POLIOMYELITIS VIRUS FLOCCULATION: THE REACTIVITY OF UNCONCENTRATED CELL-CULTURE FLUIDS

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A DIRECT flocculation reaction with poliomyelitis viruses of the 3 classical types and antisera obtained by immunization of rabbits was described by Smith, Sheffield, Lee and Churcher (1956). The reaction was shown to be strictly type-specific and to occur also with some human sera obtained from cases of poliomyelitis at early stages of the infection. It was suggested therefore that it might eventually provide a simple and rapid method of diagnosis and virus type differentiation, but that essential preliminary work must include the production of large quantities of the virus antigens of standardised potency, followed by systematic investigation of a large number of human sera of diverse provenance. A major obstacle to progress was the apparent necessity of concentrating infective culture fluids by high-speed centrifugation, giving small yields of antigens from relatively large volumes of culture ; for the unconcentrated culture fluids, with infectivity titres then considered to be the maximum attainable, were quite devoid of flocculating activity. The difficulty was partly met by the development of a micro method of flocculation requiring only 0·02 ml. of antigen per tube (Smith *et al.*, 1956). This method has proved to be invaluable in subsequent work but is less suitable than the Dreyer tube agglutination technique for routine diagnostic tests ; it is less accurate, more laborious and more dependent on subjective factors. Moreover the unpredictable instability of the finely particulate concentrated antigens introduced further problems which have not yet been solved and high-speed centrifugation of cultures of virulent poliomyelitis virus strains entails some risk of laboratory infections.

The present paper reports the use of unconcentrated virus culture fluids as flocculating antigens. The results are still of a preliminary nature, but they do indicate the possibility of obtaining antigens in quantities adequate for routine use with reduced risks of accidental infections. They thus represent a significant advance towards the ultimate objective in view.

MATERIALS AND METHODS

Poliomyelitis virus strains

Three virulent strains, Brunhilde, Y–SK and Leon, and 3 attenuated virus strains were used. The attenuated viruses were the Mahoney L–Sc 2ab, P 712 Ch 2ab and Leon 12ab strains of Sabin (1957). A seed pool of each virulent strain was prepared from cultures using a cell line Hu-Li derived from human embryo liver ; these were stored at − 20°. Seed pools of the attenuated viruses were prepared from cultures using ERK 1 cells derived from rabbit embryo kidney and stored at 4°. Both these lines of " transformed " cells were isolated and characterised by Westwood, Macpherson and Titmuss (1957).

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ERK 1 cell propagation

Two sub-lines of the ERK 1 cells were used.

Line ERK 1(*A*) was propagated serially in medium containing rabbit serum and was used for all virus infectivity titrations by the method described by Sheffield and Churcher (1957).

Line ERK 1(*D*) was used for the preparation of the flocculating antigens. It was grown in a medium consisting of bovine serum 10 per cent, tryptic digest broth 5 per cent, 5 per cent solution of lactalbumin hydrolysate 5 per cent and Earle's solution containing 0·11 per cent concentration of sodium bicarbonate 80 per cent. Penicillin 100 units per ml. and streptomycin 100 μg. per ml. were incorporated. Serial propagation was effected by trypsinisation of confluent cell cultures, resuspension of the cells in fresh medium to a cell concentration of 30,000 per ml. and distribution into either Pyrex babies' feeding bottles (10 ml. per bottle) or Roux bottles (100 ml. per bottle). Bottles were incubated in a stationary position at 36° ; the medium was changed on the 4th day and confluent cell mono-layers were obtained by the 7th day. The confluent cultures in the babies' bottles contained approximately 4×10^6 cells, those in Roux bottles approximately 40×10^6 cells.

Preparation of flocculating antigens

A two-stage method was used. For virulent strain antigens cultures of ERK 1 (D) cells grown to confluence on one side of a baby's bottle were selected. The medium was removed and the cells washed twice with Earle's solution. Medium 199, 10 ml. and virus seed, 0·1 ml. were added to each bottle. After incubation on a roller machine for 72 hr. the cultures were harvested, pooled and centrifuged at 1700 r.p.m./10 min. to remove cell debris. The fluid was then held at room temperature for several days in a lightly plugged vessel to allow escape of CO_2 with consequent increase of alkalinity. It was then used to initiate the second stage of virus production.

For the second stage ERK 1 (D) cells distributed in confluent sheets over all 6 glass faces of babies' feeding bottles were used. This was achieved by seeding each bottle with the total cell harvest from a Roux bottle confluent culture (approximately 40×10^6 cells), suspended in 50 ml. medium and placing it immediately on a roller machine at 36°. On the 2nd day the medium was removed and replaced by 4·4 ml. first-stage virus fluid plus 0·5 ml. normal rabbit serum and 0·1 ml. 5 per cent glucose solution. After 2 or 3 days' further incubation on the roller machine fluids were harvested, pooled and centrifuged. The product was a very high-titre virus fluid suitable for use as antigen in micro-flocculation tests, but too turbid for Dreyer tube tests. It was therefore clarified by filtration through Seitz FCB or GS pads which had been thoroughly washed with saline and nutrient broth.

For the attenuated strain antigens slight modifications were needed. Medium CSV 8 (Cooper, Wilson and Burt, personal communication) was substituted for Medium 199 and the primary stage virus fluid was neutralised with N/10 HCl. The inoculum per bottle for the second stage was 4·1 ml. first-stage fluid, 0·5 ml. rabbit serum, 0·1 ml. 5 per cent glucose solution and 0·3 ml. 2·75 per cent solution of sodium carbonate.

Flocculation techniques

Both the macroscopic tube and the micro methods were used. These have been fully described in our previous publications. Dilutions of both antigens and antisera were expressed as final dilutions.

Flocculating antisera

These were obtained by the intravenous inoculation of rabbits with centrifuge-concentrates of virus as described by Smith *et al.* (1956). When the sera from the primary courses of immunization were exhausted the rabbits were re-immunized with booster doses. The use of sera from different bleeds having different antibody titres therefore introduced a variable factor but this does not invalidate the conclusions to be drawn from the experimental results.

EXPERIMENTAL

Flocculation of Virulent Strain Culture Fluids

The first successful flocculation of an unconcentrated culture fluid was obtained with Brunhilde virus. The antigen was obtained by the two-stage growth tech-

nique described above. It was tested by a chessboard titration against homologous rabbit antiserum using the macroscopic tube method. Serial two-fold dilutions of antigen and antiserum were made in saline and mixtures containing 0·3 ml. volumes of each reagent were made in Dreyer agglutination tubes. These were incubated at 37° in a water bath with only the bottom third of the fluid columns immersed. Observations at frequent intervals showed that flocculation was progressive up to 6 hr. Y-SK and Leon antigens were then prepared and tested in a full cross titration.

The results of both experiments at 6 hr. incubation are recorded in Table I. They show that strictly type specific flocculation occurred with each culture fluid antigen. The antigens differed in flocculation potency which to some extent was probably a reflection of their different infectivity titres. Infectivity titres were $10^{8.9}$, $10^{8.0}$ and $10^{8.7}$ TCD_{50} per ml. for Brunhilde, Y-SK and Leon respectively. The antigen and antibody end points, however, were of the same order as those obtained previously with centrifuge-concentrated antigens and products with this degree of flocculating potency would certainly be adequate for routine use. Although the floccules are smaller than those obtained with concentrated particulate suspensions, the complete clarity of the filtered culture fluids greatly facilitates detection of " trace " reactions and difficulties arising from instability of concentrated antigens are eliminated.

Flocculation of Attenuated Strain Culture Fluids

No strictly homologous antisera were available. Culture fluids of each virus type were therefore tested against the rabbit antisera prepared against the virulent virus strains. Full chessboard cross titrations were done by the macroscopic tube method. The results, recorded in Table II show that typical type specific flocculation patterns were obtained and, unexpectedly, the antigen titres were approximately equal to those of the virulent strain fluids. Higher titres would certainly be advantageous and it is reasonable to expect that they will be attainable by further modifications of the conditions for virus growth.

Comparative Tests of Two-stage and Suspended Cell Culture Antigens by the Macroscopic and Microscopic Flocculation Techniques

Whilst all the antigens hitherto prepared by the two-stage method have had satisfactory flocculation potency, culture fluids from single-stage virus growth have mostly been completely inactive. During the progress of the work, however, we were informed that unconcentrated virus fluids derived from suspended ERK cell cultures had been found to give flocculation with our rabbit antisera (Appleyard and Westwood, unpublished) and several batches of such antigens were sent to us by these workers for test. We were also supplied with various batches of attenuated strain cultures by Cooper and his associates of the M.R.C. Virus Culture Laboratory, Carshalton.

Conservation of our potent rabbit antisera demanded preliminary screening of these potential antigens with the greatest possible economy in use of reagents. The preliminary screening of small samples of human sera is also likely to be required in future. In spite of the advantages of the Dreyer tube macroscopic flocculation method, enumerated above, it is too extravagant for this purpose. A one-dimensional test by the micro method was considered to be ideal but it was

TABLE I.—*Flocculation of Unconcentrated Culture Fluids of Virulent Strains*

Antigen	Antigen diln.	Antisera: Brunhilde: 1 in: 20	40	80	160	320	640	1280	Y-SK: 1 in: 20	40	80	160	320	640	Leon: 1 in: 20	40	80	160	320	640	Saline control
Brunhilde	½	+++	+++	+++	+++	+++	+++	−	−	..	..	..	..	..	..	−	..	..	..	..	−
	¼	++	++	++	++	++	++	+	−	..	..	..	..	..	..	−	..	..	..	..	−
	⅛	++	++	+	++	++	++	++	−	..	..	..	..	..	..	−	..	..	..	..	−
	1/16	+	−	+	−	−	+	+	−	..	..	..	..	..	..	−	..	..	..	..	−
Y-SK	½	..	..	..	..	..	..	..	++	++	++	++	++	++	−	−	..	..	..	..	−
	¼	..	..	..	..	..	..	..	+	−	+	+	+	+	−	−	..	..	..	..	−
	⅛	..	..	..	..	..	..	..	+	+	−	−	−	−	−	−	..	..	..	..	−
Leon	½	..	..	..	..	..	..	..	−	−	..	..	..	..	+++	+++	+++	+++	++	+	−
	¼	..	..	..	..	..	..	..	−	−	..	..	..	..	++	++	++	+++	+	−	−
	⅛	..	..	..	..	..	..	..	−	−	..	..	..	..	+	−	+	+	+	−	−
Saline controls	..	−	..	..	..	..	..	..	−	−	..	..	..	..	−	−	..	..	..	..	..

In all tables degree of flocculation is indicated by number of + signs; op indicates opalescence without detectable particulation; − indicates no detectable reaction.

TABLE II.—*Flocculation of Unconcentrated Culture Fluids of Attenuated Strains*

Antigen	Antigen diln.	Antisera: Brunhilde: 1 in: 40	80	160	320	640	1280	2560	Y-SK: 1 in: 40	80	160	320	640	1280	Leon: 1 in: 40	80	160	320	640	Saline
Mahoney L-SC 2ab	½	++	+++	+++	+++	++	−	−	..	−	..	..	..	..	..	−	..	..	..	−
	¼	++	++	++	++	++	++	−	..	−	..	..	..	..	..	−	..	..	..	−
	⅛	+	+	+	+	+	+	−	..	−	..	..	..	..	..	−	..	..	..	−
	1/16	+	−	−	−	−	−	−	..	−	..	..	..	..	..	−	..	..	..	−
P712 Ch 2ab	½	..	−	..	..	..	..	..	+++	+++	++	++	+	−	..	−	..	..	..	−
	¼	..	−	..	..	..	..	..	+	+	+	+	+	−	..	−	..	..	..	−
	⅛	..	−	..	..	..	..	..	+	−	−	−	−	−	..	−	..	..	..	−
	1/16	..	−	..	..	..	..	..	−	−	−	−	−	−	..	−	..	..	..	−
Leon 12ab	½	..	−	..	..	..	..	..	..	−	..	..	..	..	+++	+++	+	+	−	−
	¼	..	−	..	..	..	..	..	..	−	..	..	..	..	+++	+++	++	−	−	−
	⅛	..	−	..	..	..	..	..	..	−	..	..	..	..	+	+	+	−	−	−
	1/16	..	−	..	..	..	..	..	..	−	..	..	..	..	−	−	−	−	−	−
Saline	..	−	..	..	..	..	..	..	−	..	..	..	..	..	−	..	..	..	..	..

essential first to establish its validity by a direct comparison of the two methods. The experiment was designed to compare also the flocculation behaviour of virus fluids obtained by the two-stage and by the suspended cell culture techniques.

Two type 1 Brunhilde antigens were used, one freshly prepared by the two-stage culture method, the other prepared by Appleyard and Westwood from suspended cell cultures of ERK cells. They were each tested in full chessboard titrations by both macro and micro flocculation methods. The tests were done on the same day with serum dilutions made in bulk to eliminate possible misinterpretation from errors of dilution.

Progressive development of the reaction in macroscopic tube flocculation was observed by readings at 1, 4, 6 and 24 hr. incubation. This unfortunately is not possible in the micro method because of the time involved in the serial examination of hanging drop preparations by dark ground microscopy. A single reading of the micro tests was therefore made after 4 hr. incubation. The results are recorded in Table III, but the 24 hr. readings are omitted because by this time the reaction in every tube showing any degree of flocculation at 6 hr. had progressed to total flocculation, but without any appreciable extension of the flocculation " area " as a whole.

The superiority of the two-stage culture antigen is clearly apparent. It gave more rapid development and extension of flocculation, larger aggregates in corresponding serum dilution tubes and larger flocculation areas with reactions at higher dilutions of both antigen and antiserum. This is in line with the respective infectivity titres of the two fluids which were $10^{8.5}$ and $10^{7.8}$ TCD_{50} per ml. It should be noted, however, that the antigens supplied by Appleyard and Westwood were the earliest batches to be produced by their suspended cell culture technique so that this relatively lower potency does not necessarily indicate any intrinsic inferiority of the method. On the contrary, it is reasonably certain that, with modifications, suspended cell cultures will eventually yield antigens fully equal to those obtained by the two-stage virus growth method with the advantage of much greater flexibility for large scale production. The 4 hr. readings of macro and micro tests show closely similar reaction patterns, but with an indication that the micro test may be slightly more sensitive. Any advantage from such increased sensitivity is offset by the difficulty of detecting trace reactions by microscopic observation of single drop samples. The validity of the micro method, however, for screening tests is fully established.

Flocculability of Formalinised Culture Fluid Antigens

In previous work with centrifuge-concentrated antigens little trouble was encountered from bacterial contamination, probably because of lack of nutrient materials in the saline virus suspensions. On the other hand the unconcentrated culture fluids, containing cell breakdown products in a serum enriched medium, promote luxuriant growth of any bacterial contaminants, entry of which is difficult to prevent over long periods of use as flocculating antigens. The effect of formalin on flocculation potency was therefore investigated with a view to its incorporation as a bacteriostatic agent. Formalin was chosen because its employment in poliomyelitis virus vaccine production pointed to its possible future use in the development of flocculating antigens completely free from living infective virus.

A type 1 Brunhilde virus fluid obtained from monolayer ERK cell cultures was used in preference to a more potent two-stage virus growth antigen. To

TABLE III.—*Comparison of Tube and Micro Flocculation Techniques Using Brunhilde Tissue Culture Fluid Antigens Prepared by Two Different Methods*

			Monolayer antigen (2-stage method)											Suspended cell antigen										
			Brunhilde antiserum : 1 in :								Y-SK	Leon		Brunhilde antiserum : 1 in :								Y-SK	Leon	
Technique	Time (hr.)	Antigen diln.	15	30	60	120	240	480	960	1920	60	60	Saline	15	30	60	120	240	480	960	1920	60	60	Saline
Tube flocculation	1	1/2	++	++	++	−	−	−	−	−	−	−	−	op	op	op	−	−	−	−	−	−	−	−
		1/4	+	+	−	−	−	−	−	−	−	−	−	op	op	−	−	−	−	−	−	−	−	−
		1/8	−	−	−	−	−	−	−	−	−	−	−	op	−	−	−	−	−	−	−	−	−	−
		1/16	..	..	..	..	..	..	..	..	..	..	..	−	−	−	−	−	−	−	−	−	−	−
		1/32																						
	4	1/2	+++	+++	++	+	++	+	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−
		1/4	++	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−
		1/8	++	+	+	+	+	+	−	−	−	−	−	op	−	−	−	−	−	−	−	−	−	−
		1/16	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		1/32	op	−	−	−	−	−	−	−	−	−	−	..	..	..	..	..	..	..	..	..	..	..
	6	1/2	+++	+++	+++	+++	+++	++	+	−	op	−	−	++	++	++	++	+	+	−	−	op	−	−
		1/4	+++	+++	+++	+++	++	++	+	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−
		1/8	+++	++	++	++	+	+	+	−	−	−	−	op	op	−	−	−	−	−	−	−	−	−
		1/16	++	+	+	−	−	−	−	−	−	−	−	op	−	−	−	−	−	−	−	−	−	−
		1/32	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		Saline	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Micro method	4	1/2	++	++	++	++	++	+	+	−	−	−	−	++	+	+	++	+	+	±	−	−	−	−
		1/4	++	++	++	++	+	+	+	+	−	−	−	++	+	+	+	+	−	±	−	−	−	−
		1/8	++	++	++	++	++	+	+	±	−	−	−	+	+	±	−	−	−	−	−	−	−	−
		1/16	+	−	±	±	−	±	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		1/32	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		Saline	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..

aliquots of the fluid one-ninth volumes of saline, 1 in 400 dilution formalin, and 1 in 200 dilution formalin, were added. The mixtures were kept at 4° and tested at intervals without prior neutralisation of residual formalin. Line tests by the micro method were employed. The results summarised in Table IV show that the incorporation of formalin in a concentration of 1 in 2000 is not detrimental to the use of culture fluids as flocculating antigens over a storage period of more than 3 months. Formalinised fluids kept longer than this have not yet been tested. In this experiment no infectivity tests were done ; the production of antigens completely devoid of living virus by means of formalin and other virus-inactivating agents will be reported in a subsequent publication.

TABLE IV.—*Flocculation Reaction with Formalinised Virus-culture Fluids*

Storage 4° (days)	Culture-fluid antigen (Brun.)	Brun. antiserum : 1 in. :						Y-SK serum	Leon serum	Ant. sal. control
		20	40	80	160	320	640	20	20	
10	Untreated	+	+	+	±	−	..	..	..	−
	Form. 1/4000	++	+	+	±	−	..	..	..	−
	,, 1/2000	++	+	+	±	−	..	..	..	−
25	Untreated	++	+	+	+	±	..	−	−	−
	Form. 1/4000	++	+	+	+	±	..	−	−	−
	,, 1/2000	++	+	+	+	±	..	−	−	−
107	Untreated	++	++	+	+	±	−	−	±	−
	Form. 1/4000	++	++	+	+	−	−	−	±	−
	,, 1/2000	++	++	+	+	±	−	−	±	−

Heterotypic Cross Reactions

Table IV shows that in the test at 107 days, some degree of flocculation occurred in the control mixtures containing the Brunhilde antigens and Leon antiserum. The reason for this is that the Leon antiserum used was a sample obtained after re-immunisation of the rabbit with booster doses of ERK cell culture virus. Tests of serum samples from serial bleeds gave heterotypic cross reactions with all samples after re-immunisation, but not with earlier samples. Tests of Y-SK sera from serial bleeds gave a similar result. These cross reactions are not due to heterotypic poliomyelitis virus antibodies for they are abolished by absorption of the sera with ERK cells without reduction of the type specific flocculation titre (Appleyard and Westwood, personal communication). They indicate that in spite of their rabbit origin ERK cells contain a component which is immunogenic for the rabbit. The implications of this phenomenon are discussed below.

DISCUSSION

The development of techniques for producing poliomyelitis virus culture fluids, which will flocculate specifically with homologous antisera without the necessity of virus concentration or purification, should greatly increase the potentialities of the reaction. Systematic investigation of the dynamics of the reaction will be facilitated by the use of the macroscopic tube test in which continuous observation is possible but this requires considerable quantities of antigens which have been freed from visible particles. The preliminary work

reported shows it is possible to produce these for each virus type with both virulent and attenuated strains. Moreover their production by different methods of virus culture suggests that much more potent antigens will eventually result from further technical developments.

Previous failure to obtain flocculation with unconcentrated culture fluids was almost certainly due mainly to their relatively low content of virus particles, as reflected by their infectivity titres. It is doubtful, however, whether this is the sole determinant factor. Attempts to obtain flocculating antigens from the virus fluids of monkey kidney culture have been unsuccessful, even with centrifuge-concentration to offset their lower initial virus concentrations. There is some evidence that poliomyelitis virus flocculation may be prevented by the presence of non-specific inhibitors so that the type of cell used for virus propagation may be crucial. It may be significant that all the flocculating antigens produced as yet, either with or without centrifuge-concentration, have been derived either from HeLa cells or from one of the transformed cell lines of Westwood, and there is now little doubt that HeLa cells have also undergone the phenomenon of transformation. It is true that gel-diffusion precipitation is given by virus grown in monkey kidney cells, but diffusion may be an effective way of separating virus from inhibitor substances.

HeLa cells were originally used for production of our flocculating antigens. They were replaced by ERK cells for several reasons, as soon as these were found to be susceptible to infection with poliomyelitis viruses. ERK cells are more easily maintained in serial passages and grow well in bovine or rabbit serum-containing media. They are less liable to spontaneous degenerative changes and are thus superior for virus infectivity titrations. They yield higher titre virus fluids under comparable conditions (Sheffield and Churcher, 1957). The chief reason for their adoption, however, was because it was thought that their rabbit origin would obviate the elicitation of antibodies against any cell material which might remain in virus suspensions used for rabbit immunisation. In view of recent studies by several independent workers it is not now surprising that such antibodies do appear in the course of rabbit hyperimmunization. The phenomenon of cell transformation appears to be associated with a change of antigenic structure reflected by loss of the original species markers and acquisition, or unmasking, of an antigen common to transformed cells whatever their species origin (Westwood *et al.*, 1957 ; Melnick and Habel, 1958). This must be taken into account in any use of antisera against viruses grown in cells of this type. It is not likely to affect tests of human sera from cases of poliomyelitis but may conceivably do so with post-vaccination sera. Fortunately removal of the cross reacting antibodies by absorption with cells presents no difficulty.

The exceptionally high virus infectivity titres attained by the two-stage cultivation technique and the successful use of suspended ERK cells for production of flocculating antigen are cogent arguments for the adoption of these cells for virus vaccine production, if and when the hypothetical risks associated with potential malignancy are judged to be negligible.

SUMMARY

A two-stage method of poliomyelitis virus cultivation in ERK cells is described which yields virus fluids of exceptionally high infectivity titres.

The virus fluids are effective as flocculating antigens without prior virus concentration. They can be freed from visible particles and rendered bacteriologically sterile by Seitz filtration without loss of potency.

Effective flocculation antigens can be obtained with either virulent or attenuated strains of the 3 virus types.

Preliminary experiments indicate that formalinised antigens stored at 4° retain flocculation potency for over 3 months.

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