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Antibodies to endothelial cells in Kawasaki disease lyse endothelial cells without cytokine pretreatment

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SUMMARY

Kawasaki disease (KD) is characterized by diffuse vasculitis and marked immune activation. To confirm the presence of antiendothelial cell antibodies (AECA) and cytotoxicity of AECA, we investigated the presence of AECA using ELISA and cytotoxicity of AECA in KD. Sera from patients with acute KD were assessed for binding to human umbilical vein endothelial cells (HUVEC) using a cell-based ELISA, and for cytotoxicity against HUVEC as indicated by the conversion of a tetrazolium salt (MTT) into a coloured product. IgM AECA were detected in 8/19 KD sera, IgG AECA were detected in 5/19 KD sera. Significant differences in both AECA titres existed between patients and febrile and afebrile controls. Six out of 19 patients showed complement-dependent cytotoxicity against HUVEC. Cytotoxicity was significantly enhanced by pretreating HUVEC with tumour necrosis factor (TNF), and reduced by incubation with gammaglobulin. Serum titres of IgM AECA in the KD patients were positively correlated with cytotoxicity. Findings suggest that IgM AECA mediates complement-dependent cytotoxicity against endothelial cells in patients with KD, and gammaglobulin may reduce complement-dependent cytotoxicity of AECA against endothelial cells.

Keywords Kawasaki disease antiendothelial cell antibody endothelial cell injury gammaglobulin

INTRODUCTION

Kawasaki disease (KD) is an acute vasculitis of childhood of unknown etiology. Although the disease is self-limited, serious complications such as coronary artery aneurism or ectasia occur in about 20% of children affected. The following observations suggest that immunoregulatory abnormalities are involved in the pathogenesis of KD: the acute phase of this disease is characterized by a deficiency of suppressor and cytotoxic T cells; increased numbers of activated helper T cells and monocytes; polyclonal B cell activation [1,2]; and increased levels of serum cytokines such as IL-1 [3], tumour necrosis factor (TNF) [4], interferon-gamma (IFN- γ) [5], IL-6 [6]; and the appearance of cytotoxic antibodies against vascular endothelial cells [7–9].

The administration of an i.v. high dose of gammaglobulin (IVGG) effectively reduces the incidence of coronary artery lesions (CAL) in KD patients [10]. However, the mechanism underlying this effect is currently unknown.

We evaluated the binding of antiendothelial cell antibodies (AECA) to resting or cytokine-treated endothelial cells, cytotoxicity of these antibodies, and inhibitory effect of gammaglobulin (γ-Glb) on AECA binding to endothelial cells and cytotoxicity against endothelial cells, in samples obtained from patients with KD and control subjects.

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MATERIALS AND METHODS

Patients and sera

Nineteen Japanese children (10 males and nine females aged 0·5–5 years, mean 2·1 years) who met the diagnostic guidelines for KD were studied. Sera were obtained only during the acute phase of illness, i.e. within 5 days of onset. All patients were administered IVGG (200 mg/kg per day) for 5 days within 6–9 days of disease onset. Sera were also obtained from 10 febrile children (five males and five females aged 0·3–15 years, mean 3·8 years). Three of them were diagnosed with pneumonia, four with meningitis, two with infectious mononucleosis and one with septicaemia. Control sera were also obtained from 20 afebrile children (10 males and 10 females, aged 0·2–15 years, mean 3·5 years). Informed consent was obtained from patients and/or their parents before the study.

Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated as previously reported [11] and serially cultured. Confluent monolayers at subculture levels 2–3 were used for ELISA and

cytotoxicity studies on gelatin-coated 96-well microtitre plates at a concentration of 1×10^4 cells/well.

Activation of HUVEC

For some experiments, HUVEC were activated with recombinant human TNF-α (Genzyme, West Malling, UK) (specific activity 2×10^7 U/mg) at a final concentration of 100 U/ml for 4 h at 37°C. Activated HUVEC were fixed with glutaraldehyde for use in the ELISA assay, or were used immediately in cytotoxicity assays.

AECA ELISA

AECA were detected using the method previously described [12,13]. In brief, glutaraldehyde-fixed monolayers of HUVEC were washed with PBS and incubated with blocking buffer (1% bovine serum albumin (BSA)). After two washes, 100 μl serum (diluted 1 : 2000 with 1% BSA–0·1 M Tris–0·5 M NaCl (1% BSA–TBS)) were added to each well. After 18 h, incubation at room temperature and three washes with 1% BSA–TBS, 100 μl of horseradish peroxidase-conjugated goat anti-human IgG (diluted 1 : 5000 with 1% BSA–TBS) or anti-human IgM (diluted 1 : 6000 with 1% BSA–TBS) (Cappel, Durham, NC) were added to each well and incubated for 1 h. After five washes, 3,3′,5,5′-tetramethyl benzidine (TMB; Vector Labs, Burlingame, CA) was added and the plates were measured in an ELISA reader at 450 nm. As positive control, we used pooled serum from two patients with systemic lupus erythematosus (SLE) (optical density: 1·05, 1·31 for IgG AECA; 0·92, 0·79 for IgM AECA); as negative control we used the pooled serum from seven normal adults.

The AECA titre was calculated as AECA titre (%)$= 100 \times (S - A)/(B - A)$, where S is the absorbance of the sample and A and B are the absorbances of the negative and positive controls, respectively.

Cytotoxicity assay

Cytotoxicity against HUVEC in the absence or presence of complement was detected as described previously [14]. In brief, HUVEC monolayers in 96-well plates were incubated with 50 μl of sera (diluted 1 : 100 in PBS) or PBS for 6 h at 37°C, followed by 10 μl of human complement from fresh human AB serum or PBS for 30 min at 37°C. Subsequently, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) (5 mg/ml) (Research Organics, Cleveland, OH) were added to each well. After 4 h incubation followed by one wash with PBS, 100 μl of dimethylsulfoxide (DMSO) were added and the plates were measured in an ELISA reader at 570 nm [15].

The cytotoxicity against HUVEC was calculated as follows: cytotoxic activity (%) $= 100(1 - S/P)$ where S is the absorbance of sample, and P the absorbance of PBS.

Specific binding and cytotoxicity against HUVEC

To determine the specificity of AECA binding HUVEC and AECA cytotoxicity against HUVEC, IgM-rich and IgM-poor fractions of four KD sera and two normal control sera were prepared by separation on a Sephadex G-300 chromatography column. The serum fractions were used to assess both binding and cytotoxicity against HUVEC and human fibroblasts (Cell System Corp., Kirkland, WA).

Inhibition of AECA binding and cytotoxicity by γ-Glb

To determine the effect of γ-Glb on AECA binding to HUVEC and AECA cytotoxicity against HUVEC, different concentrations of intact γ-Glb (Takeda Chemical Industries, Tokyo, Japan) were added to the sera in both the ELISA and the cytotoxicity assays. In addition, inhibition of AECA cytotoxicity against HUVEC by different concentration of BSA was also studied by the same method as γ-Glb. The degree of inhibition of cytotoxicity was determined as the percentage reduction compared with that without γ-Glb or BSA.

Statistical analysis

Data are reported as mean ± s.d.. Wilcoxon's signed rank test was used to compare paired groups, and Wilcoxon's rank sum test to compare non-paired groups. Spearman's rank correlation test was used to evaluate relationships. A level of $P < 0{\cdot}05$ was considered statistically significant.

RESULTS

Incidence of AECA

ELISA for AECA. Serum titres for IgG and IgM AECA were determined in the 19 KD patients and 10 febrile and 20 afebrile control subjects by ELISA. Only sera that showed titres greater than the mean ± 2 s.d.of the afebrile control sera value (i.e. >40%) were considered AECA-positive. Five of the KD samples, but none of the febrile and afebrile control sera, were positive for IgG

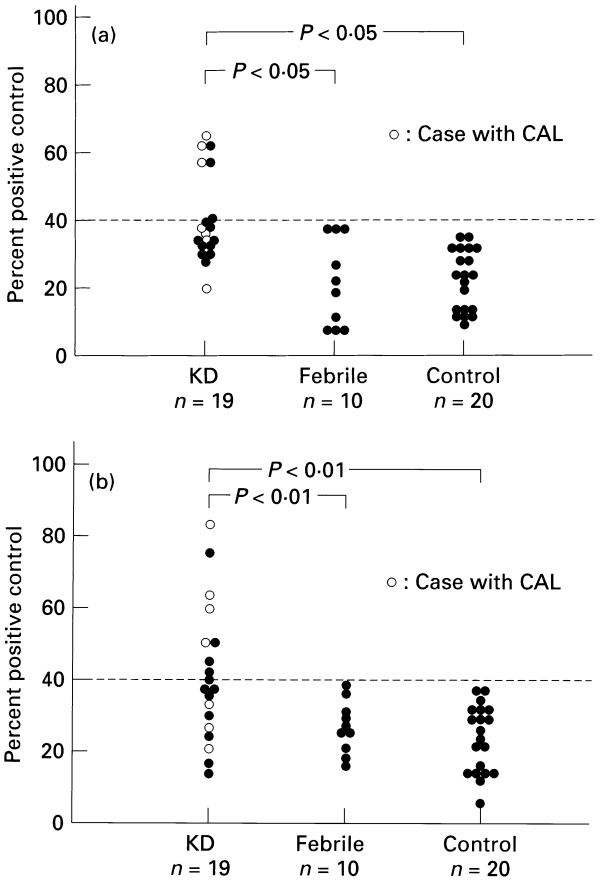

Fig. 1. Titres of IgG antiendothelial cell antibodies (AECA) (a) and IgM AECA (b) in patients with Kawasaki disease (KD). The dashed line represents the mean + 2 s.d. of the afebrile control subjects. Febrile, Febrile controls; control, afebrile controls; CAL, coronary artery lesions.

Table 1. Effect of pretreatment with tumour necrosis factor-alpha (TNF-α) on antiendothelial cell antibody (AECA) binding to human umbilical vein endothelial cells (HUVEC)

Serum donor		HUVEC treatment	
		None	TNF-α
IgG-AECA (% positive control)			
Acute KD	1	32·0	39·0
	2	62·1	63·5
	3	33·7	39·1
	4	35·0	38·2
	5	66·0	66·7
	6	48·2	50·0
	7	39·0	36·1
	8	32·5	35·2
	9	22·0	20·5
	10	29·6	33·8
		40·0 ± 14·3	42·2 ± 14·1
Control ($n = 20$)		26·3 ± 6·8	26·8 ± 5·0
IgM-AECA (% positive control)			
Acute KD	1	76·2	77·0
	2	60·0	61·2
	3	70·4	68·5
	4	64·0	84·2
	5	40·0	41·5
	6	41·2	42·0
	7	63·1	64·2
	8	23·4	23·0
	9	22·6	21·7
	10	20·0	20·8
		48·1 ± 21·3	50·4 ± 23·8
Control ($n = 20$)		28·4 ± 5·7	29·8 ± 5·0

AECA. Mean IgG AECA titres were significantly higher in the KD patients (40·6 ± 12·8%) than in febrile or afebrile control subjects (27·1 ± 7·4% and 26·3 ± 6·8%, respectively) (Fig. 1a).

Eight KD serum samples, but none of the febrile or afebrile control samples, were positive for IgM AECA. KD sera showed significantly higher mean titres of IgM AECA (41·2 ± 18·5%) than the febrile or afebrile controls (26·0 ± 7·8%, 28·4 ± 5·7%, respectively) (Fig. 1b).

Effect of HUVEC activation with TNF-α on AECA binding. We evaluated the binding of AECA to TNF-α-activated HUVEC for 10 of the KD samples. Activation of HUVEC increased the mean titres of IgG and IgM AECA. Differences in titres determined with activated and resting HUVEC did not differ significantly (Table 1).

Specificity of AECA binding to HUVEC. We analysed the specificity of AECA binding to HUVEC using the IgM-rich and IgM-poor fractions from four KD sera and two control sera. IgM-rich fractions from all four KD sera were positive for AECA, and bound to HUVEC as well as to fibroblasts. Among the IgM-poor fractions, one of four KD sera demonstrated binding to HUVEC, and none bound to fibroblasts. Neither the IgM-rich nor the IgM-poor fractions of the two control sera bound to the HUVEC or fibroblasts (Table 2).

Table 2. Binding of IgM-rich and IgM-poor fractions from Kawasaki disease (KD) and afebrile control sera to human umbilical vein endothelial cells (HUVEC) and human fibroblasts

Serum donor		AECA titre (% positive control)	
		HUVEC	Fibroblasts
IgM-rich fractions			
KD	1	44·0	37·5
	2	47·2	42·3
	3	50·1	41·0
	4	62·1	44·0
Control	1	18·3	1·1
	2	12·4	0
IgM-poor fractions			
KD	1	20·2	4·1
	2	15·3	3·1
	3	20·9	5·3
	4	41·0	10·0
Control	1	10·5	0
	2	7·6	0

Cytotoxicity studies

Cytotoxicity of KD sera. None of the KD patients or control subjects demonstrated cytotoxicity in the absence of complement against resting and TNF-α-activated HUVEC (data not shown). Sera from the 19 KD patients and from the 10 febrile and 20 afebrile control subjects were evaluated for complement-mediated cytotoxicity against resting HUVEC. Only the samples whose cytotoxicity exceeded the mean $\pm$ 2 s.d. of the afebrile control value (i.e. >37·2%) were considered to be positive for cytotoxicity. According to these criteria, six of the KD samples, but none of the samples from the febrile and afebrile controls, demonstrated cytotoxicity (Fig. 2a). The mean cytotoxic activity against resting HUVEC was significantly higher in KD sera (36·6 $\pm$ 14·8%) than in febrile (21·2 $\pm$ 12·1%) or afebrile (20·2 $\pm$ 8·5%) controls (Fig. 2a). Furthermore, the cytotoxicity of 10 KD sera was significantly higher against TNF-α-activated HUVEC than against resting HUVEC (Fig. 2b).

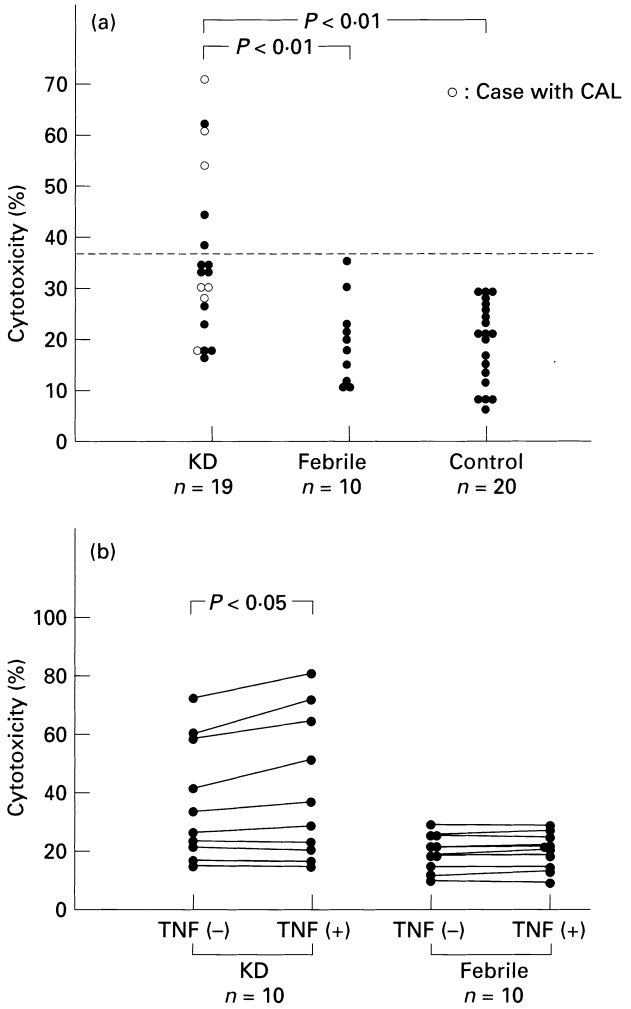

Fig. 2. (a) Complement-dependent cytotoxicity against human umbilical vein endothelial cells (HUVEC) of patients with Kawasaki disease (KD) and febrile or afebrile control subjects. Dashed line indicates the mean $\pm$ 2 s.d. of the value observed in afebrile control subjects. (b) Complement-dependent cytotoxic effects of 10 KD sera and 10 febrile control sera on HUVEC treated or untreated with tumour necrosis factor-alpha (TNF-α). CAL, coronary artery lesions.

Specificity of AECA cytotoxicity against HUVEC. IgM-rich fractions from the four KD sera analysed demonstrated complement-mediated cytotoxicity against the resting and the TNF-α-activated HUVEC. The same fractions, however, were not cytotoxic to fibroblasts (Table 3). In contrast, the IgM-poor fractions of these sera and the IgM-rich as well as IgM-poor fractions of two normal control sera did not demonstrate cytotoxicity.

Correlation analyses

A significant correlation was observed between IgM AECA titres and complement-mediated cytotoxicity against HUVEC ($r = 0{\cdot}543$, $P < 0{\cdot}05$) in KD sera (Fig. 3). In contrast, IgG AECA titres were not correlated with cytotoxicity.

Effect of γ-Glb or BSA on AECA binding to HUVEC and cytotoxicity

Effect of γ-Glb on AECA binding. To determine whether γ-Glb would modulate the AECA binding to HUVEC, AECA ELISA was performed in the presence of γ-Glb. Final concentrations from 0·1 mg/ml to 10 mg/ml of γ-Glb changed neither IgG nor IgM AECA binding activities (Fig. 4).

Effect of γ-Glb or BSA on IgM AECA cytotoxicity against HUVEC. To determine whether γ-Glb could affect IgM AECA complement-dependent cytotoxicity against HUVEC, the cytotoxicity assay was performed in the presence of γ-Glb. A higher concentration of γ-Glb gave higher percentage inhibition of cytotoxicity. Final concentrations from 0·1 mg/ml to 50 mg/ml of BSA did not significantly change IgM AECA complement-dependent cytotoxicity (Fig. 5). γ-Glb reduced IgM AECA complement-dependent cytotoxicity against both resting and TNF-α- activated HUVEC in all four KD sera tested (Table 4).

Association of clinical parameters with AECA binding and cytotoxicity

Seven of the 19 acute KD sera were derived from patients who developed CAL. IgG and IgM AECA titres in these sera were not higher than those of patients with an uncomplicated course of KD. On the other hand, sera from these seven patients had slightly higher complement-dependent cytotoxic activities (40·1 $\pm$ 18·1%) than those from the other 12 patients (37·0 $\pm$ 15·7%). Of the six KD patients whose sera were positive for cytotoxicity against HUVEC, three patients eventually developed CAL, whereas the other three did not.

DISCUSSION

We detected complement-dependent cytotoxic AECA in sera from KD patients. Although this observation generally confirms the findings of Leung *et al*. [7,8] and Kaneko *et al*. [9], there are several differences. For example, the incidence of IgG AECA was higher in our sample (five out of 19 KD sera) than in Kaneko's study (one out of 19 sera). However, the incidence of cytotoxic AECA was lower in this study (32%) than in the previous studies (50–80%). Such differences could be due in part to the time of blood sampling (within 5 days of onset) and the dilution titre (at 1 : 200 for IgG) used here. Leung *et al*. demonstrated that acute KD sera can lyse HUVEC that have been treated with cytokines, including TNF, but do not lyse untreated HUVEC [7,8]. The authors speculated that the cytotoxic antibodies target antigens on the endothelial cells that are induced by the cytokines [7,8,16].

Table 3. Cytotoxicity of IgM-rich and IgM-poor fractions obtained from sera of patients with Kawasaki disease (KD) and from afebrile control sera against human umbilical vein endothelial cells (HUVEC) with or without pretreatment with tumour necrosis factor-alpha (TNF-α), and against human fibroblasts

		Cytotoxicity (%)		
Serum donor	Target cells	HUVEC		Fibroblasts
		TNF (−)	TNF (+)	
IgM-rich fractions				
KD	1	55·1	60·7	3·7
	2	42·3	43·0	1·3
	3	58·4	60·2	5·6
	4	40·8	38·1	0·7
Control	1	5·6	6·2	1·2
	2	4·3	4·0	0
IgM-poor fractions				
KD	1	3·2	3·5	4·3
	2	1·4	1·0	0·8
	3	4·4	6·1	2·6
	4	0·8	1·2	2·7
Control	1	0·5	1·2	1·1
	2	0·2	0·7	0·1

Our study, in contrast, found that while TNF-α treatment could increase cytotoxicity, AECA (particular IgM AECA) did not require TNF-activated cells for complement-dependent cytotoxicity. These findings, together with the evidence presented by Kaneko *et al*. [9], strongly suggest that the antigens recognized by AECA in KD sera are constitutively expressed on endothelial surfaces. It is possible, however, that TNF up-regulates the expression of these antigens or induces functional changes in the endothelial cells, thus enhancing the efficiency of complement-dependent cytotoxicity.

The circulating AECA observed in the present study did not appear to be specific for endothelial cells, since they also bound to fibroblasts. Only the IgM-rich fractions, however, caused complement-dependent damage to endothelial cells, in contrast to the IgM-poor fractions, which contained IgG AECA. Furthermore, a significant positive correlation was observed between titres for IgM AECA and the extent of complement-dependent cytotoxicity. These findings, which are consistent with previous reports [7–9], demonstrate that IgM AECA can significantly contribute to vascular injury.

The exact nature of the surface antigens recognized by AECA

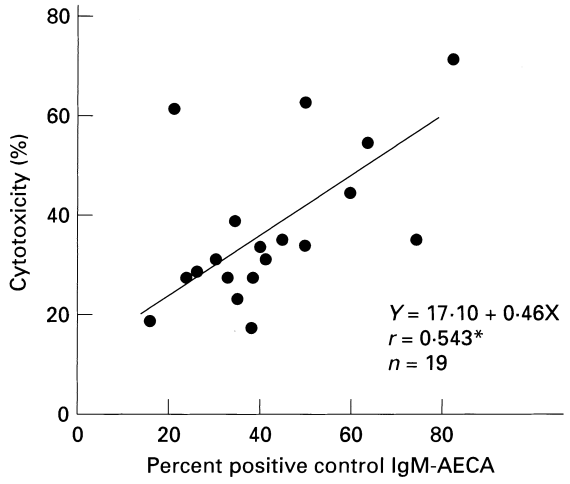

Fig. 3. Correlation between complement-dependent cytotoxicity and IgM antiendothelial cell antibody (AECA) titres. *$P < 0{\cdot}05$.

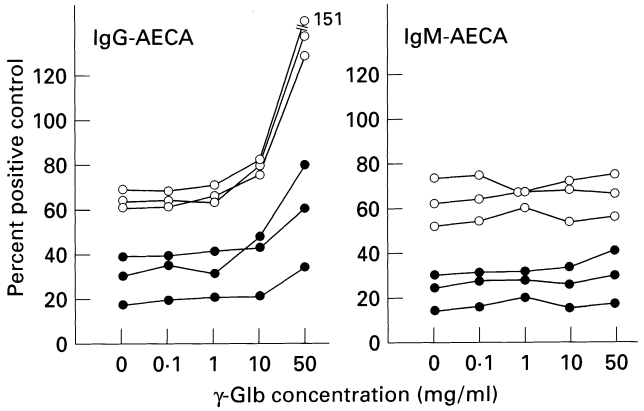

Fig. 4. Effect of gammaglobulin (γ-Glb) on antiendothelial cell antibody (AECA) binding to human umbilical vein endothelial cells (HUVEC). ○, AECA-positive sera from Kawasaki disease (KD); ●, AECA-negative sera from afebrile control subjects.

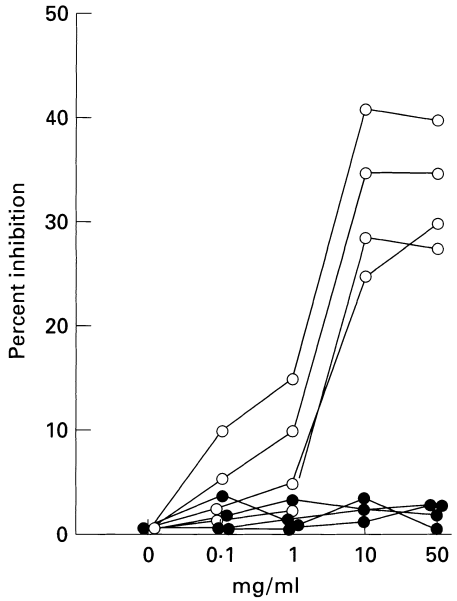

Fig. 5. Effect of gammaglobulin (γ-Glb) or bovine serum albumin (BSA) on complement-dependent cytotoxicity of IgM-rich serum fractions from four Kawasaki disease sera. ○, γ-Glb; ●, BSA.

could not be clarified in this study. The fact that HUVEC normally do not possess Fc receptors [17] and findings that IgM-poor fractions did not contain cytotoxic antibodies suggested that IgM AECA does not induce vascular injury via Fc-mediated binding. However, IgG AECA can induce an antibody-dependent cell-mediated cytotoxicity (ADCC) in primary vasculitis [18]. We could not detect ADCC against HUVEC in KD sera (data not shown). Thus, IgG AECA binding to HUVEC in our study may have been a non-specific result of the activation of polyclonal B cells.

We did not observe significant association between AECA binding, cytotoxicity and CAL. This may be due to differences in endothelial cell antigens of HUVEC and the coronary arteries. In addition, underlying anatomical abnormalities, genetic factors, or differences in the degree of local immune activation may predispose certain patients to the development of CAL [7].

This i.v. administration of γ-Glb has been shown to decrease the incidence of CAL [10]. Several studies suggest that such therapy corrects the consequences of immune activation, such as a decrease in the number of $CD8^+$ cells, spontaneous immunoglobulin synthesis and spontaneous IL-1 secretion [19,20]. In our study, IgG AECA binding was raised in the presence of extra high concentrations of γ-Glb. The fact that the same phenomenon was observed even in the absence of the serum, and γ-Glb had no cytotoxicity against HUVEC, may suggest that extra high concentrations of γ-Glb do not bind to HUVEC specifically (data not shown). High concentrations of γ-Glb reduced complement-dependent cytotoxicity against HUVEC without inhibition of IgM AECA binding to HUVEC. These findings may suggest that high doses of γ-Glb block autoantibody activity via anti-idiotypic antibodies. Alternatively, high doses of γ-Glb may inhibit complement activation [21] and/or complement attachment to AECA or HUVEC.

In conclusion, we have demonstrated the presence of AECA in KD sera that bound to endothelial cells in the absence of pretreatment with TNF. The detection of complement-dependent cytotoxic IgM AECA and the ability of TNF activation to increase cytotoxicity suggest that AECA recognize normally expressed endothelial antigens. Association of these antibodies with complement, which is enhanced considerably by TNF, may induce injury of the vascular endothelium, and thus in part contribute to the vasculitis that occurs in KD. In addition, the inhibitory effect of γ-Glb on complement-dependent cytotoxicity suggests that γ-Glb may suppress activation of the antibody and complement systems.

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Table 4. Effect of gammaglobulin (γ-Glb) on cytotoxicity of IgM-rich serum fractions from patients with Kawasaki disease (KD) and afebrile control subjects against human umbilical vein endothelial cells (HUVEC) with or without treatment with tumour necrosis factor-alpha (TNF-α)

Serum donor		Cytotoxicity (%)			
		HUVEC treatment			
		None		TNF-α	
		γ-Glb (−)	γ-Glb (+)	γ-Glb (−)	γ-Glb (+)
IgM-rich fractions					
KD	1	60·0	40·8	67·8	43·5
	2	40·1	36·5	43·7	40·7
	3	59·2	50·3	63·8	56·2
	4	33·6	26·9	36·2	33·8
Control	1	18·2	16·4	20·4	19·0
	2	14·0	12·7	14·3	13·4

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