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Tissue Culture and Circulating Myeloblasts of Avian Leukemia Studied in Electron Micrographs of Ultrathin Sections.* (23895)

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In blood of chickens with virus-induced myeloblastosis, there occur primitive cells of myeloid origin(1,2). Recent studies(3) have shown that such cells can be maintained for long periods in tissue culture. Under these conditions virus particles characteristic of the disease are liberated in large numbers into the culture fluid, as estimated by direct particle count in electron microscope and by measurement of enzymatic activity of the agent to dephosphorylate adenosine triphosphate. The source of the virus was presumably the primitive cells. In this report there are described the results of studies made by means of technics of ultrathin sectioning† with the object of obtaining information about the cell-virus relationships involved in the process. The principal materials examined were myeloblasts taken directly from the circulation and the cells after various intervals of culture. For purposes of comparison, there were investigated, also, sections of spleen, bone marrow and liver.

Material and methods. The materials were obtained from birds with avian myeloblastosis produced with virus of the B.A.I., a strain studied(4) for several years in this laboratory. Experimental induction of the disease was effected by intravenous injection of 0.1-ml volumes of filtered whole or diluted plasma from previous passage chickens. Myeloblasts were obtained as already described(3) by centrifugation of heparinized blood. Donor birds, in advanced stages of the disease, were selected for high blood content of myeloblasts (about 1 to 2 million/mm^3) as judged from routine blood smears and high plasma virus concentration (about 10^{12} particles/ml) determined by enzyme activity(5). Tissue cultures of the myeloblasts were established and maintained in the usual way(3) in medium consisting of Gey's saline solution containing 5 to 20% chicken serum and added glucose in 0.5% concentration. The cultures were prepared in 5-ml volumes with about 10^7 cells/ml in 50-ml flasks and incubated at 37°C on the gyratory shaker. Fluids were changed at 2 to 4-day intervals. Two kinds of samples of cells were taken for thin sectioning; namely, free floating cells collected by centrifugation of culture fluid; and attached cells scraped from walls of flasks. Samples were taken from 4 different series of cultures at intervals of 0 to 77 days. Each sampling was from 2 or 3 flasks carried in parallel in each series. All were fixed for 3 hours in the cold in 2 ml of 1% osmic acid buffered to pH 7.4. Myeloblasts from centrifuged blood were delivered in drops of about 1 mm diameter from vaselined fine-tipped pipettes directly into the osmic acid solution. Pieces of spleen, bone marrow and liver of about 1 mm^3 were taken from 5 diseased birds in late stage of disease. Studies were made, also, on spleen, liver, bone marrow and white blood cells from 2 untreated birds of comparable age. Procedures for preparation of all specimens for thin sectioning were essentially those of Palade(6). The fixed samples were dehydrated with alcohol, imbedded in methacrylate and sectioned with

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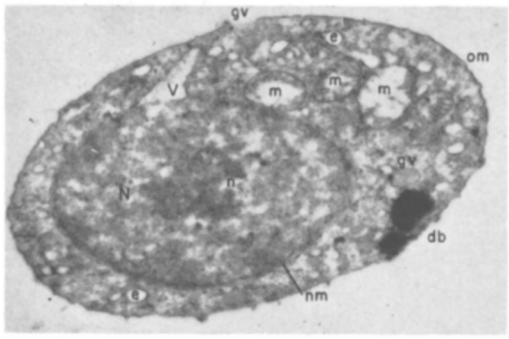

N = nucleus, n = nucleolus, nm = nuclear membrane, pm = plasma membrane, m = mitochondria, e = ergoplasmic reticulum, gv = grey vacuole, db = dense body, om = osmophilic plasma membrane, p = absent plasma membrane, V = vacuole, v = virus, b = inclusion of small virus-like particles, ib = inclusion bodies.

FIG. 1. Thin section of a typical myeloblast from peripheral blood of chicken in late stage of myeloblastosis. Magnification 10,550 ×

the Porter-Blum microtome.

Results. Circulating myeloblasts. Examination has been made of about 2500 thin sections of myeloblasts taken from blood without treatment other than centrifugation at low speed to separate primitive elements from red blood cells. The findings were quite uniform from one donor to another and are illustrated in the micrograph of the myeloblast in Fig. 1. The nucleus, surrounded by a double membrane, contains a prominent nucleolus and a loose chromatin structure showing a tendency to margination. Such cells from diseased birds differed in several respects from myeloblasts in bone marrow of apparently normal chickens. Cells in the former case showed swelling and fragmentation of mitochondria and vacuolization of cytoplasm. Densely staining bodies were of frequent occurrence in the cytoplasm, sometimes attached to the plasma membrane. While in this section the bodies were stained as densely as fat vacuoles, in others, they seemed less osmophilic and contained structures resembling virus identical in morphology with virus particles seen outside the cells. In 66% of the sections from this source bird, there were 2 or 3 of the bodies, and the ratio in sections from other birds was directly related to virus content of the plasma. Vacuoles containing lightly osmophilic material and surrounded by a single wall ("grey vacuoles") are scattered in the cytoplasm. These, on high magnification, contained many small round particles with a dense center. As shown, too, in Fig. 1, a part of the plasma membrane was frequently abnormally osmophilic.

The most remarkable feature of the findings was the *very low incidence of typical virus inside the myeloblasts.* Although virus particles were seen in areas away from cells, in only 4 instances of all sections of these myeloblasts were such particles seen inside the cells. In these cases the virus was situated in vacuoles close to the plasma membrane.

Tissue culture myeloblasts. In preparation for tissue culture, the myeloblasts were washed several times with Gey's salt solution to eliminate plasma virus. The efficacy of the procedure was shown by absence of enzyme activity of samples of fluids taken at 0 culture time. Thin sections of cells taken at 0 time were of essentially the same appearance as that of cells in the circulation as shown in

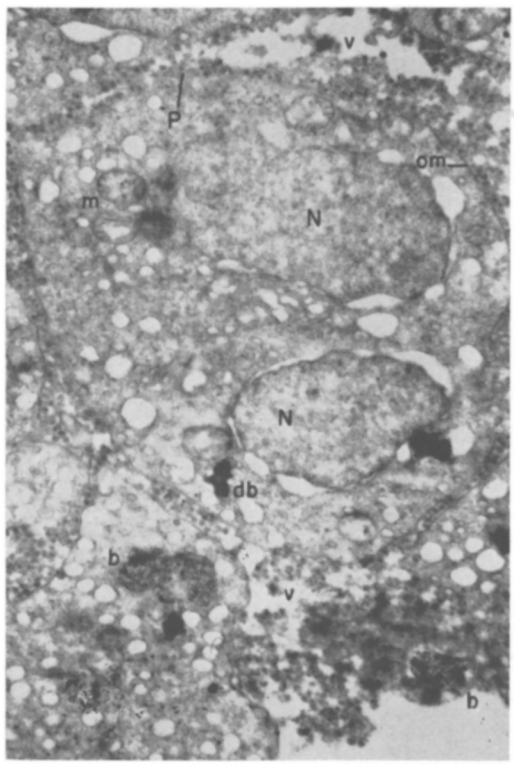

FIG. 2. Cells from a culture of myeloblasts after 77 days. A large amount of virus (v) surrounds the cells. Magnification 13,000 ×.

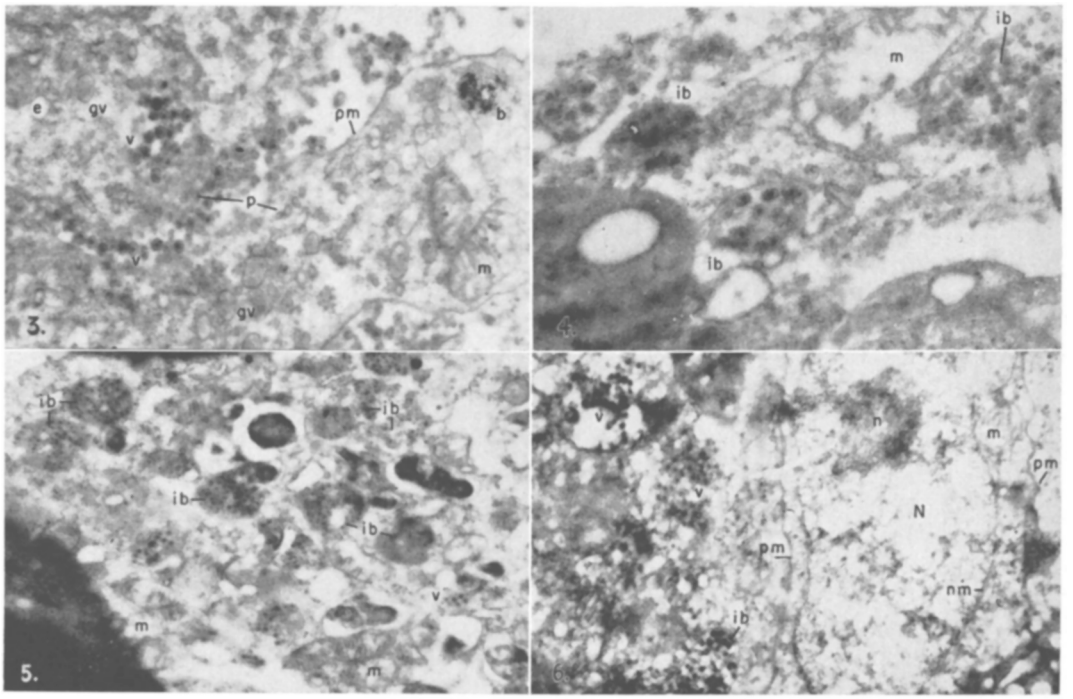

FIG. 3. Enlargement of a portion of a cell from a 58-day tissue culture of myeloblasts of the same series as Fig. 2. Virus (v) has accumulated both around the border of the cell and also inside the cytoplasm. The plasma membrane (pm) is missing in places (p). Magnification 20,000 ×.

FIG. 4. Portion of cytoplasm of an unidentified cell from bone marrow in myeloblastosis. Several inclusion bodies (ib) containing virus are shown. Magnification 30,400 ×.

FIG. 5. Portion of macrophage-like cell from bone marrow in myeloblastosis showing many inclusion bodies (ib) and free virus (v). Magnification 10,000 ×.

FIG. 6. Cells resembling reticular cells in spleen of the same bird as Fig. 5 showing similar inclusion bodies (ib) and free virus (v). Magnification 10,500 ×.

Fig. 1. Vacuolization of the cells seemed greater after washing. Virus of characteristic appearance was rarely seen either inside or outside the cells.

Cell specimens taken at intervals after 2 to 77 days of culture were of similar appearance whether they were free-floating in the culture fluid or had been attached to the flask walls. An example of findings in some sections is shown in Fig. 2 (floating cells after 77 days culture). The outstanding finding here is accumulation of large amounts of virus of characteristic appearance outside but in close association with the surface of the cells. In other sections, much less virus was accumulated about the cells although the concentration in fluid was high as indicated by enzyme activity. The factors determining distribution of particles in the culture are not yet known. Little of the agent, clearly recognizable as such, was within the cytoplasm as illustrated in Fig. 3, which is a higher magnification of a cell (from the flask wall) from the same culture series taken after an interval of 58 days of incubation.

A number of the dense bodies demonstrated in Fig. 1 are present in Fig. 2. Structures similar to the "grey vacuoles" already mentioned but containing more osmophilic small virus-like particles are present (b) in Fig. 2 and also in Fig. 3. The plasma membrane was osmophilic in some areas (om) and missing in others (p). Where the cell membrane was absent, virus particles were seen often apparently imbedded in the cytoplasm near the cell edge. In general the morphologic features of the cultured myeloblasts are recognizable, but variations in the appearance of the cells and in the number of degenerating cells were observed in different specimens. In Fig. 2,

the nucleus of the central cell is bilobed, and the perinuclear space, endoplasmic reticulum and mitochondria are more swollen than in the normal cells of the bone marrow. Some of the cells in other sections contained large amounts of osmophilic material in both the cytoplasm and the nucleus. On higher magnification of these denser areas, entities similar in shape to small virus particles could be made out.

Cells of the bone marrow. In contrast to the findings with the myeloblasts, many cells in the bone marrow contained very large accumulations of virus. An unidentified cell (not a myeloblast) in Fig. 4 contains inclusions of characteristic virus particles. As was usual with this material, much of the virus was of indistinct morphology. Dense bodies and swollen mitochondria were also present in a neighboring cell. In the bone marrow virus could also be found in association with very vacuolated myeloblasts with ruptured plasma membranes. Large cells resembling macrophages or reticular cells were crowded with large numbers of inclusion bodies filled with virus (Fig. 5). Structures resembling virus were not seen in any intact myeloblast in the bone marrow in this series.

Cells of spleen. In Fig. 6 there are inclusions of virus in cells with a large amount of cytoplasm, presumably reticular cells in the spleen of the same bird that provided the bone marrow of Fig. 5. Although a large part of the spleen was occupied by degenerating myeloblasts, no virus was positively identified in them.

Liver. The liver in some birds showed extensive changes. Very large numbers of myeloblasts were observed, and there was some necrosis of liver cells. Inclusion bodies filled with typical virus and virus free in the cytoplasm were present in large degenerate cellular elements which may have been Kupffer cells. These contained much osmophilic material, which under high resolution, appeared to be made up of small virus-like particles. The liver cells contained the same kind of grey vacuoles as those described already in myeloblasts and in a few instances, virus was seen in vacuoles in cells resembling liver cells.

No evidence was seen of virus-like particles in the spleen, bone marrow or liver of uninfected birds of the same age and strain as those diseased with myeloblastosis.

Discussion. The present observations are of significance in 2 principal aspects: (1) the infrequent demonstration of typical virus particles in myeloblasts either those in circulation or those in tissue cultures; and (2) the great difference between this lack of virus in myeloblasts and the amounts of agent found in cells other than myeloblasts in bone marrow, spleen and liver.

That virus is regularly liberated into the fluid of tissue cultures of myeloblasts has been well established(3). In some cultures, the agent shed into the fluid numbered as many as 2000 particles/cell in a 48-hour culture period. The findings provide additional substantiation of the results obtained by enzyme measurement and particle count. Because of this, it has been the more remarkable that practically none of the agent was present in distinctive form within these cells either before or after they were cultured. The studies have not been carried far enough to give a clear indication of the nature of virus precursors in the cells or the site of virus synthesis. There may be mentioned for further consideration the frequently occurring dense bodies, which usually represent fat, and greyish vacuoles in which there could be seen suggestions of ovoid structures or very small particles that might be the very early forms of the agent. Virus was occasionally found in structures with the appearance of vacuoles, but whether the particles had been formed there or simply taken up by the cell could not be determined.

With affected cells other than myeloblasts, the findings were quite different. Virus of distinctive morphology was frequently to be seen in very large amounts inside such cells. More often than not it was present in clearly defined walled structures with the appearance of inclusion bodies, though some appeared scattered in the cytoplasm. The structures observed in the non-myeloblastic cells were like those which have been seen by other investigators in the liver and spleen in lymphomatosis(7) and in sections of tissues from birds diseased with the same strain of myeloblas-

tosis virus(8) employed in the work described here. Virus particles of morphology identical with that of the agent of myeloblastosis studied in the present work have been seen in the spleen and bone marrow of chickens with erythroblastosis(9). In myeloblastosis and erythroblastosis, the agents of which are related though distinct(4,10), the virus particles observed inside the cells have been identified with the infectious entity in isolated and purified preparations(11).

The intracellular particles seen in the present work are of particular interest since the virus of myeloblastosis has been identified in purified preparations by physical, chemical and immunologic procedures(2). Studies of ultrathin sections of such purified virus have revealed(11) an essentially homogenous population of particles of characteristic structure. Particles seen inside the cells exhibited the same morphology as the particles in ultrathin sections of the purified preparations. In addition, the morphology of intracellular particles was identical with that outside the cell in the same micrographs. Because of the identity in morphology(11), of the characteristics of the disease occurring in the donor chicks, and of the biologic and morphologic characteristics of the agent liberated in tissue culture, there would appear to be a sound basis for judging that the intracellular structures are representative of the agent of myeloblastosis.

From these results it may be judged that the myeloblasts not only liberate virus but actively support the proliferation of the agent in tissue culture. It is further indicated that the final conversion of precursor substance (not recognizable in the micrographs) to virus like the extracellular particles occurs rapidly and that the agent quickly leaves the cell. The lack of fully formed virus particles in the cytoplasm suggests that a large part of at least the final process may occur close to or at the plasma membrane. This possibility has already been considered(12) in relation to the Rous sarcoma in which virus particles were present mostly at the surface outside the cell. In some micrographs(12) the particles of Rous sarcoma virus appeared to be imbedded in the most superficial cytoplasm in a manner very similar to that observed with the myeloblasts. In types of cells other than myeloblasts the processes of proliferation and liberation appear to differ from those in myeloblasts in degree if not in kind. Virus was not all liberated immediately from such cells but might be crowded in large amounts in inclusion structures and as individual particles throughout the cytoplasm. In view of this evidence of massive cellular involvement, it is small wonder that the virus may reach concentrations of more than a trillion particles/ml plasma.

The findings with myeloblasts, especially those from blood, are of much practical significance in relation to studies on possible relationships of viruses to malignancy of presently unknown etiology. The myeloblasts are malignant derivatives of virus-infected precursor cells. That they, themselves, are infected and can support virus liberation at high levels was demonstrated by the tissue culture experiments. Yet this state and activity might well have been overlooked in a cursory examination of the circulating cells. Analogous conditions have been encountered not only with the Rous sarcoma(12,13) but with the Murray-Begg endothelioma(14). In the former few particles are to be seen inside the cells, and the studies thus far have shown only extracytoplasmic agents in association with the latter. It is well to recognize probable major differences in the mechanism of proliferation of the various tumor viruses, evidence for which is already considerable. Much virus is seen in the cytoplasm of the cells of the mouse mammary carcinoma(7,15) and in those of mouse lymphatic leukemia(7); the antigen of the rabbit papilloma virus appears to be concentrated in the cell nucleus(16); and the results of the present work demonstrate considerable differences in the reaction of different types of cells to the same virus in the same host. The variations serve well to emphasize the difficulties to be encountered in the search for viruses in association with tumors not known to be caused by filterable agents.

Summary. Studies have been made by electron microscopic examination of ultrathin sections of circulating myeloblasts of avian

myeloblastosis and of these cells in tissue culture. Examinations were made, also, of tissues of spleen, bone marrow and liver of the diseased birds. The results showed that intracellular virus of typical appearance was very rarely seen in myeloblasts of the circulation and only very infrequently in the cells after culture *in vitro*. This was the remarkable feature of the findings, since the cells liberate virus at a high rate for long periods in tissue culture, and large amounts of *extracellular* virus were associated with the cultured cells. In contrast, affected cells not myeloblasts in spleen, bone marrow and liver contained intracytoplasmic virus in high concentrations, most of it in inclusion-like bodies. The significance of the results was discussed in relation to similar findings with the Rous sarcoma and Murray-Begg endothelioma and to exploratory studies for the demonstration of virus in tumors of presently unknown etiology.

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Excretion of 17-Hydroxycorticosteroid Hormones by Pantothenic Acid Deficient Guinea Pigs.* (23896)

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Although recent investigations have clearly established the important relationship of pantothenic acid to adrenal steroid secretion in the rat(1-5), the role of this vitamin in adrenocortical function in other animals has not been studied. The purpose of this report is to describe investigations of secretion of adrenal steroid hormones by guinea pigs made deficient in pantothenic acid. The results demonstrate a diminished excretion of 17-hydroxycorticosteroids in response to ACTH administration.

Materials and methods. Young, male guinea pigs (Hartley Strain) weighing 150 to 250 g were used. Animals were housed individually in metabolism cages, weighed frequently, and 24-hour urine samples collected. Each urine specimen was diluted to 100 ml with distilled water and a 5 ml aliquot used for determination of 17-hydroxycorticosteroid hormone content by the method of Silber and Porter(6). To obtain biochemical evidence of pantothenic acid deficiency, coenzyme A analyses

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