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DETECTION AND CHARACTERIZATION OF IMMUNE COMPLEXES BY THE PLATELET AGGREGATION TEST

II. CIRCULATING COMPLEXES

G. MYLLYLÄ, A. VAHERI AND K. PENTTINEN

Department of Virology, University of Helsinki, and Finnish Red Cross Blood Transfusion Service

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SUMMARY

Model experiments with preformed antigen–antibody complexes have shown that the platelet aggregation test can be used to characterize immune complexes. The platelet aggregating activity of four sera behaved like antigen–antibody complexes with respect to the effect of added antigen, added antibody and sedimentation in density gradient centrifugation. Two of the sera were from patients with fever of unknown origin. The findings suggest that the two sera contained circulating immune complexes which had both measles hyperimmunization induced and normal specificities. The serum of a patient with fatal subacute hepatitis seemed to contain immune complexes with Australia antigen. It was also positive in many of the autoimmunity tests. One of the sera was from a patient with haemorrhagic varicella in the acute phase of the disease. The patient had received large amounts of γ-globulin. The results suggested the transient presence of circulating immune complexes with varicella specificity.

INTRODUCTION

The pathogenic role of immune complexes has been firmly established in animal experiments. The Arthus reaction and the classical 'one-shot' serum sickness are the most clear-cut examples. Experimental antigen–antibody nephritis is perhaps the one with an apparent counterpart in human pathology (Dixon, Edgington & Lambert, 1967; Christian, 1969). That immune complexes may be implicated in the pathogenesis of many autoimmune conditions is increasingly being realised. Their role in acute and chronic infectious diseases has been considered less often. However, immunological factors seem to be more important in the pathogenesis of some viral infections than is generally recognized (Coombs, 1968). It is obvious that immune complexes are formed in the host especially during chronic infections and reinfections. During that time there may be large amounts of avid antibodies while microbial antigens are being produced. Their presence and role during infections have not been properly studied, mostly due to methodological difficulties.

Correspondence: Dr Kari Penttinen, Department of Virology, University of Helsinki, Haartmaninkatu 3, Helsinki 29, Finland.

The platelet aggregation (Pl.A.) test could be used to demonstrate soluble immune complexes in model experiments. We have now used the technique to study supposed circulating immune complexes in four sera with direct Pl.A. activity. Two of the sera were from patients with acute infections, and the two others from patients with more prolonged febrile disease of unknown origin. The results support the suggestion that the direct Pl.A. activities of the sera were due to immune complexes.

MATERIALS AND METHODS

Antigens

Measles antigen was prepared in VERO cell roller cultures maintained in BME medium supplemented with 0·2% bovine serum albumin. After a high multiplicity of infection with measles virus the cells degenerated in 3–5 days. The whole culture was frozen and thawed three times, treated with Tween 80 and ether, and clarified by low speed centrifugation.

Measles control antigen was prepared from similarly treated uninfected cultures.

Varicella-zoster antigen (kindly provided by Dr T. Palosuo of our Department) was prepared with a GMK-AH-1 adapted strain of *Varicella-zoster* virus (strain obtained from Dr A. Svedmyr, Stockholm), and further adapted to BS-C-1 cells. Infected cells at the third passage in BS-C-1, together with the medium (MEM + 0·2% bovine serum albumin) were harvested when the cytopathic effect was almost complete (on the 12–14th day after infection). The culture was frozen and thawed twice, concentrated 7-fold by forced dialysis against vacuum and clarified by centrifugation for 90 min at 35,000 rev/min in a Spinco rotor 40.

Herpes simplex antigen (kindly provided by Dr T. Palosuo) was prepared from BS-C-1 cell cultures infected with Type 1 virus (strain E115, Tyler) by freezing and thawing three times, twenty-fold concentration by forced dialysis against vacuum, clarification at 24,600 ***g*** for 30 min and dialysis against phosphate buffered saline.

Tick-borne encephalitis virus antigen (kindly provided by Dr P. Saikku of our Department) was prepared from virus-infected (strain Kumlinge A 52) continuous human amnion cell (strain Utrecht) cultures maintained in MEM + 0·2% bovine serum albumin. The cell free supernatant of cultures with extensive cytopathic changes was treated by protamine sulphate precipitation and inactivated by treatment with Tween 80 and ether.

Au(1) *antigen* was obtained from the serum of a patient with Down's syndrome with a strong positive precipitating line in immunodiffusion against reference anti-Au(1) serum.

Reagent sera

Measles hyperimmune monkey serum (Lot 3-2017, Microbiological Associates, Bethesda, Md) with measles antibody titre 1/32 in CF and 1/320–1/640 in HI tests.

Measles negative monkey serum (Lot No. 60-L) was kindly provided by Dr H. Koprowski, Wistar Institute, Philadelphia.

Varicella-zoster serum was from a patient (E.H.) at the convalescent stage of herpes zoster.

Au(1) *antibody serum* was from a haemophiliac (M.P.) who has had multiple blood transfusions. The other sera were SSPE serum (M.H.) with high measles antibody titres and eight normal sera from healthy people (Table 3).

Sucrose gradient fractionation and the platelet aggregation test were as described in the adjacent report (Penttinen, Vaheri & Myllylä, 1971).

RESULTS

Case 1 (*M.K.*)

The patient is a 6-year-old boy, who has been treated with prednisolone because of juvenile rheumatoid arthritis. He was admitted to the Hospital for Rheumatic Diseases on 17 March 1969 because of carditis suspicion. The medication was increased to 30 mg of prednisolone per day. 20 days later varicella eruptions appeared and he was given 2 ml of 16% γ-globulin on the same day. The same dose was given on the following day (8 April) and 4 ml of γ-globulin on 10 April. On the following day (11 April) petechial eruptions appeared, but the platelet count was still normal (400,000/mm^3) as was the thromboplastin time and whole blood clotting time. On the following day a severe bleeding tendency appeared. The platelet count dropped to 11,000/mm^3, and fibrinogen level to 75 mg/100 ml, which suggested consumption coagulopathy. The bleeding tendency could be managed during the following days and the patient survived.

TABLE 1. A case (M.K. 160463) of haemorrhagic disorder associated with varicella infection. The effect of varicella antigen on the direct Pl.A. titre of the 14 April serum specimen

		Dilution of antigen									
		5	10	20	40	80	160	320	640	s.c.	pl.c.
Dilution of serum	10	+	+	+	+	+	+	+	+	+	−
	20	+?	+	+	+	+	+	+	+	+	−
	40	−	−?	+	+	+	+	+	+	+	−
	80	−	−	−?	+	+	+	+	+	+	−
	160	−	−	−	−	−?	+?	−?	+?	−?	−
	320	−	−	−	−	−	−	−	−	−	−
	v.c.	−	−	−	−	−	−	−	−		

Measles, cytomegalo, herpes and tick-borne encephalitis antigen were ineffective.
s.c. = serum control, pl.c. = platelet control, v.c. = varicella antigen control.

Immunological studies. Serum taken on 14 April was tested for varicella antibodies. No antibodies were detectable by the usual CF or Pl.A. tests, but the serum showed direct Pl.A. activity without added antigen. In a checkerboard titration, varicella antigen decreased the direct Pl.A. titre by a factor of 4–8 (Table 1). This effect corresponds to that of specific antigen on the Pl.A. titre of preformed immune complexes. Measles, cytomegalo, herpes and tick-borne encephalitis antigens did not decrease the direct Pl.A. titre at any dilution. The serum contained herpes and cytomegalovirus antibodies but no antibodies against measles and tick-borne encephalitis. The serum was not anticomplementary.

None of the serum samples taken after 16 April showed any direct Pl.A. activity. Varicella antibodies were first detectable by the CF test 3 weeks after the rash and by the Pl.A. test a week later. The antibody response was weak as measured by the Pl.A. test.

In sucrose gradient centrifugation (Fig. 1) no direct Pl.A. activity was demonstrable in any of the fractions, but with added antibody high Pl.A. activity was observed in fractions sedimenting slower than 19S. This resembles the behaviour of complexes produced in high antigen excess; these complexes could not be separated from free antigen. There was not enough serum to study using other techniques.

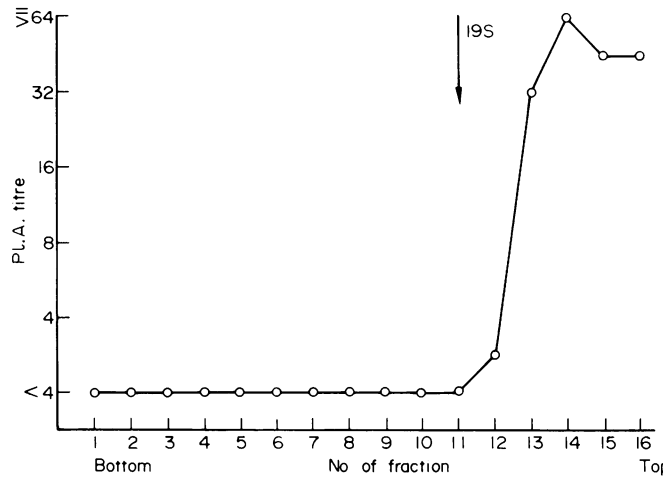

FIG. 1. A case (M.K. 160463) of haemorrhagic disorder associated with varicella. Sucrose gradient centrifugation of the 14 April serum specimen with direct (without antigen) Pl.A. activity. ○ = Pl.A. titre with added varicella antibody. No direct activity of any fraction was demonstrable. No Pl.A. activity with added antigen was demonstrable.

Case 2 (*A.V.*)

This patient is a 62-year-old female, who was admitted to the Municipal Hospital for Infectious Diseases because of icterus. On admission on 15 October she was icteric and had arthralgic pains but was otherwise in good general condition. Serum bilirubin was 16·4 mg/100 ml and SGOT was 305 mU/ml. Many findings in the laboratory tests suggested the presence of autoimmune disease: serum complement (β_{1c}) was decreased and IgG and IgA immunoglobulins were markedly increased in immunoelectrophoresis. Tests for glomerular antibodies, anti-nuclear antibodies and LE cells were all positive as was the Latex test.

She was treated with prednisolone but the general condition of the patient worsened slowly, hepatic coma developed and the patient died 5 weeks after admission.

At autopsy a small atrophic liver with large necrotic areas was found and histological diagnosis of subacute hepatitis was made.

Immunological studies. The immunodiffusion test for Au(1) antigen was negative. In the Pl.A. test the serum sample taken on 3 December showed direct Pl.A. activity (titre 640 without added antigen).

After sucrose density gradient centrifugation the direct Pl.A. activity was found in fractions sedimenting faster than the 19S marker, with a peak corresponding to about 30S. Addition of anti-Au(1) serum to the fractions greatly enhanced the Pl.A. activity and also revealed free antigen at about 7–10S (Fig. 2). On the other hand, Au(1) antigen markedly decreased the direct Pl.A. titre of the fractions, and did not reveal any free antibody at 7S. A control serum did not reduce or increase the Pl.A. activity. The findings suggested the recovery of antigen excess immune complexes containing Au(1) antigen.

Case 3 (*S.R.*)

This patient is a 26-year-old taxidriver. He was in hospital in September 1968 and again

in October 1968 because of fever of an unknown origin. On both occasions the fever continued for several weeks, it was not affected by antibiotics and subsided by itself. The aetiology remained unsettled despite very thorough examinations. In August 1969 he was again admitted to the 3rd University Clinic because of fluctuating fever, which had continued for 3 weeks.

Physical examination did not show any abnormalities. The erythrocyte sedimentation rate was greatly increased (101–127 mm/hr), and the haemoglobin value was 10·1 g/100 ml.

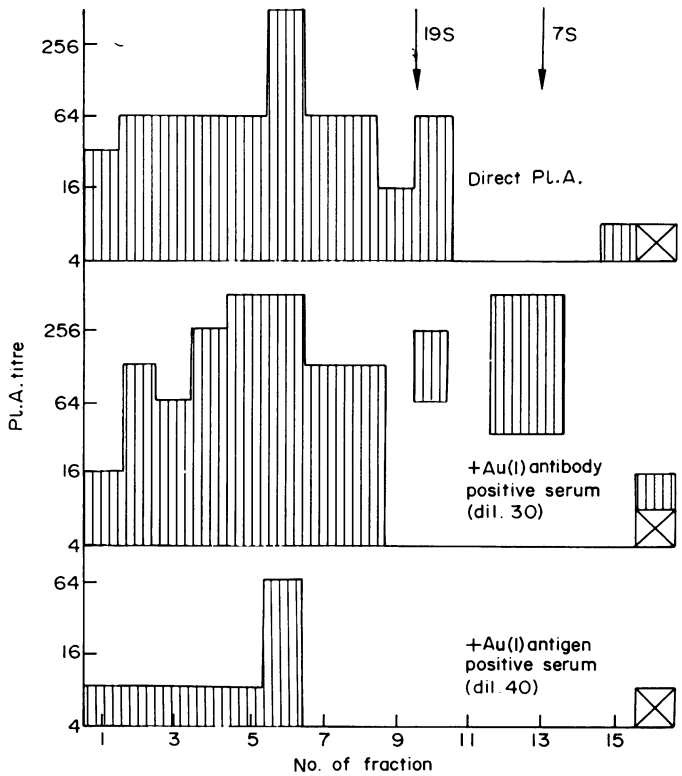

FIG. 2. Sucrose gradient fractionation of a hepatitis serum with high direct Pl.A. titre.

α-2-globulins were slightly increased in electrophoresis, but no other abnormalities could be found in laboratory or röntgenological examinations. Immunoelectrophoresis and all tests for autoimmune diseases gave normal results. Blood culture, tests for tubercle bacilli and all other bacteriological examinations were repeatedly negative. Histological diagnosis of an extirpated lymph node was nonspecific lymphonoditis. According to lymphography, the retroperitoneal nodules were slightly enlarged, but the finding did not allow any specific diagnosis. The Mantoux test was negative up to 1:10,000.

Because no causative factor could be detected and the fever continued, ex juvantibus-treatment was initiated. Anti-tuberculosis medication of 3 weeks duration had no effect on the fever. Cyclophosphamide had no more favourable effect. However, when 15 mg of prednisolone daily was begun the fever disappeared, and haemoglobin and red cell sedimentation rate rapidly became normal.

Immunological studies. During the routine studies for virus antibodies an exceptionally high CF titre of measles antibodies was found (256). The serum was not anticomplementary. The HI titre was 1280. In the Pl.A. test the patient's serum had direct Pl.A. activity (titre 40–160 without added antigen) and an anti-measles titre of 1280–2560 (with added antigen (Table 3)). We investigated the nature of the direct Pl.A. activity further.

With a checkerboard titration (Table 2) the measles antigen (derived from infected monkey kidney cells) in high dilutions decreased the direct Pl.A. activity of the serum by a factor of 64. More concentrated measles antigen produced a positive area as in normal titrations for measles antibodies. The effect of high dilutions of the measles antigen on the

TABLE 2. The effect of added measles antigen on the direct Pl.A. titre of serum S.R.300443

	Dilution of measles antigen									
	5	10	20	40	80	160	320	640	1280	s.c.
Dilution 5	–	–	–	–	+	+	+	+	++	+
of serum 10	–	–	–	–	++	++	++	++	++	++
20	–	–	–	–	–	–	+	++	++	++
40	++	+	+	–	–	+	+	++	++	++
80	++	++	++	–	–	–	–	–	++	++
160	++	++	++	+	–	–	–	–	+	+
320	++	++	++	–	–	–	–	–	+	–
640	++	++	++	+	–	–	–	–	–	–
1280	++	++	+	–	–	–	–	–	–	–
2560	+	+	–	–	–	–	–	–	–	–
5120	–	–	–	–	–	–	–	–	–	–
a.c.	–	–	–	–	–	–	–	–	–	

a.c. = antigen control. s.c. = serum control.

direct Pl.A. activity suggested that antigen(s) of the culture cells were involved. This was supported by the finding that 'control' antigen derived from non-infected monkey kidney cells had the same effect (factor = 32). Antigens derived from other virus infected cell cultures (varicella-, cytomegalovirus), and control antigen from human embryonic skin cells had no effect on the direct Pl.A. titre. However, herpes antigen from infected BS-C-1 cells decreased the direct titre by a factor of 4.

After sucrose density gradient centrifugation the direct activity was found mostly in the fractions sedimenting faster than 19S (Fig. 3). The addition of hyperimmune anti-measles monkey serum (1/100 dilution) clearly enhanced the Pl.A. activity of the fractions. The serum from an SSPE patient with high anti-measles titre had the same effect. Anti-measles antibody titres were highest in 7S fractions, but antibody was also detected in faster fractions. Refractionation of the fractions (Fig. 4) showed, however, that the distribution of the activity in the faster fractions was due to overloading of the system. The anti-measles antibodies were actually 7S. Measles-negative monkey serum did not enhance the activity of '20–30S'-complex, but inhibited slightly. Refractionation of the fractions with direct Pl.A. activity showed no or only weak direct Pl.A. activity in fractions sedimenting faster than 19S. However, addition of hyperimmune serum revealed clear Pl.A. activity in the fractions around 19S marker (Fig. 4).

TABLE 3. Results with Pl.A. technique in investigating sera from two patients with elevated measles antibodies and eight healthy persons

Code	Age	HI	CF	Pl.A.	Direct Pl.A.	Inhibition of direct Pl.A. by: Measles Ag	Inhibition of direct Pl.A. by: Control Ag	Stimulation of '20–30S complex' by measles Ab	Diagnosis
1 S.R.	26	1280	256	2560	80	+	+	+	Febris incertae causae LED?
2 L.E.	43	320	64	320*	80	+	+	+	Infiltratio pulmonis. Pneumonia virosa?
3 A.K.	27	160	<8	<10	<10			+	Healthy
4 L.K.	31	80	8	<10	<10			−	Healthy
5 M.V.	21	40	<8	<10	<10			−	Healthy
6 M.V.	21	20	<8	<10	<10			−	Healthy
7 A.V.	32	20	<8	<10	<10			−	Healthy
8 K.P.	52	20	<8	<10	<10			−	Healthy
9 R.L.	29	20	<8	<10	<10			−	Healthy
10 S.L.	28	<10	<8	<10	<10			−	Healthy

* Weak reaction.

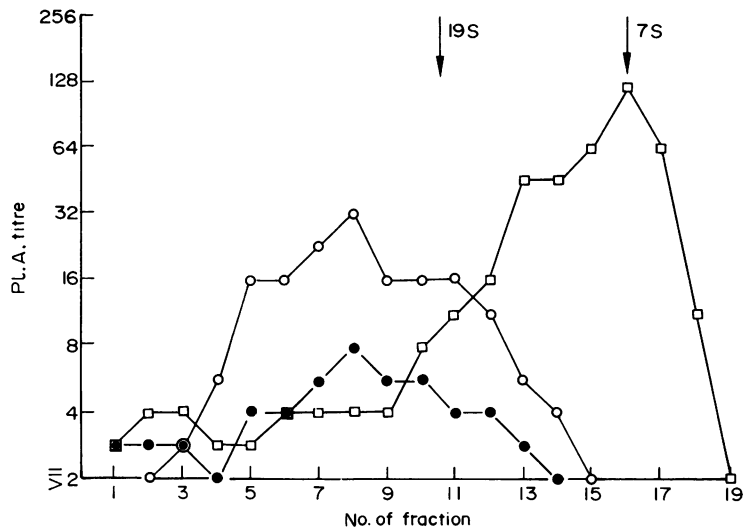

FIG. 3. Sucrose gradient fractionation of S.R. 300443 serum (030969). ●, direct Pl.A. titre; ○, Pl.A. titre with added measles monkey hyperimmune serum (dilution, 100); □, Pl.A. titre with added measles antigen.

During prednisolone treatment the direct Pl.A. activity of the serum decreased slightly Anti-measles titres remained unaffected.

A kidney biopsy showed immunoglobulin deposits without complement along glomerular basement membrane. (Preparation kindly provided by Dr E. J. Jokinen.)

Case 4 (*L.E.*)

This patient is a 43-year-old inspector, who was admitted to the University Clinic for Pulmonary Diseases because of high fever and cough. X-ray examination showed interstitial pneumonitis and preliminary diagnosis was sarcoidosis. Spirometry showed a severe restrictive ventilatory deficiency and CO_2, O_2 and pH measurements of the blood indicated an interstitial process in the lungs.

Thorough investigations did not reveal any cause for the disease: no pathogenic bacteria were found in the sputum, blood culture and culture for tubercle bacilli were negative. Kweim test was negative and apart from the pulmonary findings no other signs suggestive of sarcoidosis were detectable. The infiltration began slowly to decrease and the general condition of the patient to improve.

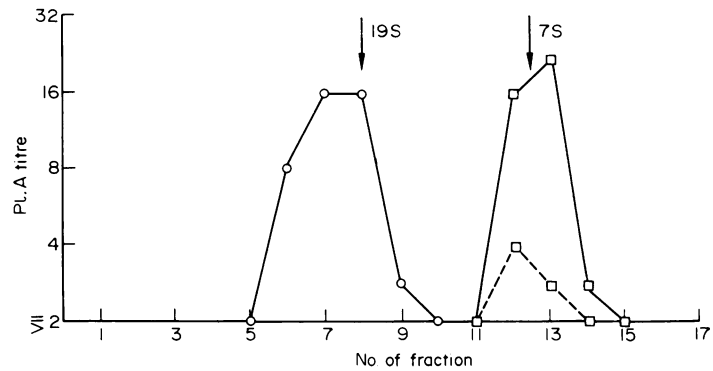

FIG. 4. Refractionation of combined and concentrated fractions 7,8 and 9,12 and 16 from Fig. 3. ○, 7,8 and 9 combined with measles hyperimmune monkey serum (dilution, 100); - - □ - -, 12 with added measles antigen; —□—, 16 with added measles antigen.

Immunological studies. As in the preceding case direct Pl.A. activity (titre 80) and exceptionally high anti-measles titres were found in the patient's serum (Table 3). In a checkerboard titration the antigen derived from measles infected monkey kidney cells had the same effect as on the serum of patient 3. That is, in high dilutions the antigen decreased the direct Pl.A. titre by a factor of 16, but in a more concentrated form it produced a positive area as in a normal titration for measles antibodies. The 'control' antigen also decreased the direct Pl.A. titre by a factor of 16. In sucrose density centrifugation the Pl.A. activity was distributed in the fractions around the 19S marker. The effect of added measles hyperimmune serum on the activity of the fractions was not so pronounced as in the preceding case. The anti-measles antibodies were found in the fractions around the 7S marker.

Normal sera. Table 3 also shows the results for eight normal sera taken from healthy laboratory personnel. The anti-measles titres with all the three techniques were below those in cases 3 and 4. It should be noted, however, that the stimulation with measles hyperimmune serum of the 20–30S fraction of serum A.K. produced slight but distinct Pl.A. activity.

DISCUSSION

Investigations of the presence and importance of immune complexes in different pathological states have been hampered by technical difficulties. Anticomplementary activity of

serum may indicate the presence of immune complexes, but it may be due to other factors. The methods of analysing sera that may contain immune complexes are quite elaborate. Model experiments with preformed complexes (Penttinen *et al.*, 1969 and 1970) and the present findings indicate that the platelet aggregation test has considerable potential for detecting and analysing immune complexes. It has some advantages over the CF test: with most of the complexes it is more sensitive, it also detects complexes that do not fix complement, and it detects complexes formed in high antibody excess. As with anticomplementarity the direct Pl.A. activity of serum, as such, does not indicate that circulating immune complexes are present. The activity must show other properties ascribed to immune complexes on the basis of model experiments.

The direct Pl.A. activity of the four sera we studied behaved like that of soluble immune complexes as regards the effect of added antigen, added antibody and behaviour in gradient centrifugation. The presence of circulating immune complexes during acute infection would not be surprising. It should be possible to find circulating complexes if the techniques are sensitive enough. Apart from the properties of the immune complexes (e.g. complement activation, size, stability, avidity of the antibodies involved, etc.) their pathological significance will depend on how long they are present. The 'equivalence *in vivo*', the phase when biologically active complexes are formed, is obviously soon over in most acute infections. In case 1 it is possible that the administration of γ-globulin with avid antibodies to a patient during active antigen production resulted in the transient appearance of immune complexes in the circulation. However, the phase continues for a longer period if both the antigen and its antibody are produced at equivalent rates. This condition might prevail during some forms of chronic infections and 'autoimmune' states. If circulating immune complexes are pathogenic the immunological specificity of antibodies (and autoantibodies) may not determine the site where they cause most damage, but they may give rise to various seemingly unrelated manifestations.

The behaviour of the direct Pl.A. activity in the sera of patients 3 and 4 suggested the presence of circulating immune complexes with measles hyperimmunization induced specificity. The exceptionally high anti-measles titres of the sera suggested the diagnosis of chronic or recurrent measles. The inhibitory effect of control antigen on the Pl.A. titre suggested the presence of normal cell specificities in the immune complexes. It is obvious that virus infection in the cell cultures (as in our measles antigen) and in patients or in the hyperimmunized monkey may give rise to new antigenic specificities of cellular as well as viral derivation.

The possible ways in which virus infections may lead to autoimmunization have been extensively discussed. Recent sero-epidemiological studies indicate elevated titres to measles, rubella and some other viruses in systemic lupus erythematosus (Phillips & Christian, 1969; Hollinger, Sharp & Rawls, 1970). From this point of view the high anti-measles titres of sera from patients 3 and 4 and the composition of the immune complexes are of special interest. However, only the prolonged follow-up of the patients may reveal the significance of the findings.

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ABBREVIATIONS

Au(1)	Australia
BME	basal medium Eagle
BS-C-1	Biological Standard Cercopithecus Monkey kidney cell line
GMK-AH-1	African Green Monkey kidney cell line
HI	haemagglutination inhibition
Pl.A.	platelet aggregation
S	Svedberg unit of sedimentation coefficient
SGOT	serum glutamic oxaloacetic transaminase
SSPE	subacute sclerosing panencephalitis
VERO	African Green Monkey cell line