J. Hyg., Camb. (1983), **91**, 113–130
Printed in Great Britain

Diagnostic assays with monoclonal antibodies for the human serum parvovirus-like virus (SPLV)

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(*Received* 21 *March* 1983; *accepted* 5 *April* 1983)

SUMMARY

Monoclonal antibodies to the serum parovirus-like virus (SPLV) were prepared by the hybridoma technique. They provided an antibody reagent which was used to develop solid phase antibody-capture assays for anti-SPLV IgM and IgG and for SPLV antigen. These assays were more sensitive than those based on human convalescent antibody as a reagent, and were more economical in the use of SPLV antigen. Their use enabled the serological responses to SPLV to be studied more fully and their sensitivity revealed the extent of SPLV infection.

SPLV antigen was detected in four patients by both counter-immuno electrophoresis (CIE) and radioimmunoassay (RIA) and in two others by RIA alone. Parvovirus particles were seen in all six by electron microscopy. The anti-SPLV IgM response was measured in patients infected by SPLV. It was strong 5–18 days after the onset of illness, then declined and was only detectable in trace amounts after 6 months. Anti-SPLV IgG was also formed early, and persisted for at least 6 months. In a survey of 310 blood donors anti-SPLV was detected in 134 (43%) by CIE, but in 190 (61%) by IgG antibody capture RIA.

INTRODUCTION

The human serum 'parovirus', B19, was discovered by Cossart and co-workers in 1975. Using counter-immunoelectrophoresis (CIE), they demonstrated the presence of the virus in human serum and the development of antibody to it. They showed that the prevalence of antibody increased with age, and that about 30% of adults were sero-positive. More recently CIE has been used to show that the virus can give rise to a febrile illness in normal subjects (Shneerson, Mortimer & Vandervelde, 1980) and to a severe but self-limiting anaemic episode, the aplastic crisis, in patients with chronic haemolytic anaemia (Serjeant *et al.* 1981; Kelleher *et al.* 1983). The agent has been difficult to name. It has the morphology and buoyant density of a parvovirus, but definitive biochemical evidence is still wanting, and we refer to the virus as the serum parvovirus-*like* virus (SPLV).

An IgM-antibody capture radioimmunoassay (MACRIA) has already been developed to diagnose SPLV infection in children with sickle cell anaemia recovering from aplastic crisis (Anderson *et al.* 1982). This method for detecting specific IgM is less cumbersome and probably more sensitive than CIE testing of gradient-separated IgM fractions of serum (Kelleher *et al.* 1983) but, lacking optimum reagents, it fails to take full advantage of the MACRIA system. It also uses large amounts of antigen.

These problems have now been overcome by preparing monoclonal antibodies to SPLV. This has permitted the development of more sensitive assays not only for specific IgM but also for specific IgG and SPLV antigen. The assays allow recent infections to be identified, discriminate clearly between anti-SPLV positive and negative specimens and detect SPLV with great sensitivity. The antibody assays can also be applied more freely than previous methods to diagnostic and epidemiological studies because they are sparing of SPLV antigen, scarcity of which has previously limited such investigations.

MATERIALS AND METHODS

Antigens

The source of SPLV antigen (SPLV Ag) for immunizing mice was the serum of one of the blood donors studied by Cossart *et al.* (1975) and later referred to as serum B19 (Paver & Clarke, 1976). It had been stored at −20 °C since it was originally found to contain SPLV. Antigen was purified by gel filtration followed by pelleting through sucrose. Two ml of serum B19 was applied to a 2·9 × 32 cm column of Sephacryl S-300 (Pharmacia Fine Chemicals Ltd) and eluted with phosphate buffered saline (PBS), pH 7·2, containing 0·1 % bovine serum albumin (BSA). Fractions (2·5 ml) were collected, monitored for optical density and tested for SPLV Ag by CIE. Antigen was eluted in the first protein peak. Two ml amounts of pooled antigen positive fractions were layered on to 3 ml of 20 % (w/v) sucrose and centrifuged for 4 h at 40000 rev./min (150000 ***g***) in a Beckman L3-50 ultracentrifuge using a SW50.1 rotor. The resulting pellet was resuspended in 0·2 ml PBS using a bath sonicator, distributed into 0·1 ml aliquots and stored at −20 °C. The purified SPLV Ag had a titre of 1/16 by CIE compared with a titre of 1/4 in the starting material.

Supplies of serum B19 were limited and it was only used for immunizing mice. In subsequent tests (except where stated) SPLV Ag was obtained from a unit of plasma (Br I) collected from a blood donor at the South West Regional Transfusion Centre, Bristol (kindly provided by Mr A. Archer). Antigen from 2 ml aliquots of the Br I plasma was prepared by centrifuging through sucrose as described above, the pellet being resuspended in 0·5 ml PBS containing 0·5 % BSA (PBS/BSA). A negative control antigen was prepared in the same way from the plasma of a blood donor negative for SPLV Ag.

Counter-immunoelectrophoresis

CIE was carried out as described by Cohen, Hewish & Mortimer (1981) using serum 'P' (Cossart *et al.* 1975) to detect SPLV Ag and serum 'W' from a SPLV Ag positive blood donor (kindly supplied by Mr B. Combridge, Blood Products Laboratory, Elstree) to detect antibody to SPLV. Both reagents were used undiluted.

Preparation of monoclonal antibodies

A non-secreting mouse myeloma cell line of BALB/c origin, Ag 8653, (given by Dr B. Askonas, National Institute for Medical Research, London) was grown in

Dulbecco's modified Eagle's medium with 15% fetal calf serum. The cells were fused with immune spleen cells according to the method of Köhler & Milstein (1976). The spleen cell donor was a BALB/c mouse immunized by intraperitoneal injection with 0·1 ml of a suspension of SPLV Ag purified from serum B19. A boosting dose of 0·1 ml of the same material was given into the tail vein 6 months later and the spleen removed after a further 3 days. Hybridoma cells producing antibody to SPLV detected by enzyme linked immunosorbent assay (ELISA) were cloned by limit dilution and inoculated intraperitoneally into pristane-primed BALB/c mice. Both hybridoma cell culture fluids and mouse ascitic fluids were used for further tests.

ELISA screening test for monoclonal anti-SPLV

Preparation of solid phase SPLV and control antigens. Antigen was bound to the solid phase with IgG of anti-SPLV specificity. Flexible P.V.C. microtitre plates with flat bottomed wells, or flat bottomed 'removawells' (Dynatech Ltd) were used as the solid phase. The IgG fraction of a human serum ('P') positive for anti-SPLV by CIE was prepared by ion-exchange chromotography using DE-52 (Whatman Ltd) and 0·02 M phosphate buffer, pH 8·0. It was coated on to the solid phase in 0·1 ml volumes, diluted in 0·05 M carbonate-bicarbonate buffer, pH 9·6 by incubation at 4 °C for 18 h. The plate was then washed three times with PBS containing 0·05% tween 20 (PBST), and 0·2 ml PBS/BSA was added to each well. The plate was incubated for 2–3 h at room temperature and washed again. Test and control antigens were diluted in PBST containing 10% fetal calf serum (PBST/FCS) and 0·1 ml added to each well of the anti-SPLV IgG-coated plate. After incubation at 4 °C for 18 h the plate was ready to use as the solid phase for ELISA screening for monoclonal antibody to SPLV.

Performance of ELISA screening test. Antigen was aspirated from the wells of the microtitre plate, and the plate was washed. A 0·1 ml volume of the tissue culture or ascitic fluid from the hybridoma under test was added to SPLV- and control antigen-coated wells. The tissue culture fluids were screened neat and the ascitic fluids were tested at dilutions in PBST/FCS from 1/100. They were incubated with the solid phase antigens for 2 h at 37 °C and the plate was then washed. A 0·1 ml volume of goat anti-mouse IgG peroxidase conjugate (TAGO Reagents, Tissue Culture Services Ltd.) was added to each test well. The conjugate was diluted in PBST/FCS to a dilution of 1/300 as recommended by the manufacturer. After a further incubation period of 2 h at 37 °C the plate was washed to remove unbound conjugate. The ELISA reaction was developed by adding 0·1 ml of a substrate solution consisting of 0·05 M citric acid/0·1 M disodium hydrogen phosphate buffer, pH 5·0, containing 40 mg orthophenylenediamine and 0·04 ml of 30% H_2O_2 per 100 ml of buffer. After 30 min incubation in the dark at room temperature colour development was stopped by adding 0·025 ml 2·5 M sulphuric acid. The optical densities at 492 nm were measured in a 'Multiskan' ELISA reader (Flow Laboratories).

Once monoclonal anti-SPLV had been identified in a hybridoma tissue culture fluid by the method described above, it was possible to standardize the reagents used to prepare the solid phase antigen for the ELISA screening test. This was

done by a 'chessboard' titration of the coating anti-SPLV IgG and SPLV Ag. The results indicated that the optimal dilution of anti-SPLV IgG was 1:100 and of SPLV Ag 1:30.

Immune electron microscopy

Immune electron microscopy (IEM) was carried out to confirm the presence of anti-SPLV in tissue culture fluids from selected ELISA positive hybridomas. A 0·2 ml volume of the tissue culture fluid was mixed with 0·1 ml of the Br I plasma containing SPLV Ag and incubated at room temperature for 1 h. The mixture was diluted to 2·5 ml with PBS and centrifuged at 18000 rev./min (40000 ***g***) for 1 h in a Sorvall RC2-B centrifuge. The resulting pellet was negatively stained with 3% phosphotungstic acid, pH 6·3 and examined in an AEI 801 electron microscope for the presence of aggregates of parvovirus-like particles.

IEM was also used to confirm RIA and CIE results for SPLV Ag on clinical specimens. The serum under test (0·1 ml) was mixed with 0·1 ml of a standard anti-SPLV serum ('P') and incubated 1 h at room temperature. Pelleting and staining were then carried out as described above.

Development of the assay for anti-SPLV IgM

The principle of 'M'-antibody capture (Flehmig *et al.* 1979) was adopted to measure anti-SPLV IgM. As solid phase, 6·4 mm diameter polystyrene beads (Northumbria Biologicals) were used. They were coated by immersion in goat anti-human μ chain serum (TAGO reagents) diluted in 0·05 M carbonate/bicarbonate buffer, pH 9·6, at room temperature for 2–3 h and then at 4 °C until needed. The optimal dilution of the anti-μ serum was 1 in 3000 (Dr D. W. G. Brown, personal communication). Prior to use the anti-μ coated beads were washed with PBST and then incubated in reaction trays (Abbott Laboratories) for 3 h at 37 °C with 0·2 ml volumes of the sera under test, diluted in PBST/FCS (the optimal dilution for screening the sera was 1 in 300–see Results). The beads were washed, and 0·2 ml of SPLV Ag, diluted in PBST/FCS to its working concentration, was added for a further 3 h at 37 °C. After washing, 0·2 ml of monoclonal anti-SPLV was added to each bead. The source of monoclonal antibody for the assay was the IgG fraction of ascitic fluid collected from a mouse which had been inoculated with a clone of anti-SPLV-producing hybridoma cells. The IgG fractions of ascitic fluids with anti-SPLV titre of $\geqslant 10^{-5}$ by ELISA were separated by ion-exchange chromatography on DE-52 using 0·01 M, 0·03 M and 0·06 M phosphate buffers, pH 8·0, as described by Tedder, Yao & Anderson (1982). It was diluted in PBST/FCS to its optimal concentration and incubated with the beads for 18 h at room temperature. The beads were then washed and 0·2 ml of ^{125}I-labelled sheep anti-mouse Ig (Amersham International, code IM.131) was added. The radiolabel was diluted in PBST/FCS with 5% human serum negative for anti-SPLV (NHS), to give approximately 50000 c.p.m. per 0·2 ml. After 2 h incubation at 37 °C the beads were washed again with PBST and the bound radioactivity measured over five minutes in a NE 1600 gamma counter (Nuclear Enterprises Ltd). Anti-SPLV IgM was quantified by the method described for anti-hepatitis B core IgM (Tedder & Wilson-Croome, 1980) using a strongly positive control serum (J.H.) that was arbitrarily regarded as containing 100 units of anti-SPLV IgM.

It was also possible to perform the assay as an ELISA in microtitre plates with peroxidase-conjugated anti-mouse IgG in place of the ^{125}I-labelled antiserum. Procedure and conditions were as described for MACRIA but it was necessary to use the reagents at approximately ten-fold lower dilutions.

Development of the assay for anti-SPLV IgG

Anti-SPLV IgG was measured by a method analogous to that for detecting anti-SPLV IgM. IgG was 'captured' on to a solid phase coated with anti-human IgG (γ chain specific) and the performance of the assay then followed that of the anti-SPLV IgM RIA.

In a preliminary investigation solid phase coatings by sheep (TAGO Reagents), goat (Abinc) and rabbit (DAKO Reagents) anti-human γ chain sera were compared. The rabbit antiserum gave the best discrimination between anti-SPLV IgG positive and negative sera. Polystyrene beads were coated by immersion in a 1 in 1000 dilution of this serum in 0·05 M carbonate/bicarbonate buffer, pH 9·6, for 2–3 h at room temperature and for 18 h, or until needed, at 4 °C.

Prior to use the beads were washed with PBST and added to a 0·2 ml volume of a dilution of each specimen in PBST/FCS, in the well of a reaction tray. The effect of varying the specimen dilution was investigated (see Results) and a 1 in 30 dilution found to be convenient. After 3 h at 37 °C, the beads were washed and 0·2 ml of SPLV Ag, diluted in PBST/FCS to its working dilution (see Results), was added. After a further 3 h at 37 °C the beads were washed three times with PBST and 0·2 ml of the monoclonal antibody added.

Both tissue culture fluids and ascitic fluids derived from anti-SPLV positive hybridomas were investigated as monoclonal antibodies for the assay (see Results) and, as in the anti-SPLV IgM RIA, a 1/3000 dilution of IgG purified from ascitic fluid was chosen. After 18 h at room temperature the monoclonal antibody was aspirated and the beads washed.

The assay was completed by adding ^{125}I-labelled anti-mouse Ig, as for the anti-SPLV IgM RIA. After 2 h at 37 °C the beads were washed and the bound radioactivity was measured. Anti-SPLV IgG was quantified in the same way as anti-SPLV IgM with 100 unit control serum (K.M.) that was strongly anti-SPLV IgG positive.

Assays for SPLV antigen using monoclonal antibody

An antigen assay was set up by using the MACRIA system with a serum known to be positive for anti-SPLV IgM (diluted 1 in 300). Dilutions of the test specimen (antigen status unknown) were inserted in place of SPLV Ag and the assay was completed as described above. A similar antigen assay was set up using IgG antibody capture with a serum known to be strongly anti-SPLV positive, diluted 1 in 3000.

Sera tested

Sequential sera from two soldiers with febrile illness due to SPLV infection (Shneerson *et al.* 1980) and serum from two contacts in the same unit were examined. Sera from one of the patients were also tested by a MACRIA based on human convalescent serum as described by Anderson and co-workers (1982).

Five sera from a patient with haemolytic anaemia due to pyruvate kinase deficiency in aplastic crisis were kindly made available by Dr J. B. Kurtz, Public Health Laboratory, Oxford. Two sera from family contacts of this patient were also examined.

Sera from two SPLV outbreaks in families with hereditary spherocytosis were examined. Details of these outbreaks are reported elsewhere (for family H, Kelleher *et al.* 1983; for family S, Harvey *et al.*, personal communication).

Sera collected from 310 blood donors at North London Blood Transfusion Centre in July 1982 were provided by the director, Dr T. D. Davies. All were tested for anti-SPLV by CIE and for anti-SPLV IgG by RIA. The first one hundred were also tested for anti-SPLV IgM by RIA.

A panel of 80 sera from conditions unrelated to SPLV infection were examined for anti-SPLV IgM by RIA to assess the specificity of the assay. We thank Drs W. Knowles, S. D. Gardner and J. M. B. Edwards for giving some of these specimens, and for making their diagnostic findings available.

RESULTS

Production of monoclonal antibodies to SPLV

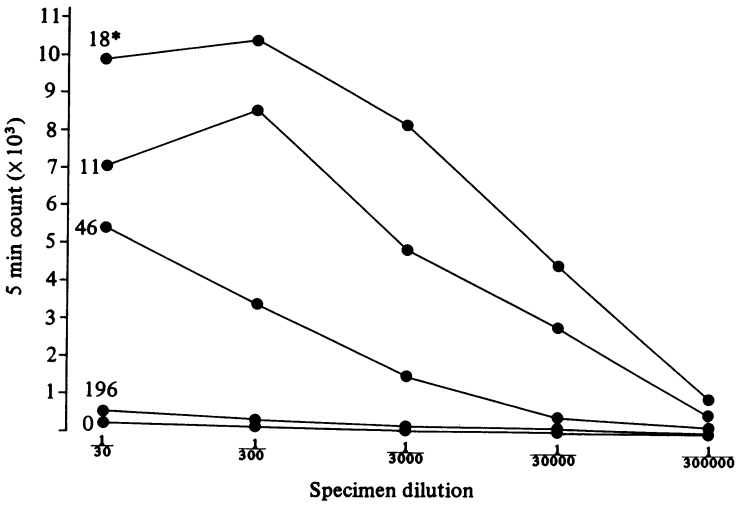
The fusion of immune spleen cells with mouse myeloma cells resulted in the formation of 24 hybridomas, of which eight were identified as secretors of anti-SPLV by the ELISA screening test. This was confirmed in selected cases by immune electron microscopy (Plate 1 A). Tissue culture fluids from ELISA positive hybridomas aggregated SPLV particles whereas ELISA negative fluids did not. Anti-SPLV in tissue culture fluids from ELISA (and IEM) positive hybridomas did not, however, react in the CIE test for anti-SPLV.

Two of the eight positive hybridomas (VRL/B19/7 and VRL/B19/11) were cloned, and the remaining six were stored at −180 °C. Clones derived from the two parent hybridomas were inoculated into mice and the resulting ascitic fluids collected. It was necessary to separate IgG from the ascitic fluid because, without further purification, it bound the labelled anti-mouse Ig reagents non-specifically. Ion exchange chromotography of the ascitic fluids resulted in two protein peaks obtained with 0·03 and 0·06 M phosphate buffers, pH 8·0. Most anti-SPLV activity (determined by ELISA) was present in the 0·03 M peak, and material from this peak was used as a monoclonal antibody reagent.

Development of RIA for anti-SPLV IgM

In the development of RIA for anti-SPLV IgM the effect of varying the concentration of reactants at four stages of the assay were studied.

Test specimens Five sera from one of the two patients with febrile illness due to SPLV were diluted in 10-fold steps in PBST/FCS from 1/30 to 1/30000 and tested for anti-SPLV IgM (Fig. 1). The best discrimination between strongly positive and negative reactions was obtained with sera diluted 1 in 300. Weakly positive specimens collected 6 weeks or more after the onset of illness gave stronger reactions when tested at a dilution of 1 in 30. A similar result was obtained with the set of sera collected from the other patient. A dilution of 1 in 300 was therefore adopted for testing specimens for anti-SPLV IgM.



* Number of days after first specimen with SPLV Ag

Fig. 1. Anti-SPLV IgM RIA: effect of varying specimen dilution of five sequential sera from a patient (M.D.) with SPLV infection. The admission specimen (day 0) was SPLV Ag positive. Subsequent specimens collected on day 11, 18, 46 and 196 were SPLV Ag negative.

Table 1. *Effect of varying SPLV Ag concentration*

	ANTI-SPLV IgM		ANTI-SPLV IgG	
	Label*		Label	
Antigen dilution	binding (%)	P/N† ratio	binding (%)	P/N ratio
10^{-2}	4·20	30·0	4·62	75·8
$10^{-2·5}$	3·98	50·0	3·78	68·2
10^{-3}	2·90	47·5	2·99	51·3
$10^{-3·5}$	1·75	39·2	1·26	17·0

* ^{125}I-labelled anti-mouse Ig: % binding with 100 unit control.
† Ratio of 100 unit positive control/negative control counts.

SPLV antigen. SPLV antigen was diluted in 0·5 $\log_{10}$ steps and tested in the anti-SPLV IgM assay using known positive and negative sera (Table 1). Although maximum label binding occurred with antigen diluted 10^{-2}, the highest P/N ratios were obtained when antigen was diluted $10^{-2·5}$ or 10^{-3}. This reflects the lower non-specific binding by negative serum when higher dilutions of antigen were used. A dilution of 1 in 1000 was used in subsequent assays as it gave good discrimination between positive and negative specimens and was economical of antigen.

Monoclonal anti-SPLV. Using the 100 unit anti-SPLV IgM positive specimen diluted 1 in 300 and SPLV Ag diluted 1 in 1000, an anti-SPLV IgM assay was set up with varying concentrations of monoclonal anti-SPLV in the form of either purified IgG from ascitic fluid or untreated tissue culture fluid. Material derived from the two hybridomas studied gave similar results and those obtained with hybridoma VRL/B19/11 are illustrated (Fig. 2). Maximum label binding was obtained with purified IgG from ascitic fluid diluted between 1 in 100 and 1 in

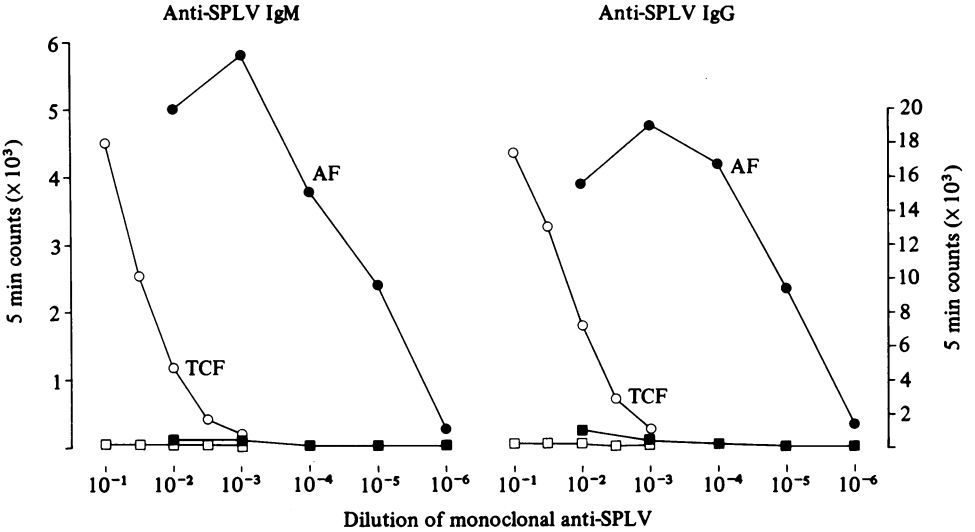


Fig. 2. Effects of varying dilution of monoclonal anti-SPLV in RIA for specific IgM and IgG. Different concentrations of tissue culture fluid (TCF) and ascitic fluid (AF) derived from hybridoma VRL/B19/11 were tested with the appropriate 100 unit positive control serum (○—○, ●—●) and NHS (□—□, ■—■).

Table 2. *Effect of adding normal human serum to diluent for ^{125}I labelled anti-mouse Ig*

	^{125}I-labelled anti-mouse Ig			
	With 5% NHS		Without 5% NHS	
	% label binding	P/N	% label binding	P/N
Anti-SPLV IgM Units				
100	3·06	114·6	3·12	29·7
1	0·85	32·0	0·87	8·25
0	0·03	—	0·11	—
Anti-SPLV IgG Units				
100	3·75	39·5	5·51	17·4
1	0·30	3·1	0·60	1·9
0	< 0·1	—	< 0·1	—

10000. Less label was bound when tissue culture fluid was used, even at a low dilution. A 1 in 3000 dilution of the IgG from ascitic fluid was chosen as it gave both good label binding and high P/N values.

Radio-labelled anti-mouse Ig. The effect of adding 5% NHS to the PBST/FCS label diluent is shown in Table 2. Lower label binding, but higher P/N values were obtained with diluent containing NHS.

Development of RIA for anti-SPLV IgG

The concentration of reactants at four stages of the anti-SPLV IgG RIA test procedure were studied using the 100 unit positive serum (K.M.) and, as negative control serum, NHS. The results of these studies are set out below.

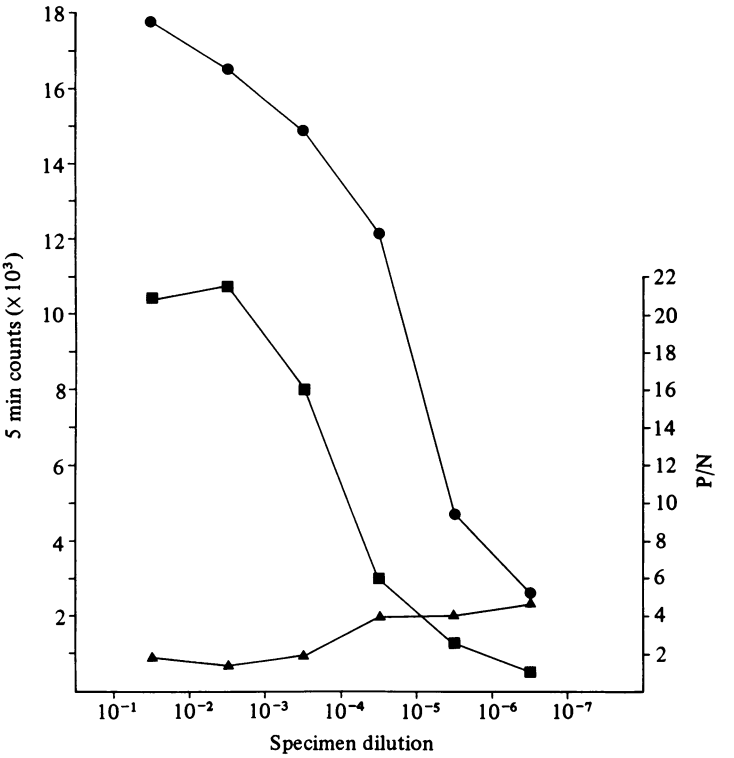


Fig. 3. Anti-SPLV IgG RIA: effect of varying specimen dilution. A 100 unit positive control serum (●—●) and NHS (▲—▲) were tested at dilutions ranging from $10^{-1.5}$ to $10^{-6.5}$. The ratio of counts given by the positive serum to those given by NHS (P/N) is plotted at each dilution (■—■).

Test specimen. The effect of varying the dilution of the specimen under test is shown in Fig. 3. Pelleted antigen from Br I plasma was used at a dilution of 1 in 1000 for this experiment. The best discrimination between antibody positive and negative sera was obtained with specimens diluted between $10^{-1.5}$ and $10^{-2.5}$. For convenience a screening dilution of $10^{-1.5}$ was adopted. With negative sera more radio-label was bound with increasing dilutions of the specimen.

SPLV antigen. The results obtained with varying dilutions of SPLV Ag are shown in Table 1. As in the anti-SPLV IgM assay, an antigen dilution of 10^{-3} gave satisfactory results with the 100 unit positive control. Higher P/N values were obtained with antigen diluted $10^{-2.5}$, and as it was helpful in testing weakly reactive specimens, this dilution of SPLV Ag was chosen for anti-SPLV IgG assays.

Monoclonal anti-SPLV. Best results were obtained with IgG purified from ascitic fluids, diluted between 10^{-2} and 10^{-4} (Fig. 2). As in the anti-SPLV IgM assay, a dilution of 1 in 3000 was chosen for the anti-SPLV IgG test. Tissue culture fluids diluted up to 1 in 30 gave adequate results.

Radio-labelled anti-mouse Ig. The effect of adding 5% NHS to PBST/FCS for the label diluent is shown in Table 2. Lower label binding but higher P/N values were achieved with diluent containing NHS.

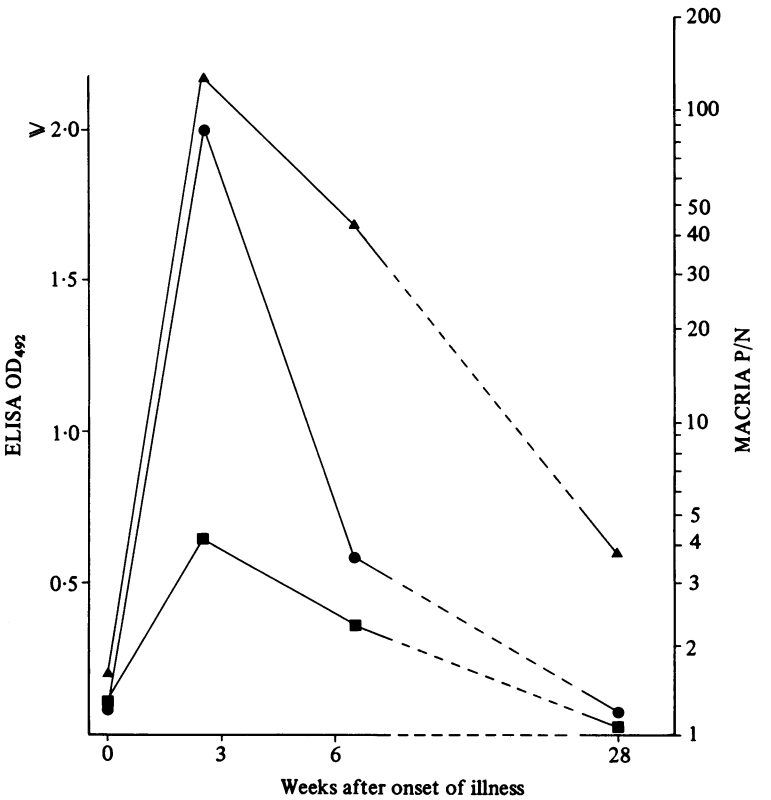


Fig. 4. Anti-SPLV IgM response in patient M.D. measured by three assays: MACRIA (▲—▲) and ELISA (●—●) using monoclonal anti-SPLV, and MACRIA (■—■) based on human convalescent anti-SPLV.

Detection of anti-SPLV IgM

Two patients with febrile illness. The production of anti-SPLV IgM by one of the two patients (M.D.) with febrile illness due to SPLV infection is illustrated in Fig. 1. The specific IgM response was similar in the second patient. Specimens collected on admission to hospital, which were SPLV Ag positive, contained no anti-SPLV IgM. Specimens collected 11 days later, which were SPLV Ag negative, were anti-SPLV IgM positive. After a further 7 days the specific IgM peaked, and then declined and was virtually undetectable in specimens collected at day 196.

The two contacts were both anti-SPLV positive by CIE when tested 11 days after admission to hospital of the index cases. One of them was strongly positive for specific IgM by RIA and ELISA with monoclonal anti-SPLV, indicating recent infection. The other had anti-SPLV IgG but not IgM.

The specific IgM response in patient M.D. was measured by ELISA as well as by RIA using the monoclonal antibody, and also by MACRIA with a radio-labelled polyclonal anti-SPLV of human origin. Although the pattern of response measured by all three assays was similar (Fig. 4) the assays based on monoclonal anti-SPLV discriminated more clearly between positive and negative sera.

Aplastic crisis in a patient with pyruvate kinase deficiency. The markers of SPLV

Table 3. *Aplastic crisis in pyruvate kinase deficiency: markers of SPLV infection in a patient and contacts*

Date of specimen	Anti-SPLV IgM (units)	Anti-SPLV IgG (units)	Other findings*
		Patient T.T.	
5.v.82†	N/A	N/A	SPLV EM positive
10.v.82‡	40·0	16·7	Anti-SPLV IEM postive
17.v.82	76·0	71·0	Anti-SPLV IEM positive
7.vi.82	8·1	37·5	—
25.vi.82	1·4	62·0	—
15.vii.82	1·1	> 100·0	—
		Sister J.T.	
13.v.82	4·6	50·0	Anti-SPLV IEM positive
		Grandfather J.M.	
7 vi. 82	< 1	31·0	Anti-SPLV IEM positive

* Courtesy of Dr. J. B. Kurtz, PMLS Oxford.
† Not available for testing by RIA.
‡ SPLV Ag negative by RIA.

Table 4. *Aplastic crisis in hereditary spherocytosis: markers of SPLV infection in two family outbreaks*

	Date of specimen	SPLV Ag CIE	SPLV Ag RIA T/N ratio	Anti-SPLV IgM (units)	Anti-SPLV IgG (units)
(*a*) Family S					
Sibling Ad.S*	23. iv. 79	–	5·1†	1·2	< 1
	4. v. 79	–	< 2	> 100·0	27·4
P.S.*	23. iv. 79	–	< 2	1·9	< 1
	4. v. 79	–	< 2	> 100·0	31·0
K.S.	23. iv. 79	–	< 2	7·4	1·6
	4. v. 79	–	< 2	35·8	28·0
S.S.*	23. iv. 79	–	< 2	> 100·0	53·0
	4. v. 79	–	< 2	29·0	76·0
Ag.S.*	4. v. 79	–	< 2	14·0	27·0
Grandmother B.S.	23. iv. 79	–	< 2	< 1	17·5
	4. v. 79	–	< 2	< 1	22·0
Father	23. iv. 79	–	< 2	< 1	5·3
	4. v. 79	–	< 2	< 1	7·4
(*b*) Family H					
Sibling J.H.*	7. v. 82	+	21·5†	< 1	< 1
	12. v. 82	–	< 2	75·0	8·3
	15. vi. 82	–	< 2	16·0	24·3
S.H.*	7. v. 82	–	< 2	74·0	16·0
	15. vi. 82	–	< 2	10·5	> 100
Mother E.F.	14. v. 82	–	31·7†	< 1	< 1
	15. vi. 82	–	< 2	53·0	46·0

* Hereditary spherocytosis.
† Presence of SPLV confirmed by electron microscopy.

Table 5. *Anti-SPLV IgM in sera from conditions unrelated to SPLV infection*

	Number of sera with anti-SPLV IgM (units)			
	1	1·0–2·9	3·0–9·9	⩾ 10·0
Hepatitis A*	9	1	0	0
Acute hepatitis B*	9	0	1	0
Chronic hepatitis B	10	0	0	0
Cytomegalovirus infection*	9	1	0	0
Rubella*	10	0	0	0
BK virus reactivation*	10	0	0	0
Infectious mononucleosis (Monospot positive)	10	0	0	0
Rheumatoid factor positive	6	2	1	1

* Specfic IgM positive.

infection detected in the patient with pyruvate kinase deficiency are shown in Table 3. Parvovirus-like particles were seen by electron microscopy in a specimen taken 3 days after the onset of illness. The first specimen available for RIA testing, collected 5 days later, was SPLV Ag negative, but positive for anti-SPLV IgM and IgG. Higher levels of anti-SPLV IgM and IgG were present in a serum obtained 7 days later. Declining amounts of specific IgM, but persistently high levels of specific IgG, were found in three other specimens collected during the following 2 months. Recent SPLV infection was also diagnosed in this patient's sister by detection of anti-SPLV IgM. Another family contact was anti-SPLV IgG positive but IgM negative.

Two SPLV outbreaks in families with hereditary spherocytosis. Anti-SPLV IgM was detected in all five siblings of family S, including one unaffected by spherocytosis, but not in their father or grandmother. Significant changes in the titre of anti-SPLV IgM were observed in paired sera obtained from four of the five children (Table 4). In one child, Ad.S., low levels of specific IgM (1·2 units, P/N = 7·0) were found together with SPLV Ag (Plate 1 B).

Anti-SPLV IgM was detected in the two siblings and mother of family H (Table 4). Follow-up sera from the children showed declining levels of anti-SPLV IgM, but increasing levels of anti-SPLV IgG.

Blood donors. None of the 100 donors tested was anti-SPLV IgM positive.

Sera from patients with conditions unrelated to SPLV infection. Ten sera from each of eight conditions were examined. Of the eighty specimens, seven reacted in the anti-SPLV IgM RIA (Table 5). Six of these specimens were weakly positive, but one serum from a patient with rheumatoid factor contained more than 10 units of anti-SPLV IgM.

Detection of anti-SPLV IgG by RIA

Survey of blood donors. By RIA, 190 (61 %) out of 310 donors were positive for anti-SPLV IgG. The quantity (units) of specific IgG in each sample of donor serum was estimated, and the distribution of results is shown in Fig. 5.

By CIE, 134 (43 %) donors were anti-SPLV positive. All of these donors were anti-SPLV IgG positive by RIA. A further 56 RIA positive donors were CIE

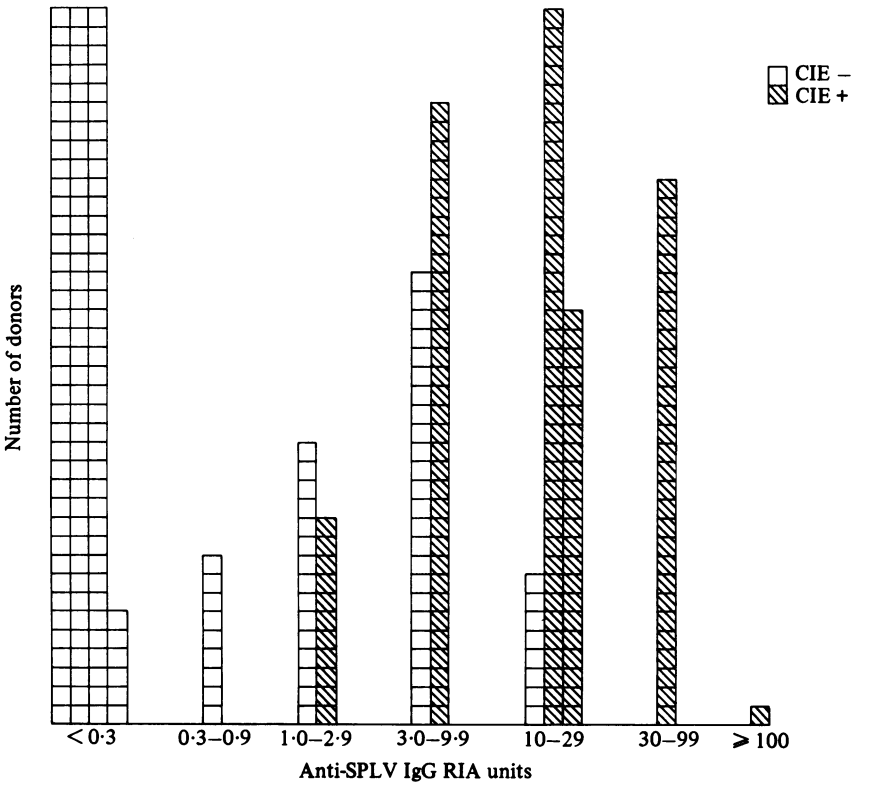


Fig. 5. Antibody to SPLV in 310 blood donors.

negative. RIA strongly positive donors were much more likely to be CIE positive, so that all donors with ⩾ 30 RIA units of anti-SPLV IgG were CIE positive.

Seroconversion in patients infected by SPLV. Patient M. D. (Fig. 1) was anti-SPLV IgG negative on admission to hospital. Subsequently, anti-SPLV IgG was detected on day 11 (46 units) and through to day 196 (39 units). The specific IgG response was similar in the other patient with a febrile illness due to SPLV infection. Conversion from anti-SPLV IgG negative to positive was also observed in the outbreaks of SPLV infection in the two families with hereditary spherocytosis (Table 4). In family S two siblings (P.S. and Ad.S.) seroconverted and another (K.S.) showed a significant rise in anti-SPLV IgG titre. In family H, one child (J. H.) and the mother seroconverted; the second child showed a significant rise in titre.

Detection of SPLV antigen

Titration of antigen Br I. Dilutions of the BrI plasma in PBST/FCS were tested from SPLV Ag by μ- and γ-capture RIA. Antigen was detected at dilutions up to 1 in 10000 by both assays (Fig. 6). This compared with a titre of 1 in 4 when Br I was tested by CIE with a human anti-SPLV serum.

Antigen detection in clinical specimens. Sera from the two outbreaks of SPLV infection in hereditary spherocytosis were tested for antigen by μ-capture RIA (Table 4). Antigen was detected by RIA in two sera that had previously been found

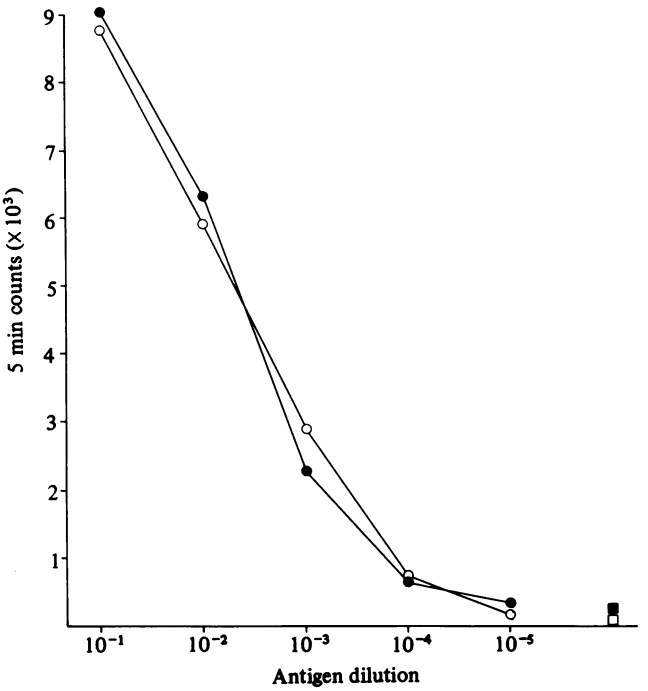


Fig. 6. Titration of SPLV Ag by RIA. Dilutions of plasma Br I were prepared in PBST/FCS buffer and examined by M (○—○) and G (●—●) antibody capture RIA. Samples of buffer containing no antigen were also tested in the two assays (□, ■).

SPLV Ag negative by CIE. In both cases the presence of SPLV Ag was confirmed by IEM. One of these sera, from patient Ad.S., contained large complexes with numerous SPLV particles which could be seen *without* the addition of anti-SPLV. The cross-linking antibody in these complexes had the appearance typical of IgM (Plate 2). Serum from the other patient in this category (E. F.) contained much smaller numbers of SPLV particles which could only be identified readily after the addition of specific antibody.

DISCUSSION

Diagnostic tests for SPLV have been inadequate until now because of both shortage of reagents and lack of sensitivity. The scarcity of purified antigen has prevented specific hyperimmune serum being raised in laboratory animals, and serological studies have had to be based on insensitive tests using as antibody human convalescent serum. However, antibodies with the necessary specificity have now been prepared from small quantities of only partially purified antigen by the hybridoma method of Köhler & Milstein (1976). With these monoclonal antibodies solid phase assays for anti-SPLV IgM and IgG and for SPLV antigen have been developed. All the assays are based on the principle of antibody capture, and thus problems associated with the narrow specificity of monoclonal antibodies are not relevant (Tedder *et al.* 1982). The two antibodies used in the assays, produced by clones VRL/B19/7 and VRL/B19/11, are equally satisfactory. Further studies on the specificity of these two antibodies, and of antibodies produced by other clones, are in hand.

In the assays described, monoclonal anti-SPLV has been used with radio-or enzyme-labelled anti-mouse Ig sera as indicator reagents. As these labelled antisera are available commercially and are of high quality, they provide a convenient means of completing the assays, though the additional step involved does result in somewhat cumbersome tests. In an attempt to overcome this problem monoclonal anti-SPLV was directly labelled in work not reported here. Assays for SPLV Ag with this directly labelled monoclonal anti-SPLV were satisfactory and are being developed. Assays for anti-SPLV IgM and IgG however, were inferior to those based on labelled anti-mouse Ig as described above.

Monoclonal anti-SPLV has helped to alleviate the main problem associated with SPLV studies – shortage of antigen. Because the solid phase assays based on monoclonal anti-SPLV are so sensitive the test is satisfactory even if SPLV Ag is used below its optimal concentration, and this is less than that required for CIE, or for RIA with human convalescent serum as the source of anti-SPLV.

The increased sensitivity of the assays using monoclonal antibodies was apparent in both RIA and ELISA, but the working dilution of antigen was at least ten-fold higher in RIA than ELISA, and this was therefore chosen as the method for diagnostic assays. Though it requires more antigen, ELISA for anti-SPLV IgM is otherwise a satisfactory alternative to RIA for diagnostic work. For the screening of hybridoma tissue culture fluids ELISA was actually preferred, because its microtitre format was compatible with that used to propagate the clones and it was convenient to read results by eye.

Both the tissue culture fluids and ascitic fluids derived from anti-SPLV producing clones could be used in the assays described. Tissue culture fluid could be incorporated into the assay without further treatment, but antibody from ascitic fluid had to be purified to reduce non-specific activity. However, anti-SPLV purified from ascitic fluid gave higher binding of the ^{125}I-labelled anti-mouse Ig and higher test:negative count ratios than the tissue culture fluid-derived antibody. Moreover, ascitic fluids, having higher titres of antibody than tissue fluids, were more convenient for storage. For these reasons, ascitic fluid was the preferred source of monoclonal anti-SPLV.

Sensitive assays such as described here will certainly be needed to find new supplies of SPLV Ag. Originally the antigen was obtained from viraemic blood donors detected by chance during hepatitis B screening (Cossart *et al.* 1975; Paver & Clarke, 1976); but as hepatitis testing is now done by more specific methods it is unlikely that SPLV Ag will often be found in this way again. Several alternatives exist. Screening donors for SPLV Ag by a solid phase assay compatible with the automated procedures now in use at transfusion centres might be possible. The incidence of SPLV antigenaemia in donors is likely to be low, however, and it may only be found when SPLV is epidemic. Other sources should therefore be sought. Since SPLV is readily transmissible, antigen might be obtained from the healthy contacts of patients with SPLV-related aplastic crises. Other possible sources include the laboratory production of SPLV, either by propagation in cell culture or by the application of recombinant DNA techniques. The production of SPLV Ag from *in vitro* sources can now be monitored by the sensitive assays based on monoclonal anti-SPLV.

Serological diagnosis of SPLV infection may be made in four ways: by detecting SPLV antigen, anti-SPLV IgM, a seroconversion or a rising titre of anti-SPLV IgG.

Illustrations of all these processes are provided by the patients tested in this study. Antigen was demonstrated in six patients, all of whom subsequently developed anti-SPLV IgM and IgG. Antigen was found by CIE and RIA in four of the patients, the other two being CIE negative but RIA positive. In all six the presence of SPLV was confirmed by electron microsopy. Anti-SPLV IgM and IgG developed in a further ten subjects in whom SPLV Ag was not found, probably because the antigenaemic phase was over before a specimen could be taken. However, the observation of numerous SPLV particles by electron microscopy in one patient's serum that was antigen negative by CIE and only weakly positive by RIA (Ad.S., Table 4) should prompt a re-examination of other convalescent sera. It is possible that early specific antibody masks SPLV Ag, making it difficult to detect by CIE and RIA while it can still be readily seen in the electron microscope. Only small numbers of SPLV particles were seen in the specimen from the other patient who was antigen negative by CIE but positive by RIA (E.F., Table 4), though the RIA test: negative count ratio was higher in this sample (31·7) than in that from patient Ad.S. (5·1). Unlike the specimen from Ad.S., that from E.F. contained no anti-SPLV IgM and no visible complexes of virus particles. It apparently contained more free antigen and was probably collected in the early phase of this asymptomatic infection.

Though intense, SPLV antigenaemia is relatively brief and it may precede onset of symptoms by several days. More infections, therefore, are likely to be diagnosed by detecting specific IgM than by detecting antigen. During this study several observations were made about the anti-SPLV IgM response. There was no detectable gap between the clearance of SPLV Ag and formation of specific antibodies. The formation of anti-SPLV IgM slightly preceded anti-SPLV IgG (patients P.S. and K.S., Table 4). The specific IgM response peaked at 5–18 days, had declined considerably by 50 days, but could still be measured in trace amounts for up to 6 months, especially if the specimen was examined at a lower dilution. Demonstration of anti-SPLV IgM was particularly valuable when only a single specimen was available from, for example, asymptomatic contacts of index cases. There was no evidence that the specific IgM response in patients with chronic haemolytic anaemia differed from that in normal subjects though the clinical features were so different. Tests for specificity of the RIA for anti-SPLV IgM were carried out on 100 blood donors and on 80 patients with conditions unrelated to SPLV infection. None of the donors reacted in the assay, but seven of the patients were positive. One patient with rheumatoid factor reacted strongly (> 10 units) in the anti-SPLV IgM assay.

Seroconversion or rising anti-SPLV IgG titres, although observed in the present study, were not needed to diagnose infection. When they were found, SPLV Ag or anti-SPLV IgM was also detected. For longitudinal studies, however, seroconversion would be a useful indicator of intervening infection.

The prevalence of anti-SPLV IgG in the survey of blood donors, measured by RIA, was 61 %. This is higher than that found by CIE in this (43 %) and in previous (30–40 %) studies of donors (Cossart *et al.* 1975; Paver & Clarke, 1976). Although CIE was less sensitive than RIA and gave many presumably falsely negative results, there were no CIE positive, RIA negative results. We had no means of confirming weak RIA reactions for anti-SPLV IgG, but as almost all were clearly

distinguishable from negative results there is no reason to doubt their specificity. The IgG antibody-capture RIA provided a means by which monoclonal antibody could be employed to measure anti-SPLV IgG. It gave a more accurate measure of the extent to which SPLV infects the community than previous methods for SPLV testing.

Assays using monoclonal antibody therefore confirmed and extended the findings made by methods based on human convalescent serum as source of anti-SPLV (reviewed by Anderson, 1982). The sequence of serological events in SPLV infection is that of a massive, but transient antigenaemia followed by the development of specific IgM and IgG. Compared with the results of earlier methods, the assays using monoclonal antibodies detected antigen in more cases and for longer. They distinguished between anti-SPLV IgM positive and negative sera readily and determined anti-SPLV IgG status precisely. In addition these assays had the practical advantage of being economical in the use of SPLV antigen.

It will now be possible to investigate the epidemiology and pathogenic potential of SPLV more effectively. Because of the easily recognized complication that arises, more is known of the effect of SPLV infection in a group of uncommon haemolytic anaemias than in otherwise normal patients. However, the high prevalence of antibody to the virus in adults indicates that the infection is widespread, and it should now be possible to identify more infections in normal subjects. Some of these may be subclinical, but others are probably associated with outbreaks of febrile illness. Significant fever and malaise without localising signs are likely features of infection in normal patients, just as aplastic crisis is in patients with haemolytic anaemia. The SPLV assays described above will prove valuable in investigating these conditions and in testing asymptomatic contacts of the cases identified.

We thank Mr R. Studd and Mr P. Gerson for their help in raising the monoclonal antibodies. We also thank Dr S. Pugh for advice on setting up the ELISA screening test, Drs J. F. Kelleher, J. W. Shneerson J. B. Kurtz and T. C. Harvey for providing clinical specimens, and Miss B. Mandalia for preparing the manuscript.

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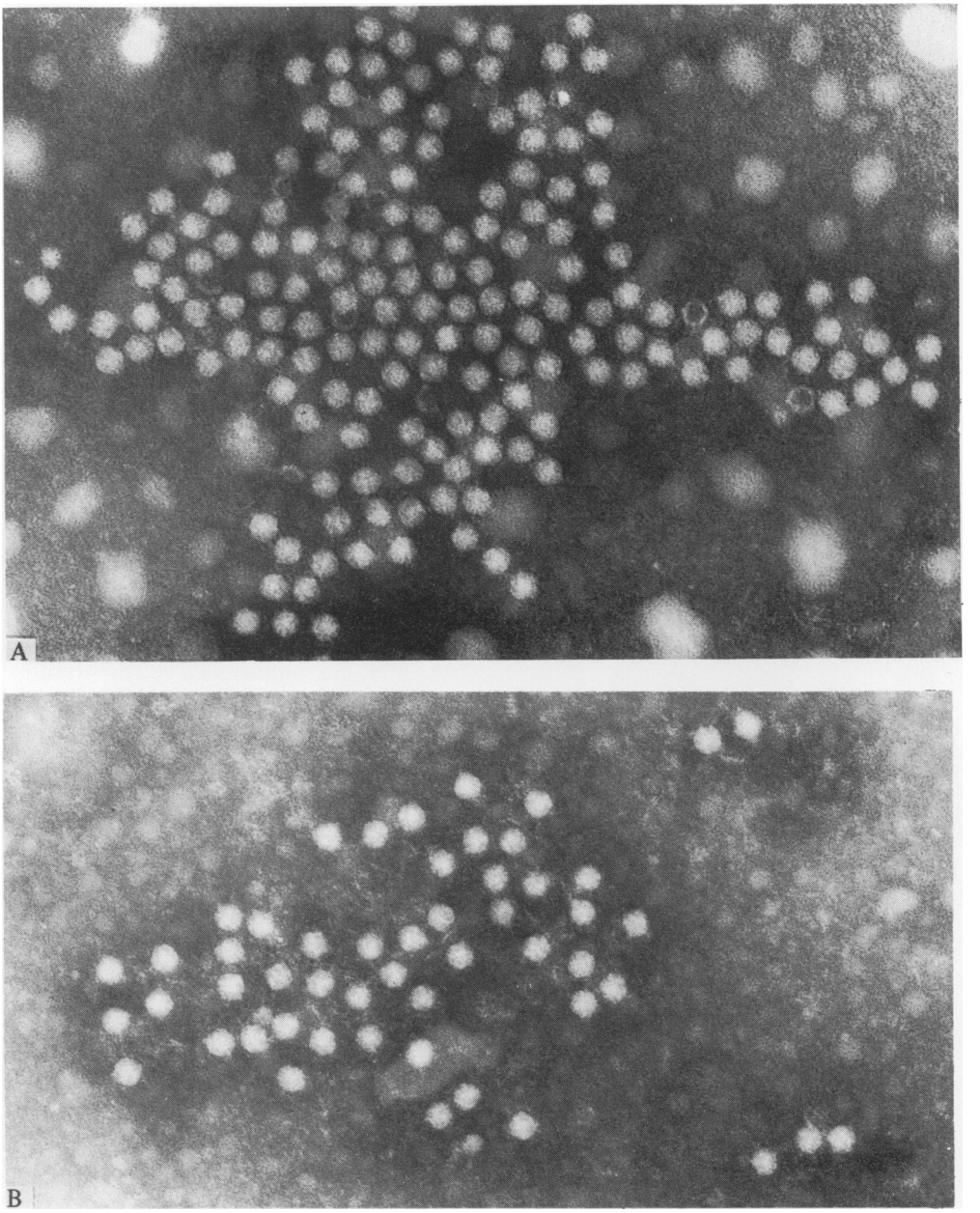
EXPLANATION OF PLATE

(A) Immune electron microscopy of SPLV particles aggregated by monoclonal anti-SPLV (× 150000). (B) Electron micrograph of serum from patient Ad.S. (Table 4) showing an immune complex of SPLV particles aggregated by IgM-like antibody (× 150000).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