Technical methods

Enterovirus typing by immune electron microscopy using low-speed centrifugation

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Anderson and Stanley¹ carried out an electron microscopic study of the reaction between tobacco mosaic virus and its antibody, but practical applications of the technique developed only after the introduction of negative staining.²

Since the advent of immune electron microscopy (IEM)³ the technique has been used extensively to confirm the structural identity of virus material,^{4 5} to determine antibody class participating in the reaction, to identify the aetiological role of virus-like particles in association with clinical syndromes,^{6 7} and as a routine method of detecting specific virus directly or as a means of serotyping viruses after their growth in tissue culture.⁸ Existing methods rely on pelleting substantial quantities of virus by high-speed centrifugation, reacting the resuspended pellet

with antisera, repelleting, and demonstrating the reaction by negative staining on dipped grids. We have developed a method of increased sensitivity which greatly reduces the amounts of material required and shortens the time taken to obtain a result.

Material and methods

We studied routinely isolated and typed enteroviruses (Table 1). These were passed into 4 oz bottles of Bristol HeLa monolayers and incubated at 37°C until an obvious cytopathic effect (CPE) was observed, usually after 24-48 hours. The cultures were harvested by shaking the cells off the glass into the overlying tissue culture fluid, which was dispensed in 2-ml volumes and stored at -40°C as stock, to avoid repeated freeze-thawing. The virus titres were determined by dilution in microplates seeded with Bristol HeLa cells and incubated at 37°C for 48 hours (Table 1). Monospecific neutralising antisera were obtained from the Public Health Laboratory Service Standards Laboratory, Colindale. Dilutions of both virus suspensions and antisera were made in phosphate buffered saline (PBS)—the latter up to the limit of their nominal neutralising titre (Table 1). For control purposes virus suspensions were mixed with at least one or

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Table 1 *Single dilution of antigens of known titre used with various dilutions of monospecific neutralising antiserum*

Virus	Tissue culture dose (TC ₅₀)	Antisera dilution					Antibody neutralising titre
		1/16	1/32	1/64	1/128	1/256	
Coxsackievirus B ₁	10 ⁻¹	+	+	+	+	+	1:200
	10 ⁻²	+	+	+	+	+	1:400
	10 ⁻³	+	+	+	+	+	1:200
	10 ⁻⁴	+	+	+	+	+	1:400
	10 ⁻⁵	+	+	+	+	+	1:400
	10 ⁻⁶	+	+	+	+	+	1:100
Polio type 1	10 ⁻³	+	+	+	+	+	1:2000
	10 ⁻⁴	+	+	+	+	+	1:200
	10 ⁻⁵	+	+	+	+	+	1:200
ECHO	10 ⁻¹	+	+	+	+	+	1:100
	10 ⁻²	+	+	+	+	+	1:200
	10 ⁻³	+	+	+	+	+	1:500
	10 ⁻⁴	+	+	+	+	+	1:100
	10 ⁻⁵	+	+	+	+	+	1:800
	10 ⁻⁶	+	+	+	+	+	1:50
	10 ⁻⁷	+	+	+	+	+	1:400
	10 ⁻⁸	+	+	+	+	+	1:1600
	10 ⁻⁹	+	+	+	+	+	1:400

+ indicates agglutinated virus particles seen.

more neutralising sera (Table 2) of different specificity or PBS. In the test, 25 μ l of antibody were added to 75 μ l of the antigen dilution in microtitre plates. The mixtures were shaken and left to react at room temperature. Grids for electron microscopy were prepared as described previously.⁹ Briefly, 6 mm diameter flat-bottomed polythene tubes were cut to a height of 15 mm, 200 μ l of distilled water was added, and carbon/formavar-coated grids were placed, membrane side up, on the bottom of the tube. Samples of 5 μ l of the virus/antibody mixtures were transferred to these tubes at $\frac{1}{2}$, 1, 2, 4, 6, and 24 hour intervals. Immediately after the test samples had been added, the tubes were centrifuged at 3700 rpm (2100 *g*) for 30 minutes in a MSE bench centrifuge. Grids were also prepared in some cases after centrifugation for 5, 10, and 15 minutes. The grids were removed, dried, negatively stained with phosphotungstic acid (PTA), pH 6.6, and examined by EM at a standard magnification of $\times 25\,000$.

Results

In most instances, large clumps of agglutinated virus particles were seen in the first grid square examined (Figure). Often many of these clumps consisted of over 100 particles. The virus aggregates displayed the typical morphological features seen in immune electron microscopy, including the separation of particles by fine electron-dense material consistent with the appearance of surface antibody coating (Figure). Using a reaction time of 30 minutes, some preparations did not show satisfactory clumping or the clumps were small. However, after 1 hour maximal clumping had occurred (Figure); no apparent improvement was obtained by incubating the virus/antibody mixture for a longer period. Specific agglutination was seen over a wide range of antiserum dilutions up to the nominal neutralising titre (Table 1). At a relatively high antibody concentration

Table 2 *Antigen dilutions against 1/64 dilution of antiserum*

Virus		Antigen dilution				
		Neat	1/4	1/8	1/16	1/32
Polio	I	+	+	+	+	—
Coxsackie	B ₁	+	+	+	—	—
	B ₂	+	+	+	+	—
	B ₃	+	+	+	+	—
	B ₆	+	+	+	+	—

+ aggregates of virus particles seen.

the particles became excessively coated with antibody so that it became difficult to make out the virus structure. Four of the stock virus culture types were diluted in PBS and reacted with only one dilution of 1:64 specific neutralising sera for 1 hour (Table 2). Except where the initial virus titre was low, all specimens were positive at a dilution of 1:16 antigen (Table 2). Irrespective of whether the spinning time was 5 or up to 30 minutes, a large number of clumps of virus were seen on all the grids.

Throughout the study, agglutination occurred only when a virus suspension was mixed with its specific antiserum. Controls of virus suspensions mixed with heterologous neutralising sera and of virus suspensions with PBS were consistently negative (Table 3).

Discussion

Conventional IEM technique requires a substantial volume for concentration of virus by high-speed centrifugation before reacting with typing antisera. Resuspension of the initial pellet may occasionally be incomplete and lead to non-specifically clumped virus appearing on the grids. The resuspended second pellet, after antibody treatment, tends to contain smaller clumps, or possibly if larger ones are present

Table 3 *Single dilution antisera of virus specificities against neat virus suspension*

Virus		Antisera at 1/16 dilution							
		P ₁	P ₂	P ₃	B ₁	B ₂	B ₃	B ₄	B ₆
Polio	1	—	—	—	—	—	—	—	—
	2	—	+	—	—	—	—	—	—
	3	—	—	+	—	—	—	—	—
Coxsackievirus	1	—	—	—	+	—	—	—	—
	2	—	—	—	—	+	—	—	—
	3	—	—	—	—	—	+	—	—
	4	—	—	—	—	—	—	+	—
	5	—	—	—	—	—	—	—	+
	6	—	—	—	—	—	—	—	+

Results indicate specific reaction (agglutination +) of the antisera.

Immune electron microscopy of ECHO II. Note the antibody between and on the surface of virus particles (arrows). Agglutinated by antibody for 1 hour.

they do not readily stick to the grids on dipping. Dipping, though widely used, is a relatively inefficient means of sampling particles in liquid suspension since the amount taken by the grid is variable and its distribution uneven. In consequence, much time may be taken examining the grids in the EM to find agglutinated virus or to exclude their presence. For these reasons, immune electron microscopy for routine typing of isolates has not been widely accepted.

Our method differs in a number of respects and overcomes these shortcomings. The main advantage is that only small volumes of material are required. The appearance of a cytopathic effect denoting virus infection in a single tissue culture tube provides sufficient material for passing the isolate into fresh tissue culture tubes and serotyping the virus on the same day by IEM. The results show that the method is specific, and the viruses do not agglutinate with non-specific antisera (Table 3).

Most of the material sediments directly on to the grid, and large clumps of virus are usually easily seen in the first grid square. Almeida and Waterson⁴ stated that polio antigen-antibody aggregates require approximately 1 hour of spinning at 15 000 rpm. However, in our study, a large number of virus aggregates were seen after a spinning time of only 5 minutes at 2100 *g* in a clinical type bench centrifuge, but it is likely that spinning for a full half-hour would be necessary to sediment small clumps of virus into the grids. These aggregates consisted of both empty and core particles, free of cell debris, showing typical electron microscopic antigen-antibody reaction.

A further feature of the technique described is that a quite low concentration of antibody may be used; around four times the neutralising titre is optimal. In addition the positive results obtained from diluted virus suspensions (Table 2) suggest that virus may be detected in cultures of low infective titre. It is important to avoid taking too much sample, otherwise an unacceptable quantity of debris, mostly from tissue culture cells, is deposited on the grid. It is likely that this method could be used satisfactorily for the typing of other viruses.

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A technique avoiding carcinogens for the demonstration of myeloperoxidase in blood and bone marrow smears

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For the demonstration of myeloperoxidase in leucocytes in haematological smears of blood or bone marrow a widely used current technique is a modification of that of Kaplow¹ where the substrate 3,3 di-amino-benzidine (DAB) is substituted for the original benzidine dihydrochloride. As the recognised carcinogenic properties of benzidine and its derivatives result in their production and distribution

becoming increasingly limited, 2,7-fluorenediamine has been used as an alternative substrate^{2,3} which, while apparently satisfactory in demonstrating the enzyme, is still not free of potential carcinogenicity.⁴

We have adapted a histochemical method which employs pre-mixed *p*-phenylenediamine and pyrocatechol⁵ and which totally avoids the use of known or suspected carcinogens. This technique has been tried before without success,³ but when modified as we suggest it seems to give perfectly satisfactory results.

Material and methods

Peripheral blood films were obtained from healthy controls, and bone marrow smears were examined from patients with acute leukaemia at diagnosis and in remission. Slides were fixed for 1 minute in 10% formaldehyde in 95% ethanol and washed under running tap water for 2 minutes. They were then

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