Electron Microscope Study of a Cell-Free Induced Leukemia of the Mouse: A Preliminary Report*

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PLATES 76 TO 84

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ABSTRACT

Preliminary results of an electron microscope study of a leukemia of the mouse transmissible by cell-free filtrates are reported. In twelve out of forty-seven specimens examined, virus-like particles were observed. So far, these particles have not been observed in control, non-leukemic animals. They are located inside the cytoplasm of leukemic cells which infiltrate the spleen or the liver. Their diameter is approximately 78 mμ. Their resemblance to other particles recently described in different mouse tumors is stressed. Their significance and, in correlation with ultrafiltration data, their possible etiological meaning are discussed.

A leukemia which is serially transmissible to adult Swiss or DBA/2 mice by cell-free filtrates was recently described (8). Since this neoplastic disease is apparently induced by a virus, it was of interest to examine material from infected mice for the presence of submicroscopic particles of viral appearance.

The terminal stage of the disease is characterized by a typical leukemic blood picture, with an almost complete replacement of the parenchymal tissues of the greatly enlarged spleen and liver by leukemic cells. Filtrates are infective when prepared from blood, spleen, kidney, liver, brain, lung, or lymph nodes. Localized tumors do not appear throughout the evolution of this leukemia. This makes the morphological recognition of the virus more difficult because it is impossible to get a specimen containing only tumor cells. On the other hand, evidence for the viral character of the agent is obtained from filtration data, which demonstrate infectivity in material passing filters having a pore size of 200 mμ. Infectivity of the filtrate is lost with filters of smaller pore size.

In an attempt to study the morphological character of these particles, filtrates of spleens of infected mice were submitted to ultracentrifugation. The resulting pellet proved to be suitable material for preliminary electron microscopical investigation. The findings obtained from the study of the particles in these pellets, as well as in tissue sections from the organs of leukemic mice, are presented in this communication.

Materials and Methods

Tissue specimens were taken from thirty-three Swiss and fourteen DBA/2 leukemic mice. In the experiments in which pellets of cell-free filtrates were used, the spleens of five to six additional Swiss mice were pooled for each preparation. The method of preparation of ultrafiltrates and of transmission of the disease have been described elsewhere (8). Twenty-two tissue specimens were taken from untreated control mice. The spleens of fifteen to twenty normal mice were pooled for control pellets.

Preparation and Fixation of Pellets for Electron Microscopy.—Freshly prepared spleen filtrate (10.5 ml.) was centrifuged at 40,000 R.P.M. (approximately 110,000 *g*) in the Spinco model L for 60 to 90 minutes. The pellet was quickly removed from the tube with a

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small spatula, covered with a drop of fixative, and cut with razor blades into cubes approximately 1 cubic mm. in size. The specimens were then treated in the manner described below for tissues.

Preparation of Tissue Specimens for Electron Microscopy.—The organs were taken out of ether-anesthetized mice. The composition of the fixative was as follows: stock buffer solution (4 ml.), 1.0 molar sucrose (4 ml.), 0.2 N hydrochloric acid (2 ml.), and 2 per cent osmic acid solution (10 ml.). The stock buffer solution was composed of: sodium acetate (9.714 grams), veronal (14.714 grams), and distilled water to 500 ml. The pH of this fixative was between 7.40 and 7.45. Fixation time was one hour at room temperature, or (in summertime) in the refrigerator. After fixation, the specimens were rinsed quickly in 50 per cent alcohol, then passed through 70 per cent alcohol (two times for 10 minutes each), 95 per cent alcohol (two times for 10 minutes each), and finally through absolute alcohol (three times for 30 minutes each). After 30 minutes in a 50/50 mixture of absolute alcohol and methacrylate, the specimens were embedded in methacrylate which had been prepolymerized at 60°C. to a syrupy consistency, and completely polymerized by overnight exposure to a 30 watt ultraviolet lamp at a distance of 20 cm. The methacrylate used was a mixture of one part of methyl and nine parts butyl methacrylate, freshly dried on "molecular sieve" 4A, 1/16 inch pellets (10).

For sectioning, a Porter-Blum microtome was used, equipped with a glass knife or, more recently, with a diamond knife.[1] Sections were mounted on "Athene—new 200" grids, and coated with a carbon film.

A Siemens electron microscope (Elmiskop I) was used for all the pictures presented in this study. The working conditions were as follows: condenser 1, off; condenser 2, on; fixed condenser aperture, 600 μ; mobile condenser aperture, 200 μ; mobile objective aperture, 20 μ; filament voltage, 2.7 volts; beam current, 10 ma.; acceleration tension, 60 kv. Micrographs were taken at 10,000 or 20,000 direct magnification on Kodak contrast or Gevaert contrast plates.

OBSERVATIONS

Fig. 1 is a micrograph of a thin section from a pellet obtained after centrifugation of a filtrate prepared from an extract of leukemic mouse spleens. Although mixed with a variety of cellular debris, it is possible to recognize a large number of particles, regular in size and in morphological character. The ovoid shape of these bodies is believed to be a sectioning artifact. If we consider the true external diameter as the average between the longest and the shortest axis, the external diameter in this particular case is 110 mμ. The structure of these particles consists of two concentric spherical shells, the inner one having an approximate diameter of only 50 mμ.

It was possible to repeat this observation on another pellet prepared from a filtrate of an extract of a second pool of leukemic mouse spleens. So far, control pellets from spleens of normal mice have not revealed the particles. This preliminary observation was helpful for further interpretation of the virus-like particles found in the tissue sections taken from leukemic mice.

Fig. 2 represents a portion of the cytoplasm of a spleen cell from a leukemic mouse. Approximately twenty virus-like particles scattered between cytoplasmic components are recognizable. Their similarity to those of Fig. 1 is to be stressed. It is important to note, however, that the external average diameter is only 65 mμ, the inner spherule having a diameter of 40 mμ. Despite the differences in size, which might possibly be attributed to variations in procedure before fixation, the morphological features of these particles suggest that they are of the same nature as those observed in the leukemic spleen pellet. The resolution of this micrograph permitted the observation of a fine structural detail of the two membranes of the virus body. These membranes are apparently double (arrow), and are separated by a less dense interspace measuring approximately 30 A in breadth. Observations on similar particles in the cells of Ehrlich ascites tumor have recently been published (1).

In Fig. 2 the relationship of the particles to cytoplasmic components is not clear. Some particles are inside a cytoplasmic vacuole, and some are surrounded by the cytoplasmic matrix. On the other hand, Fig. 3 shows a typical intravacuolar localization of about fourteen virus-like particles. Here again, the cell observed was from the spleen of a leukemic mouse.

Particle-containing vacuoles, though frequently observed are not always so isolated. Fig. 4 gives an example of multiple vacuoles in the same cell. It is of interest to note that the number of particles observed per vacuole profile is between ten and fifteen. This constancy in the number of particles was a frequent observation in the leukemic material. The only exceptions were observed in cells which appeared to be megakaryocytes. An example of this is seen in Fig. 5, taken from a section of a

[1] We are grateful to Dr. H. Fernández-Morán for supplying the diamond knife with which a part of the ultrathin sections used in this study were cut.

liver highly infiltrated with leukemic cells. The cytoplasm of this cell is rich in mitochondria, Golgi apparatus, and a large variety of vacuoles. A small pseudopod emerges from the cell surface. The nucleus cannot be seen in the section. A very large vacuole, approximately 2 microns in length, contains a great number of virus-like particles. Smaller vacuoles scattered in the cytoplasm contain only one or two. It is possible, of course, to interpret this cell as a macrophage and the big vacuole as a product of phagocytic activity. Nevertheless, the cytoplasm resembles that of megakaryocytes observed in spleen or in bone marrow. One of these cells found in the bone marrow of a DBA/2 mouse 30 days after inoculation with a filtrate of a leukemic spleen in shown in Fig. 6. Only a small portion of the cytoplasm of the cell is shown. On direct examination, however, the size, the complex shape of the nucleus, and the details of the cytoplasmic components suggest the megakaryocytic nature of this cell. Here again, the cytoplasm shows a certain number of small vacuoles containing only one, two, three, or four virus-like particles. The nature and significance of these vacuoles will be discussed later.

Before considering some different aspects of this material, it seems appropriate to note that in a certain number of spleen cells from infected animals the cytoplasm contains large bodies, the contents of which are rather complex, surrounded by a dense membrane (Fig. 7). The background consists of a finely granular material, moderately electron dense. Some extremely dense particles 60 A in diameter are dispersed in this material. Finally, several blocks of a very dense substance in an extremely regular crystalline pattern seem to be characteristic of these bodies. The significance of this finding will be discussed later.

As far as the observation of infiltrating leukemic cells is concerned, a special problem arises in the liver. Although this organ is extensively infiltrated with leukemic cells in the terminal stages of the disease, there may be small areas in which only hepatic cells can be observed. In cutting thin sections for electron microscopy, the chance of observing these infiltrating cells may be small. In future studies it is planned to make a preliminary light microscope check on semithin methacrylate sections.

Studies with a light microscope revealed a marked atrophy of hepatic cords in mice in the advanced stages of the disease (8). In the electron microscope images this seems to correspond to a variable extent with the disappearance of ergastoplasm which is observable as early as a few days after inoculation (Fig. 8). Virus-like particles were observed only in infiltrating cells, never in the hepatic cells. Nevertheless, it seems necessary to pinpoint the morphological differences between virus-like particles and some dense droplets frequently observed in topographical relation with Golgi structures in hepatic cells (2). Fig. 8 gives an example of such particles. In small cytoplasmic vacuoles near a biliary canalicule, one can distinguish a few dense osmiophilic droplets, 50 mμ in diameter. These structures were observed at a similar frequency in the control animals. The vacuoles containing them are believed to be Golgi components, and their apparent high osmiophily suggests a lipide nature. In Fig. 9 the relationship of these structures is more complex. Here, some of these dense granules are seen packed inside a smooth membrane system (arrow), but in this case some are also visible scattered in the hepatic cytoplasm. These scattered granules are sometimes surrounded by a spherical membrane, 80 mμ in diameter, and look quite similar to the particles previously described. From a purely morphological viewpoint, the difference is essentially one of density. Virus-like particles appear more like a system of two concentric spherical shells, the inner one seeming empty, or containing only a moderately dense substance, but never presenting the density of the particles observed in hepatic cytoplasm.

Two important questions can be asked in relation to these preliminary observations. They concern the average diameter of these virus-like particles and the frequency of their occurrence. Obviously, the number of observations here does not permit a true statistical evaluation of particle size. However, a preliminary quantitative approach may be useful for further studies. Though the majority of the measurements of the external diameters of the particles fell between 62 and 96 mμ, they ranged from 53 to 115 mμ. The average was 78 mμ. The inner spherule had an approximate diameter of 30 to 40 mμ.

How frequently were the virus-like particles observed in the material studied? Forty-seven specimens from twenty-nine different leukemic mice, both Swiss and DBA/2, were examined. The percentage of positive results was apparently higher in the DBA/2 specimens than in those from the

Swiss mice. It was possible to recognize the virus-like particles in a total of twelve specimens from ten different mice. In such positive specimens, only a very small percentage of the cells contained particles. No attempt has been made as yet to put this ratio of positive cells to negative cells on a quantitative basis.

Thus far, no correlation has been detected between the cytological relationship of the viral particles and the age of the experimental infection. The ten positive mice had been inoculated from 20 to 66 days before the tissues were examined.

DISCUSSION

The particles described in this paper are fundamentally different from any known components of the normal cytoplasm. Their morphological features, however, are becoming more and more familiar to cytologists interested in the cytopathology of virus-induced tumors. Fortunately, numerous technical improvements have been made in the last few years, allowing a much more precise knowledge of virus ultrastructure. It is, therefore, interesting to compare our results with those obtained to date with different pathological material.

The particles seen in our material are essentially composed of two concentric spherical shells. Comparable, but not identical structures have been observed in many pathological cells derived from Rous chicken sarcoma (9), mouse mammary carcinoma (3), mouse Ehrlich ascites tumor (1, 6, 7, 14), frog renal adenocarcinoma (5), cells infected with influenza virus (11), and HeLa cells infected with RI-APC virus (12).

The present discussion will be limited to the virus-like particles observed in mouse tumors: the mammary adenocarcinoma, the Ehrlich ascites tumor (EAT), and the leukemia presently under study. Since the EAT was originally derived from a mouse mammary carcinoma (1), and since the leukemia originally had occurred in a mouse which had been inoculated with a cell-free preparation of EAT (8), the observation of identical particles in these three diseases would not be surprising. There is, however, an important difference in the transmissibility of these tumors. The mammary carcinoma and the leukemia are transmissible by cell-free material, whereas the EAT can be transmitted only by cells. Nevertheless, the particles observed by Adams and Prince (1) in EAT cells are quite similar to those found in our leukemic material. In both cases the particles have an outer diameter of around 70 mμ, and they have a similar morphology, with the same dual structure of the membranes. Their exclusively cytoplasmic localization, and their frequent association with vacuoles make the resemblance more complete. In EAT cells these vacuoles containing virus-like particles are considered by the authors (1) as units of the endoplasmic reticulum. Whether these vacuoles are of the same nature in our leukemic material is not yet clear, except in the case of a few megakaryocytes. It seems reasonable, indeed, to identify the particle-containing vacuoles present in these highly differentiated cells with some endoplasmic reticulum derivative. Certainly, these vacuoles are not damaged mitochondria. They are never surrounded by a double membrane, and no intermediary stages with remnants of cristae were observed. The apparent affinity of the virus-like particles for the megakaryocytes must be stressed and requires more extensive investigation.

Finally, the curious crystalline structure seen in Fig. 7 must also be discussed. If one admits that such structures are related to some synthetic process, the question arises as to what biochemical compound is involved. The proximity of particles resembling ferritin might suggest a relation with hemoglobin synthesis. These structures were observed, however, in cells entirely different from erythroblasts. Such crystalline structures might also be related to protein synthesis in virus-infected cells (13), but no direct topographical relations were observed between the virus-like particles and the crystals. In one case, the cytoplasm of the same cell showed one particle-containing vacuole and one crystal-containing body. Extensive studies and control experiments will be necessary to clarify the cytochemical significance of this crystalline structure.

In seeking the general physiopathological meaning of the virus-like particles observed in our leukemic material, three possibilities must be considered: (1) The particle is a contaminant. (2) The particle is a normal cell component. (3) The particle is the etiological infective factor. It has recently been demonstrated that lymphocytic choriomeningitis (LCM) virus frequently contaminates mouse neoplasms (15). This virus has a diameter of 40 to 60 mμ and is thus fairly similar in size to the particle studied here. Unfortunately, no thin section study of the LCM virus has been done up to now, so that no structural comparison is possible. Nevertheless, a lack of immunological

relationship between the agent of the leukemia and the LCM virus has been demonstrated (8). Such a contamination thus seems unlikely. Contamination by other viruses is also difficult to imagine in the light of the experimental data on transmission and pathology.

The possibility that the virus may be a normal component of the cells is difficult to check in the EAT, since there is no adequate control tissue for comparison. In our material, this hypothesis is difficult to accept, since thus far, similar particles have never been observed in material from the control non-leukemic animals. This conclusion is based on the study of only twenty-two control specimens, and must therefore be considered with caution.

The last possibility is concerned with the virus as an infective particle responsible for the presently studied disease. Ultrafiltration data on "gradocol" membranes indicate that the infective particles pass through pores of 220 mμ in diameter. Using smaller pore size, the infectivity of the resulting filtrate greatly decreases and is completely lost when the average pore diameter is between 100 and 50 mμ. From the studies of W. J. Elford (4), it is well known that particles passing through a gradocol membrane with an average pore diameter of 220 mμ, for example, must have a much smaller diameter, approximately one-half that of the pore size. The observed average diameter of the virus (78 mμ) seems therefore to correlate with the filtration data. The difference in the size of the particles found in the tissue sections (78 mμ) and those found in the filtrate pellet (110 mμ) may be due to the fact that the tissue is fixed immediately upon removal from the animal, whereas there is a lapse of several hours during the preparation of the pellet before fixation is possible.

Finally, it must be stressed that nearly all the leukemic organs examined in this study were also biologically tested and proved to transmit the disease by cell-free filtrates. This is particularly suggestive in the case of pellets prepared from leukemic mouse spleens in which "sister-pellets," prepared from the same filtrates, were resuspended and proved to be highly infective.

It seems, therefore, that this last hypothesis represents the most reliable guide for further studies of this leukemia.

Although there is no doubt that this mouse leukemia is caused by a virus, it is important to correlate the particles observed by means of the electron microscope with the etiological agent of the disease. The similarity of the particles observed in our material to those previously described in other mouse tumors should be stressed. One can only hypothesize as to the relationship of these particles to one another. Are they identical, their different effects being due to host reactions, or are they separate entities, undistinguishable by present electron microscope techniques, each causing specific neoplastic lesions?

A great deal of work is still necessary to clarify these questions. The natural history of the host-cell virus relationship must still be examined by following the entire course of the disease.

Further studies on this subject are now in progress.

We are pleased to express our sincere gratitude to Dr. C. P. Rhoads, Dr. A. J. Dalton, Dr. J. J. Biesele, and Dr. W. Bernhard for helpful advice and encouragement throughout the course of this study.

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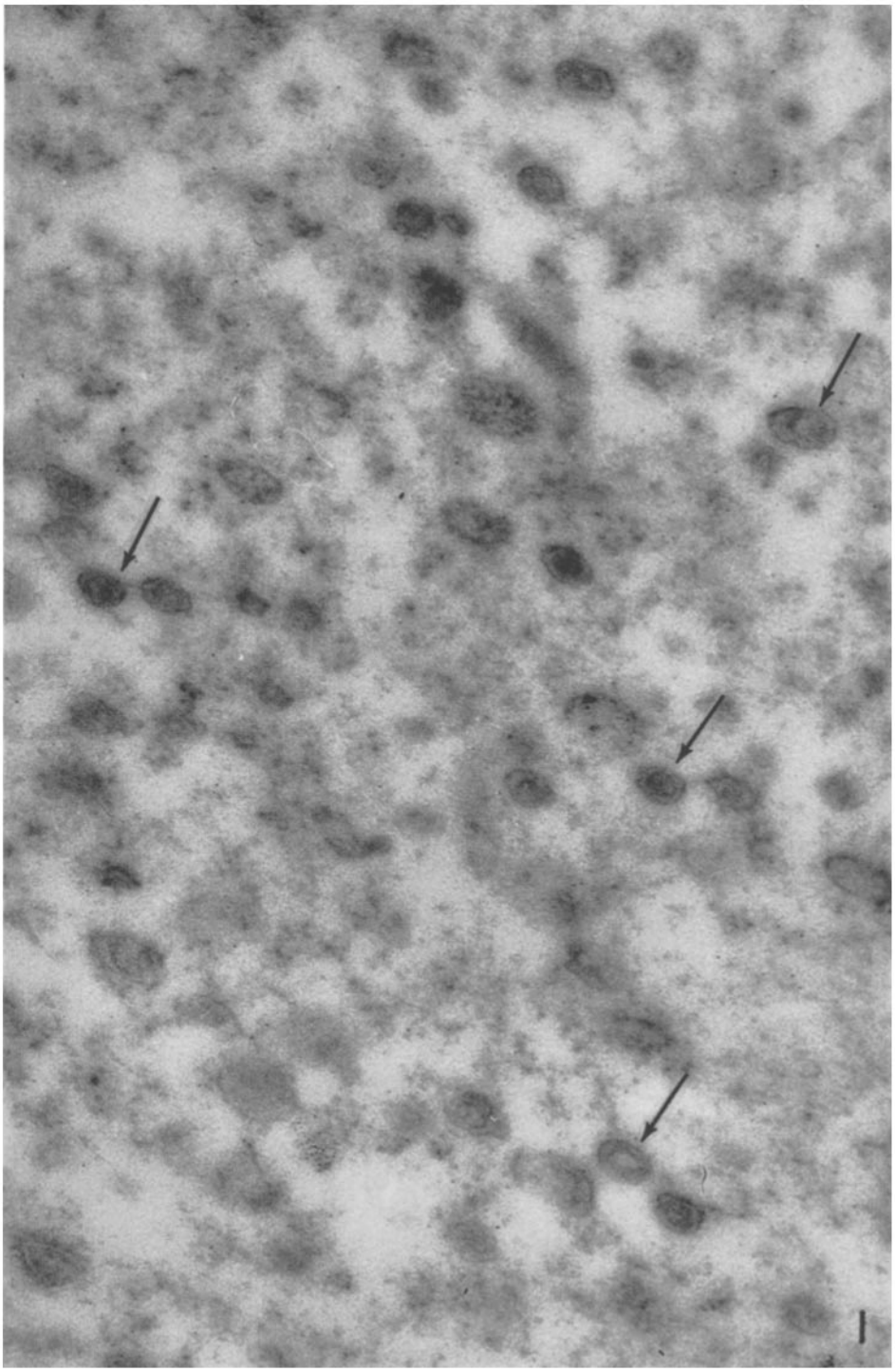
Explanation of Plates

Plate 76

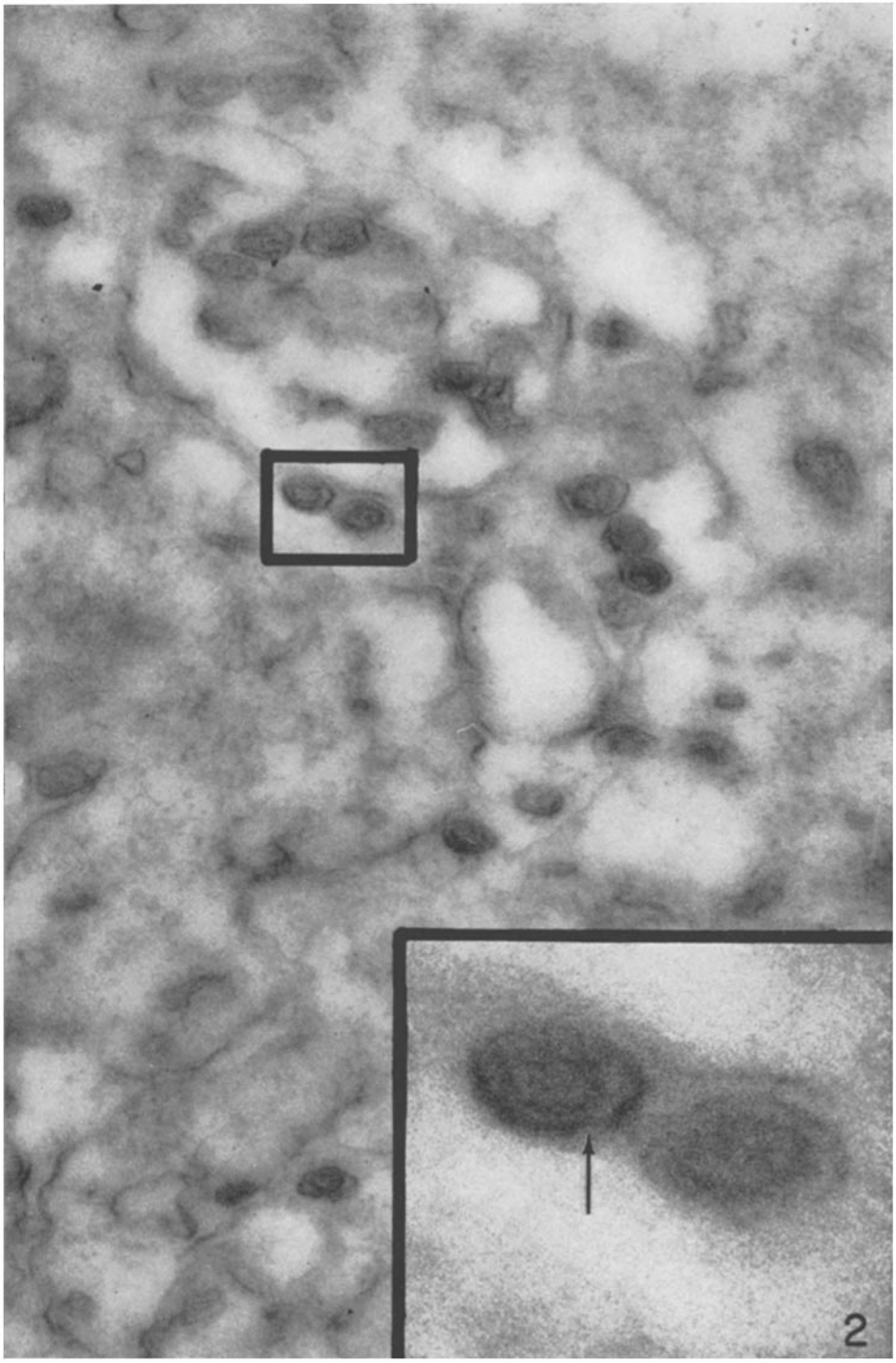
Fig. 1. Section through a pellet obtained by ultracentrifugation of a filtrate of spleens from leukemic mice. Several particles are recognizable (arrows). × 70,000.



(de Harven and Friend: Cell-free induced leukemia of mice)

PLATE 77

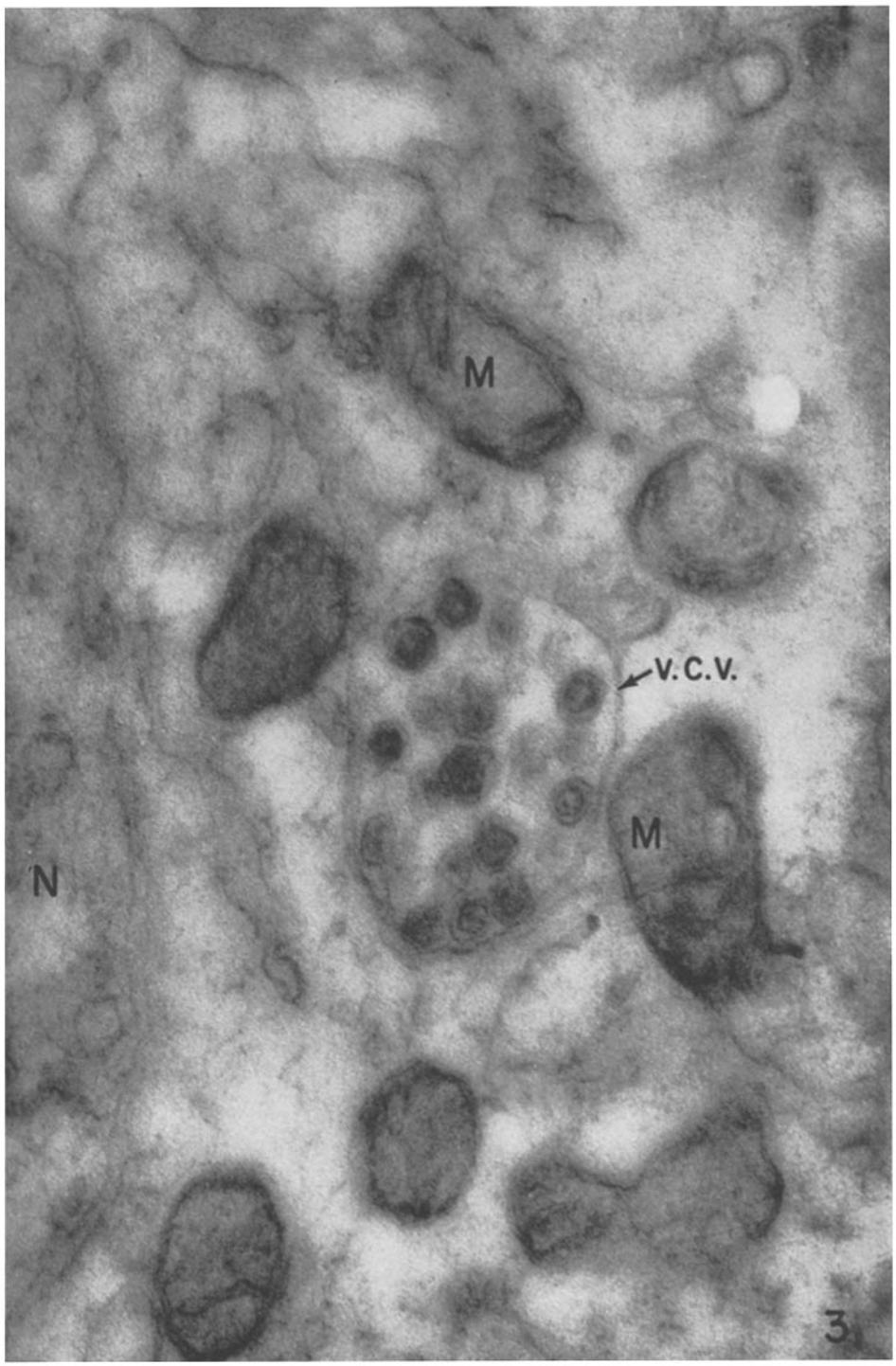
FIG. 2. DBA/2 mouse spleen, 61 days after inoculation. Some typical virus-like particles are seen in the cytoplasm of this cell. One of these (arrow) shows a dual structure of the membranes (enlarged in the corner of the plate to 350,000 magnification). × 114,000.



(de Harven and Friend: Cell-free induced leukemia of mice)

PLATE 78

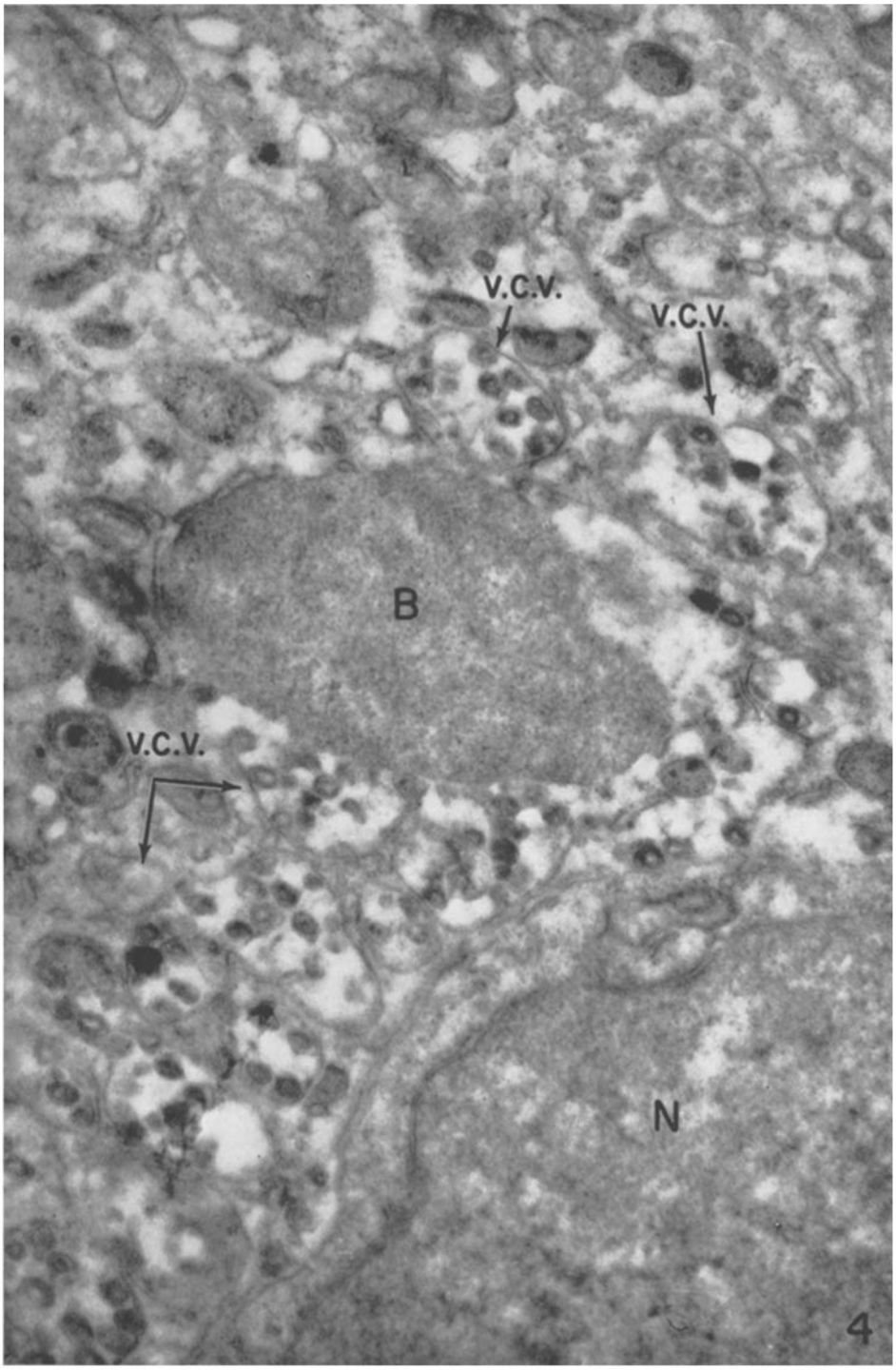
FIG. 3. The same spleen as Fig. 2. Virus-like particles are clustered in a cytoplasmic vacuole, *v.c.v.* Such vacuoles were never observed in relation with the plasma membrane, so that the extracellular localization of these particles appears unlikely. Mitochondria (*M*) and part of the nucleus (*N*) are present. × 120,000.



(de Harven and Friend: Cell-free induced leukemia of mice)

PLATE 79

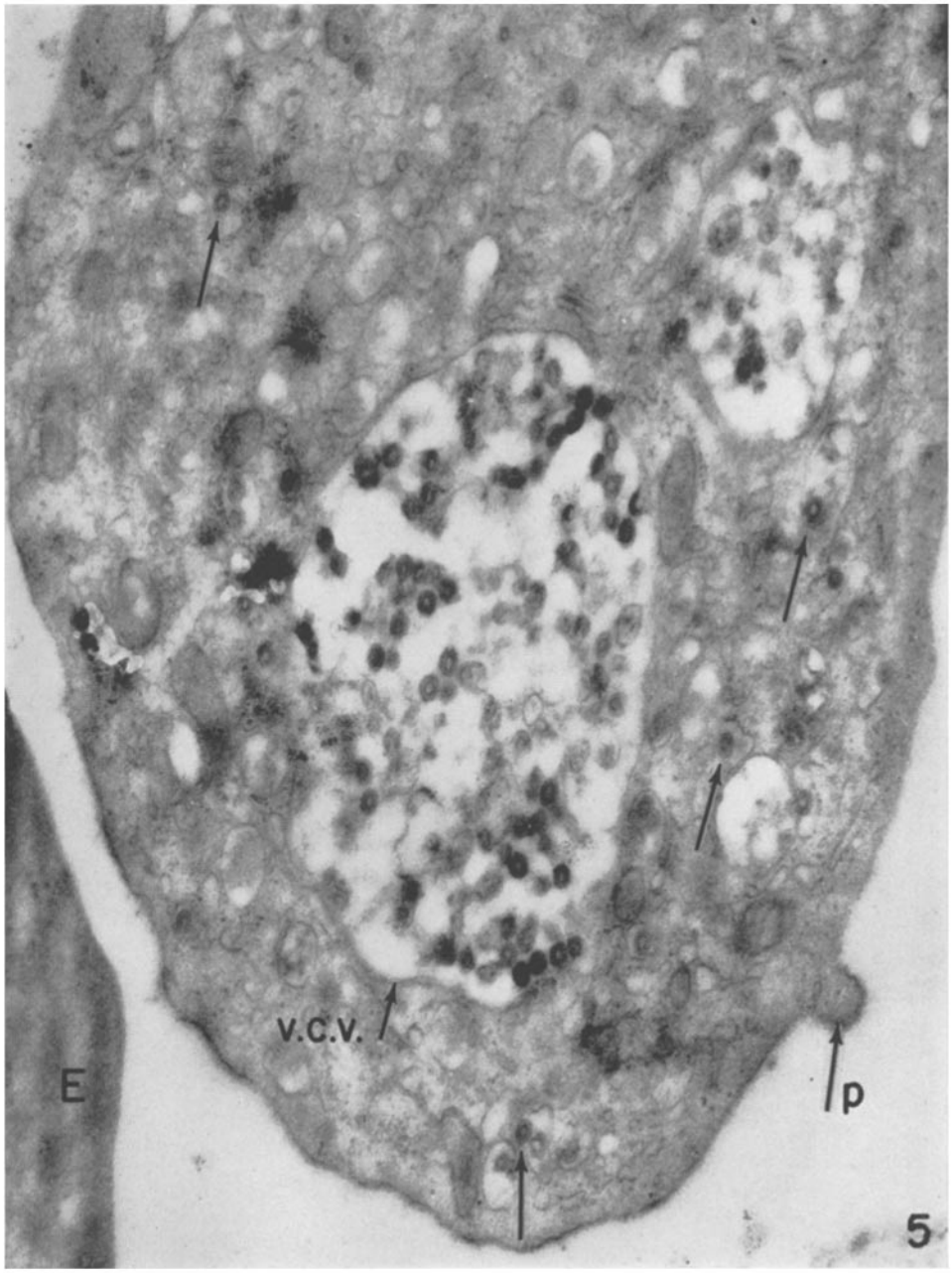
FIG. 4. Spleen of a DBA/2 mouse 66 days after inoculation. About 15 vacuoles containing virus-like particles (*v.c.v.*) are seen in the cytoplasm of the same cell. The size of these vacuoles and the number of particles in each of them appear fairly regular. In the middle of the micrograph, a large granular body (B) is seen. The lack of nuclear membrane would suggest that it is not of nuclear origin. Part of the nucleus (*N*) is seen in the lower right corner. × 43,000.



(de Harven and Friend: Cell-free induced leukemia of mice)

PLATE 80

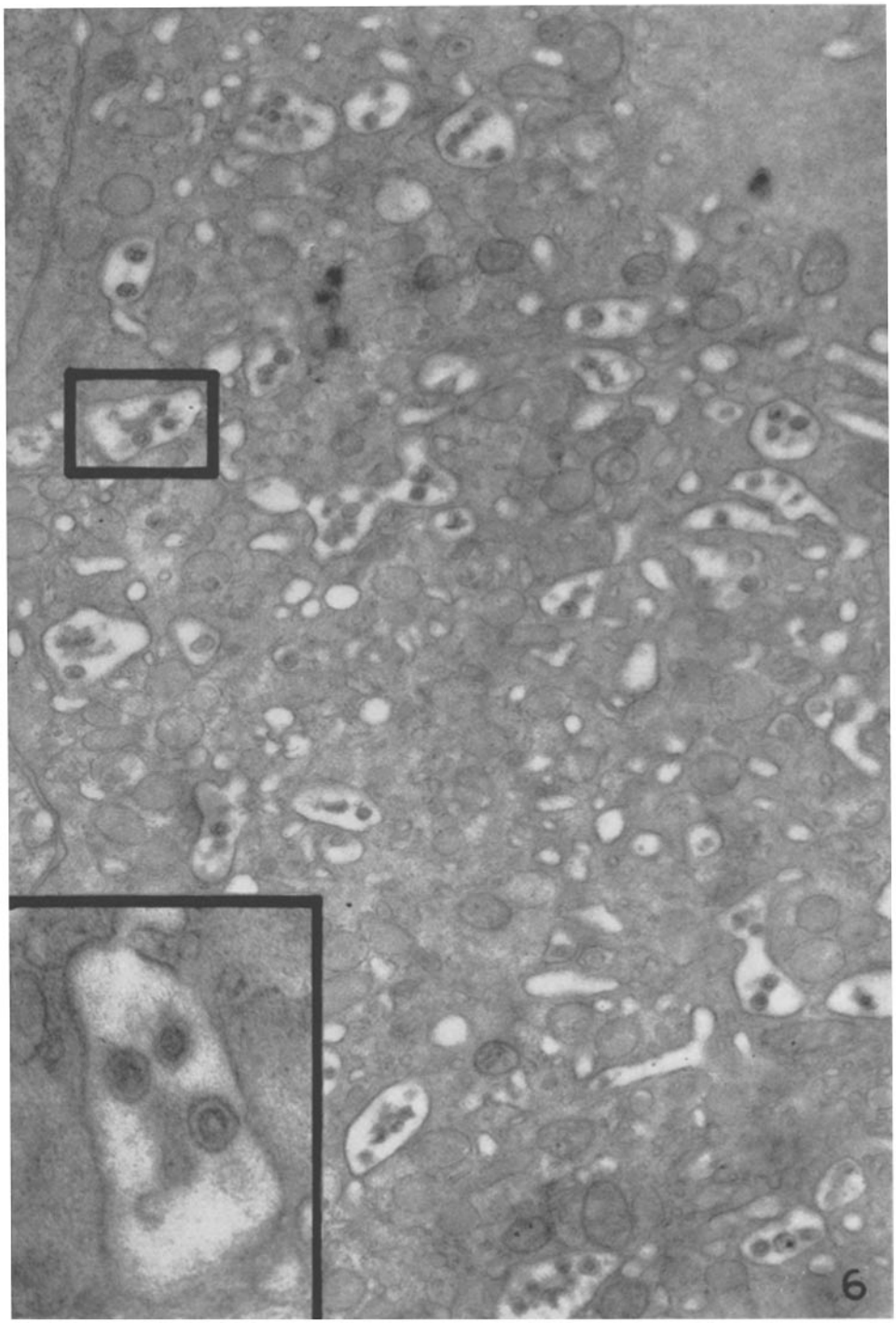
FIG. 5. Liver of a DBA/2 mouse 35 days after inoculation, highly infiltrated with leukemic cells. This image is considered to be a part of a megakaryocyte. The cytoplasm shows many particle-containing vacuoles, *v.c.v.*, of which one is of an exceptional size and contains a large number of particles. Note that the cores of the particles are particularly transparent in this case. A pseudopod (*p*) emerging from the cell surface and a portion of an erythrocyte (*E*) are also seen. × 43,000.



(de Harven and Friend: Cell-free induced leukemia of mice)

PLATE 81

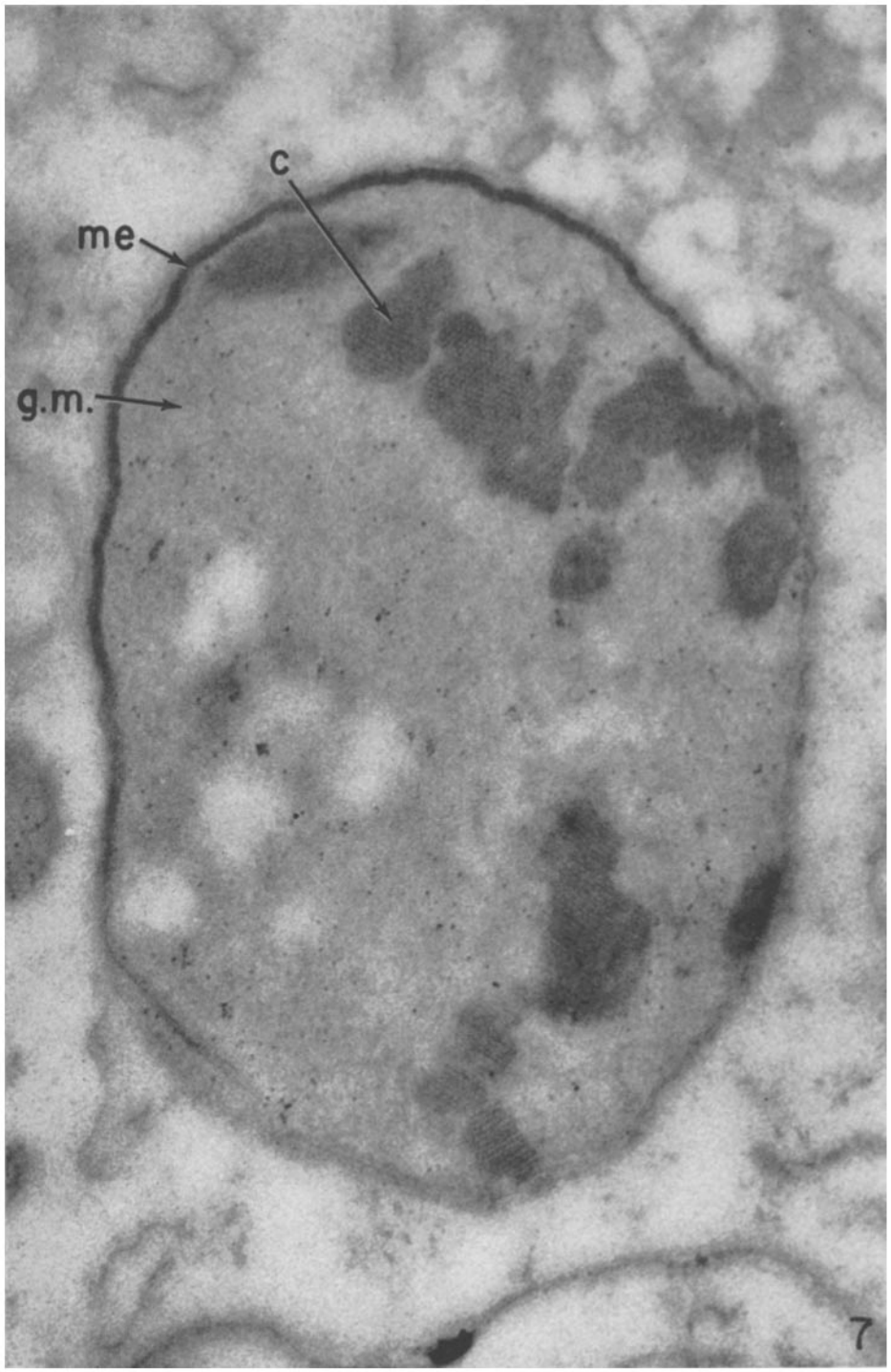
FIG. 6. Bone marrow of a DBA/2 mouse 30 days after inoculation. Many particle-containing vacuoles in the cytoplasm of a megakaryocyte are seen. These vacuoles are extremely irregular in shape and, except for the particles, appear rather empty of electron dense material. One of the vacuoles is enlarged up to 79,000. × 27,000.



(de Harven and Friend: Cell-free induced leukemia of mice)

PLATE 82

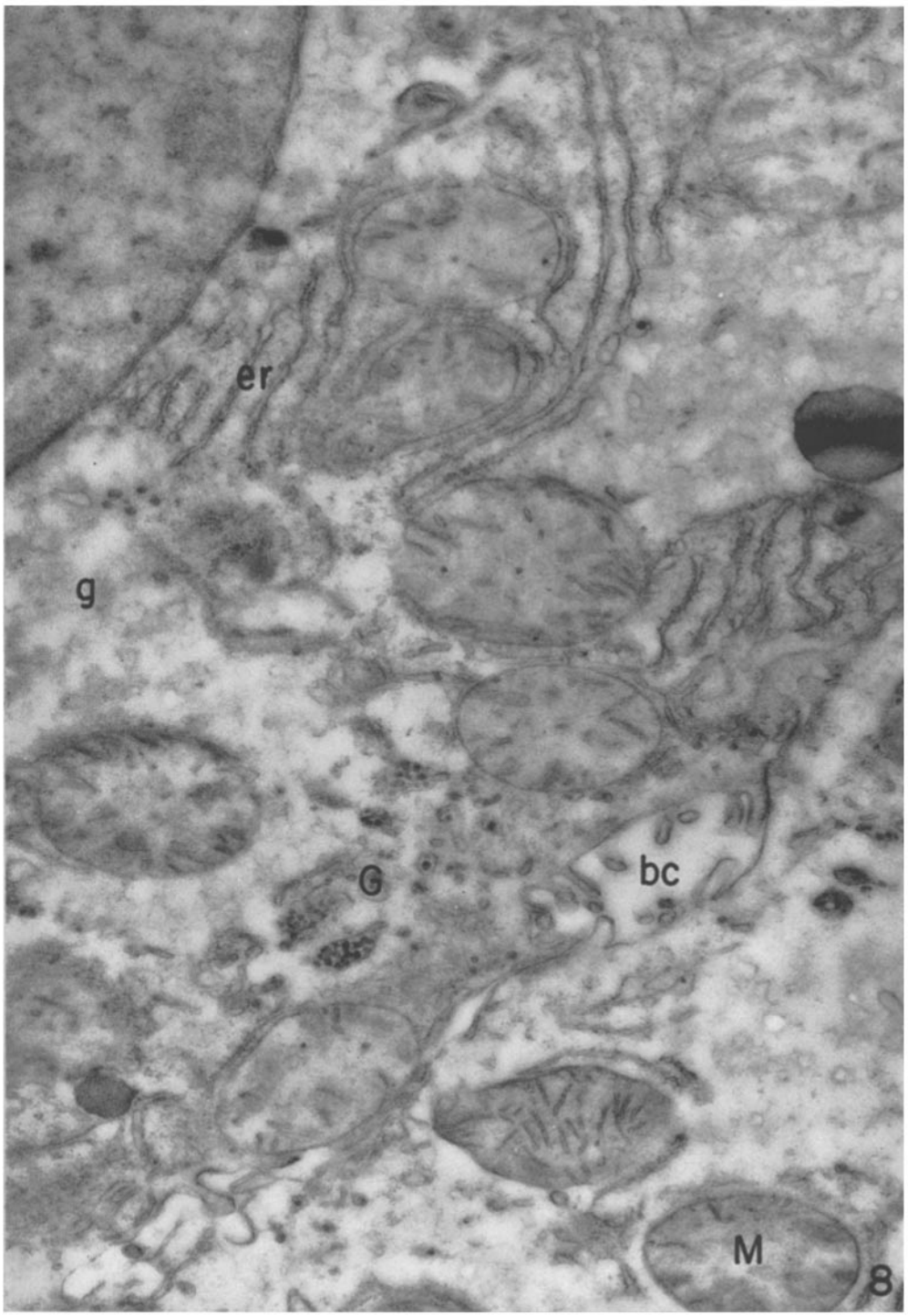
FIG. 7. Spleen of a DBA/2 mouse 61 days after inoculation. The cytoplasm of this cell shows a large body, more than 1 μ in length, particularly surrounded by a very dense 19 mμ thick membrane (*me*). This structure contains a finely granular material (*g.m.*) with a few ferritin-like particles and approximately 10 dense inclusions presenting an extremely regular crystalline lattice (*c*). The hexagonal dimensions of this lattice seem to indicate a radius of about 60 A. $\times$ 120,000.



(de Harven and Friend: Cell-free induced leukemia of mice)

PLATE 83

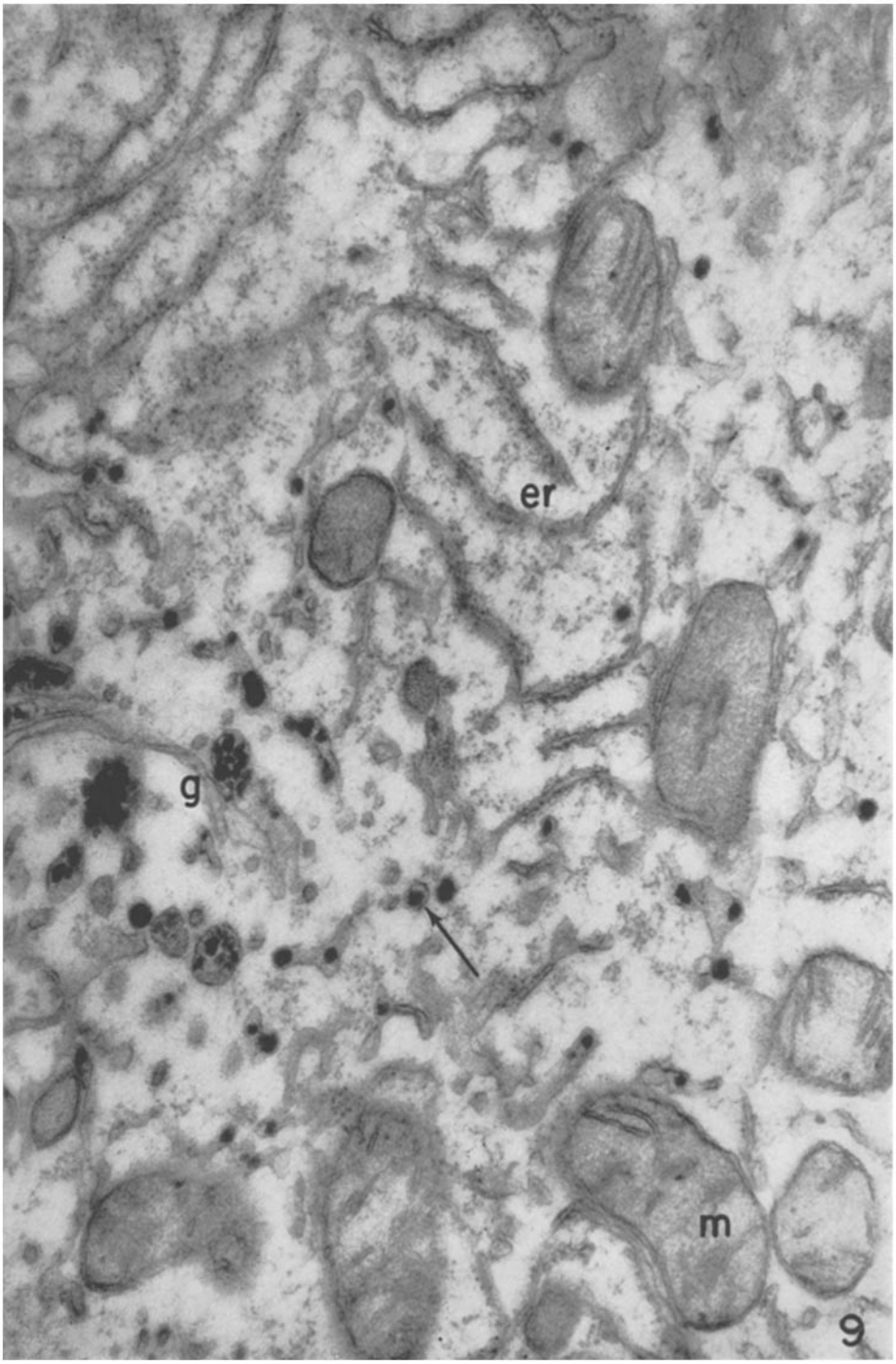
FIG. 8. Liver of a Swiss mouse 6 days after inoculation. The cytoplasm of these two cells is relatively poor in ergastoplasm (*er*). The mitochondria are well preserved (M), and some Golgi structures (*G*) near a biliary canalicule (*bc*) are full of small dense droplets 30 to 40 mμ in diameter. × 29,000.



(de Harven and Friend: Cell-free induced leukemia of mice)

PLATE 84

FIG. 9. Liver of a Swiss mouse 9 days after inoculation. Numerous osmiophilic droplets are seen scattered between mitochondria (*m*) and ergastoplasm (*er*). Some are in relation with smooth membranes and vesicles, presumably Golgi components (*g*). Others, however, are apparently free in the cytoplasm, or surrounded by a thin membrane (arrow). Similar structures were frequently observed in the control non-leukemic livers and are not believed to correspond to the virus-like particles described in this paper. × 58,000.



(de Harven and Friend: Cell-free induced leukemia of mice)