

Antiendothelial cell antibodies detected by a cellular based ELISA in Kawasaki disease

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Abstract

Kawasaki disease is an acute vasculitic illness of childhood associated with significant morbidity and mortality. A cellular based enzyme linked immunosorbent assay (ELISA) was used to demonstrate the presence of antiendothelial cell antibodies in sera from children with Kawasaki disease. Twenty one of 32 patients with Kawasaki disease had raised IgM antibody titres and four had raised IgG antiendothelial antibody titres. There was a significant difference in the IgM antiendothelial cell antibody titres when comparing the patients with normals and febrile controls. The antibody titre paralleled the disease activity in patients studied serially. There was no relative increase in binding of antiendothelial cell antibodies after cytokine stimulation. These findings may be of importance in further research into the understanding of mechanisms involved in this and other forms of vasculitis in man.

Kawasaki disease is a form of acute systemic vasculitis of childhood, which was first described in 1967.¹ Since that time over 80 000 cases have occurred in Japan. Although the disease is less common in the United Kingdom approximately 70-90 cases per year have been reported since the introduction of the active reporting system through the British Paediatric Surveillance Unit in 1986, and this is probably still an underestimate.² The disease manifests itself as a febrile illness associated with mucosal inflammation, lymphadenopathy, rashes, and peripheral desquamation. In approximately 20% of cases cardiovascular complications develop secondary to coronary vasculitis. The presence of coronary artery aneurysms may lead to coronary thrombosis, myocardial infarction, and in approximately 1% of cases there may be a fatal outcome.³

The aetiology of this disease is still unknown but an infectious agent is suspected. Numerous organisms, including retroviruses, have been implicated but none confirmed.⁴ The infectious aetiology theory is supported by the seasonal incidence and the epidemics that have occurred in some countries.⁵

In the absence of a specific laboratory test the diagnosis at present depends on the presence of five out of six of the established clinical criteria providing other illnesses are excluded.⁶ There is evidence that immunological mechanisms are activated in Kawasaki disease. In vitro studies have demonstrated an increase in the number of activated lymphocytes spontaneously secreting

IgG and IgM, increased activated T helper cells, and a deficiency of circulating suppressor/cytotoxic cells in children with this illness.⁷ Recently antibodies cytotoxic to cytokine stimulated endothelial cells have been demonstrated in the acute phase of Kawasaki disease.^{8,9}

Using a cellular based enzyme linked immunosorbent assay (ELISA) system¹⁰ we have studied the presence of antiendothelial cell antibodies and their binding to cytokine stimulated endothelial cells in serum from children with Kawasaki disease.

Patients and methods

PATIENTS

Thirty two cases which fulfilled the clinical criteria for Kawasaki disease were evaluated. These included either patients referred to the Hospital for Sick Children or patients presenting at other hospitals with Kawasaki disease whose paediatricians sent serum samples to us. Sera were collected prospectively and in most cases from children as early as possible in the disease and in some cases serial samples were measured. Initial samples range from day 6 to day 51 of the illness and were stored at -70°C before testing.

Two control groups were also studied both of whom needed blood samples for routine clinical purposes. The first consisted of normal children admitted to the Hospitals for Sick Children for routine surgery or those undergoing investigation of short stature, and the second including children admitted with acute febrile illnesses.

The age range of the patients was 0.3-12.6 years (mean 2.34) and included 18 boys and 14 girls. The majority of patients were white with five West Indians and four Japanese. The age range of the febrile controls was 0.66-10.25 years (mean 3.05) and that of the normal controls 1.08-16.7 years (mean 7.41). The control groups were mainly white children.

Coronary artery abnormalities occurred in 15 of the 32 patients. Coronary artery aneurysms were found in 10/32 (31%) and coronary artery dilatation in a further 5/32 (16%).

Approval was obtained from the ethical committee of the Hospitals for Sick Children and the Institute of Child Health for collecting serum samples from patients and controls.

ENDOTHELIAL CELL CULTURE

Human umbilical vein endothelial cells were obtained by collagenase (Sigma) digestion using a modified method as described by Jaffe.¹¹ Unpooled cells were grown to confluence in 25

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ml flasks (Nunc) precoated with 1% gelatin solution (Sigma) in Roswell Park Memorial Institute (RPMI) medium 1640 with glutamine (Gibco), 20% heat inactivated fetal calf serum (Gibco), and 100 U/ml penicillin and streptomycin (Flow). The cells were incubated at 37°C, 5% carbon dioxide, and fed at three day intervals. When confluent they exhibited a typical cobblestone appearance by phase contrast microscopy and 95% positivity for factor VIII, by immunofluorescence. Cells from individual flasks were transferred to a precoated 96 well gelatin microtitre plate (Nunc) after removal with a 0.2% trypsin EDTA solution (Flow). Cells were plated at 2×10^4 per well at which density they were seen to be confluent by phase contrast microscopy after an overnight incubation. They were used in the ELISA within four days and plates where cell growth was found to be uneven were discarded.

ANTI-ENDOTHELIAL CELL ELISA

All stages of the ELISA were performed at 37°C in 5% carbon dioxide using medium RPMI 1640 with glutamine, 10% fetal calf serum, and 10% adult bovine serum (Imperial) diluent. The plates were checked for confluence before the start and throughout the ELISA by phase contrast microscopy. Initially 180 μ l of diluent was added to block non-specific binding sites. After two washes with sterile phosphate buffered saline, test sera diluted one in 50 were added to five adjacent wells. After a three hour incubation the plate was again gently washed twice with sterile phosphate buffered saline and 120 μ l/well of goat antihuman polyvalent, IgG or IgM antibody (Sigma), at a dilution of 1:350, or 1:500 respectively, added to each well. Samples were run in quintuplicate. After a two hour incubation the plates were washed twice with phosphate buffered saline and 75 μ l of nitrophenolphosphate substrate in diethanolamine buffer at pH 9.8 added. The reaction was stopped after 90 minutes by the addition of 75 μ l/well of 3M sodium hydroxide and the plates were read using a Titertek multiscan with a 405 nm filter.

Initial screening of patients with systemic lupus erythematosus had identified a patient with high binding who was adopted as the positive standard for all experiments. Similarly a normal control sera with relatively low reactivity was selected to be a negative control for all experiments (background levels were assessed using diluent alone).

For each serum tested a binding index ratio was calculated using the following formula (OD, optical density):

$$\frac{(\text{OD test sera} - \text{OD negative standard})}{(\text{OD positive sample} - \text{OD negative standard})} \times 100$$

Initially 65 blood donors were screened (arithmetic mean (SD) 11 (9)) and results were taken as abnormal if they were greater than 3SD above the mean (40%) for this group. Only one patient was positive at this level. The coefficient of variation between plates was 7.5%.

CYTOKINE PRESTIMULATION

(1) Interferon gamma

Altogether 96 well microtitre plates were prepared as for the ELISA studies and checked for confluence of endothelial cells using phase contrast microscopy. The medium was then aspirated and 200 μ l/well of fresh medium added to half of the plate. Finally 200 μ l/well of recombinant interferon gamma at a dilution of 100 U/ml was added to the remaining half of the plate. The plate was then incubated for 48 hours before running the ELISA, which was modified to allow identical samples in quintuplicate to be run concurrently in both stimulated and unstimulated halves of the plate. Interferon gamma activity was initially investigated by studying the dose response expression of class I and class II molecules. One aliquot of interferon gamma was divided into separate aliquots at -70°C to be used in all experiments, and therefore was not checked on each occasion.

(2) Tumour necrosis factor

Plates were prepared as for the experiments with interferon gamma, tumour necrosis factor- α (AMGEN) at a dilution of 50 U/ml being substituted for interferon gamma to prestimulate the endothelial cells on half of the plate. Plates were then incubated for 90 minutes before being used in the modified ELISA as detailed above. As with the interferon gamma, tumour necrosis factor was initially aliquoted and frozen until used in individual assays.

Statistical analysis was performed using the Mann-Whitney U test.

Results

Twenty one of the patients with Kawasaki disease demonstrated titres of IgM antiendothelial cell antibodies greater than 40% of the standard positive control. This level was opted for being the mean +3SD of the standard positive control when evaluated on blood donor sera. There was a significant difference between the Kawasaki disease group and the normal controls ($p < 0.001$) and also the febrile controls ($p = 0.001$) (fig 1). Four children also showed raised titres of IgG antiendothelial cell antibodies but this was not significant

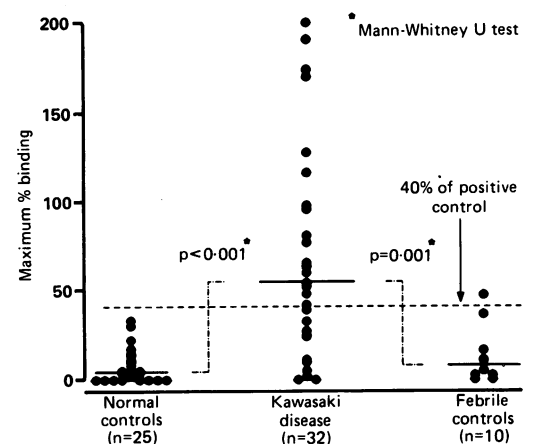


Figure 1 IgM antiendothelial cell antibodies in Kawasaki disease.

(fig 2). Serial measurements of IgM antiendothelial cell antibodies in six patients showed an initial rise in antibody titre with a fall in the convalescent period (fig 3). In one patient IgG antiendothelial cell antibodies were seen to rise as the level of IgM fell (fig 4). In this patient Sandoglobulin (Sandoz) had been given as part of the treatment but the IgG antiendothelial cell antibodies titre continued to rise long after the administration of gammaglobulin.

CYTOKINE STIMULATION EXPERIMENTS

After 24 hours of stimulation of the endothelial cells with recombinant interferon gamma a morphological change in the cells was seen with the endothelial cells becoming elongated. ELISAs

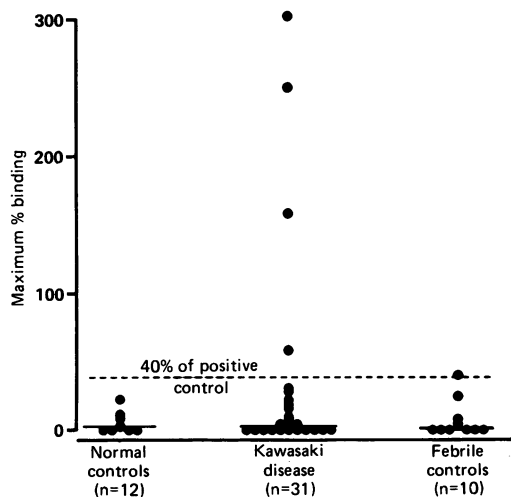


Figure 2 IgG antiendothelial cell antibodies in Kawasaki disease.

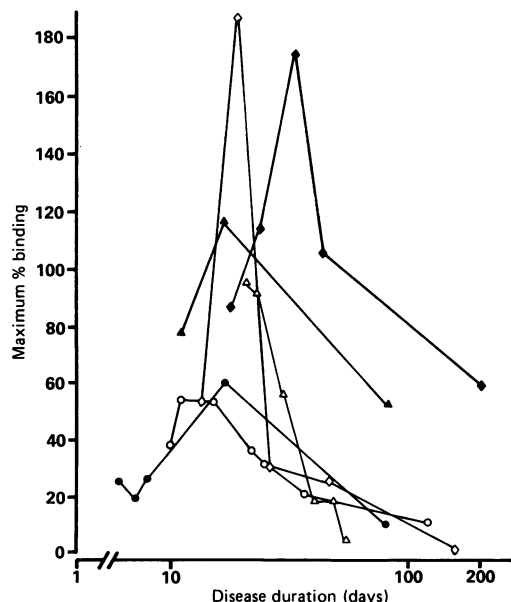


Figure 3 Serial IgM antiendothelial cell antibodies in six patients with Kawasaki disease.

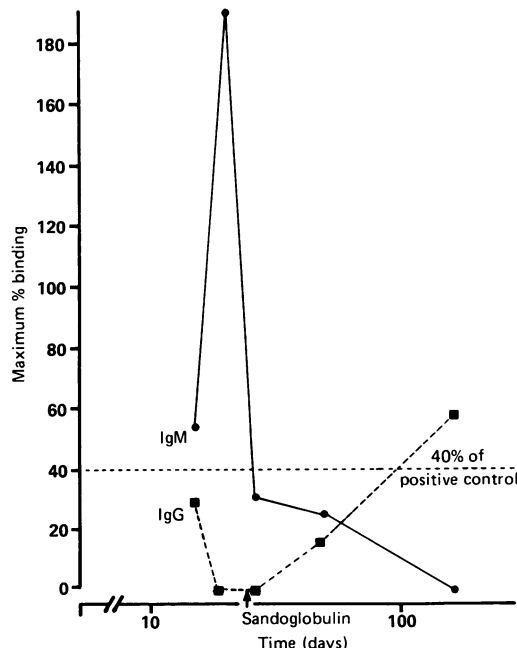


Figure 4 Serial IgG and IgM antiendothelial cell antibodies in one patient with Kawasaki disease.

using mouse monoclonal antibodies to class I antigens (W6 32) and class II antigens (Royal Free DR) confirmed significant stimulation of the endothelial cells in a dose related response (table 1). Similarly tumour necrosis factor was shown to be effective at 90 minutes incubation.

In 15 patients tested for IgM antiendothelial cell antibodies no enhancement of the binding was seen after prestimulation of the endothelial cells with interferon gamma. There was also no enhancement found in 10 patients tested for IgG antiendothelial cell antibodies. Three of four patients showed increased binding of polyvalent antisera after stimulation of the endothelial cells with interferon gamma. Two of four patients exhibited enhanced binding of IgG antiendothelial cell antibodies after a 90 minute prestimulation of the endothelial cells with tumour necrosis factor. This was not observed in four patients tested for IgM antiendothelial cell antibodies binding after stimulation by tumour necrosis factor (table 2).

Table 1 Expression of class I and II antigens correlated with dose and time of incubation with interferon gamma

Dose interferon gamma (U)	Optical density (SD)	
	Royal Free DR (class II)	W632 (class I)
Incubation time:		
24 Hours		
0	0.037 (0.101)	0.140 (0.014)
10	0.360 (0.048)	0.380 (0.046)
100	0.323 (0.037)	0.468 (0.062)
48 Hours		
10	0.568 (0.101)	0.389 (0.064)
100	0.773 (0.068)	0.421 (0.075)

Table 2 Effect of cytokine stimulation on binding of antiendothelial cell antibodies in Kawasaki disease

No of patients	Antibody + cytokine	Results
10	IgG + interferon gamma	No change
15	IgM + interferon gamma	No change
4	Polyvalent antisera + interferon gamma	Binding ↑ in three
4	IgG + tumour necrosis factor	Binding ↑ in two
4	IgM + tumour necrosis factor	No change

CLINICAL ASSOCIATION OF ANTI-ENDOTHELIAL CELL ANTIBODIES

Of the 15 patients with cardiac sequelae nine had raised titres of IgM anti-endothelial cell antibodies. Twelve patients without coronary artery lesions also demonstrated raised titres.

Discussion

Using a cellular based ELISA system we have been able to demonstrate antibodies, predominantly of the IgM class, which bind to the surface of umbilical vein derived human endothelial cells. These antibodies were found in children with Kawasaki disease, but not febrile controls or normals. Serial studies in six positive patients showed an association between the antibody titre and the stage of the disease and this may explain some of the negative results in patients either in whom samples were taken too early to detect antibody production or in whom sampling was delayed.

Previous work has suggested the presence of anti-endothelial cell antibodies in Kawasaki disease which are cytotoxic to endothelial cells prestimulated with cytokines, interferon gamma, tumor necrosis factor, and interleukin 1.^{8,9} It is of interest that we were unable to demonstrate enhancement of binding after prestimulation of the endothelial cells with interferon gamma and tumour necrosis factor in the majority of samples. This discrepancy may in part be explained by the method of calculating the binding index of the tested sera. In the calculation the binding of the negative sample is subtracted from that of both the positive and test sera. We observed an increase in the antibody binding (that is, optical density) of the samples after cytokine stimulation when compared with the identical samples on the unstimulated cells. Because of an equal increase in both negative and positive sera, however, the net binding observed was unchanged. This enhancement may be sufficient to cause the cytotoxicity seen in the experiments of Leung *et al.*^{8,9} Attempts were made to reproduce the cytotoxicity observed by Leung *et al.*, albeit by a slightly different technique, but unfortunately complement in the recommended dilution caused cell lysis. Further experiments are needed in this area. We do, however, feel that our results are consistent with those of Leung *et al.*

It might have been hoped that we would be able to demonstrate an association between anti-endothelial cell antibodies and serious cardiovascular sequelae. However, Kawasaki disease affects many other arteries and it is also possible that other factors are important such as anatomical variation that predispose certain vessels to be involved. Another reason for the lack of association between anti-endothelial cell antibodies and cardiac involvement may be due to the failure to collect samples at the peak of antibody production. The ELISA assay for detecting anti-endothelial cell antibodies may, however, be of particular use in identifying children with incomplete Kawasaki disease in whom the awareness of potential cardiac complications may be important.

The exact nature of the antigen against which the antibodies are directed is not yet known. It is possible that they are directed against epitopes on the as yet unidentified infectious agent and cross react with antigenic sites on the endothelial cell, thus leading to endothelial damage in the presence of cytokine stimulation. The identification of this

antigen might aid the discovery of the infecting agent or vice versa.

Recently the expression of endothelial cell activation antigens has been demonstrated in skin biopsy specimens of children with Kawasaki disease.¹² After treatment with gammaglobulin, which has been shown to reduce the incidence of coronary artery aneurysms in Kawasaki disease,^{13,14} Leung *et al* demonstrated the disappearance of endothelial activation in four of six patients.¹² In the remaining two the persistence of endothelial activation was associated with continuing symptoms. There also appeared to be an association between clinical improvement and reduction in cytokine secretion from peripheral blood mononuclear cells, and persistently high levels were associated with coronary artery abnormalities. Thus although we were able to demonstrate binding of anti-endothelial cell antibodies to unstimulated cells, it is likely that cytokines influence the cytotoxic effect of the anti-endothelial cell antibodies on the endothelial cells.

Taken in conjunction with the cytotoxicity studies of Leung *et al*^{8,9} it appears that these antibodies may have an important role in the pathogenesis of Kawasaki disease. The ELISA method is simpler than the cytotoxic assay, described by Leung *et al.*, for determining the presence of these antibodies and although anti-endothelial cell antibodies are not specific for Kawasaki disease, they may be a useful tool in understanding the pathophysiology of this and other forms of vasculitis.

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