Platelet immune complex interaction in pathogenesis of Kawasaki disease and childhood polyarteritis

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Abstract

The role of platelets in the pathogenesis of vasculitis and the formation of coronary artery aneurysms was studied in 19 children with Kawasaki disease and five with polyarteritis. All patients with Kawasaki disease developed thrombocytosis in the third week of illness. The peak platelet count was significantly correlated (p < 0.005) with the subsequent development of coronary artery aneurysms. The rise in platelet count was associated with the appearance in the circulation of a factor that induced aggregation and serotonin release in normal platelets. This factor was shown to be of high molecular weight, and its activity was lost at low pH—features suggestive of an immune complex. Immune complexes, detected by precipitation with polyethylene glycol, also appeared in the circulation as the platelet count increased. These complexes induced platelet aggregation, and there was a significant correlation (p < 0.001) between the concentrations of IgG and IgA in the polyethylene glycol precipitated material and the platelet aggregating activity. Similar platelet aggregating activity was also detected in patients with polyarteritis but followed a different time course, persisting in the circulation for several months in association with continued disease activity.

These findings imply that different mechanisms have a role in distinct phases of Kawasaki disease. The initial feverish phase (probably infective) is probably followed by an immune complex vasculitis that occurs when antibodies to the initiating agent appear in the circulation. The immune complexes aggregate platelets and induce release of serotonin. Platelet derived vasoactive mediators may increase vascular permeability and facilitate further deposition of complexes in the tissues.

Introduction

Kawasaki disease, or the mucocutaneous lymph node syndrome, is an acute, feverish illness affecting young children, with prominent mucocutaneous and cardiovascular manifestations. Since its description in 1967 many thousands of cases have been seen in Japan, and the disease is recognised increasingly often in many other countries. 1, 2

Kawasaki disease is often a benign and self limited illness. Arteritis affecting the extraparenchymal muscular arteries is, however, common and results in the development of coronary artery aneurysms in 20-60% of cases. 3, 4 Death due to coronary artery thrombosis, myocardial infarction, or aneurysm rupture occurs in 1-2%, of patients in the first three months of the illness, 3, 4-6 and there are reports of sudden death due to myocardial infarction occurring months to years after the initial illness. 3

Coronary artery aneurysms and myocardial infarction are most commonly detected after the second week of the illness and usually occur at a time when the fever and mucocutaneous manifestations are subsiding and the child appears to be convalescing. 3, 4 Thrombocytosis also occurs at this time, with the platelet count often exceeding 1000 × 10^9/L. 4 This puzzling temporal association of thrombocytosis with the development...
of coronary artery aneurysms and thrombosis led us to investigate the role of platelets in the pathogenesis of Kawasaki disease and also in the related disorder, childhood polyarteritis.

Patients and methods

Nineteen children fulfilling the criteria for Kawasaki disease laid down by the mucocutaneous lymph node syndrome study group were studied after admission to the Hospital for Sick Children, London, between June 1981 and June 1983. Five other children were studied in whom polyarteritis had been diagnosed on the basis of multisystem disease and the exclusion of other known infective, neoplastic, and immunological diseases. The diagnosis of polyarteritis was confirmed arteriographically in two of these patients and histologically in another two. Table I shows clinical details of both groups of patients.

| Table I—Clinical features of patients with Kawasaki disease or polyarteritis |
|----------------|----------------|
|                 | Kawasaki disease (n = 19) |
|                 | Polyarteritis (n = 5) |
| Median (range) age (years) | 1-3 (0-3-3) | 6-7 (1-6-14) |
| No of boys       | 12               | 1               |
| Median (range) duration of symptoms (days) before admission to: | 6 (1-15) | 32 (14-180) |
| Preston hospital | 13 (6-23) | 183 (30-260) |
| Hospital for Sick Children | 13 (6-23) | 183 (30-260) |
| No of children with: |              |
| Fever > 38°C     | 19              |
| Polymorphous rash| 15              |
| Mucous membrane changes | 18              |
| Conjunctival hyperaemia | 17              |
| Cervical lymphadenopathy | 15              |
| Generalised lymphadenopathy | 0              |
| Oedema and erythema of hands and feet | 18              |
| Vasculitic skin ulcers | 0              |
| Coronary aneurysms | 0              |
| Heart affected in other ways | 2              |
| Afferred kidneys  | 2               |
| Hypertension      | 8               |

Venous blood was collected from patients on admission and twice each week throughout the illness. Citrated plasma (one volume 3/8th, trisodium citrate to nine volumes blood) was prepared within one hour after collection by centrifugation at 1300 g for 15 minutes, and both serum and plasma were stored at −70°C until required. Similar samples were obtained from 30 healthy controls. Serial platelet counts were performed twice a week.

**DETECTION OF PLATELET AGGREGATING ACTIVITY**

Platelet aggregating material was detected in serum with a modification of the platelet aggregating titre of Penttinen and Myllä. Platelets were prepared from the blood of a group of Rh negative donor by centrifuging blood, collected into one tenth the volume of acid citrate dextrose, at 140 g for 15 minutes. The platelet rich plasma was layered on to a gradient of 10%, and 25%, metrizamide (Nyegaard Sigma) and centrifuged at 1000 g for 15 minutes. The plasma and 25% metrizamide were removed from above, and the 10% metrizamide from below, the layer of platelets. The platelets were recovered from the interface, resuspended in calcium free phosphate buffer pH 7.1, washed three times in the same way, and finally resuspended in calcium free phosphate buffer pH 7.1. Volumes of patients' or controls' serum of 100 µl were serially diluted in barbitone buffered saline pH 7.4 in U bottomed microtitre plates. Washed platelets, 50 µl, were then added to each well. After incubation at 4°C for 18 hours agglutination of platelets was detected macroscopically against a direct light source. Heat aggregated IgG was used as a positive control and buffered saline as a negative control, and normal control sera were included in each plate. Results were expressed as the log2 multiple of the highest titre at which aggregation was detected in normal serum.

In eight patients platelet aggregating activity was also determined in plasma using a modification of the dual channel aggregometer coupled to a Rikadenki recorder. Test plasma 200 µl was added to 200 µl washed normal platelets (final platelet count 150-300 × 10^9/l) in an aggregometer cuvette. The mixture was stirred at 900 rpm for five minutes and the aggregation recorded. We determined the effect on aggregation of prostacyclin 10 ng/l, albumin 2 g/l, 5 mM edetic acid, and 2.5 mM calcium solution. Plasma from a control was tested simultaneously in each experiment.

**RELEASE OF SEROTONIN**

The effect of patients' or controls' plasma on serotonin release from normal platelets was assessed as follows: platelets from a control were washed with metrizamide and labelled at room temperature with serotonin, labelled with carbon-14, (5-hydroxytryptamine), side-chain-2(14C) creatinine sulphate (Amersham International), specific activity 222 × 10^3 Bq (60 mCi)/mmol, final concentration 0.84-0.95 µM. Labelled platelets 200 µl were added to test or control plasma 200 µl to give a final platelet count of 200-300 × 10^9/l. The proportion (q) of 14C labelled serotonin released into the surrounding medium after stirring for three minutes in the aggregometer was determined as follows: 100 µl of the platelet suspension was added to 400 µl 1.5%, formaldehyde at 0°C and centrifuged for 30 seconds in an Eppendorf microcentrifuge; supernatant 200 µl was transferred to scintillation fluid 2.5 ml for counting on an LKB β-counter.

**ISOLATION OF PLATELET AGGREGATING MATERIAL**

Serum or plasma from two patients with Kawasaki disease and one with polyarteritis, each of whom had a greatly raised platelet aggregating titre, was fractionated by sucrose density gradient ultracentrifugation. The test samples were diluted with 12 volumes of phosphate buffered saline pH 7.2 and ultracentrifuged at 150 000 g for 18 hours at 4°C in an MSE 65 prepin ultracentrifuge on a linear 10-40% sucrose gradient. IgG and IgM were used as external markers. Fractions were collected by downward displacement, diluted to the original volume with phosphate buffered saline, and then tested for platelet aggregating activity on both the platelet aggregating titre test and aggregometry. Fractions were also prepared after acidification to pH 3.0 and 0.1M glycine hydrochloride. Fractions prepared at low pH were neutralised with 0.2M potassium phosphate before assay.

**POLYETHYLENE GLYCOL PRECIPITATION OF IMMUNE COMPLEXES**

Serum was incubated with polyethylene glycol (20%, final concentration) at 4°C for 12 hours. The precipitate was washed with 2% polyethylene glycol, resuspended in the original volume of phosphate buffered saline, and tested for platelet aggregating activity. The IgG, IgA, IgM, and IgD content of the polyethylene glycol precipitate was measured by an enzyme linked immunosorbent assay (ELISA) technique. After incubating the precipitate with specific anti-immunoglobulin labelled with peroxidase the unbound antibody was measured by its binding to immunoglobulin coated ELISA plates. Standard curves were determined using known quantities of immunoglobulin for each ELISA plate.

**Results**

**SERIAL PLATELET COUNTS**

All 19 children with Kawasaki disease developed thrombocytosis (platelet count 500 × 10^9/l) in the second and third weeks of illness as the fever and mucocutaneous manifestations subsided. Figure 1 shows the course in a representative patient and figure 2 serial platelet counts for all 19 patients. After reaching a peak in the third or fourth week (often exceeding 1000 × 10^9/l) the platelet count fell rapidly in the fifth week and then declined gradually towards normal in the subsequent weeks.

Cross sectional echocardiography was undertaken each week in all patients with Kawasaki disease, and the peak platelet count was significantly higher (P < 0.005; Student's t test) in those who developed aneurysms than in those who did not (fig 3).

Of the patients with polyarteritis, four developed thrombocytosis and one thrombocytopenia. The platelet counts in the patients with polyarteritis, however, showed a persistent mild increase for many months rather than the transient rise seen in Kawasaki disease.
The aggregation was blocked by the addition of prostacyclin (10 ng/l) or edetic acid (5mM) but was unaffected by the addition of albumin. Increasing the calcium concentration to 2.5mM enhanced the aggregation. No aggregation was detected on addition of normal control plasma to either normal or patients' washed platelets.

**RELEASE OF SEROTONIN**

Plasma from two patients with polyarteritis and three with Kawasaki disease was tested and in each case induced release of ^14^C labelled

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**DETECTION OF PLATELET AGGREGATING ACTIVITY**

The rise in platelet count was closely associated with a rise in serum platelet aggregating activity (as detected by platelet aggregating titre) in patients with Kawasaki disease (fig 2) and in four of the five with polyarteritis. The peak level of platelet aggregating material coincided with a peak in the platelet count, and platelet count and platelet aggregating titre were significantly correlated (p<0.001; Spearman rank test). In contrast with the transient rise in platelet aggregating material seen in Kawasaki disease, the increase in platelet aggregating titre persisted in the patients with polyarteritis, often for many months in association with continued disease activity.

Plasma from each of the eight patients tested (four with polyarteritis and four with Kawasaki disease) aggregated washed normal platelets on stirring in the aggregometer. Figure 4 shows a typical example.
serotonin from labelled normal platelets (table II), whereas release did not occur using normal plasma.

**ISOLATION OF PLATELET AGGREGATING MATERIAL**

The fractions of serum and plasma from patients with Kawasaki disease or polyarteritis prepared by sucrose density gradient ultracentrifugation were tested for platelet aggregating activity by both the platelet aggregating titre test (serum) and aggregometry (plasma). Aggregating activity was detected in the high molecular weight fractions (>-19 S) prepared at neutral pH. Fractions prepared under dissociating conditions for immune complexes at pH 3 contained no aggregating activity (fig 5).

**TABLE II—Release of serotonin, labelled with carbon-14, from labelled platelets induced by plasma from normal subjects or patients with polyarteritis or Kawasaki disease.**

<table>
<thead>
<tr>
<th>Serotonin release (%/a)</th>
<th>IgG complexities (% of normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled normal plasma</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Normal control plasma</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Plasma from patients with Kawasaki disease:</td>
<td></td>
</tr>
<tr>
<td>(Case 1)</td>
<td>27</td>
</tr>
<tr>
<td>(Case 2)</td>
<td>21</td>
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<tr>
<td>Plasma from patients with polyarteritis:</td>
<td></td>
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<tr>
<td>(Case 1)</td>
<td>20</td>
</tr>
<tr>
<td>(Case 2)</td>
<td>28</td>
</tr>
<tr>
<td>(Case 3)</td>
<td>39</td>
</tr>
</tbody>
</table>

**FIG 5—Fractionation of serum from patients with Kawasaki disease by sucrose density gradient ultracentrifugation. IgM and IgG were used as molecular weight markers. Aggregating activity was detected in fractions 1-6 (molecular weight > IgM) separated at pH 7 (●). No aggregating activity was detected in fractions prepared at pH 3 (●).**

The precipitate of serum from patients with Kawasaki disease or polyarteritis obtained after incubation with 2°, polyethylene glycol induced platelet aggregation when tested by the platelet aggregating titre test in the presence of calcium (n=5). IgG immune complexes were raised in 13 (68%) of 19 tests in patients with Kawasaki disease. There was a highly significant correlation (p<0.001; Spearman rank correlation) between the rise in platelet aggregating titre and the rise in IgG complexes (fig 6). IgA complexes were detected in 85% of patients with Kawasaki disease, and there was a significant correlation between the presence of IgA complexes and the rise in platelet aggregating titre (p<0.001). A similar correlation was not observed for IgM (raised in 10 patients (55%) or IgE complexes.

All patients with polyarteritis had appreciably raised concentrations of IgG, IgA, and IgM complexes. The concentrations of IgG and IgM complexes were significantly higher in the patients with polyarteritis than in those with Kawasaki disease.

**Discussion**

The pathogenesis of Kawasaki disease remains unknown despite its worldwide occurrence and the intensive investigation of many thousands of cases. The clinical features of the illness, with fever, leucocytosis, and raised acute phase reactants, suggest an infective process, a possibility that is supported by the occurrence of epidemics,13 geographical and temporal clustering,14 seasonal variation,15,16 and occasional simultaneous occurrence in siblings.14 Despite recent reports, however, implicating a variant strain of the aerobic organisms Propionibacterium acnes transmitted by a mite18 and earlier studies suggesting infection with rickettsia like organisms19 20 attempts to isolate the causative agent have been unsuccessful. Furthermore, the failure to respond to antibiotics (including those active against rickettsia and anaerobes), the lack of case to case transmission,12 13 and the progression of the disease despite negative cultures suggest that factors other than infection play a part.

An alternative hypothesis is that the disease is due to an immunological reaction to either a ubiquitous micro-organism or some other environmental agent. This is supported by the systemic vasculitis, a reported association with atopy, raised IgE concentrations,14 and the presence of immune complexes,18 19 20 which have been detected in some but not all studies.21

A role has been suggested for platelets in the pathogenesis of the lesions in the coronary arteries and other vessels: platelets are hyperaggregable in both acute and convalescent phases of the disease,21 22 and circulating in vivo platelet aggregates have been detected42 as have raised plasma concentrations of β thromboglobulin,22 platelet factor 4,23 and thromboxane B2.24 The mechanisms by which platelets take part and their relation to the immunological or infective processes have not been documented previously.

Our findings suggest that both infective and immunological processes are important in the pathogenesis of Kawasaki disease but that they occur at different phases of the illness and that interaction between immune complexes and platelets may be important in mediating the vascular complications.

The thrombocytosis occurring in the third to fifth weeks of the illness is associated with the appearance in the circulation of material that induces aggregation and release of serotonin from normal platelets and is highly correlated with both the platelet aggregating titre and the subsequent development of coronary artery aneurysms. The platelet aggregating material is of high molecular weight (>19 S), and labile at low pH, all features suggesting an immune complex. Immune complexes also appear in the circulation simultaneously with the platelet
aggregating activity, and there is a highly significant correlation between the amount of IgG and IgA that can be precipitated with polyethylene glycol and the platelet aggregating activity. Similar platelet aggregating material is also detected in patients with polyarteritis but follows a different time course, persisting in the circulation for months or years in association with continued disease activity.

These findings suggest that Kawasaki disease has three distinct pathophysiological phases. During the first fever, lymphadenopathy, and mucocutaneous manifestations occur, the platelet count is normal, and neither platelet aggregating activity nor immune complexes are detectable in the circulation.

In the second, in the third to fifth weeks, when the fever and systemic symptoms are subsiding and desquamation has started, a dramatic rise in the platelet count occurs and platelet aggregating material and immune complexes become detectable. (It is during this phase that aneurysms are most often found and death from coronary artery thrombosis is most likely to occur.1,2) Finally, the platelet count decreases and platelet aggregating material and immune complexes become undetectable. Patients who have survived the first two phases generally recover, but those with coronary artery aneurysms remain at risk from late complications of myocardial infarction or coronary thrombosis.

Although the features of the first two weeks of Kawasaki disease suggest an infective origin for this feverish phase, the subsequent temporal relations are similar to those of experimental immune complex vasculitis, in which platelets play an important part in starting the vascular damage.25-27 In the serum sickness model (in which the coronary artery is often affected) antigen antibody complexes are formed in the second week after injection of heterologous protein and interact with platelets to cause aggregation and release of vasoactive mediators such as histamine and serotonin.28 These mediators increase vascular permeability and permit deposition of immune complexes in the subendothelial tissues.25-28 The pathologic importance of platelet derived vasoactive substances has been shown by the enhancing effect of histamine or serotonin on immune complex deposition in the tissues27 and the inhibition of immune complex deposition and tissue damage by platelet depletion and histamine or serotonin antagonists.25-27,28 Ultimately, continued production of antibodies results in elimination of the antigen and resolution of the vasculitis.

The association of thrombocytopenia with the presence of circulating platelet aggregating material is paradoxical. Aggregated or damaged platelets are normally rapidly removed from the circulation by the reticuloendothelial system, and thus thrombocytopenia rather than thrombocytosis might have been the expected consequence. In animal models saturation of the reticuloendothelial system appreciably delays the clearance of injected colloidal carbon aggregates.29 Similar observations have been made in patients with immune complex diseases in whom delayed reticuloendothelial system clearance of heat damaged or IgG coated red cells has been shown.30 Small immune complexes may saturate the reticuloendothelial system and prevent the clearance of larger immune complexes31 or platelet aggregates. Thrombocytopenia may therefore be an indication of saturation of the reticuloendothelial system by immune complexes, a possibility substantiated by the positive correlation between platelet count and immune complex titles. An additional possibility is that thrombocytosis may be stimulated by a product released from aggregated platelets, such as platelet derived growth factor.

The question of whether Kawasaki disease, infantile polyarteritis, and adult polyarteritis represent different diseases or overlapping end results of the same process has caused considerable debate.32 Our findings suggest that interactions between immune complexes and platelets are important in both disorders. In contrast, however, with the transient rise and fall of platelet aggregating complexes in Kawasaki disease, polyarteritis the platelet aggregating immune complexes persist in the circulation for months or years, in association with active disease. Whether this persistence is due to a defect in immune elimination of antigen or to a continuing antigenic exposure is not known. A key question requiring further study is whether Kawasaki disease is due to a single (infective) antigen or an immune response to various antigens and whether these antigens are distinct from those in polyarteritis.

Finally, the pathogenic mechanisms we have described have several clinical and therapeutic implications. Patients with the highest platelet counts also have the highest titres to immune complexes and are most likely to develop coronary artery aneurysms. The peak platelet count may therefore be a marker of severe disease and could be used to identify a group of patients requiring more aggressive treatment. Antiplatelet agents may be beneficial in reducing not only the risk of thrombosis but also the severity of the vasculitis.

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