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INTERACTION BETWEEN HUMAN BLOOD PLATELETS, VIRUSES AND ANTIBODIES

IV. POST-*RUBELLA* THROMBOCYTOPENIC PURPURA AND PLATELET AGGREGATION BY *RUBELLA* ANTIGEN–ANTIBODY INTERACTION

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SUMMARY

A new method of measuring antibodies by observing sedimentation patterns of platelets has been compared with the complement fixation and haemagglutination inhibition techniques in ten cases of *Rubella* and seven cases of post-*Rubella* thrombocytopenic purpura. The method is based on the aggregation of platelets by the joint action of antibody and small size antigens. The platelet aggregation method gave exceptionally high titres in cases of post-*Rubella* thrombocytopenic purpura. Other serologic methods did not give these high titres. The hypothesis that small size virus antigen and antibody against it are both needed to induce thrombocytopenia during the recovery period is discussed. Large amounts of both may result in clinical symptoms.

INTRODUCTION

Post-infectious thrombocytopenic purpura seems to be a clinical entity and distinct from other haemorrhagic complications of virus diseases. This type of thrombocytopenia may follow acquired post-natal *Rubella* (Ackroyd, 1953; Tadzen, 1958; Adkins & Fernbach, 1965; Bayer *et al.*, 1965; Morse, Zinkham & Jackson, 1966; Staub, 1968). The thrombocytopenic purpura in *Rubella* develops abruptly during the 1st week after the rash, and usually disappears within a few weeks, but it may last for months. Although post-infectious thrombocytopenic purpuras are a rare complication of *Rubella* or any other virus infection, they form a major part, up to 80%, of 'idiopathic' thrombocytopenic purpuras in childhood (Bentegeat *et al.*, 1965, Luscher & Zuelzer, 1966).

An increased destruction of circulating platelets seems to be the most important pathogenic mechanism in post-infectious thrombocytopenia. Injury to platelets may be caused by a direct effect of virus on platelets or by some immunological mechanism (Bayer *et al.*, 1965;

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Morse *et al.*, 1966; Staub, 1968). Neither the time of onset nor the duration of thrombocytopenia in *Rubella* suggest that damage to platelets is directly caused by virus. Thrombocytopenia develops at about the same time as the specific antibodies appear in the circulation.

Thrombocytopenic purpura that appears to be caused by viral infections is usually transient. However, apart from the case history, there is no method of separating them clinically from other forms of acute idiopathic thrombocytopenic purpura, and it has not been possible to confirm the causal relationship between infection and the following thrombocytopenic purpura. This paper will describe an approach to differential diagnosis by a new laboratory method.

We found recently (Penttinen & Myllylä, 1968) that certain small size ('soluble') virus antigens together with antibodies against them aggregate human platelets very effectively *in vitro*. This gave us the opportunity to study whether there are differences in this kind of platelet aggregating antibodies (which act jointly with antigens) in thrombocytopenic purpura and uncomplicated *Rubella*, and to characterize the antibodies and antigens concerned to some extent.

MATERIALS AND METHODS

Rubella antigens

Suspension cultures of BHK21/13S cells infected with the RA 27/3 strain of *Rubella* virus (Vaheri, Sedwick & Plotkin, 1967) were used. For the platelet aggregation by antigen–antibody interaction (PA) and complement fixation (CF) tests the antigen was prepared from infected cells by sonication or alkaline extraction (Halonen *et al.*, 1967), or from the cell-free supernatant by thirty-fold concentration using forced dialysis against a vacuum. In the early studies the antigens were used without clarification (Furukawa, Vaheri & Plotkin, 1967). In later preparations the antigens were clarified by (low-speed) centrifugation. This removed most of the direct aggregating activity on thrombocytes caused apparently by coarse BHK21 cell debris. *Rubella* virus was removed from some small size antigen preparations by three consecutive sedimentations at 40,000 *g* for 120 min. In most of the present experiments the antigen was a sonicate of 10% BHK21/13S packed cells in Dulbecco's phosphate-buffered saline. Identically prepared control antigen preparations of non-infected BHK 21/13S cells had no PA or CF activity with *Rubella* sera. The haemagglutinating antigen was prepared by Tween-ether treatment of virus-containing tissue culture fluid.

Titrations

Rubella haemagglutination inhibition test: according to the modification by Halonen *et al.* (1967).

Rubella complement fixation test: as originally described by Sever *et al.* (1965).

Platelet aggregation test: according to the micromethod described earlier (Penttinen & Myllylä, 1968) using various serum and small size antigen dilutions. The test is based on the sedimentation pattern of a fresh suspension of washed human platelets at +4°C on microtitre U-plates. In the early studies sera were both heat-inactivated and kaolin-treated before test. The kaolin treatment was found to be unnecessary if alkaline buffer (pH 7·8) was used as diluent. A study of the factors affecting the PA test will be published separately

(Myllylä & Penttinen, 1969). The PA titre is the highest dilution of serum that, together with the optimum concentration of small size *Rubella* antigen, induces platelet aggregation.

Sedimentation analysis

The separation of IgM and IgG *Rubella* antibodies by sedimentation in sucrose gradients was described earlier (Vesikari & Vaheri, 1968).

Analysis of homogenates of *Rubella*-infected BHK21 cells by gradient centrifugation is described in the text.

Patients

Normal population: Sera of fifty apparently healthy 15-year-old school girls were collected in March 1967 and stored at −20°C until titration.

Rubella patients: Serial serum samples were taken from three *Rubella* cases (age 3–5 years) at the children's hospital Lastenlinna, and seven at an Army base near Helsinki.

Congenital Rubella patients: Serum specimens were obtained from five cases of congenital infection; the diagnosis was verified by virus isolation.

Patients with post-Rubella thrombocytopenic purpura: The seven patients were admitted to various local hospitals during 1967–68 because of thrombocytopenic purpura. All of them had had a preceding infection, which was classified clinically as *Rubella*. In all cases serological tests suggested a recent *Rubella* infection. Haemorrhagic manifestations appeared 2–10 days after the rash. The lowest platelet counts ranged between 1600 and 18,000, and the duration of thrombocytopenia varied from 2 weeks to 3 months. Aspirated bone marrow contained normal or slightly increased numbers of megakaryocytes in all cases. Steroids were given to the two patients with the most severe bleeding tendency.

RESULTS

Comparison of the serum PA, haemagglutination inhibition (HI) and CF titres in cases of Rubella and post-Rubella thrombocytopenic purpura

We studied the development of PA, HI and CF activity in ten cases of acute *Rubella* infection. The results are shown in Figs. 1, 2 and 3, where each measurement is plotted on a time–titre scale. The HI and CF antibodies appeared as described previously (e.g. Lennette *et al.*, 1967), i.e. HI titres rose rapidly from zero to a high value, whereas CF titres usually increased more slowly. In a few cases low CF activity was detected as early as at the onset of the rash. PA activity followed the CF rather than the HI titre. However, the PA titres were about five times higher than the CF titres, which suggests that the PA test is more sensitive than the CF test.

Serum samples were obtained from seven convalescent cases of clinically overt post-*Rubella* thrombocytopenic purpura. The HI and CF titres were well within the normal range, but the PA titres were consistently at the upper limit of normal variation (Figs. 1, 2 and 3). Thus it appeared that an increase in the serum PA titre was associated with post-*Rubella* thrombocytopenic purpura. However, we did not find any such correlation in sporadic serum samples from cases of congenital *Rubella* with thrombocytopenic purpura, in which low PA as well as low CF titre at birth were detected.

Comparison of Rubella PA, HI and CF titres in the normal population

We studied serum samples from fifty normal 15-year-old girls to evaluate the *Rubella* PA test as a measure of *Rubella* immunity. The results in Table 1 show that the PA test

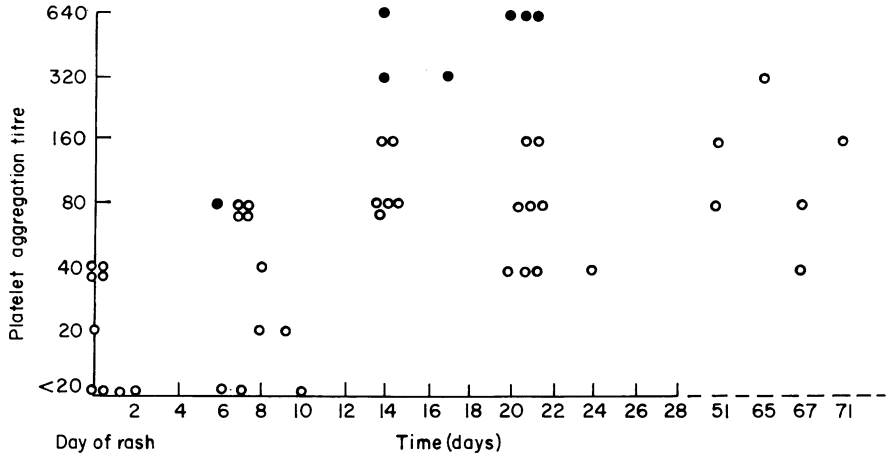

FIG. 1. Platelet aggregation titres in cases of acquired *Rubella* (○) and post-*Rubella* thrombocytopenic purpura (●).

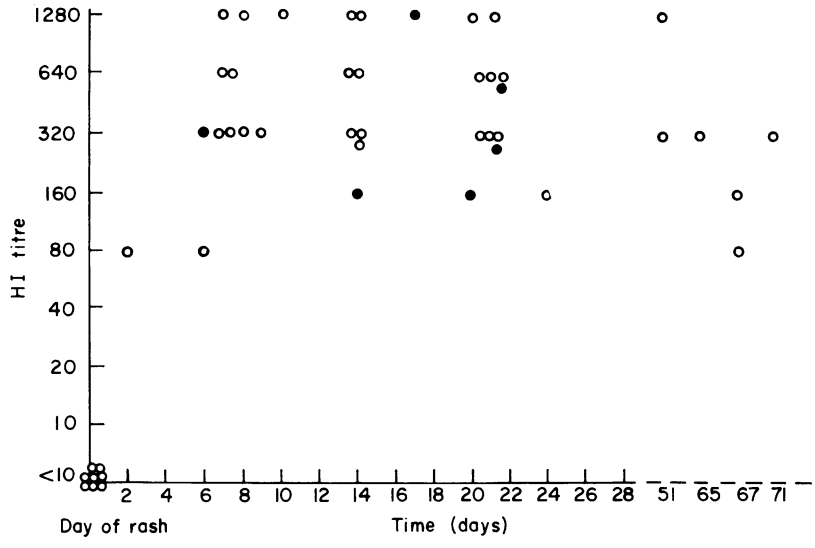

FIG. 2. HI titres in cases of acquired *Rubella* (○) and post-*Rubella* thrombocytopenic purpura (●).

was as sensitive as the HI test. However, the CF test using 2 units of complement, as was expected from earlier studies, gave a considerably smaller number of seropositives. The coincidence of sera positive in both the PA and HI tests was complete in this small series. In no case was the CF test positive in HI-negative sera. In positive cases the serum titres

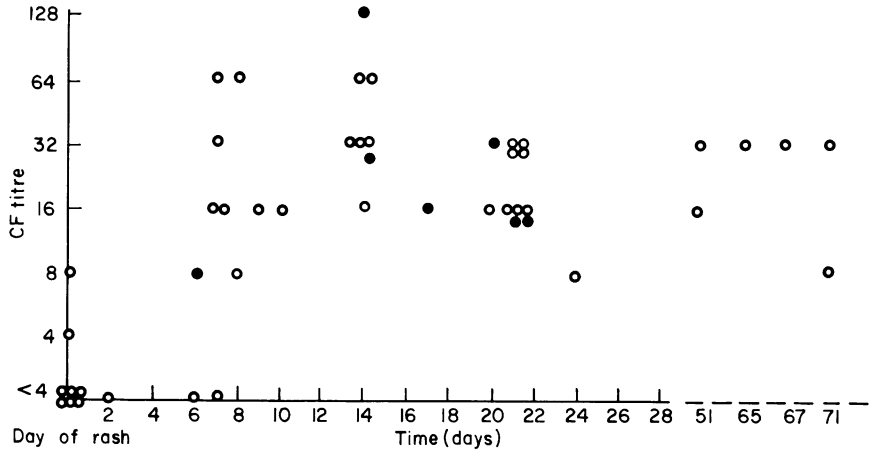

FIG. 3. CF titres in cases of acquired *Rubella* (○) and post-*Rubella* thrombocytopenic purpura (●).

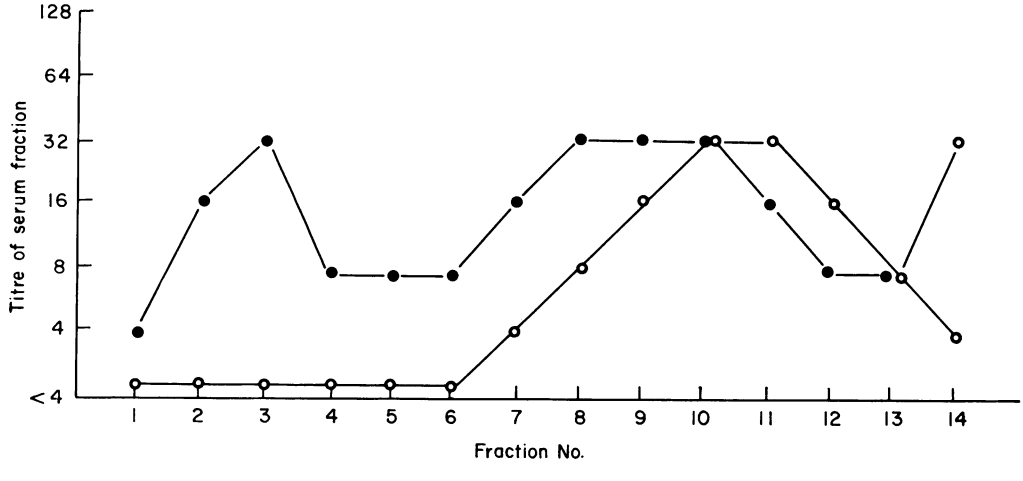

FIG. 4. Fractionation of serum sample from post-*Rubella* thrombocytopenic purpura patient (A.A.) by sedimentation analysis in sucrose gradients. ●, Titre of serum fraction in HI test; ○, titre of serum fraction with optimal antigen dilution in PA test. 37–12·5% w/v, Spinco SW65 Rotor at 35,000 rev/min for 14·5 hr at +4°C.

TABLE 1. Incidence of *Rubella* antibodies among a group of normal 15-year-old girls as by HI, CF and PA techniques

Total No. studied	50	
Positive in HI (titre >10)	36	72%
Positive in CF (titre ⩾ 4)	17	34%
Positive in PA (titre >10)	36	72%

in the PA test varied between 1:40 and 1:320. These findings suggest that antibodies against small size *Rubella* antigens persist for a long time in the serum.

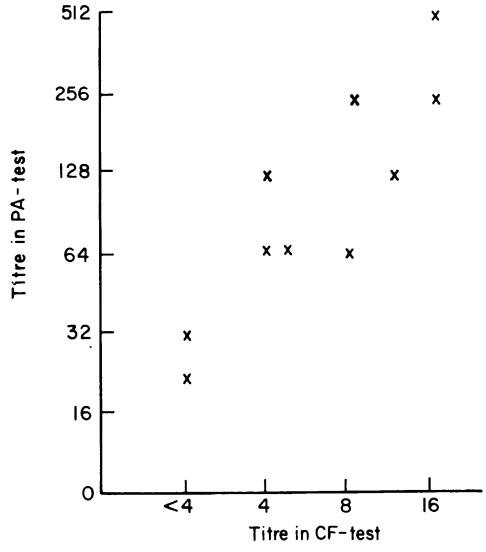

FIG. 5. Comparison of the PA-titres of different antigen lots to their CF-activities.

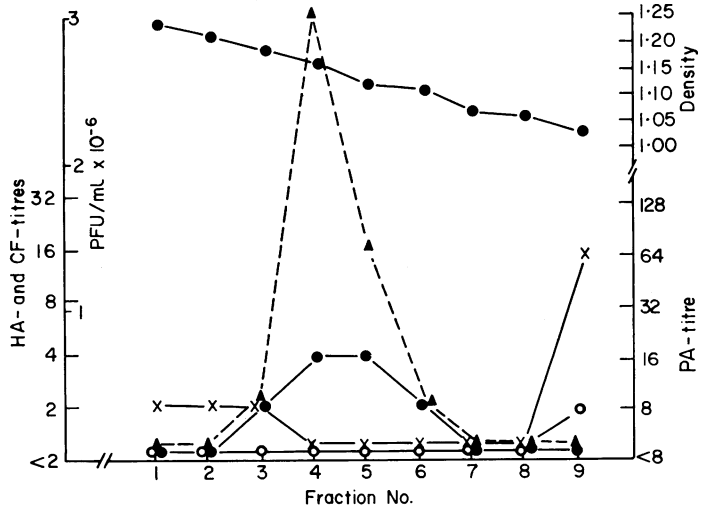

FIG. 6. Sedimentation analysis of a homogenate of *Rubella* infected BHK21 cells. A 0·5-ml sample was layered on top of a 4·2-ml linear 5–50% sucrose gradient, buffered with 0·05 M-Tris -Cl, 0·001 M-EDTA, pH 7·3. After centrifugation for 60 min at 35,000 rev/min in a Spinco SW65 rotor the fractions were collected dropwise through a hole punctured in the tube bottom. ●, HA-titre; ○, CF-titre; ×, PA-titre; ▲, PFU-titre.

Comparison of IgG and IgM activities in the PA, HI and CF tests

In order to characterize the antibodies involved in the platelet-aggregation reaction, sera were fractionated in sucrose gradients, and the PA and HI activities of the fractions were subsequently determined. Fig. 4 shows the sedimentation analysis of serum from a patient 3 weeks after post-*Rubella* thrombocytopenic purpura. *Rubella* HI antibodies were found in both the IgM and IgG fractions (Vesikari & Vaheri, 1968), whereas all the PA activity seemed to be associated with the IgG region. The CF activity also seemed to be located solely in the IgG fraction, but the test was unreliable because the sera fractionated in our gradients were highly anti-complementary. Essentially similar results have been obtained with five other sera.

Comparison of the activity of the antigen preparations in PA, CF, haemagglutination (HA) and infectivity tests

Slight direct thrombocyte-aggregating activity (without antibody) was detected in our early *Rubella* antigen and control antigen preparations. This could be removed by low-speed sedimentation (see 'Materials and methods'). Antigens with high HA activity did not cause platelet aggregation. Thus in our conditions *Rubella* virus did not aggregate platelets as for instance myxo- and reoviruses do (Penttinen & Myllylä, 1969).

A positive correlation between the PA and CF activities was evident in the different lots of antigen (Fig. 5).

High speed centrifugation (40,000 *g* for 180 min) of crude *Rubella* antigen preparations, such as cell homogenates or thirty-fold concentrates of medium, removed 95% of viral infectivity and HA activity. This did not appreciably alter the activity in the PA test. Newcastle Disease virus antigen preparations give similar results (Penttinen & Myllylä, 1968).

Sedimentation analysis by rate zonal centrifugation (Fig. 6) also separated the two properties of *Rubella* virus particles—HA and infectivity—from the small size antigen fraction containing the PA and CF activities.

DISCUSSION

Thrombocytopenic purpura may form part of the congenital *Rubella* syndrome and here defective platelet production is apparently the main cause (Banatvala, 1965; Bayer *et al.*, 1965; Vossaug *et al.*, 1966). Thrombocytopenic purpura associated with some acquired virus diseases, such as dengue fever, has also been ascribed to suppression of megakaryocytes (Nelson & Bierman, 1964). However, in patients with post-*Rubella* thrombocytopenic purpura, as in most other types of post-infectious thrombocytopenic purpura, increased disappearance of circulating platelets seems to be the main pathogenic event. This is indicated by the very short survival of transfused platelets in these patients (Hirsch & Gardner, 1952; Morse *et al.*, 1966).

The basic mechanism of the increased destruction of platelets is not known. It has been thought that circulating viruses might damage platelets and shorten their life-span. Although this may be important in other haemorrhagic complications of viral diseases it does not seem to be the main cause of injury in post-infectious thrombocytopenic purpura. In *Rubella* viraemia can be detected between 7 days before and 2 days after the rash (Green *et al.*, 1964). However, purpura appears 2–14 days after the rash, and the degree of thrombo-

cytopenia does not appear to be related to the severity of the preceding infection (Ackroyd, 1953; Lokietz & Reynolds, 1965; Adkins & Fernbach, 1965; Morse *et al.*, 1966). These facts together with the long duration of thrombocytopenic purpura do not support the concept of a direct virus effect on platelets. In addition the incidence of post-infectious thrombocytopenic purpura is not correlated with the affinity of the viruses for platelets (Penttinen & Myllylä, 1969).

It has also been suggested that platelets are destroyed by virus–antibody interaction when virus particles are adsorbed onto their surface. However, we have demonstrated that specific antibodies, when added to purified virus particles, do not increase platelet aggregation *in vitro* but inhibit it, as they do virus haemagglutination (Penttinen & Myllylä, 1968). We found that certain small size virus antigens, together with their specific antibodies, interact with platelets and produce platelet aggregation *in vitro*. This interaction might also cause platelet damage *in vivo*. Such a mechanism would be analogous to that in quinidine purpura, with the small size virus antigen in place of the haptenic drug (Shulman, 1963). In the present study we found higher titres of PA antibodies in patients with post-*Rubella* thrombocytopenic purpura than in patients with uncomplicated *Rubella*. The HI and CF titres were of the same order in both groups. The higher PA antibody titres in the thrombocytopenic group were not due to variations in the residual serum prothrombin concentration (Myllylä & Penttinen, 1969) nor otherwise connected with thrombocytopenia *per se*, but were due to specific antibody. This is indicated by studies of a great number of sera from many kinds of thrombocytopenias. Sera from patients with post-*Rubella* thrombocytopenic purpura aggregated platelets only occasionally and in low titres with other virus antigens (e.g. herpes and cytomegalovirus). On the other hand, sera from patients with thrombocytopenic purpura following other infections aggregated platelets only sporadically in low titres with *Rubella* antigen.

After *Rubella* PA and CF titres increased at the same time (Fig. 1). However, CF and PA titres in individual sera were not always correlated. It could suggest that two distinct antibodies are involved. PA activity was found only in the antibodies of IgG region in the six cases so far studied and not in IgM (Fig. 4). This finding is interesting but its significance is uncertain so far. However, complement-independent opsonization of antigens for attachment to macrophages is mediated only by IgG antibodies (reviewed by Rabinovitch, 1968). The interaction of virus antigen–antibody complexes with human platelets in the PA test, which is independent of complement *in vitro* at least, may involve the same kind of mechanism. Further comparisons with the opsonizing IgG antibodies and more detailed characterization of PA antibodies remain to be done.

The small size viral antigen which is active in the PA test seems to be easily separable from the infective and haemagglutinating particle, and to be similar to small size *Rubella* CF antigen (Schmidt, Lennette & Gee, 1966). A more detailed analysis of small size *Rubella* PA, CF and immunoprecipitin antigens, their inter-relationships and formation in *Rubella*-infected BHK21 cells will be of interest.

The PA test can be used to detect small size virus antigens, and its major advantage over the CF test is the great sensitivity (Table 1 and Figs. 5 and 6). It appears that one CF unit equals approximately 10 PA units of *Rubella* antigen. It is somewhat surprising that the PA test is as sensitive as the HI test, which involves antibodies against viral surface proteins. Sera positive in the PA test were also positive in the HI test. This indicates the great specificity of the PA test.

According to our concept of platelet damage in post-*Rubella* thrombocytopenic purpura the time of onset and duration of purpura would depend on the availability of antibodies in the circulation. The antibody titres in thrombocytopenic purpura and uncomplicated cases of acquired *Rubella* suggest that the severity of thrombocytopenic purpura depends on the magnitude of the antibody response. Thus thrombocytopenic purpura is connected with a certain type of hyperimmune state if the results of our small series have general validity. According to our concept small size virus antigens as well as antibody are required for platelet destruction, and the duration of thrombocytopenic purpura would depend on persistence of both. Although very little is known about the fate of viral antigens in the organism it is possible that they occur in the circulation during active virus disease. Accordingly a slight decrease in platelet count should occur often during active diseases, when there are the necessary antigens and antibodies in the circulation. Moderate thrombocytopenia without clinical symptoms is in fact a common phenomenon in patients with *Rubella* (Ackroyd, 1953; Tadzen, 1958; Wallace, 1963). However, in the case of post-*Rubella* thrombocytopenic purpura the persistence of small size virus antigens for weeks and sometimes even for months must be postulated. Evidence suggesting that this is so in the more chronic forms of common virus diseases has been presented. For example the subacute sclerosing panencephalitis, recently described as a possible late complication of measles, suggests the production of measles antigens years after the acute disease.

It is of interest that sera from patients with the thrombocytopenic congenital *Rubella* syndrome had only low titres of PA antibodies. This also suggests differences in the mechanism of thrombocytopenia in acquired and congenital *Rubella*, as do the bone marrow findings. Our results with congenital *Rubella* patients are in contradiction to those of Bayer *et al.* (1965). They found that the sera of patients with thrombocytopenic congenital *Rubella* aggregated platelets both with and without *Rubella* virus. The reason for this discrepancy is not evident, but the quality of the platelet aggregating activity was apparently different in their conditions.

In the patients with post-*Rubella* thrombocytopenic purpura there was no evidence of any changes in coagulation factors which would suggest intravascular coagulation (Tadzen, 1958). Intravascular coagulation is known to have a central role in the production of other severe and more acute haemorrhagic complications of many virus diseases (McKay & Margaretten, 1967). Perhaps the slighter injury to platelets caused by antigen–antibody interaction in post-infectious thrombocytopenic purpura is sufficient to shorten platelet life-span but not to initiate intravascular clotting. However, large amounts of antigen–antibody complexes are known to cause consumption coagulopathy, and therefore their role in this syndrome should be taken into account.

The very recent studies of S. B. Halstead (Eighth International Congress on Tropical Medicine and Malaria, September 1968, Teheran) on the occurrence of dengue haemorrhagic fever suggested the hyperimmune nature of the syndrome. It seemed to occur particularly when abundant antibodies and virus antigens were simultaneously present.

Whether virus antigen–antibody interactions are important in other manifestations and late complications of virus illnesses may be worth considering.

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