ≥13% inhibition. The

Table 1. *Inhibition of A toxin mitogenicity by Kawasaki disease patient's sera*

	cpm (mean ± SD)*				
	A toxin, pI 5.0†	A toxin, pI 5.2	Crude toxin	B toxin	Con A
Mitogen stimulated	17564 ± 1359	21973 ± 4087	21593 ± 5962	7309 ± 1067	35760 ± 8098
+ patient's serum,‡ I.D.	6143 ± 1235 (66)§	7842 ± 3508 (65)	14750 ± 731 (32)	10668 ± 4137	39412 ± 699
U.S.	1443 ± 314 (93)	3129 ± 364 (87)	6675 ± 1940 (70)	10176 ± 2943	39257 ± 956
S.M.	7572 ± 873 (58)	11715 ± 3343 (48)	15900 ± 3133 (27)	12787 ± 3734	39091 ± 1504
T.M.	2878 ± 164 (85)	2776 ± 416 (89)	10861 ± 1785 (51)	12708 ± 1162	35732 ± 2588

* cpm in control wells without mitogen: 194 ± 89 in experiments tested streptococcal erythrogenic toxins, and 246 ± 55 in experiments tested Con A.

† Doses of mitogen: A toxin, both pI 5.0 and 5.2, 0.1 ng/mL of culture, B toxin and crude toxin 1.0 ng/mL of culture, Con A 5 μg/mL of culture.

‡ Ten μL of 20 times diluted serum added to each well containing 200 μL of rabbit lymphocyte culture.

§ Numbers in parentheses are percent inhibition.

Table 2. *Age distribution of AMA-positive sera in Kawasaki disease and control patients*

No. of patients tested	Age								Total	
	≦ 3m	4-11m	1y	2y	3y	4y	5y	≧ 6y	All patients*	Age matched†
Kawasaki disease										
Total	1	13	21	12	4	4	4	2	53‡	52‡
AMA+	0	11	10	6	1	3	0	0	28‡	28‡
(% positive)	(0)	(85)	(48)	(50)	(25)	(75)	(0)	(0)	(53)	(54)
Control patients										
Total	9	24	31	16	21	13	13	34	160‡	118
AMA+	3	4	4	0	3	1	0	5	20	12
(% positive)	(33)	(17)	(13)	(0)	(14)	(8)	(0)	(15)	(13)	(10)

* The difference of frequency of AMA+ individuals between KD and all control patients is significant at $p = 0.001$ (28/53 *versus* 20/160, $\chi^2 = 37$, $\phi = 3$).

† Patients or specimens at or less than 3 mo of age and at or more than 6 y of age are excluded. The difference of frequency of AMA^+ individuals is significant at $p = 0.001$ (28/52 *versus* 12/118, $\chi^2 = 38$, $\phi = 3$).

‡ Some specimens from the same patient are included in different age groups.

mean ± SD percent inhibition of the 52 positive specimens was 52 ± 28. In contrast, only 13 specimens were positive against B toxin (mean ± SD percent inhibition: 36 ± 12) Only one serum of 160 sera tested contained a weak inhibitory activity against Con A (26% in average) together with those against A toxin (100%) and B toxin (49%). Concerning the number of individuals tested, 53% (28 patients) of 53 KD patients had serum inhibitory activity against A toxin (Table 2), whereas only 15% (eight patients) reacted against B toxin with (five of eight) or without (three of eight) anti-A toxin activity. Inasmuch as specific inhibition of A toxin mitogenicity is evident in KD patients, the following experiments will be described only about A toxin.

The degree of the inhibition against A toxin varied according to the duration of illness. AMA-positive sera were most frequently found between 11th and 30th d of illness. Prevalence of strong inhibitory activity was also seen in the same period (Table 3).

We next correlated the presence of inhibitor in sera of KD patients with the results of measurement of IgG antitoxin antibody by ELISA. Thirty-seven AMA-positive sera from 22 patients were also positive in ELISA (Fig. 1). Values of percent inhibition in AMA in these specimens correlated with OD of ELISA ($r = 0.68$, $p = 0.001$). Fifteen specimens were positive by AMA, but not by ELISA. Their degrees of inhibition in AMA were significantly lower than those of the ELISA-positive specimens (respective means ± SD of 26 ± 12 *versus* 63 ± 26, $p = 0.001$). Thirty-eight specimens were only positive by ELISA. These AMA-negative specimens showed lower IgG antibody titers by ELISA than the AMA-positive sera (respective means ± SD of 0.44 ± 0.33 *versus* 0.72 ± 0.42, $p = 0.01$). However, several specimens showed high titers of more than 1.00 in OD. Of 169 specimens tested, 75 were positive by ELISA, involving 35 of 53 patients. Seventy-nine specimens were negative by both AMA and ELISA.

Table 3. *Occurrence of AMA+ sera according to day of illness in 53 patients with Kawasaki disease and in 130 control patients with acute disease onset*

No. of specimens tested	Day of illness				Total
	1-10	11-20	21-30	≧ 31	
Kawasaki disease					
Total	43	42	29	55	169
AMA+*	10	21	11	10	52
(% positive)	(23)	(50)	(38)	(18)	(31)
+	7	7	6	7	27
2+	3	7	1	2	13
3+	0	7	4	1	12
Control patients					
Total	92	54	15	19	180
AMA+	10	8	2	2	22
(% positive)	(11)	(15)	(13)	(11)	(12)
+	2	4	0	2	8
2+	2	1	0	0	3
3+	6	3	2	0	11

* Percent inhibition in AMA: +, <50%; 2+, 50–79%; 3+, ≧80%.

AMA and ELISA in control patients and comparison with those in KD. A total of 214 serum specimens from 160 patients with various infections and allergic diseases was tested as controls. AMA was positive in 27 specimens from 20 patients (mean ± SD percent inhibition 61 ± 34) of which 17 specimens from 13 patients were also positive by ELISA. However, 56 AMA-negative specimens from 49 patients were positive by ELISA. Table 2 shows the age distribution of serum specimens tested from KD and control patients. The prevalence of AMA-positive sera was relatively high in control infants up to 3 mo old and in

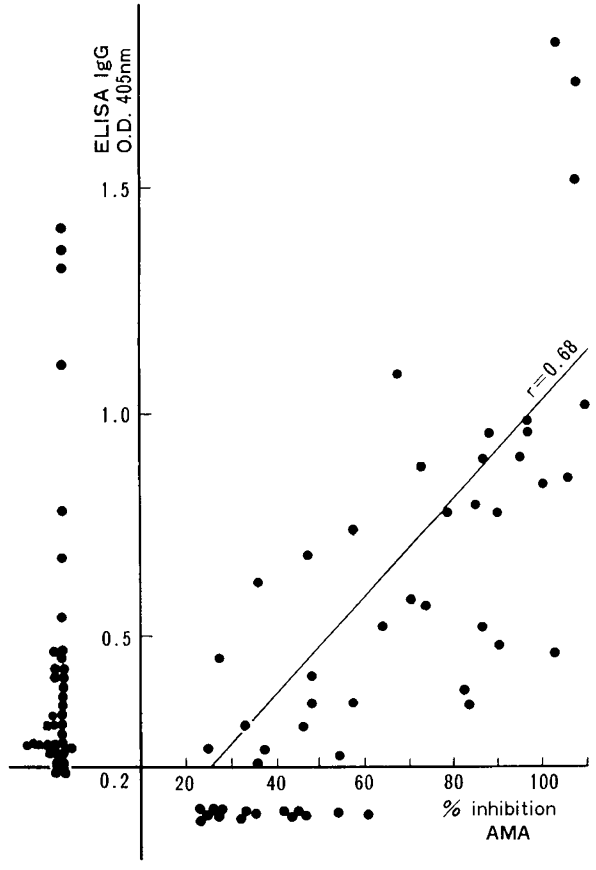

Fig. 1. Relationship between AMA and ELISA in KD patients' sera. AMA + ELISA + 37 specimens, AMA + ELISA - 15 specimens and AMA - ELISA + 38 specimens are shown. AMA - ELISA - 79 specimens are not included. Statistical significance: r at $p = 0.001$.

control older children at 6 y of age or older; age groups in which KD is relatively infrequent. Also, among the control patients strong inhibitory activity was predominantly found in neonates and older children (mean ± SD percent inhibition: 79 ± 28, significant at $p = 0.05$ *versus* positive controls in the other age groups). Except for infants ≤3 mo and children ≥6 y the percentage of KD patients who were AMA positive was larger than in the age-matched controls ($p = 0.001$). However, among positive individuals, the inhibitory activity was similar in KD patients as in age-matched control patients (respective means ± SD of 52 ± 28 *versus* 48 ± 33). Occurrence of AMA-positive sera in 130 control patients with acute disease onset was slightly different from that in KD patients (Table 3). Variation according to the day of illness was not as apparent as in KD patients, and the prevalence of strong activity in the control patients was seen predominantly in the first 10-d period.

Among 102 control patients with respiratory diseases, 11 were AMA positive (two of 31 patients with pneumonia, two of 13 with bronchopneumonia, three of 12 with bronchitis, one of three with acute tonsilitis, and one each with laryngeal neurofibroma, or pulmonary hypertension combined with patent ductus arteriosus, both with fever of unknown etiology, and herpetic stomatitis). Anaphylactoid purpura (two of two), atopic dermatitis (three of three), hepatitis (two of four), aseptic meningitis (one of two), and one patient each with protracted nephritis and newborn pseudomonas infection, although small in number, were also noteworthy. Negative control patients included 17 with acute upper respiratory infection, 12 with asthmatic bronchitis and bronchial asthma, 14 with acute gastroenteritis including rotavirus infection, and 10 with various other viral infections, including one patient each with adenovirus infection, rubella, and hand-foot-and-mouth disease, two patients each with mumps and varicella, and three patients with Epstein-Barr virus infection.

β-Hemolytic streptococcal infection. Among 23 serum specimens from 19 patients with β-hemolytic streptococcal infection, only three specimens from three patients were AMA positive. ELISA was positive in 12 specimens from 11 patients including the three with AMA-positive sera. The low frequency of AMA-positive sera suggests a relatively low prevalence of the toxin-producing strains among β-hemolytic streptococci.

Healthy children. Among 39 specimens from 24 individuals, nine specimens from seven healthy individuals were positive. The ages of these 24 subjects ranged from birth to 4 y old. AMA-positive sera were only found among cord blood (four of seven) and sera from infants less than 3 mo old (five of 13). This finding strongly suggests passive transfer of the antibody from mothers. ELISA was positive in 21 specimens from 12 individuals including all of the AMA-positive sera.

AMA and ELISA with serum fractions separated by protein A Sepharose column. To obtain additional evidence that the human serum inhibitor might represent antitoxin antibody, IgG fractions were separated from individual AMA-positive sera by protein A Sepharose column chromatograph and tested for AMA. The IgG fractions included 10 specimens from KD patients, 10 specimens from control patients, two specimens from patients with β-hemolytic streptococcal infection, and three specimens from healthy neonates and one cord blood specimen. All the IgG fractions were as positive by both AMA and ELISA as the respective original sera. Comparable results were obtained in AMA with those by original sera in six specimens from KD patients (mean ± SD percent inhibition: 90 ± 9 by IgG *versus* 92 ± 5 by serum), in eight specimens from control patients (92 ± 7 by IgG *versus* 96 ± 4 by serum), and in all six specimens from patients with β-hemolytic streptococcal infection, from healthy neonates and cord blood (97 ± 5 by IgG *versus* 98 ± 3 by serum). AMA was only positive in three specimens of the effluent fraction from the column: one each from KD (17% inhibition in average) and control (44%) patients, both among specimens that showed decrease in AMA of IgG fractions (from 80% by serum to 56% by IgG, from 70 to 35%, respectively), and the other with strong activity in both IgG (100% in average) and effluent (97%) fractions from one patient with β-hemolytic streptococcal infection. ELISA for IgG antibody was negative in all the effluent fractions tested. Although the data are limited, these findings suggest the existence of the antibody belonging to classes other than IgG.

DISCUSSION

AMA described above is specific and sensitive to the mitogen used and economic in the sense that it requires only small amounts of purified toxin and test sera. A single 20-fold dilution of test serum, used in our experiments, was used because of the availability of only small quantities of sera in most cases. Further increase in positive results might be expected by addition of a higher volume or higher concentrations of test serum. However, in several attempts, addition of twice the volume (20 μL) of the AMA-negative sera resulted in enhancement of the mitogenic activity of both A and B toxins and of Con A (data not shown). Similar nonspecific enhancement was also occasionally noted when standard volume (10 μL) of sera was used (Table 1). The nonspecific enhancement seems to be a barrier for further improvement of AMA. By using 10 μL of a 1:20 dilution of test sera, the ELISA against A toxin remained negative in most specimens. Therefore, we increased the volume of serum in the ELISA to 20 μL, and this appears to increase the sensitivity of the assay without decreasing specificity. The apparently higher frequency of positive results in ELISA than by AMA may be explained in part by the use of the larger amount of sera in the

ELISA. However, some discrepancy between AMA and ELISA results may be caused by possible impurities of the antigen used, although a highly purified toxin preparation was used throughout the experiment. Another possibility is that there exist two kinds of the anti-toxin antibody, one blocking lymphocyte mitogenicity, and the other binding to the toxin but not blocking mitogenicity. The latter may represent at least a portion of the ELISA-positive, AMA-negative sera. This possibility is now under investigation.

We have no direct evidence that the serum inhibitor measured by AMA is antibody. However, indirect evidence is that most of the AMA-positive sera were also positive by ELISA, and that, in general, there was a good correlation between the magnitude of results in the two assays. Furthermore, IgG fractions prepared from AMA-positive sera gave similar results as the respective sera by AMA and ELISA. Thus, it seems reasonable that the AMA-positive sera contain the specific antibody against the toxin used as the mitogen.

Alternatively, the inhibition in AMA might be explained by the presence of immune complexes in test sera. Presence of circulating immune complexes has been reported in KD (29). Inhibition by immune complexes of mitogenic stimulation of lymphocytes, both antigenic and nonspecific, has been described (30–33). Whether the mitogenicity of SET is nonspecific or antigenic has not been settled (34). However, we are inclined to favor the nonspecific nature of the mitogens. Lymphocytes from normal rabbits without any prior immunization respond well to both A and B toxins, and the degree of blastogenic response is higher than that usually induced by antigenic stimulation and comparable to that by Con A, a notable nonspecific mitogen. Addition of heat-aggregated human or rabbit IgG induced no effect on blastogenic response of rabbit lymphocytes to SET in our assay system. Small amounts of goat antiserum against rabbit IgG, IgM or IgA occasionally induced a slight degree of variable enhancement but no inhibitory effect (Y. Abe and S. Noma, unpublished observation). Thus, we have no evidence for nonspecific inhibition of SET mitogenicity by insoluble or soluble immune complexes. These results, however, do not exclude a role of immune complexes in the pathogenesis of KD. Questions about immune complexes in KD must be considered due to the wide range of their influence, including the relation to vasculitis.

Culture of β-hemolytic streptococci was unsuccessful from throat swabs in all of KD patients whose sera were examined in this study. Also, serum anti-streptolysin O antibody, a sensitive indicator of previous β-hemolytic streptococcal infection, remained in low titers in the same patients. These findings, when taken together with the results of AMA and ELISA to SET, suggest the possibility that toxin-producing bacteria other than *S. pyogenes* may play a role in the etiology or pathogenesis of KD. This possibility requires further investigation.

REFERENCES

1. Kawasaki T 1967 M.C.L.S. Clinical observation of 50 cases (in Japanese). Jap J Allerg 16:178–222
2. Kawasaki T, Kosaki F, Okawa S, Shigematsu I, Yanagawa H 1974 A new infantile acute febrile mucocutaneous lymph node syndrome prevailing in Japan. Pediatrics 54:271–276
3. Yanagawa H, Shigematsu I, Kusakawa S, Kawasaki T 1979 Epidemiology of Kawasaki disease in Japan. Acta Paediatr Jap 21:1–10
4. Shigematsu I, Tamashiro H, Shibata S, Kawasaki T, Kusakawa S 1980 Worldwide survey on Kawasaki disease. Lancet 1:976–977
5. Morens DM, Anderson LJ, Hurwitz ES 1980 National surveillance of Kawasaki disease. Pediatrics 65:21–25
6. Bell DM 1981 Kawasaki syndrome: description of two outbreaks in the United States. N Engl J Med 304:1568–1575
7. Melish ME 1981 Kawasaki syndrome: a new infectious disease. J Infect Dis 143:317–324
8. Yanagisawa M, Kobayashi N, Matsuya S 1974 Myocardial infarction due to coronary thromboarteritis, following acute febrile mucocutaneous lymph node syndrome in an infant. Pediatrics 54:277–281
9. Tanaka N, Sekimoto N, Naoe S 1976 Kawasaki disease: relationship with infantile periarteritis nodosa. Arch Pathol Lab Med 100:81–86
10. Landing BH, Larson EJ 1977 Are infantile periarteritis nodosa with coronary artery involvement and fatal mucocutaneous lymph node syndrome the same? Pediatrics 59:651–662
11. Amano S, Hazama F, Kubagawa H, Tasaka N, Haebara H, Hamashima Y 1980 General pathology of Kawasaki disease. On the morphological alterations corresponding to the clinical manifestations. Acta Pathol Jap 30:681–694
12. Fujiwara H, Fujiwara T, Ta-Chirn Kao, Ohshio G, Hamashima Y 1986 Pathology of Kawasaki disease in the healed stage. Relationships between typical and atypical cases of Kawasaki disease. Acta Pathol Jap 36:857–867
13. Tanaka N, Naoe S, Masuda H, Ueno T 1986 Pathological study of sequelae of Kawasaki disease with special reference to the heart and coronary artery lesions. Acta Pathol Jap 36:1513–1527
14. Shulman ST 1987 Kawasaki Disease. Alan R Liss, New York, pp 73–147
15. Schlossberg D, Kandra J, Kreiser J 1979 Possible Kawasaki disease in a 20-year-old woman. Arch Dermatol 115:1435–1436
16. Person JR 1980 Kawasaki disease and staphylococcal exotoxins. Arch Dermatol 116:986
17. Milgrom H, Palmer EL, Slovin SF, Morens DM, Freedman SD, Vaughan JH 1980 Kawasaki disease in a healthy young adult. Ann Intern Med 92:467–470
18. Milgrom H, Palmer EL, Slovin SF, Morens DM, Freedman SD, Vaughan JH 1981 Toxic shock syndrome or Kawasaki disease. Ann Intern Med 94:138
19. Kim YB, Watson DW 1972 Streptococcal exotoxins: biological and pathological properties. In: Wannamaker LW, Matsen JM (eds) Streptococci and Streptococcal Diseases. Academic Press, New York, pp 33–50
20. Alouf JE 1980 Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). Pharmacol Ther 11:661–717
21. Hudson L, Hay FC 1980 Practical Immunology, 2nd ed. Blackwell Scientific Publications, Oxford
22. Kamezawa Y, Nakahara T 1989 Purification and characterization of streptococcal erythrogenic toxin type A produced by *Streptococcus pyogenes* strain NY5 cultured in the synthetic medium NCTC 135. Comparison with the dialyzed medium-derived toxin. Microbiol Immunol 33:183–194
23. Gerlach P, Köhler W, Knöll H, Moravek L, Weeks CR, Ferretti JJ 1987 Purification and characterization of streptococcus pyogenes erythrogenic toxin type A produced by a cloned gene in streptococcus sanguis. Zbl Bakt Hyg A 266:347–358
24. Weeks CR, Ferretti JJ 1986 Nucleotide sequence of the type A streptococcal exotoxin (erythrogenic toxin) gene from *Streptococcus pyogenes* bacteriophage T12. Infect Immun 52:144–150
25. Cunningham CM, Barsumian EL, Watson DW 1976 Further purification of group A streptococcal pyogenic exotoxin and characterization of the purified toxin. Infect Immun 14:767–775
26. Barsumian EL, Cunningham CM, Schlievert PM, Watson DW 1978 Heterogeneity of group A streptococcal pyrogenic exotoxin type B. Infect Immun 20:512–518
27. Abe Y, Alouf JE 1976 Partical purification of exocellular lymphocyte mitogen from *Streptococcus pyogenes*. Jpn J Exp Med 46:363–369
28. Abe Y, Alouf JE, Kurihara T, Kawashima H 1980 Species-dependent response to streptococcal lymphocyte mitogens in rabbits, guinea pigs and mice. Infect Immun 29:814–818
29. Sawa F 1979 Circulating immune complexes in MCLS. Acta Paediatr Jpn 21:82
30. Lee JC, Sigel MM 1974 A differential effect of IgG and IgG antibodies on the blastogenic response of lymphocytes to rubella virus. Cell Immunol 13:22–31
31. Ryan JL, Arbeit RD, Dickler HB, Henkart PA 1975 Inhibition of lymphocyte mitogenesis by immobilized antigen-antibody complexes. J Exp Med 142:814–826
32. Stout RD, Herzenberg LA 1975 The Fc receptor on thymus-derived lymphocytes II. Mitogen responsiveness of T lymphocytes bearing the Fc receptor. J Exp Med 142:1041–1051
33. Dickler HB 1976 Lymphocyte receptors for immunoglobulin. Adv Immunol 24:167–214
34. Barsumian EL, Schlievert PM, Watson DW 1978 Nonspecific and specific immunological mitogenicity by group A streptococcal pyrogenic exotoxins. Infect Immun 22:681–688