NEGATIVE STAINING OF THINLY SPREAD CELLS AND ASSOCIATED VIRUS

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The first demonstration of intracytoplasmic virus by negative staining was that of Horne and Nagington (9) who subjected poliovirus-infected cells to freezing and thawing and negatively stained a suspension of the fragments. Dales (5) examined the attachment of some viruses to the cell surface by the negative staining of whole mounts of cells subjected to osmotic shock. Whittaker and Horne (15) examined normal cell components by the negative staining of fractions of tissue homogenates separated by centrifugation. Methods of negative staining of thin sections of frozen or frozen-dried tissue have been developed by Fernández-Morán (7). Almeida and Howatson (1, 2) have recently developed a method of studying viruses and virus-cell relationships by negative staining of frozen sections.

The technique of spreading the cytoplasmic structures of cells on a water surface, for electron microscope examination, was originated by Fernández-Morán (6). A needle was inserted into the tissue and then dipped into distilled water or saline. Surface tension drew the cells off the needle and spread the cytoplasmic contents into a very thin film. Nuclei were unaffected. In the method to be described, cells are spread by a similar method and then treated with potassium phosphotungstate (PTA). The negative contrast

so obtained greatly improves the visibility of normal cell components and abnormal structures such as virus particles. A preliminary account of the application of the technique to the study of Gross virus has already been published (13).

MATERIALS AND METHODS

The basic procedure involved three simple steps. First, a needle was pushed into the tissue in order to pick up a small number of cells. Second, the cells were floated off the needle by dipping it into phosphotungstate solution, and third, coated specimen grids were floated on the surface in order to pick up the film of thinly-spread cells. The grids were dried and then examined in the electron microscope.

A small container (depression slide or plastic-tube top) was filled, to give a level surface, with the spreading solution (substrate). Most conveniently this was 1 to 2 per cent potassium phosphotungstate (PTA) at pH 6.0 or 7.3, to which 0.005 to 0.010 per cent bovine serum albumin (BSA) had been added. The addition of BSA was necessary to ensure that the specimen grids were wetted by the surface film and to give an even distribution of cell components and PTA on the grid. The BSA (roughly estimated on a spatula after an initial weighing) was added daily to stock PTA since it was unstable in solution. For general use, the best pH for the PTA was 6.0. At 7.3 the contrast was lower and a granular background (presumably of protein) was sometimes visible. Phosphate-buffered saline (PBS) pH 7.0, or normal saline, also containing 0.005 to 0.010 per cent BSA, sometimes gave improved spreading. The procedure was observed through a 20× binocular dissecting microscope arranged in a way similar to that used for viewing thin sections in the trough of a microtome knife.

A steel sewing needle (about 1 mm thick) was cleaned by rubbing with dry lens paper and pushed a few millimeters into the exposed tissue of an anaesthetized or freshly killed animal. It was found important either to remove the capsule (if present) or expose the inner tissue by an incision. The needle was removed from the tissue and dipped slowly into the spreading fluid. Cellular material was observed to flow off the needle onto the surface. Most of the material instantly became invisible but a few scattered clumps of cells usually remained. A larger area of the spread-cell film could be seen by phase microscopy. The spreading properties of different cells could be readily evaluated with the phase microscope.

Specimen grids were carefully placed, film downwards, on the surface adjacent to, but not including, one of the visible cell clumps. Forceps with the tips bent to about 110° were convenient for this step. The specimen grids were covered with a formvar film slightly thicker than normal, and strengthened with the usual light coating of carbon. This reduced film breakage. If the substrate was PTA, the grids were picked off the surface (without wetting the non-filmed side with PTA), excess PTA removed with a fragment of filter paper, and the grids allowed to dry. If PBS was used, the grids were floated on PTA for a few seconds and then dried as before. The thickness of the spread film could be judged by the way the excess PTA was removed from the grid by the filter paper. If fluid was withdrawn slowly and sticky strands attached to the filter paper, the cell material was too thick and nucleoprotein from broken nuclei had been precipitated. Excess PTA withdrew rapidly from thin preparations leaving the grid covered by a very thin moist layer. No serious deterioration occurred if the grids were kept for a few hours in a moist atmosphere but most grids were examined as quickly as possible.

Tissue culture cells or cells isolated by trypsin or ethylene diamine tetra-acetic acid (EDTA) treatment were spun to a pellet and then treated as for a tissue. If the cells did not spread readily, a larger number were carried over to the surface by means of a wider instrument (*e.g.*, a sealed-off glass Pasteur pipette). Nuclear components and virus in the nucleus could be examined as follows: tissue culture or cells isolated by trypsin or EDTA were broken by ultrasonic vibration or freezing and thawing several times. Whole tissue was partly broken up by a single stroke of a Potter-Elvejhem homogenizer. The larger cell fragments, including fragments of nuclei, were spun down into a pellet, and the needle pushed into the pellet in the same way as for a tissue.

Fixation of the film of spread-cell material could be carried out with osmium tetroxide acid vapour (5 to 10 seconds) or the cells were spread directly onto Palade's osmium tetroxide (5 to 30 minutes). Also cells could be spread onto a 10 per cent solution of formalin in PBS (10 to 60 minutes). Frequently, fixed cell components could not be readily picked up on the grid. Somewhat better results were obtained by spreading the cells first on PBS, picking them up on a grid and transferring the grid to the fixative. After fixation, the grid was floated on PTA and dried as before.

RESULTS

A variety of normal, neoplastic, and virus-infected tissues was examined. Amongst normal tissues, mouse and rat liver, kidney and brain spread particularly well, lung poorly, and muscle not at all. Failure to obtain satisfactory spread-cell preparations was sometimes due to poor transfer of cells to the needle and in other cases to poor spreading of the cells on the surface. The neoplastic cells examined (lymph nodes and thymus from

leukemic mice and rats, C3H mouse mammary tumors, X5563 plasma-cell tumor in C3H mice, and PC5 plasma-cell tumor in BALB/C mice) transferred and spread very well.

The importance of the size and material of the transferring instrument was investigated. Successful transfers were made with fine glass needles, freshly flamed platinum wires, loops and foil, and small slips of freshly cleaned mica. The mica was very lightly touched to the tissue and obliquely immersed into the spreading fluid. Transfer with mica appeared to be useful where it was important to preserve the anatomical arrangement of the spread cells. For general work the steel sewing needle was used.

At this time, little can be said about the influence of pH and ionic strength of the substrate on the morphology of cytoplasmic structures. Structures appeared satisfactorily preserved in the pH range of 5.5 to 7.5. In future work, careful consideration should be given to the formation of artifacts due to distortion, denaturation effects produced by surface spreading, and extraction of substances by the substrate. The biggest difficulty in handling films of cytoplasmic components fixed by formaldehyde and osmium tetroxide was their poor affinity for the filmed specimen grids, but structures that were picked up looked identical to fresh unfixed structures.

The step of transfer of the surface film to the specimen grid was a critical one. For routine work, placing the film directly onto the grid was satisfactory. Since the area of the grids (diameter 3 mm) was small in comparison with that of the surface film (diameter 1 to 2 cm) large changes in surface pressure did not occur. However, in order to preserve an even distribution of spread-cell components it would be preferable to transfer to the grid under constant surface pressure (3, 8).

In Fig. 1, part of the cytoplasm of a rat liver cell is shown. The endoplasmic reticulum is present as branching elongated saccules. Identification rests on the abundance of these structures, especially in plasma-cell tumors, and the fact that a virus in BALB/C plasma-cell tumors is specifically associated with the endoplasmic reticulum (see below). However, ribosomes were never seen on these membranes. This may be because of their destruction by PTA as is the case for *E. coli* ribosomes (10). Alternately, the ribosomes may be hidden in a protein layer surrounding the outside of the endoplasmic reticulum as suggested by the cryo-fixation experiments of Bullivant (4).

The mitochondria present a variety of appearances depending on the penetration of PTA and their degree of flattening. However, they are easily recognized when the PTA penetrates to show the two outer membranes and the cristae (Figs. 1 and 2). In these preparations the inner membranes usually appear thicker than the outer ones. This is due to the location of particles of 75 A diameter with a narrow neck of 35 A on the cristae (Fig. 3). The structures appear identical with those recently demonstrated by Fernández-Morán (7) using his technique of negative staining of frozen or freeze-dried thin sections.

FIGURE 1

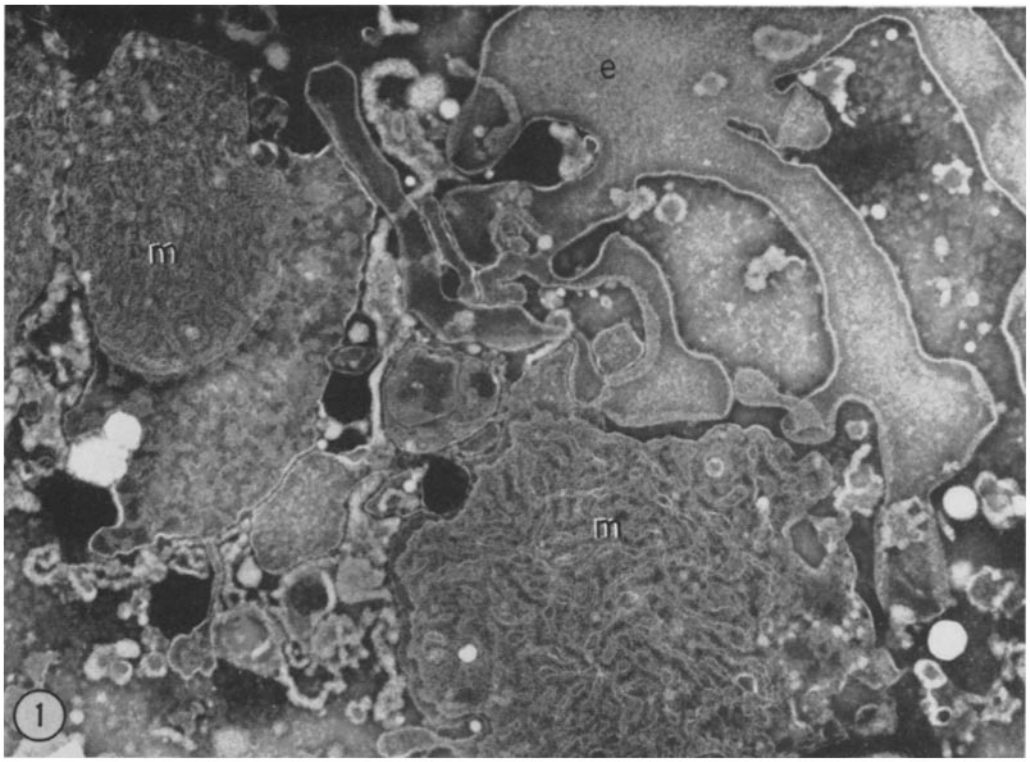
Portion of the cytoplasm of a normal rat liver cell spread on PTA, pH 6.0. The mitochondria (*m*) show recognizable cristae. The branching saccules shown at *e* represent smooth or rough portions of the endoplasmic reticulum. The ribosomes are not visible in spread-cell preparations. × 32,000.

FIGURE 2

Mitochondrion from a spread-cell preparation of a leukemic mouse lymph node. The general appearance is quite similar to that of mitochondria in thin sections but the inner membranes are thicker than the external ones. The mitochondrion is too thick to show details of the structure of the cristae. × 80,000.

FIGURE 3

Higher magnification of a mitochondrion from a mouse plasma-cell tumor. The mitochondrion has flattened sufficiently to show details of the structure of the cristae. Projections consisting of a head of 75 A diameter and a neck of 35 A diameter are present in the cristae. × 132,000.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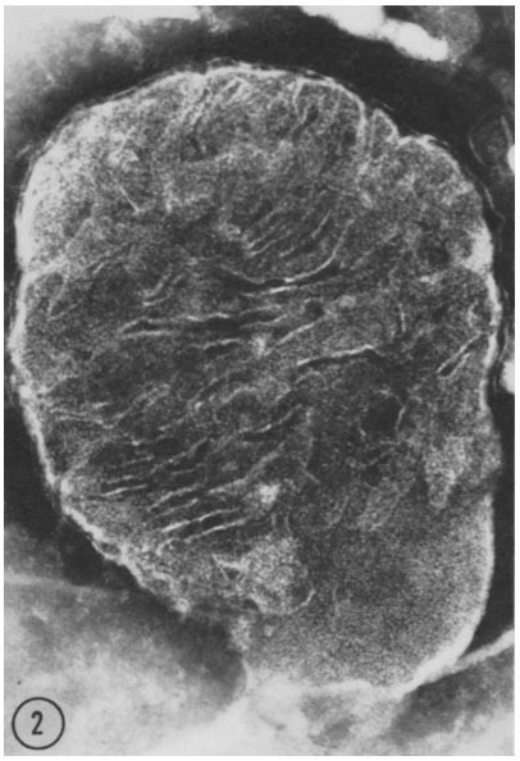
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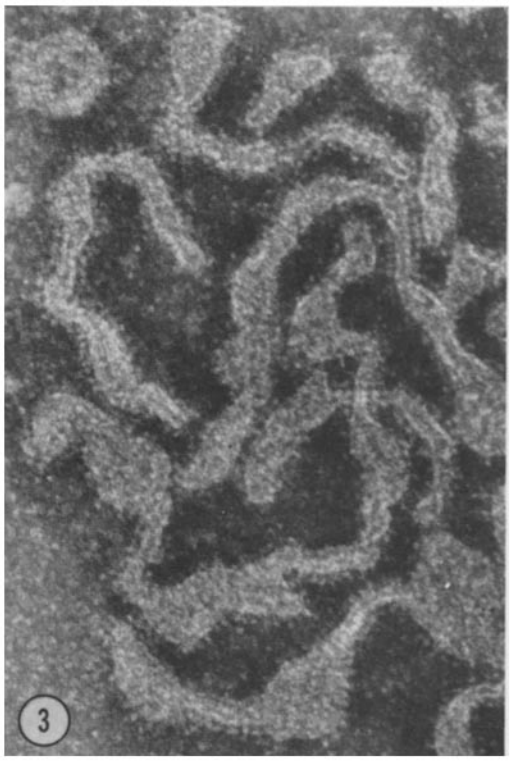
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A few examples of the use of the method to visualize intracellular virus are given. In Fig. 4, part of the cytoplasm of a mouse mammary tumor cell is shown. Type A particles consisting of a single shell structure may be compared with Type B particles which are covered with spines 45 A thick and 100 A long (12). A more complex form consisting of 4 or 5 concentric shells is also shown.

In Fig. 5, a portion of the endoplasmic reticulum from a plasma-cell tumor (PC5 carried in BALB/C mice, donated by Dr. M. Potter) is shown. Type A particles may be seen budding from the surface in a manner that could easily be correlated with the thin section appearances.

In Fig. 6, a large group of K virus particles is shown in the nucleus of a mouse lung endothelial cell.

DISCUSSION

The simple technique described here readily allows the examination of cytoplasmic structures and virus in the cytoplasm of a variety of types of cells. Undoubtedly the method is capable of further development. With this in view, some of the underlying principles will be mentioned.

The attachment of cells to the needle and the separation of clumps of cells on the surface is dependent on the adhesive properties of the plasma membrane. This is known to depend on the presence of calcium, amongst other factors. The reduced calcium content of tumor tissues (14) may, in part, be responsible for the particular ease of transfer and spreading of the tumor cells examined.

Spreading on the surface is related to several factors. The degree of flattening of cells with a small volume of cytoplasm (*e.g.*, lymphoblasts) or with a cytoplasm containing filaments, etc. (muscle cells) is presumably less, on depositing on the surface, than that of cells with a larger volume of mobile cytoplasm. In the latter case, the contact angle is smaller and a greater stretching force is exerted.

Spreading of the nucleus does not occur and the study of viruses developing in the nucleus is probably best carried out by the sectioning techniques of Almeida and Howatson (2) or Fernández-Morán (7), since some of the original architecture of the nucleus is retained. Spreading of the nucleus may, however, prove possible if the cells are first subjected to hypotonic swelling or mechanical squashing.

The substructure on the surface of mitochondrial cristae (Fig. 3) provides one example of the additional information that can be obtained by the negative staining of normal cell components. The described procedure often makes it unnecessary to purify viruses that develop in the cytoplasm. The viruses are exposed to the minimum of manipulation and are more likely to remain intact. The spread-cell method, because of its rapidity, simplicity and ability to handle only a small number of cells, would seem to be especially indicated for the diagnosis of virus diseases that give rise to superficial lesions. The method also lends itself to enzyme digestion, and other cytochemical studies of cytoplasmic organelles or associated virus.

FIGURE 4

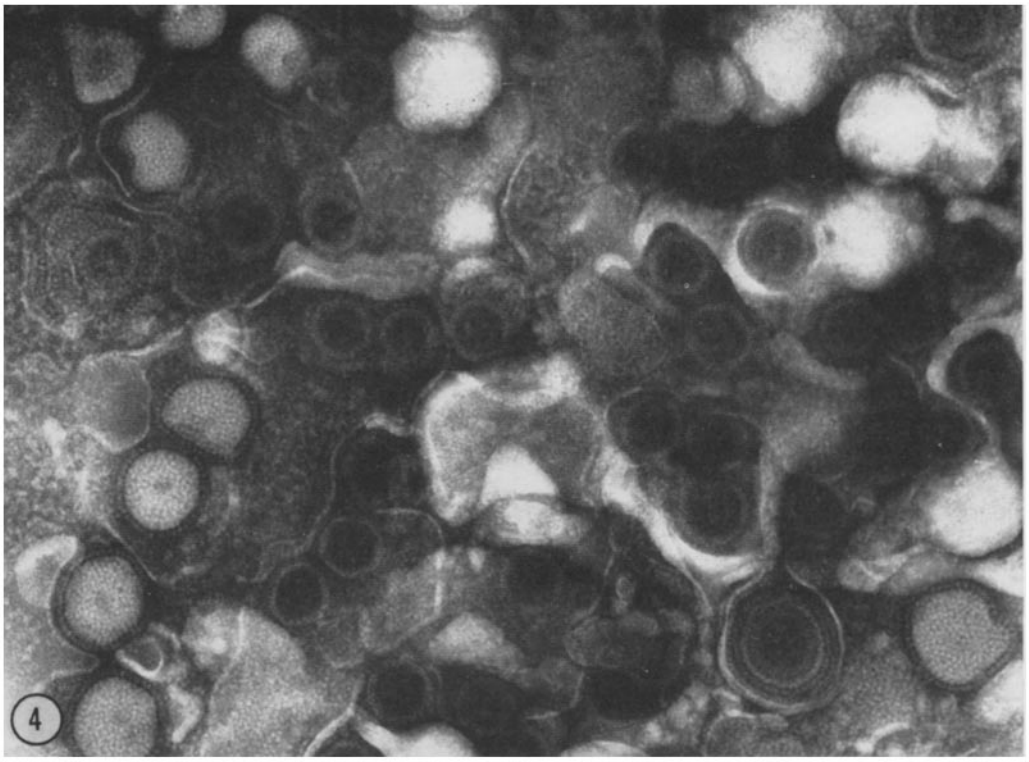
Portion of an inclusion of developing virus particles in a C3H mouse mammary tumor. The Type A particles seen inside the cytoplasm in sections are seen in spread-cell preparations as a single outer shell. The Type B particles are covered with spines (45 A thick, 100 A long). A particle consisting of 5 concentric shells is also shown. × 119,000.

FIGURE 5

A portion of endoplasmic reticulum from a PC5 plasma-cell tumor carried in BALB/C mice is shown. Type A particles are seen budding from the wall of the endoplasmic reticulum. × 131,000.

FIGURE 6

A large aggregate of Kilham's mouse pneumonitis virus (K-virus, (11)) is shown. Infected mouse lung tissue was lightly broken up in a Potter-Elvejhem homogenizer and the fragments spread on PTA. × 60,000.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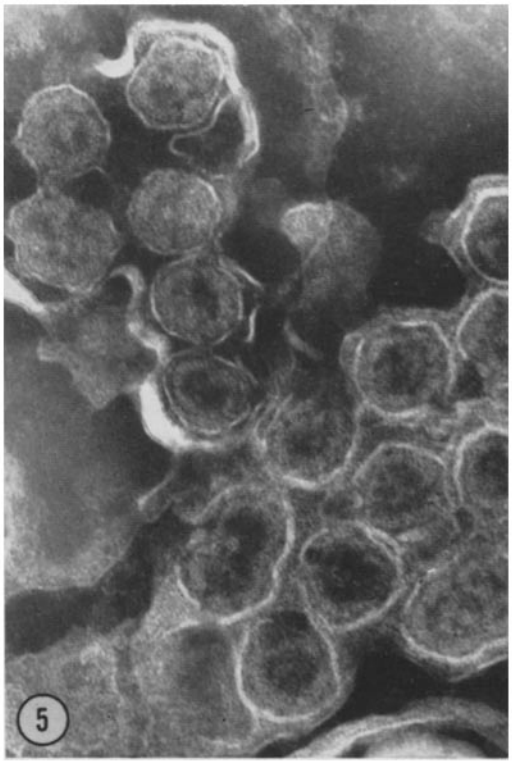
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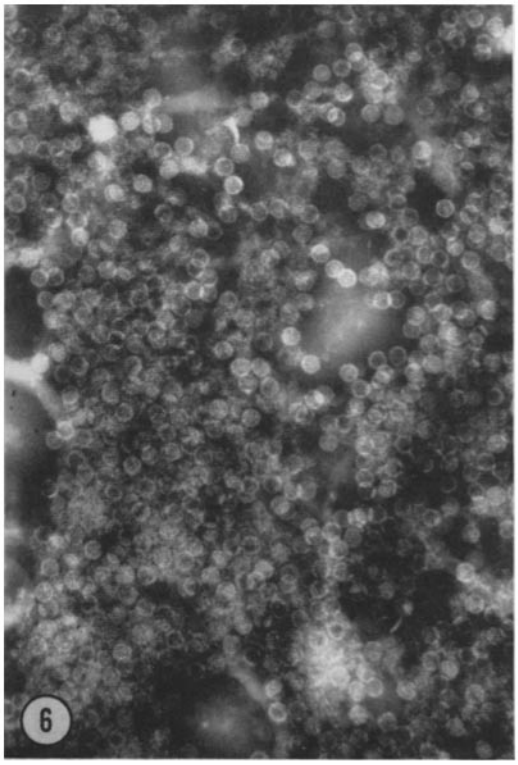
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