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8 June 1962

Recognition and Quantitation of Herpesvirus Particles in Human Vesicular Lesions

Abstract. Herpesvirus particles from crude vesicular fluid of a patient were stained with uranyl acetate and potassium phosphotungstate, and then were identified and counted by electron microscopy. Virus was seen and quantitated in all the samples taken from five vesicles. Specimens from human lesions can be prepared and examined within 3 hours, permitting rapid presumptive identification of herpesvirus.

Van Rooyen and Scott (*1*) observed elementary bodies of smallpox, taken from pustular material, directly in the electron microscope and suggested the use of such examination as an aid in diagnosis. Evans and Melnick (*2*) described the appearance of herpes simplex, varicella and herpes zoster particles obtained from vesicular and spinal fluids. Melnick *et al.* (*3*) found characteristic particles in purified extracts of human papillomas (warts) and molluscum contagiosum lesions. All of these viruses were prepared for microscopy by metal shadowing. Almeida *et al.* (*4*) recently described the use of negative staining for the recognition of varicella virus particles from vesicle fluid. Human papova virus can be identified and quantitated directly in crude tissue homogenates (*5*). Smith and Melnick (*6*) described a method for identifying the nucleic acid type of viruses in a variety of crude materials by staining and electron microscopy. Stains which reveal identifying structures of viruses are very helpful in examining crude materials which nearly always contain substantial amounts of tissue debris. The report presented here illustrates the combined advantages of staining, to facilitate particle recognition, and counting of these particles in the study of crude unpurified vesicle fluids from a patient with a herpetic infection.

A 43-year-old male suffering from Hodgkins disease became seriously ill with a disease resembling generalized herpes. Numerous large vesicular lesions appeared over his body. Vesicular fluid was aspirated from five different lesions with fine needles and syringes. Very small volumes were obtained, about 0.01 to 0.02 ml per lesion. Each fluid was washed from the needle by withdrawing and expelling 2.0 ml of tissue culture fluid. Tissue cultures (human lung fibroblast and cercopithecus monkey kidney epithelial cells) were inoculated and were observed to develop cytopathogenic effects typical of herpesvirus within 24 hours (microplaques). Specific antiherpes serum inhibited the development of this effect.

The diluted fluids were each treated with a trypsin-chymotrypsin mixture to digest cell debris, then were diluted further and sedimented on agar in the ultracentrifuge as described previously (*6*). The collodion pseudoreplicas from these agar blocks were stained with 0.2 percent uranyl acetate or 0.2 percent potassium phosphotungstate, or both (*7*). The preparations were then examined with an RCA-EMU-3-F microscope.

Numerous particles resembling herpesvirus (*7*, *8*) were seen in each of the five specimens. Figure 1 (top) shows the morphology of these particles following the treatment with uranyl acetate. The particles are somewhat angular in outline, and they display a lightly stained capsid and a densely stained central core. Some structural detail can be seen around the less densely stained periphery of each particle. Over 80 percent of the particles stained in the core area and were therefore presumed to contain a nucleic acid core. This conclusion was supported by the observation that only a small fraction were penetrated and stained in the core following phosphotungstate treatment.

Two virus particles stained by phosphotungstate are shown in Fig. 1 (middle). These particles show the surface structure much more clearly than do the particles stained with uranyl acetate (Fig. 1, top). There is some evidence of collapse of particles treated with phosphotungstate, as manifested by the rather ragged periphery. Figure 1 (bottom) shows a particle stained first

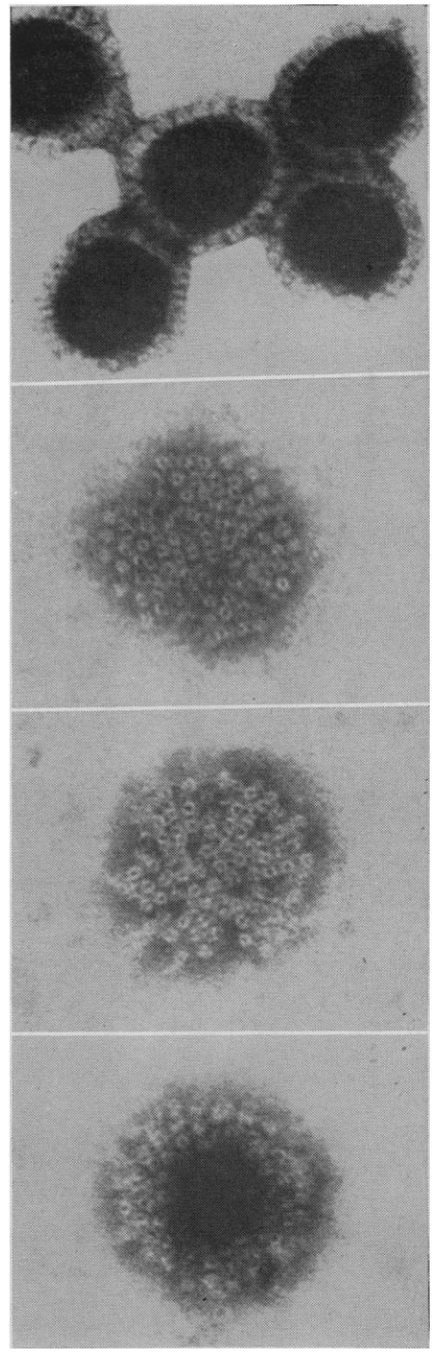

Fig. 1. Herpesvirus particles obtained directly from human vesicular fluid. (Top) Group of five virus particles stained with uranyl acetate (× 230,000). (Middle) Two virus particles stained with potassium phosphotungstate at *p*H 7.0 (× 230,000). (Bottom) Virus particle stained with uranyl acetate, then counterstained with potassium phosphotungstate (× 260,000).

with uranyl acetate, then with phosphotungstate. In this way both the core and the surface structure of a single virus particle can be seen at the same time. Particles prepared in this manner are similar in appearance to the small fraction of coreless forms when they are stained and penetrated by phosphotungstate. There is no evidence of collapse. Such particles are usually as well preserved structurally as those treated with uranyl acetate only (Fig. 1, top).

Low-magnification electron micrographs were made of random fields of each preparation, and the particles were counted. The approximate concentration in the original vesicular fluid was calculated (*6*) although the precision of the original fluid volume measurement was poor. This volume could have varied by three-fold, therefore the dilution factor was by far the most variable element in the calculation. However, each of the five vesicular fluids contained sufficient numbers of particles to make quantitation possible. Concentrations ranged from 3×10^9/ml to 7×10^{10}/ml of vesicular fluid. The morphology of the particles was essentially the same in each preparation.

The approximate time required for preparing and examining three such specimens from time of receipt in the laboratory is 3 hours. The fine structure and staining characteristics of several viruses are sufficiently well known to make their tentative recognition possible in clinical specimens. It is apparent that the concentration of virus particles in some clinical materials is more than sufficient for direct electron microscopy. The methods employed in this study were sensitive enough to detect several particles per microscope field when the original vesicle fluid had been diluted 1:1000 prior to sedimentation. As preparative techniques become more refined and simplified, and thus more widely used, examining clinical materials for virus particles may become as practical as examining Gram-stained bacterial smears for the presumptive diagnosis of certain diseases (*9*).

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12 April 1962

Protection of Rats against Adjuvant Arthritis by Bacterial Lipopolysaccharides

Abstract. Lipopolysaccharides from both gram-negative and gram-positive bacteria in incomplete adjuvant caused rats to become resistant to mycobacterial (complete) adjuvant arthritis. Lipopolysaccharides which protected rats when they were administered in adjuvant did not protect when administered in saline. Mycobacterial wax D in saline was protective, while in adjuvant it produced arthritis. The degree of stimulation of a common tissue reaction may determine whether the result is protection or disease.

The varied physiological responses of animals to lipopolysaccharide (LPS) from gram-negative bacteria have been studied extensively (*1*). Stimulation of nonspecific immunity to bacterial and viral infections (*2*) and antiallergic effects (*3*) have been included. The nonspecific defense reaction has been credited both to the reticuloendothelial system (*4*) and to the properdin system (*5*). Recent research (*6*) indicates that inoculation of LPS into mice may cause "temporary elevation of a whole array of specific antibodies rather than the appearance of non-specific or cross reacting antibodies." Most previously reported inoculations have been given intraperitoneally or intravenously in an aqueous medium, rather than intracutaneously in a Freund-type (*7*) adjuvant. The dosages have been proportionately smaller than those we used.

Adjuvant arthritis was induced in young adult rats (Long-Evans, Wistar, Lobund) used in our experiments as described elsewhere (*7, 8*) by intracutaneous inoculation superscapularly with 0.5 ml of a water-in-oil emulsion. The inoculation contained wax D fraction (1 mg/ml) from virulent Canetti and Brevannes human strains of *Mycobacterium tuberculosis* (*9*). Although the mechanisms behind this interesting arthropathy have not been irrefutably established, some evidence indicates that they involve delayed hypersensitivity reactions (*10*).

To investigate further the factors influencing the pathogenesis of adjuvant arthritis, various lipopolysaccharides (*11*) were substituted for wax D in the adjuvant. These included LPS from gram-positive *Staphylococcus* A and from gram-negative *Salmonella typhosa* 0901, *S. typhimurium*, and *Escherichia coli* 055:B2. When technique and dosage were identical with those used for the inoculations of arthritogenic wax D adjuvant, no overt symptoms of disease developed. (Minimal joint swelling was noted with *Salmonella typhosa* LPS adjuvant in only two of four rats after a second inoculation. To date, this technique has not been investigated further.)

Subsequent injection of the same animals with wax D adjuvant showed that the animals were completely or partially refractory to adjuvant arthritis. The disease was considered to have been modified by previous injection of LPS if (i) no arthritis developed after challenge with wax D adjuvant, (ii) the disease occurred in a significantly smaller number of rats than in unprotected controls, (iii) its onset was markedly delayed, (iv) its symptoms were mild, or (v) recovery was more rapid than in controls.

With LPS from *S. typhosa*, only 9 of 36 rats (25 percent) developed arthritis as compared with 30 of 35 (85 percent) of the controls. Protection was much greater when the interval between the initial inoculation and the challenge was 8 days than when longer intervals (up to 60 days) were used. With LPS from *Staphyloccocus* A and intervals of 15 to 40 days, arthritis appeared in 14 of 31 rats (45 percent). When rats were protected with LPS from *Escherichia coli* there

Table 1. Effects of bacterial fractions, in incomplete adjuvant and in saline, on arthritis in rats. A second inoculation, consisting of wax D adjuvant, was given 24 days after the first. Evaluation: +, protection; 0, no protection.

First inoculation	Effects		Evaluation
	Arthritis/total	%	
In adjuvant:			
S. typhosa LPS	2/9	22	+
Staph. A. LPS	3/10	30	+
In saline:			
S. typhosa LPS	8/10	80	0
Staph. A. LPS	6/10	60	0
Wax D	2/10	20	+
Controls:			
Nothing	24/31	77	