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Polyoma-like Virions in Human Demyelinating Brain Disease

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ABSTRACT

Specimens of brain tissue obtained at autopsy from three patients suffering from progressive multifocal leukoencephalopathy (PML) were examined by electron microscopy. In specimens from all three cases particles similar to those of the papova virus group were present, confirming previous observations. By the negative staining method it was possible to define the morphological characteristics of the particles more precisely and it was shown that they are structurally similar to virions of the polyoma-SV40-K type. The need is emphasized for obtaining fresh unfixed diseased tissue from persons suffering from PML in order that the biological properties of the particles can be investigated.

SOMMAIRE

On a examiné au microscope électronique des spécimens de tissu cérébral prélevés lors de l'autopsie de trois malades souffrant de leuco-encéphalopathie progressive à foyers multiples (LPM). Dans les spécimens des trois cas, on trouvait des particules semblables à celles des virus papova, ce qui confirmait des observations précédentes. Par la méthode de coloration négative, on est parvenu à définir avec plus de précision les caractéristiques morphologiques des particules et à montrer que leur structure était semblable aux virions du type polyome-SV40-K. Les auteurs insistent sur le besoin d'obtenir des spécimens de tissu pathologique non fixé de personnes souffrant de LPM, en vue d'étudier les propriétés biologiques des particules.

IN 1958 Aström, Mancall and Richardson[1] presented three case reports of an unusual disease of the central nervous system which they termed progressive multifocal leukoencephalopathy (PML). In these three cases and in two of five others that had been previously reported, the disease was superimposed on either chronic lymphatic leukemia or Hodgkin's disease. Three years later Richardson[2] collected data on a total of 22 cases of PML, including those previously mentioned. Of the 22 cases, 17 were associated with some form of neoplastic disease, this term being considered to include chronic leukemias, Hodgkin's disease, lymphosarcoma and carcinomatosis. Weinstein, Woolf and Meynell[3] in 1963 tabulated 31 cases and emphasized the pre-existence of either malignant or benign diseases of the reticuloendothelial system in patients with PML. Neurological symptoms in afflicted persons are progressive over an average three to four months' period and include hemiparesis, visual disturbances, aphasia, mental changes, deterioration of intellect, and in the rare cases of more extensive cerebellar involvement, vertigo and unilateral ataxia. Electroencephalography was found to be the only diagnostic procedure useful in establishing the presence of this organic brain disease.[2, 3] In some instances PML seems to have been the decisive organic disease in the death of a chronically ill patient.

Progressive multifocal leukoencephalopathy is characterized grossly by multiple, in part confluent, areas of demyelination, usually most numerous in the parieto-occipital white matter. In the light microscope the demyelination is of the sudanophilic type and does not spare the U-fibres. Most subsequent authors have accepted Richardson's[2] inter-

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pretation of the cytopathology of the disease. Invariably, nuclei of oligodendrocytes enlarge and become densely basophilic in the early lesions. Upon progression of the disease all oligodendrocytes disappear from the tissue and in a majority of cases bizarre, giant astrocytes develop which may exhibit typical or atypical mitoses. This cytologic response of the astrocytes is unique for a spontaneous, non-neoplastic brain disease. A viral etiology of PML was considered by Cavanagh *et al.*[4] on the basis of the observation of eosinophilic inclusions mainly in abnormal oligodendroglial nuclei. Richardson[2, 5] and Waksman and Adams[6] felt that the changes in both oligodendroglial cells and astrocytes were consistent with the cytopathic effects of a virus.

In an attempt to obtain further evidence concerning the viral hypothesis, Zu Rhein and Chou[7, 8] in 1964 used the electron microscope to examine demyelinated as well as normal brain tissue obtained at autopsy from two female patients with PML, 67 and 33 years of age. The former had suffered for three decades from chronic bronchitis and her final seven months' neurologic disease was thought to be due to multiple cerebral infarcts. The latter was afflicted with idiopathic thrombocytopenic purpura and lupus erythematosus for four years and her final brain disorder of four months' duration was thought to be the result of a progressive cerebellar hemorrhage. The brain tissue of both cases had been fixed in 10% formalin at pH 6.0 for about two years prior to being processed by the standard procedures used in preparing thin sections for electron microscopy. In the demyelinated tissue only, particles resembling those of the papova virus group[9] were present in great number in the nuclei of glial cells, almost exclusively oligodendroglial cells. In addition to randomly distributed spherical particles, there were observed in some nuclei crystalline arrays of similar particles and elongated cylindrical structures like those occasionally seen in cells infected with polyoma virus. In two other laboratories, virus-like particles similar to those described above have been observed by electron microscopy in thin sections of brain specimens from persons with PML.[10, 11]

The present communication reports observations made mainly by the negative staining method[12] that provide further evidence for the viral nature of the particles in tissue previously examined and also the finding of similar particles in two other brains from a different source. The particles are shown to resemble polyoma virions more closely than papilloma virions.

MATERIALS AND METHODS

The first specimen (No. 1) was obtained from formalinized autopsy brain tissue that had previously been the source of material used for electron microscope examination.[7] It consisted of small blocks (2-3 mm.) of subcortical white matter selected from regions that had shown by light microscopy a high concentration of abnormal oligodendroglial cells. The blocks were processed for thin sectioning by procedures similar to those used for fresh unfixed tissue, *viz.* overnight in 5% glutaraldehyde (phosphate buffer, pH 7.2) 1½ hours in 1% osmium tetroxide (veronal acetate buffer, pH 7.2), dehydration in acetone, and embedding in Epon 812. The sections were stained with uranyl acetate and lead citrate. Other pieces of tissue were prepared for negative staining by grinding in a mortar and adding about 1 ml. of water. An aliquot of the resulting suspension was mixed with an approximately equal volume of a 2% solution of sodium phosphotungstate (pH 6.0) and applied to a carbon-coated formvar-covered grid. The fluid was reduced to a thin layer by touching with a fragment of filter paper. The remaining liquid film was allowed to dry in air and the specimen was then examined in a Siemens Elmiskop I.

The other two specimens were obtained through the courtesy of Dr. R. Hasselback (Ontario Cancer Institute) and Dr. C. L. Dolman (Vancouver General Hospital), and were from cases diagnosed at the latter hospital but not previously studied by electron microscopy. The first of these (specimen No. 2) consisted of pieces of brain tissue taken at autopsy from a man who had PML superimposed on lymphatic leukemia and an incidental glioma. This unpublished case has been referred to and tabulated previously.[2, 3] The tissue had been stored in formalin for about four years. A few cubic millimetres of tissue were removed by means of a scalpel from selected demyelinated areas of the main blocks and prepared for thin sectioning and negative staining as described above for specimen No. 1.

The other tissue specimen (No. 3) was from a case previously reported.[13] The patient was clinically suspected of having basilar artery insufficiency due to spread of Hodgkin's disease to the dura. However, postmortem examination of the brain revealed that the patient had suffered from PML. The tissue was treated in the same way as the previous specimens.

RESULTS

Specimen No. 1.—Examination of thin sections of tissue confirmed the observations previously reported.[7] In favourable areas of the sections it was not difficult to find glial nuclei in which there were numerous, randomly scattered spherical particles of diameter about 38 mμ. (Fig. 1). Elongated structures of somewhat smaller diameter were also occasionally observed. The spherical particles were similar to those previously described and were most frequently observed within very large round nuclei with marginated chromatin which probably corresponded to the swollen oligodendroglial nuclei seen by light microscopy. They were also observed in nuclei that were smaller, denser, and less

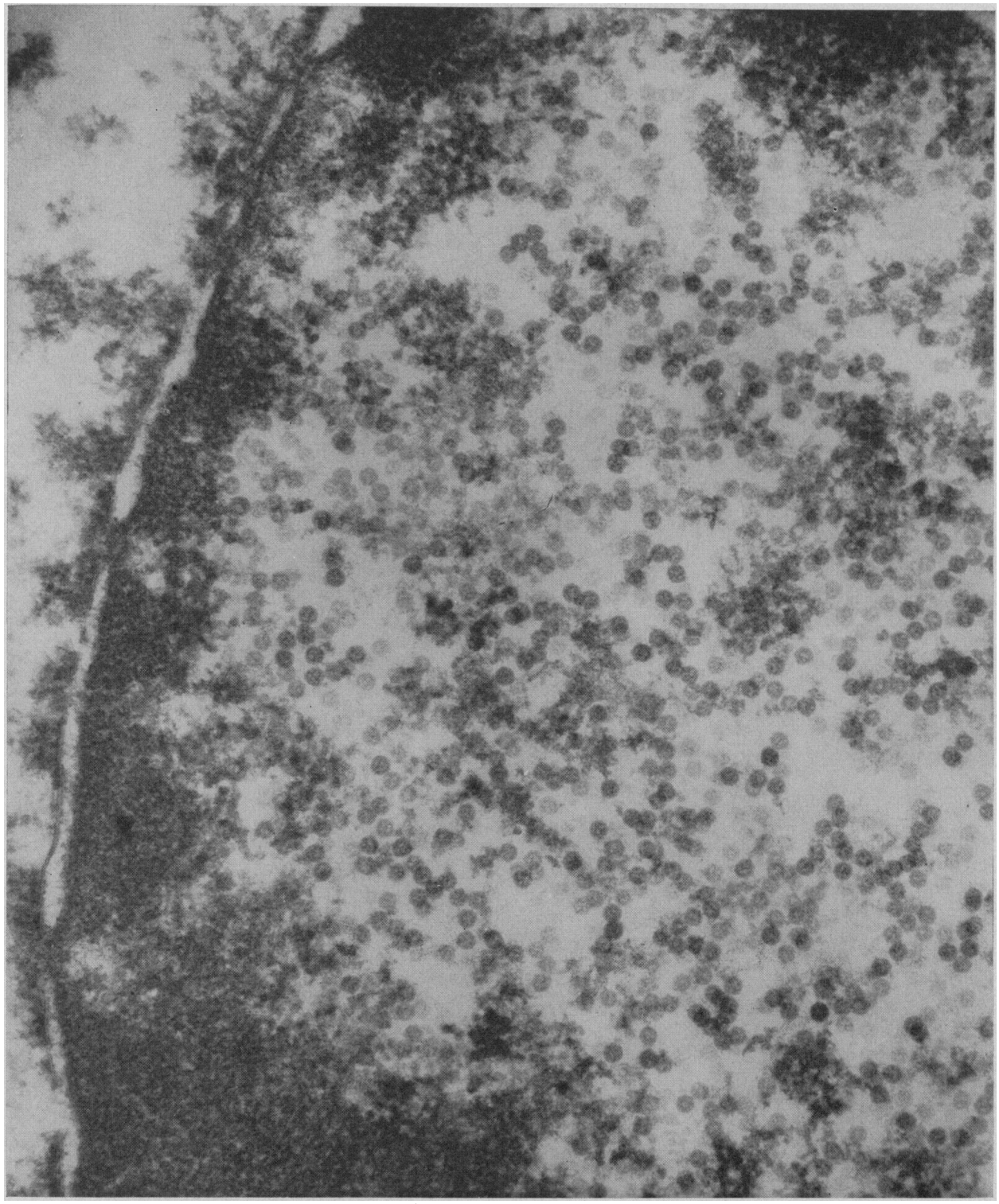

Fig. 1.—Section showing part of a glial cell from demyelinated white matter (specimen No. 1). Numerous scattered spherical particles are present in the nucleus which occupies most of the picture. Residual dense chromatin is applied to the nuclear membrane which can be seen at the left. (Magnification 80,000 ×.)

regular in outline, in association with granular, chromatin-like material. In a few nuclei of this type the particles were grouped in regular crystalloid formations. The different orientations of the crystalloids and superposition of more than one layer of particles resulted in the appearance illustrated in Fig. 2.

The negatively stained preparations consisted mainly of membranous material, fibres and particulates of various shapes and sizes. Attention was concentrated on a search for particles with distinctive features not known to be associated with any normal cell components. Careful scrutiny of the specimens at a screen magnification of 40,000

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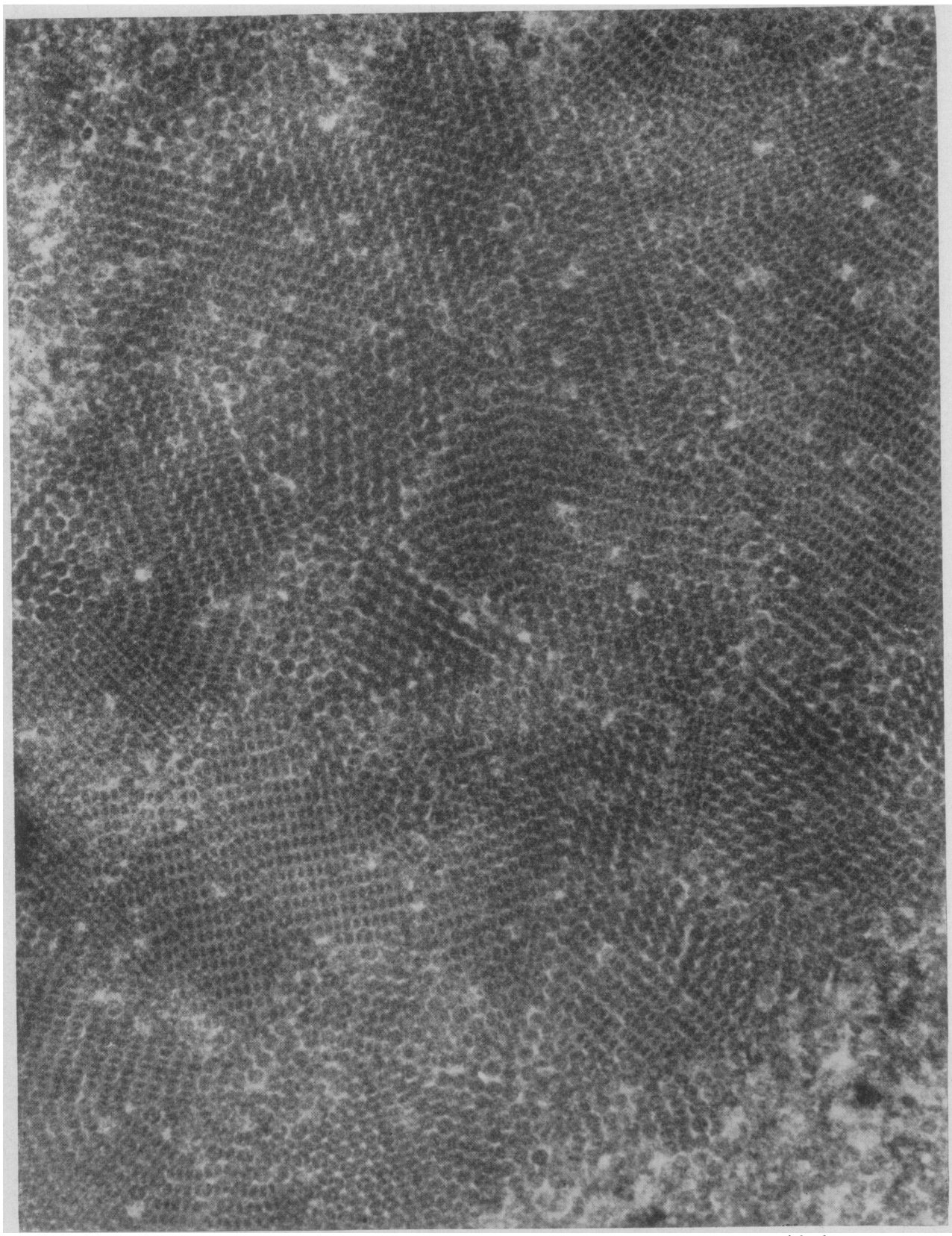

Fig. 2.—Section through a glial nucleus showing part of a large aggregate in which the particles are arranged in regular crystalloid formations. The variation in the appearances of the crystalloids is due to the different orientations and to the superposition of two or more layers of regularly arranged particles. (Magnification 80,000 ×.)

revealed particles, arranged either singly or more often in small groups, that had a distinctive morphology similar to that associated with viruses of the papova type. They were circular or somewhat hexagonal in outline, their diameters were uniform and their surfaces were studded with small projec-

tions resembling viral capsomeres. A typical group of particles is shown in Fig. 3. Their average diameter (50 particles measured) was 41 mμ. It was not possible to determine accurately the number and arrangement of the surface subunits. However, in several particles peripheral subunits were about 5 mμ. wide and they were separated by a distance of about 8 mμ.

Specimen No. 2.—This specimen was first examined by the negative staining method. Particles similar in all respects to the spherical particles described above were observed in preparations of sufficiently distinct to be counted for about half the circumference, the number being approximately eight. This indicates that the total number of peripheral subunits is about 16. The subunits cerebellar tissue. They were, however, present in fewer numbers and in smaller groups than in specimen No. 1. Their appearance is illustrated in Fig. 4.

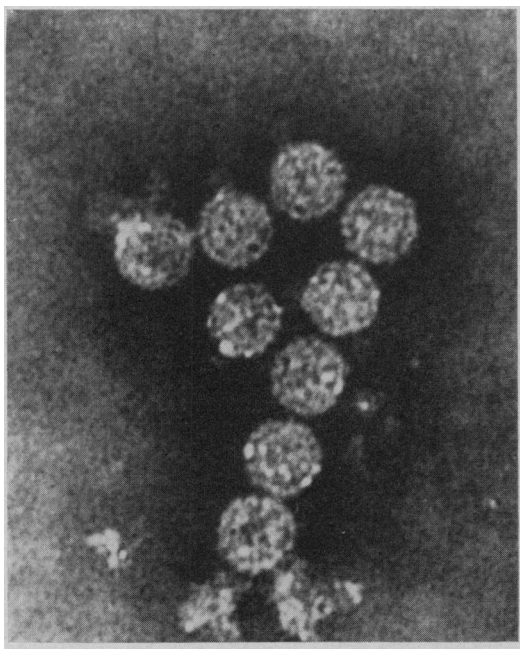

Fig. 3

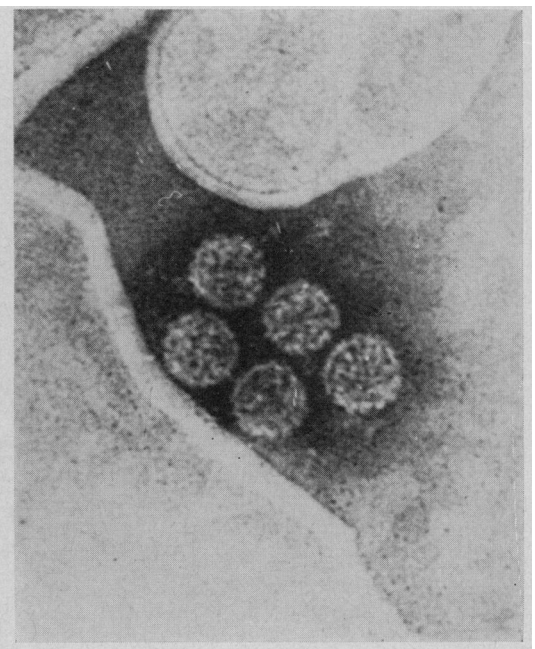

Fig. 4

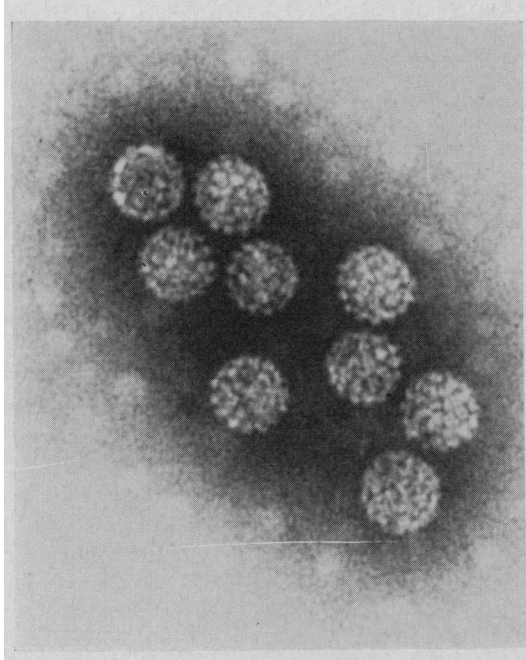

Fig. 5

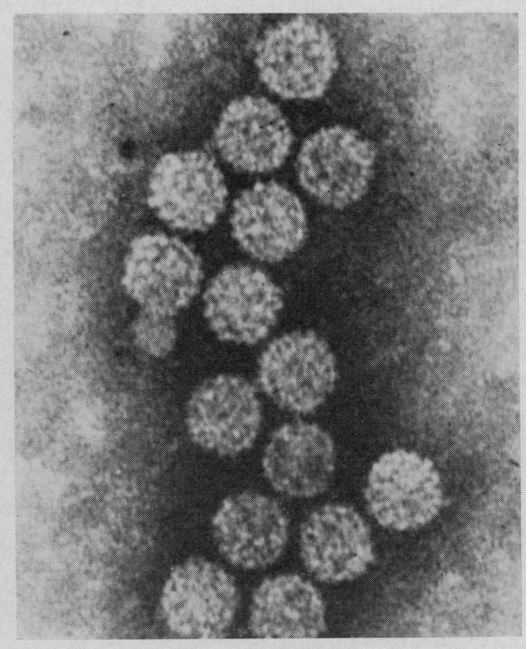

Fig. 6

Figs. 3-6.—Virions negatively stained with phosphotungstate. The group in Fig. 3 is from brain specimen No. 1 and in Fig. 4 from specimen No. 2. Fig. 5 shows an *unfixed* preparation of polyoma virus and Fig. 6 the same preparation after immersion in formalin for 10 days. (Magnification 240,000 ×.)

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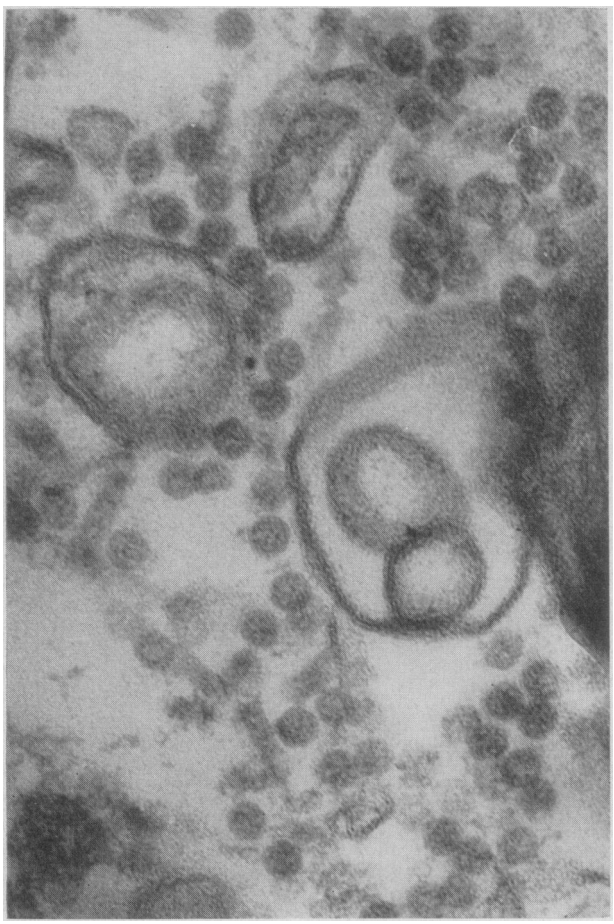

Fig. 7.—Section of demyelinated white matter (specimen No. 2) showing a group of particles similar to those shown in Fig. 8. The particles are associated with membranes suggestive of myelin figures. (Magnification 140,000 ×.)

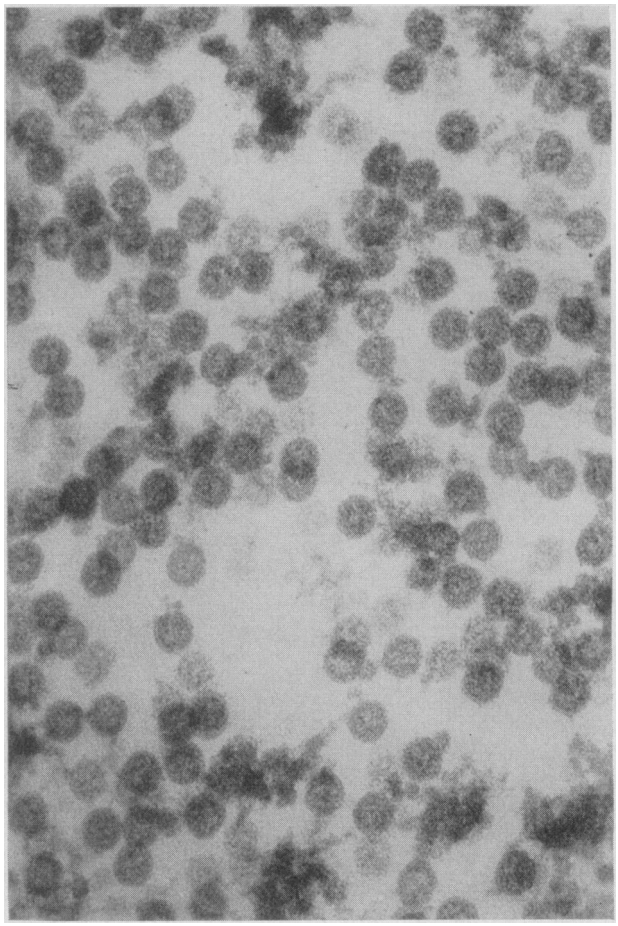

Fig. 8.—Section of specimen No. 1, similar to Fig. 1, showing intranuclear virions at higher magnification. Some show a thin peripheral zone that is less dense than the central area. (Magnification 140,000 ×.)

In thin sections, the tissues of this specimen appeared less well preserved than those of specimen No. 1 and particles were not as plentiful in the blocks that were examined. However, in sections of cerebellar white matter, scattered groups of particles identical to those seen in sections from specimen No. 1 were observed. One such group is illustrated in Fig. 7. A similar group of particles from specimen No. 1 is shown for comparison at the same magnification in Fig. 8. No crystalline arrays of particles were observed in this specimen.

Specimen No. 3.—This specimen was similar to specimen No. 2 but characteristic particles were more difficult to find. In negatively stained preparations only isolated particles or pairs of particles were observed; in thin sections, groups of particles were present in association with unidentified cell material.

DISCUSSION

The first question that arises from these observations concerns the nature of the particles that have been described in sections and by negative staining. Does the evidence justify the conclusion that they are viruses and if so can they be identified? The answer depends to a large extent on one's definition of a virus. If, as is usually the case, the definition involves demonstrable biological activity, the requirements for identifying the particles observed in human brain tissue as viruses have not so far been met. However, the situation regarding viral identification has been altered in recent years by advances in technique that allow the structural characteristics of many viruses to be defined with considerable precision. This enables such viruses to be clearly distinguished from normal cell components and other disease agents, to be characterized morphologically and allocated to a particular class of virus on the basis of their structural features alone. The term "virion" has been proposed to describe the morphologically complete particle.[14] Since this term is a morphological one and does not include the concept of infectivity it will be used in this communication to denote entities that have clearly defined structural characteristics of viruses whether or not biological activity has been demonstrated. The term "virus" will be used only in cases where infectivity of the virions can be regarded as established.

Some virions can be more readily and more precisely characterized than others. For example, those of the adenovirus group when examined by negative staining show such well-defined surface structure that there is no difficulty in identifying

even individual particles as representatives of this particular group of viruses. On the other hand, some virions have no regular symmetrical substructure and their identification in unpurified preparations presents considerable problems. It is clear that morphological identification must be used with discretion and is valid only if there are precise objective criteria for defining the structural characteristics of the particular type of virion.

The discussion so far has been concerned with the structure of individual virions, which is usually best studied by negative staining methods. Valuable information about their nature can also be obtained by studying their effects on host cells, including the site and mode of assembly of the virions. This, as a rule, is best done by thin-sectioning methods. From studies of this latter type it has been concluded that the particles observed in the human brain tissue resemble papova viruses[7, 8] in morphology and in their distribution in cells. The additional information from negative staining studies reported in this paper lends support to this conclusion and allows a more precise characterization of the virion, as will now be discussed.

The papova viruses belong to a large category of viruses that are characterized by the type of symmetry they possess. The viral protein coats or capsids are round or polyhedral in shape and are composed of projecting subunits or capsomeres that are arranged in accordance with the icosahedral or 5:3:2 type of cubic symmetry. In some viruses (e.g. herpes, adeno) the arrangement is precisely known but with the papova viruses there is still no general agreement about the exact number and arrangement of the capsomeres. Although all members of the group appear to have the same type of capsid structure they are not morphologically identical, some members having a distinctly larger capsid than others with a correspondingly larger DNA content (where this has been determined).[15] The first subgroup consists of the viruses polyoma, SV40 (vacuolating virus) and K (mouse pneumonitis virus). These are indistinguishable from one another by negative staining procedures and have mean diameters in the range 40-45 mμ. in unfixed preparations.[15-17] The second subgroup comprises the papilloma viruses affecting different species such as rabbits, dogs, cattle and man (wart virus). These are all morphologically identical but are appreciably larger than the first type, having mean diameters in the range 52-55 mμ. and about twice the DNA content of polyoma virus. The difference in size can be appreciated by referring to Fig. 9 which shows unfixed particles of two distinct sizes in a preparation of mixed polyoma and human papilloma viruses.

The resemblance of the particles obtained from human brain tissue to the papova virion is striking. Projecting subunits can be seen over at least part of the surface of most particles, though they are not as distinct as in most unfixed preparations of papova viruses (Figs. 5, 9). The effect of immersion of polyoma virus in 10% formalin for 10 days is shown in Fig. 6. By comparing Figs. 5 and 6 it can be seen that exposure to formalin does not appear to alter the surface structure to any great extent. There is some distortion of the capsomere arrangement and the individual capsomeres are not as well defined. There is also a slight diminution in particle size after fixation. The mean measured

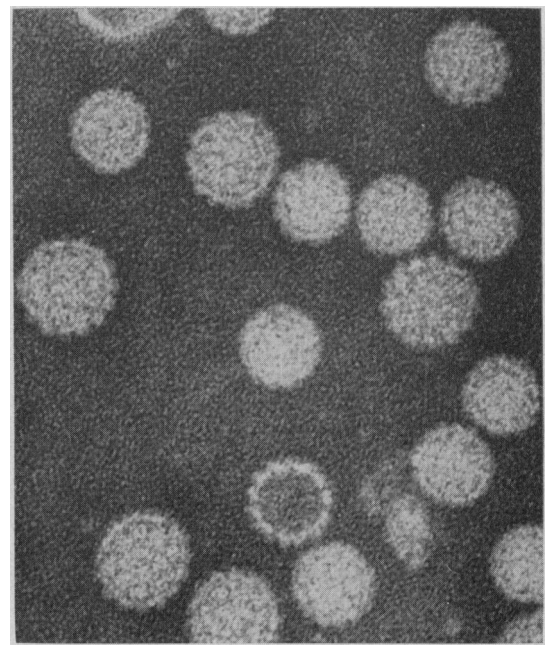

Fig. 9.—Mixture of unfixed polyoma and human papilloma viruses showing virions of two distinct sizes. (Magnification 240,000 ×.)

value of the diameter of unfixed particles was 42.5 mμ.; and of formalinized particles 40.5 mμ. The difference may be accounted for by slight shrinkage due to prolonged immersion in formalin or, more likely, by increased rigidity leading to less flattening during drying. The average diameter of the virions in the human brain tissue is not significantly different from that of polyoma virions but it is appreciably smaller than that of the papilloma virions. The size and spacing of the surface projections also correspond closely to the values observed with polyoma virions.

The evidence from negative staining, then, indicates that the virions associated with the human brain lesions are of the polyoma rather than the papilloma type. This is in accord with the observation in thin sections of long cylindrical structures in the nuclei of glial cells.[7, 8] Similar structures have been observed in sections of nuclei of renal cells infected with polyoma virus,[18] but have not been reported in cells infected with papilloma virus. It is not possible to proceed any further with the identification of the particles in the brain lesions on the basis of their structural characteristics alone. Preliminary attempts to demonstrate the presence of specific polyoma or SV40 antigen in

paraffin sections of the formalinized tissue by standard fluorescent antibody techniques gave negative results, but this could have been due to loss of antigenicity by prolonged immersion in formalin. Cells known to contain polyoma virus also gave negative results after being kept for eight days in formalin.*

Assuming, as the evidence strongly indicates, that the particles in the diseased human brain tissue are indeed viruses, what can be concluded about their relation to the demyelinating process and to the chronic diseases with which PML is almost invariably associated? From the histopathological findings in the light microscope it would appear that demyelination results from damage to or destruction of oligodendroglial cells. The electron microscope studies suggest that the cytolysis of these cells is due to extensive multiplication of virus within their nuclei. If this conclusion is correct it means that there exists a rare, potentially fatal human disease that is caused by a virus of a type not previously known to be present in human beings. Furthermore, it can be concluded from the results of negative staining that the virions seen in glial nuclei are structurally similar to the polyoma, SV40, K virus subgroup of papova viruses. It is well known that polyoma and SV40 viruses can affect cells *in vitro* and *in vivo* in one or other of two different ways. In the first of these they behave like "ordinary" viruses, multiplying within parasitized cells and destroying them. In the second type of virus-cell interaction there is little or no viral multiplication but the cells are morphologically transformed and may acquire malignant characteristics. Recently, Shein[19] has demonstrated such a dual cell response in dispersed cell cultures composed of human fetal spongioblasts and astrocytes infected with SV40 virus. The spongioblasts were destroyed after one to two weeks, but astrocytes showed evidence of transformation after one to two months. There is no direct evidence that virus-directed malignant transformation occurs in man in the situation under study. However, the unusual proliferative activity of the astrocytes in some cases of PML is reminiscent of the phenomena associated with transformation.

*We are grateful to Mr. Robert Escoffery for carrying out these experiments.

The relationship of PML to the neoplastic diseases with which it is commonly but not invariably associated, remains to be elucidated. A possibility that cannot be ignored is that they have a common cause. However, a more likely explanation is that the chronic disease lowers the immunological competence of the patient resulting in increased susceptibility to infection or allowing a latent virus to multiply. A question that remains, however, is whether there are any diseases other than the very rare PML that may be attributed to the action of the agent detected in the brain tissue. Only further studies can answer this question.

It is obvious that unfixed brain tissue from patients with PML is urgently needed in order that the viral nature of the agent can be conclusively proven and its biological properties determined. For this we depend on the interest, awareness and co-operation of our clinical colleagues who may suspect such cases during lifetime and assist in obtaining subcortical biopsy material or very fresh autopsy material for studies in suitable culture systems or animals.

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PAGES OUT OF THE PAST: FROM THE JOURNAL OF FIFTY YEARS AGO

MILLIONS OF BLUEBOTTLES

The inventive genius of our time has been employed largely in devising destructive measures: the great guns with their infernal shells, the aeroplane, and the torpedo are used to destroy human life; preventive medicine and sanitation are ranged on the other side. In spite of lectures on the means of preventing the breeding of these insects the trenches are filled with millions of bluebottles and house-flies; they infest every corner, they cover every morsel of food, and settle with avidity upon the soldier sleeping in the dug-out and upon his wounded comrade. To quote from a letter recently published in a daily paper: "If someone were to devise a means of ridding our men at the front of the millions of noisome flies which are pestering them these hot summer days he would earn the thanks of all the army."—Editorial, *Canad. Med. Ass. J.*, 5: 708, 1915.