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The platelet aggregation test in group B arbovirus infections

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SUMMARY

A new serological method, the platelet aggregation (PA) test, was used to study Group B arbovirus infections, which are often associated with thrombocytopenia. The method is based on the interaction of antigen-antibody complexes with platelets. The PA test was nearly as good as the haemagglutination-inhibition (HI) test for epidemiological studies of tick-borne encephalitis (16 out of 18 HI positive sera) and was superior to the complement-fixation test which was positive in 10 out of 18 HI positive sera. The PA test could be used in the serological diagnosis of Group B arbovirus infection. The titres with the PA test were of the same magnitude as the HI titres, and about ten times higher than the CF titres. Two pairs of dengue haemorrhagic fever sera showed high initial PA titres. The role of immune complexes in the pathogenesis of haemorrhagic fevers is discussed.

INTRODUCTION

Thrombocytopenia is often found during infections casued by the arboviruses of Group B (Clarke & Casals, 1965). In the haemorrhagic forms (yellow fever, haemorrhagic dengues, Omsk haemorrhagic fever and Kyasanur forest disease) it is an almost constant finding. Platelets can be aggregated *in vitro* by complexes of viral antigens and antibodies, and the platelet aggregation (PA) test has been developed to assay this activity (Penttinen & Myllylä, 1968; Myllylä, Vaheri, Vesikari & Penttinen, 1969). In this study the antibodies associated with Group B arbovirus infections were investigated by the PA test and by haemagglutination inhibition (HI) and complement fixation (CF). An attempt is made to use the findings for explaining some features of the pathogenesis of Group B arbovirus infections.

METHODS

Sera

The following human sera were studied: 24 from a region where tick-borne encephalitis (TBE) is endemic; 19 samples from nine virologically verified cases of clinical TBE; one sample taken 3 years after Omsk haemorrhagic fever; and samples from a man vaccinated once with yellow fever 17 D vaccine and a woman vaccinated four times with inactivated TBE cell culture vaccine (Institute of Poliomyelitis and Viral Encephalitis, Moscow). All these sera were stored at −20 °C.

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Dr T. J. Smith (SEATO) kindly sent two paired lyophilized sera obtained from two cases of dengue haemorrhagic fever in 6-year-old girls.

Antigens

TBE virus antigens were prepared from the local strain 'Kumlinge A 52' (CABV no. 188) in cell cultures of a continuous line of human amnion cells (strain 'Utrecht'), treated with protamine (Salminen, 1962), and inactivated by heat or Tween-ether treatment (Halonen, Stewart & Hall, 1967). Yellow fever antigen was prepared from 17 D vaccine strain ('Arilvax') in the same cells and inactivated with Tween-ether treatment.

Dr J. Casals (Yale Arbovirus Research Unit) kindly sent lyophilized sucrose-acetone mouse brain antigens of dengue virus types 1–4.

Haemagglutination inhibition

Standard methods (Clarke & Casals, 1958) modified for microtechnique were used. Sera, not inactivated, were treated with kaolin and absorbed with goose cells. The borate buffer contained 0·4 % bovine albumin. The reagents were diluted in tubes and then pipetted onto plates in volumes of 0·025 ml. TBE antigen was used in concentrations of 4–16 units/0·025 ml. For this, heat-inactivated antigen was diluted five times, and the Tween-ether treated antigen 50 times. Antigen-serum mixtures were incubated overnight at 4° C. or at room temperature, and 0·05 ml. of 0·3 % male goose cells at pH 6·2–6·4 were added. The control TBE immune serum gave 4–8 times higher titres with the Tween-ether treated antigen.

Complement fixation

The standard micromethod (Lennette, 1964) was used with minor alterations. Sera were heated at 56° C. for 30 min., diluted in tubes and pipetted onto plates. Two units of guinea-pig complement and two units of antigen (Tween-ether antigen undiluted) were used. Antigen-serum mixtures were incubated overnight at 4° C. before adding the haemolytic system. Haemolysis of 25 % or less was regarded as positive.

Platelet aggregation

The method has been described in detail earlier (Penttinen & Myllylä, 1968; Myllylä *et al.* 1969). Washed human platelets (200,000/mm^3.) were suspended in buffered (pH 7·4–7·8) saline solution. Serum and antigen dilutions (0·05 ml. of each) were mixed and 0·05 ml. of platelet suspension was added. The sera were heated at 56° C. for 30 min. before use. Microplates were kept overnight at 5° to 8° C., and aggregation was read the following morning. In TBE titrations Tween-ether inactivated antigen was used in dilutions of 3 and 10 (reciprocal values are given). A negative prozone was frequently found in PA titrations, and it was more marked with smaller amounts of antigen. With dengue antigens and sera 'checkerboard' titrations were used. These indicated that the antigen titre was 80, and that an antigen dilution of 1/20 gave the highest antibody titres.

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RESULTS

Sera from TBE-endemic region

One serum aggregated platelets directly without added antigen. The PA, HI and CF titres of the other 23 sera with TBE virus antigen are shown in Table 1. Eighteen of the sera were positive by HI, 16 of these were also positive by PA and 10 by CF. There was a good correlation between titres in the PA and HI tests, but

Table 1. *Antibody titres of sera from TBE-endemic region against TBE antigen using three different methods*

Serum no.	PA	HI	CF
Kn 7724	0	20	0
Kn 7804	0	0	0
Kn 7806	0	20	0
Kn 7809	0	0	0
Kn 7831	0	0	0
Kn 7852	0	0	0
Kn 7921	0	0	0
Kn 7746	40	20	0
Kn 7770	40	20	0
Kn 7792	40	40	0
Kn 7812	40	40	0
Kn 7813	80	80	20
Kn 7723	160	80	0
Kn 7749	160	80	0
Kn 7752	160	80	20
Kn 7801	160	80	10
Kn 7815	160	320	10
Kn 7820	160	80	20
Kn 7753	320	320	40
Kn 7757	320	160	40
Kn 7777	320	160	40
Kn 7791	320	320	40
Kn 7782	640	320	80

PA = Platelet aggregation; HI = haemagglutination inhibition (heat-inactivated antigen); CF = Complement fixation.

the PA test tended to give slightly higher titres than the HI test with the less sensitive heat-inactivated TBE virus antigen. Two sera were weakly positive by HI but negative by PA (the specificity of the HI reactions was verified with the tissue culture neutralization test). CF was apparently less sensitive than the other two tests.

Sera from vaccinated persons

The serum of the man vaccinated against yellow fever did not react with TBE virus antigens in any of the tests (homologous HI titre against yellow fever antigen was 160). Similarly, no reaction with TBE virus antigens could be detected by any test in the serum of the woman injected with TBE vaccine (low grade neutralizing activity was seen in the tissue culture neutralization test).

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Sera from patients

Two samples of serum from TBE patients reacted directly with platelets without added antigen. The titres of the other 17 sera in the PA, HI and CF tests with TBE virus antigen are shown in Table 2. In two cases samples taken during the early

Table 2. *Antibody titres in sera from TBE patients against TBE antigen using three different methods*

	Time	PA	HI	CF
A. A.	−0	0	0	0
	122	320	640	40
R. S.	6	0	0	0
	260	320	640	40
G. S.	15	0	320	0
	23	320	640	10
Å. R.	15	0	320	10
	28	640	1280	20
K. V.	42	320	1280	80
	52	160	640	40
	156	160	320	20
L. E.	18	320	320	20
	31	160	640	80
	52	160	2560	80
K. P.	84	1280	1280	10
U. L.	44	320	160	80
E. R.	15	320	1280	40

PA = Platelet aggregation; HI = haemagglutination-inhibition (tween-ether-treated antigen); CF = Complement fixation. Time in days after onset of disease.

stages of the disease were positive only in HI, or in HI and CF. In other samples there was a good correlation between the PA and HI titres, but with the more sensitive Tween-ether antigen HI gave higher titres. Two or more samples were obtained from six patients. Diagnostic changes in titre (at least fourfold) could be detected by PA and CF in four and by HI in five cases. Serum from the man who had Omsk haemorrhagic fever 3 years earlier had a PA titre of 320 and an HI (heat-inactivated TBE virus antigen) titre of 160. The serum was anticomplementary.

The later serum of one patient (sample no. 36945) with dengue haemorrhagic fever aggregated platelets directly to a titre of 200. Dengue type 4 antigen, which was treated with protamine as well as sucrose-acetone, reacted directly with platelets without added serum. The results with other dengue antigens and sera from patients are shown in Table 3. Even the early serum sample reacted strongly in the PA test. The PA titres were generally higher than the HI titres (Dr Smith) in early samples but lower in late samples. Cross-reactions were as wide in both tests. Dengue serum no. 33431 was fractionated in a sucrose gradient, and the antibody that was active in PA was 7S.

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Table 3. *Antibody titres in sera from two patients with haemorrhagic dengue, tested against* 3 *serotypes of dengue virus*

Serum no.	Time	Dengue 1		Dengue 2		Dengue 3	
		PA	HI	PA	HI	PA	HI
36,522	2	640	320	1,280	320	320	320
36,945	30	3,200	5,120	6,400	⩾ 20,480	3,200	10,240
33,122	3	800	640	800	320	400	640
33,431	30	6,400	10,240	1,600	10,240	1,600	10,240

Time in days after onset of disease. HI titres according to Dr T. Smith.

DISCUSSION AND GENERAL CONCLUSIONS

The results show that the PA technique can be used to measure group B arbovirus antigens and antibodies. The PA titres were higher than the CF titres, and frequently equal to the HI titres. The PA test detected antibody in nearly as many as the HI test. Similar results were found in the rubella system (Myllylä *et al.* 1969).

The results, though limited, suggest that the serological cross-reactions between members of the TBE and dengue complexes might be as extensive with PA as with HI.

The PA test can be used in the serological diagnosis of group B arbovirus infections. In TBE infections the results with a few samples suggest that PA antibodies may appear later than HI antibodies. So far only 7S antibodies seem to be active in PA. These appear later than 19S antibodies which are active in HI also (see also Myllylä *et al.* 1969; Penttinen *et al.* 1969).

In dengue the very limited results suggest that the PA technique is nearly as sensitive as the HI technique. It is interesting that the PA titres could be higher than the HI titres in early sera, but with late sera the reverse was true. The high initial PA titres might be connected with the development of thrombocytopenia in haemorrhagic dengue. However, the high PA titres do not necessarily entail platelet damage *in vivo*. In the PA test platelets are not affected by antibodies but by antigen-antibody complexes. The immune complexes may damage platelets also *in vivo*. Soluble viral antigens are probably present in the circulation during active infection, though there is no direct evidence for this. The development of thrombocytopenia in haemorrhagic forms of dengue seems to require the presence of anti-dengue antibodies at the beginning of the infection (Halstead, 1968). They may be cross-reacting antibodies acquired by preceding infection with other types of dengue (or related viruses) or by transplacental passage from the mother. Thus circulating antibodies should be present while viral antigens are being produced. In this situation soluble antigen-antibody complexes may damage platelets *in vivo*, and thus cause thrombocytopenia. The observation of direct high-titre PA activity in one dengue serum is of interest in this connexion.

The platelet aggregation technique depends on the interaction of immune complexes with target cells, and thus is a useful method for investigating the action and significance of immune complexes.

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