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DETECTION AND CHARACTERIZATION OF IMMUNE COMPLEXES BY THE PLATELET AGGREGATION TEST

I. COMPLEXES FORMED *IN VITRO*

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SUMMARY

In a model system, immune complexes formed *in vitro* (NIP–BSA conjugate/anti-NIP antibody) were analysed by platelet aggregation (Pl.A.) and gradient centrifugation. The addition of specific antibody or antigen to immune complexes alters their composition towards different Ag/Ab ratios. This may result in increased, decreased or complete loss of Pl.A. titre. Adding antibody to mixtures of free antigen and antigen excess complexes increased the Pl.A. titre greatly ($\geqslant$64-fold). The smallest complexes produced in antigen excess Ag/Ab$>$4 were only weakly Pl.A. active, and the complex could not be separated by gradient centrifugation from the free antigen. The S value of the complex with antigenic activity was greater than that of the original antigen. The titre of antibody excess complexes tended to decrease slightly after addition of low dilutions of antibody. Adding antigen to complexes produced smaller changes in Pl.A. titres than adding antibody. Pl.A. active complexes were also found after refractionation in sucrose gradient of a 20S complex (Ag/Ab$\sim$4), but most of the activity was lost during manipulations. The lost activity could be restored by adding antibody. The possibility of using the Pl.A. technique and sucrose gradient centrifugation to detect and analyse natural immune complexes is discussed.

INTRODUCTION

Platelet aggregation (Pl.A.) can be used to detect immune complexes (Penttinen & Myllylä, 1968; Penttinen *et al.*, 1969) around the equivalence zone. In diagnostic surveys of viral diseases certain sera were found to aggregate platelets without added antigen (direct Pl.A. reaction). One possible explanation for such activity is the presence of immune complexes in the blood. Therefore, the behaviour of complexes formed *in vitro* was tested as a model system (NIP–BSA conjugate/anti-NIP antibody). The purpose was to study the rules

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governing the changes in Pl.A. titre of different immune complexes caused by added antigen or antibody. The probable difference in behaviour of antigen and antibody excess complexes was especially interesting with regard to the possibility of analysing complexes found in the blood.

The effect of added antigen or antibody on the Pl.A. activity of immune complexes formed *in vitro* is described. Gradient centrifugation was used to study the stability and to characterize the soluble immune complexes separated from unbound antigen and antibody.

MATERIAL AND METHODS

Antigen. The synthetic determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) conjugated with bovine serum albumin (BSA) antigen (Brownstone, Mitchison & Pitt-Rivers, 1966) was kindly supplied by Dr O. Mäkelä (Department of Serology and Bacteriology, University of Helsinki). The preparation contained, on average, 24 NIP molecules per BSA molecule. The carrier BSA molecule has a sedimentation coefficient of about 4·5S and a molecular weight of 67,000. The NIP_{24} BSA complex sedimented at about 6–7S. However, the calculated molecular weight of NIP_{24} BSA is only about 75,000. The reason for the unexpected increase in the S value of BSA after coupling is not known. The antigen could not be kept for long at 4°C because the hapten dissociated and inhibited the Pl.A. reaction. Therefore the stocks containing 0·4% NIP_{24} BSA were stored at −70°C.

Sera and definition of equivalence. Anti-NIP sera were produced by immunizing rabbits with alum-precipitated NIP_{10}-chicken globulin (Mäkelä, Cross & Ruoslahti, 1969). Sera were inactivated (56°C, ½ hr). The anti-NIP antibody contents (μg/ml) were not determined. The equivalence in this study is the ratio of anti-NIP serum to NIP_{24} BSA which does not show measureable free antibody or antigen in gradient fractions tested by Pl.A. This equivalence corresponds well with that described in our earlier paper, which was calculated from the precipitation data (Penttinen *et al.*, 1969). Only 7S antibodies were active in these sera of hyperimmunized rabbits.

Immune complexes were prepared by mixing equal amounts of calculated dilutions of antigen and antiserum. After incubation for 1 hr at room temperature, the experiments with added antigen or antibody or with gradient centrifugation were made. Incubation at 37°C gave the same results.

Pl.A. technique. Fresh human platelets were separated by differential centrifugation (Aster & Jandl, 1964). The platelets were washed twice with physiological saline, once with buffer and were finally suspended (200,000 platelets/mm^3) in the buffer (Dulbecco's phosphate-buffered saline without Ca^{++} and Mg^{++}, adjusted to pH 7·8 with 1 M NaOH). In some experiments modified Ringer's solution adjusted to pH 6·7 with 1 M HCl was used. This buffer enhances the sensitivity of platelets (Myllylä, 1971). All media contained 0·15 mg/ml of glucose. The platelets were used on the day of preparation. A pool of three lots of platelets was always used to decrease the effect of varying sensitivity of different lots of platelets from different blood donors.

In experiments with added antigen and antibody four drops (0·1 ml) of Ag/Ab mixture were mixed on U-microplates with two drops (0·05 ml) of antigen or antibody dilution. (Dilutions in Tables and Figures are expressed as initial ones.) After 1 hr incubation at room temperature 0·05 ml of platelet suspension was added. In gradient centrifugation experiments 0·025 ml of fraction dilutions, beginning from 4 (inverse values) and 0·025 ml

of buffer, antigen or antibody dilution were mixed. Optimal dilutions were selected according to checkerboard titrations. After 1 hr incubation platelet suspension (0·05 ml) was added. The platelets were incubated overnight at a temperature of 5–8°C and the sedimentation patterns were read the following morning with dark background illumination; a smooth white button and dark even pattern on the bottom of the well indicated negative and positive results respectively. Intermediate results with different ring formations approaching the negative and positive patterns were observed in borderline dilutions. These results were also recorded and used for interpolations. Only checkerboard titrations were used in the experiments with added antigen and antibody. The sensitivity of each platelet preparation was estimated by the 'score' (number of positive Ag/Ab combinations) in a checkerboard titration of NIP_{24} BSA antigen and anti-NIP serum (Penttinen *et al.*, 1969). Some series with low platelet reactivity were rejected.

Sedimentation analysis. Samples of 0·3 ml were layered on linear 12·5–37% w/vol sucrose gradients and centrifuged in a Spinco SW50, 1 rotor for 16 hr at 30,000 rev/min and 4°C. The gradient buffer was Dulbecco's phosphate-buffered saline and contained 0·2% bovine serum albumin, which appeared to enhance the recovery of Pl.A. activity from gradients. The fractions were collected drop-wise through the tube bottom. For sedimentation coefficient determination reference markers were sedimented in companion gradients: Paul Bunnell positive serum (19S) or serum containing IgM (19S) and IgG (7S) rubella HI antibodies. Actual S values were computed according to McEven (1967). For resedimentation the complex fractions were dialysed against buffer and concentrated by dialysis against Carbowax 20 M (Fluka).

RESULTS

Effect of added antibody on the Pl.A. activity of different immune complexes in antigen/antibody mixtures

When antibody is added to immune complexes the effect on the Pl.A. titre depends on the original Ag/Ab ratio (Table 1). When the original complexes were produced in excess antigen and the possibility of free antigen was not eliminated, the addition of antibody greatly increases the Pl.A. titre. Antibody does not affect the titre of immune complexes produced at equivalence, and may decrease the titre of those produced in antibody excess. In the experiment shown in Table 1, complexes with an Ag/Ab ratio of 16 did not aggregate platelets and behaved like antigen alone. With very sensitive platelets, however, some Pl.A. was observed with these complexes thus indicating a low degree of activity.

The titres of the six different complexes cannot be compared directly as a function of the Ag/Ab ratio, because different quantities of antigen were used (Table 1). However, with the two complexes produced around equivalence with the same amount of antigen (1·67 μg) the one with 4 times more antibody had a 4 times higher Pl.A. titre. With the two antigen excess complexes made with 13·3 μg of antigen the complex with 4 times more antibody had at least an 8 times higher titre. With two large antibody excess complexes no difference could be seen.

Effect of added antigen on the Pl.A. activity of different immune complexes in antigen/antibody mixtures

Table 2 shows the change in Pl.A. titre of different complexes after addition of antigen.

The changes were small compared to those following the addition of antibody. Large amounts of antigen tended to decrease the titre. Addition of smaller amounts of antigen to antibody excess complexes increased the titre. Table 3 shows a checkerboard titration with added antigen and antibody excess complex. The effect of antigen is dose dependent.

TABLE 1. The effect of added antibody on the Pl.A. activity of different immune complexes

Concentration of Ag (μg/ml)	Ag/Ab ratio	Titre of immune complex		
		Without added antibody	With added antibody Dilution of anti-NIP serum	
			1:80	1:20
13·3	16	< 1	128	128
13·3	4	8	256	256
1·67	2	16	32	16
1·67	0·5	64	64	32
0·21	0·25	8	8	4
0·21	0·07	8	8	4

Analysis of antigen, antibody and immune complexes by gradient centrifugation

In the experiments we have described so far, antigen or antibody were added to solutions containing preformed complexes. When these were not prepared at equivalence they probably contained free antigen or antibody, whose role in the alterations in the Pl.A. titre is not clear. To clarify this point and study the stability of the complexes, they were centrifuged in sucrose gradients. Earlier it was shown that the complexes can be detected by the Pl.A. technique after gradient centrifugation (Penttinen *et al.*, 1969). We mainly studied three different complexes, Ag/Ab = 8, Ag/Ab = 2 and Ag/Ab = 0·5, made with two different doses of antigen. Some other experiments will also be mentioned.

TABLE 2. The effect of added antigen on the Pl.A. activity of different immune complexes

Concentration of Ag (μg/ml)	Ag/Ab ratio	Titre of immune complex		
		Without added antigen	With added antigen	
			0·16 μg/ml	5μg/ml
13	16	< 1	< 1	< 1
13	4	8	8	4
1·67	2	16	8	4
1·67	0·5	64	64	32
0·21	0·25	8	16	2
0·21	0·07	8	32	4

Antigen excess complexes. With Ag/Ab = 8 complex no direct Pl.A. activity was observed in any of the fractions (Fig. 1). In a few similar experiments, however, weak direct Pl.A. activity was seen. Thus, complexes formed in a large excess of antigen are not readily detectable by the Pl.A. technique, as was also seen earlier (Penttinen *et al.*, 1969).

The amounts of antigen recovered from the gradients correlated favourably with the amounts of antigen used. No free antigen separable from the activity of the supposed complex could be observed, but the complexes behaved like antigen except that they had a

TABLE 3. The effect of added antigen on the activity of antibody excess immune complex; Ag = 0·21 μg/ml Ag/Ab = 0·07

Dilution of immune complex	Concentration of added antigen (μg/ml)								
	5	2·5	1·25	0·63	0.32	0·16	0·08	0·04	c.c.
1	++	++	++	++	++	++	++	++	++
2	++	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++	++
8	−	−	++	++	++	++	++	++	+
16	−	−	−	−	+	++	++	−	−
32	−	−	−	−	−	++	−	−	−
64	−	−	−	−	−	−	−	−	−
a.c.	−	−	−	−	−	−	−	−	

a.c. = antigen control; c.c. = complex control.

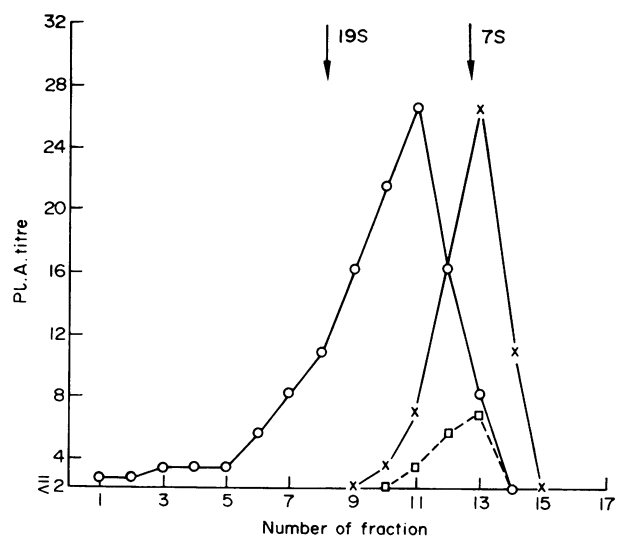

FIG. 1. Sucrose gradient centrifugation of antigen excess (Ag/Ab = 8) complex. Pl.A. titrations in phosphate buffer. Anti-NIP serum (dil. 80) added to fractions. ×, NIP_{24} BSA 6·7 μg/ml; □, NIP_{24} BSA 1·67 μg/ml; ○, Ag/Ab = 8 complex with 6·7 μgAg/ml. No direct Pl.A. activity.

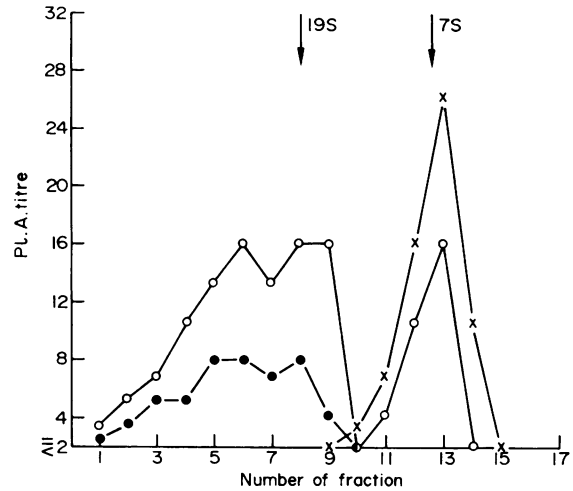

FIG. 2. Sucrose gradient centrifugation of antigen excess (Ag/Ab = 2) complex. Pl.A. titrations in phosphate buffer. ●, Ag/Ab = 2 complex with 6·7 μg Ag/ml, direct Pl.A. titre; ○, Ag/Ab = 2 complex with 6·7 μg Ag/ml + anti-NIP serum (dil. 80); ×, NIP_{24} BSA 6·7 μg/ml + anti-NIP serum (dil. 80).

higher sedimentation coefficient. The new complexes appearing after addition of antibody had at least as much Pl.A. activity as the antigen in them (plus optimal antibody). Complexes with an Ag/Ab ratio of 4 gave similar results.

When Ag/Ab = 2 complex was centrifuged there was a zone of direct Pl.A. activity around 20–30 S (Fig. 2). The activity was increased up to four-fold by added antibody. The Pl.A. activity recovered was similar in titre to that of the antigen used (plus optimal antibody). There was also a peak of free antigen at 6–7S. In some other experiments, the enhancement of the complex titre by added antibody was eight-fold in fractions sedimenting slower than the 19S marker.

Equivalence complexes. The peak Pl.A. activity of equivalence complex Ag/Ab = 1 in one experiment was at about 25S. No free antigen or antibody was detectable although a wide

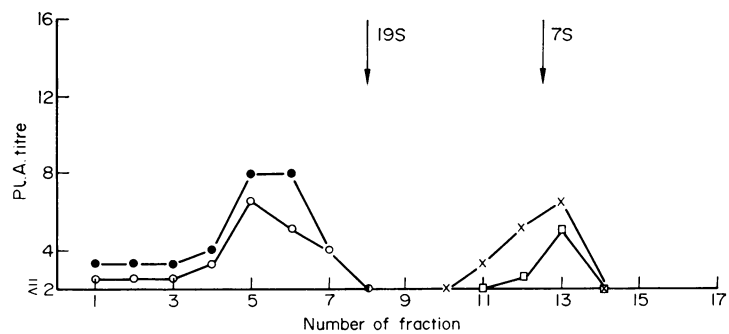

FIG. 3. Sucrose gradient centrifugation of antibody excess (Ag/Ab = 0·5) complex. ●, Ag/Ab = 0·5 complex with 1·67 μg Ag/ml, direct Pl.A. titre; ○, Ag/Ab = 0·5 complex with 1·67 μg Ag/ml + anti-NIP serum (dil. 80); □, Ag/Ab = 0·5 complex with 1·67 μg Ag/ml + 0·4 μg Ag/ml; ×, NIP_{24} BSA 1·67 μg/ml + anti-NIP serum (dil. 80).

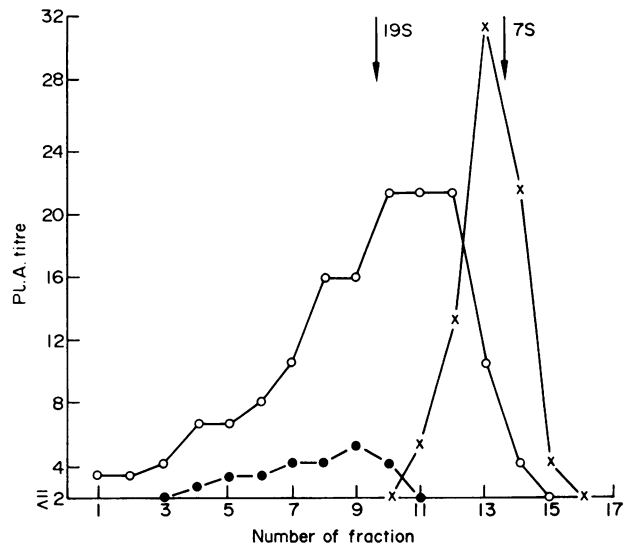

FIG. 4. Sucrose gradient centrifugation of antigen excess (Ag/Ab~4) complex. Pl.A. titrations in phosphate buffer. ●, Ag/Ab~4 complex, direct Pl.A. titre; ○, Ag/Ab~4 complex+anti-NIP serum (dil. 80); ×, NIP_{24} BSA (~3·4 μg/ml)+anti-NIP serum (dil. 80).

range of different antibody and antigen dilutions were added. This result was used to define the equivalence (see Methods). Enhancement by antibody was again apparent with slower sedimenting complexes but not with fast ones.

Antibody excess complexes. Complexes formed in slight antibody excess (Ag/Ab = 0·5) (Fig. 3) had less Pl.A. activity, but this was due to the smaller amount of antigen (1/4) used in the complex formation. The complex sediments faster than the Ag/Ab = 2 complex. Again, the Pl.A. activity of the complex was as great as that of antigen alone (plus optimal antibody). Addition of antibody did not enhance the activity, but sometimes depressed it slightly. Free antibody was found in another experiment using optimal dilution of antigen. This is presented in Fig. 3 also.

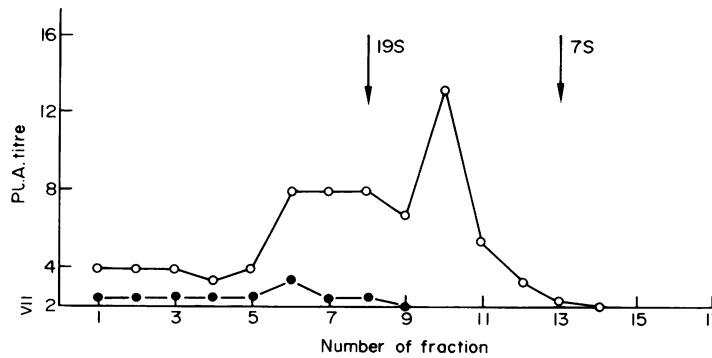

FIG. 5. Refractionation of combined and concentrated fractions 8, 9 and 10 from Fig. 4. Pl.A. titrations in carbonate buffer. ●, Direct Pl.A. titre; ○, Pl.A. titre with anti-NIP serum (dil. 80).

The effect of adding antigen to the complexes has not been presented. In general, adding antigen had a much less effect than adding antibody. Antigen increased the PA titre of large antibody excess complexes, and decreased the titre of small antigen excess complexes.

Refractionation of antigen excess complexes. In order to test the stability of Pl.A.-active complexes, an attempt to re-isolate them was made. Antigen excess complexes (Ag/Ab ~ 4) were pooled from three fractions at 20S (Fig. 4) then concentrated and refractionated (Fig. 5). The Pl.A. tests of Figs 4 and 5 were not done with the same lots of platelets and with the same buffer and thus quantitative comparisons are not accurate. According to the controls the sensitivity of the platelets used in these two experiments was of the same order. Without added antibody no clear direct Pl.A. activity could be observed. Most of the Pl.A. activity appeared to have been lost during the refractionation procedure, but was restored by the addition of antibody.

DISCUSSION

In this and the earlier study (Penttinen *et al.*, 1969) with NIP_{24} BSA conjugate/anti-NIP antibody the Pl.A. activity began at an Ag/Ab ratio of 10 and continued up to 0·02. The addition of antibody to antigen excess mixtures changed the Pl.A. titre of the mixtures considerably. This is probably partly due to the formation of new complexes and partly to the addition of more antibody to old ones. Complexes approaching the structure $Ab(IgG)Ag_2$ (the smallest possible in Ag excess) (Singer & Campbell, 1952) show decreasing Pl.A. activity. It is probable that for a positive Pl.A. reaction more than one antibody molecule attached to the same antigen molecule is needed. The Pl.A. technique may not differentiate between antigen and the smallest complexes, which have many free antigenic determinants. The only difference in behaviour may be the greater size (about 10S, Fig. 1) of the complexed antigen. The smallest complexes formed in antigen excess ($AbAg_2$) have, thus, not been 'biologically' active in this system.

The Pl.A. titre of high antibody excess complexes cannot be increased by added antibody; in fact, a slight depression may be seen. Saturation of antigen molecules with antibody probably sets the limit. Antibody excess complexes behave like antibody alone when a suitable amount of antigen is added.

Added antigen is not as effective as antibody. It may change the structure of immune complexes towards Ag/Ab ratios (> 10) which are not readily active in the Pl.A. test. None of the amounts of antigen we added abolished Pl.A. activity completely. The amounts were not great enough (Table 2) and/or it is technically difficult to utilize fully the reorganization capacity of the complexes.

The detection of free antigen or antibody by centrifugation from Ag/Ab = 2 or 0·5 complexes is evidence of the sensitivity of the Pl.A. technique. Whether or not the complexes have lost the observed free antigen or antibody during the gradient centrifugation is not clear from the present results. The antigen excess complexes are not quite stable in the centrifugation medium, as shown by the reisolation experiment. Most of the Pl.A. activity appears to have been lost during manipulations. This activity can be restored by addition of antibody.

The combination of Pl.A. technique and gradient centrifugation makes it possible to detect and characterize complexes by direct Pl.A. activity, and by changes caused by added antibody or antigen. The Pl.A. titre of an immune complex seems to depend, firstly, on its

antigen content, and, secondly, on the degree of saturation of antigen with antibody. Antigens with high determinant densities allow more antibody around the antigen molecule. The positive zone of antigens with high determinant densities is thus broader (containing more PA positive Ag/Ab combinations) than that of antigens with low determinant densities. The addition of specific antibody or antigen to immune complexes alters their composition towards different Ag/Ab ratios. This may result in increased, decreased or complete loss of Pl.A. titre.

The changes in the Pl.A. titres of complexes after addition of specific antibody or antigen are of such a magnitude that it seems reasonable to use the techniques to analyse natural complexes. There are probably many mechanisms that may disturb the relatively clear picture obtained. For example, the determinant density of the antigens, when approaching 2, may change the behaviour of immune complexes; some components of the serum (complement?) may be attached to the complexes and change their activity; the weak affinity of the antibody may make the complexes more labile.

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ABBREVIATIONS

NIP 4-hydroxy-3-iodo-5-nitrophenylacetic acid
Pl.A. platelet aggregation