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Morphology of Characteristic Particles Associated with Avian Erythroblastosis.* (21971)

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Erythroblastosis and myeloblastosis (granuloblastosis)(1) are highly malignant leukemic diseases of viral etiology in the chicken. In the latter condition involvement of the precursors of the myelocytes(2) results in the occurrence in the blood circulation of very large numbers of primitive cells of myeloblastic features. The virus of erythroblastosis presumably affects the progenitors of the red blood cells and the disease is characterized(3) by the presence of many erythroblasts in the circulating blood. That the respective viruses, likewise, are present in the circulation in both conditions is attested by transmissibility of the diseases with filtered blood plasma. By application of ultracentrifugal procedures, the virus of myeloblastosis has been obtained from the plasma of diseased chicks in appreciable amounts in purified preparations for physical, chemical and biological characterization(4,5). The findings with myeloblastosis have stimulated the undertaking of similar studies with the virus of erythroblastosis. Examination of filtered blood plasma from chicks with this disease has revealed characteristic particles, which by virtue of the experience with myeloblastosis, may represent the etiological agent of erythroblastosis. This paper describes results of preliminary investigations on the morphology of these particles observed in electronmicrographs.

Materials and methods. Erythroblastosis virus was obtained from Dr. Astrid Fagraeus of the State Bacteriological Laboratory, Stockholm, who had received it previously from Dr. J. Engelbreth-Holm at the Department of Pathological Anatomy, University of Copenhagen. The agent has been passed in this country by means of filtered plasma injected whole, or diluted, in 0.1 ml volumes in 3-day to 3-month-old chickens. The birds employed routinely were inbred White Leghorns of line 15 developed(6) at the Regional Poultry Research Laboratory, East Lansing, Mich. and the same as those used in the work with myeloblastosis(7). As will be developed in later reports, the strain of erythroblastosis appears to be a characteristic entity entirely distinct from myeloblastosis with respect both to the pathological manifestations and to host-response to the agent. Small chicks with the disease were bled by section of the sagittal sinus and larger ones by heart puncture. The blood was received in chilled tubes containing heparin and cleared of cells by 2 cycles of low speed centrifugation. The plasma was recovered in each case with fine tipped pipettes, then filtered with celite and passed through Selas filters by the technics(8) employed in the work with myeloblastosis. Donor birds were selected on the basis of the number of erythroblasts seen in routine daily blood smears, and bleeding was effected only when the chickens were near death. Once the chicks became obviously ill, as indicated by lassitude or weakness, the condition pro-

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gressed rapidly to death within a few hours. In most instances the chickens were closely watched, and bled when they became moribund. During these stages of the disease, the increase in erythroblasts was rapid, and it seemed likely that the virus content of the plasma might, likewise, reach the highest levels in the few hours before death of the host. Thus far, none of the plasmas has exhibited significant activity to dephosphorylate adenosine triphosphate in the microscreening test (9). Consequently, this criterion of plasma content of virus, so useful in the study of myeloblastosis, has not been available in the experiments with erythroblastosis. However, in contrast with host response to the virus of myeloblastosis(10), 2-month-old chickens were not greatly different in susceptibility than 3-day-old birds to infection with erythroblastosis virus. This was a most important factor, since the older birds furnished much larger volumes of plasma as source of the virus. The ages of the donor chicks used in the present work were from 3 to 60 days. Blood was drawn 7 to 15 days after inoculation of the virus. The technics employed for examination of the particles in erythroblastosis with the electronmicroscope were identical with those applied in the studies on the virus of myeloblastosis(8). Although observations were made with the particles in plasma, the principal investigations involved the particles in concentrates obtained by 1 or 2 cycles of sedimentation. The best results were obtained with individual plasmas spun, undiluted, in collodion tubes of appropriate volume. The particles were concentrated by centrifugation at 16,400 x g for 60 minutes and resuspended in small volumes, 5 to 25% of those of the initial plasmas, in normal saline solution containing 0.001 M phosphate of pH 7.0. These concentrates from individual plasmas or, more often, in pools (pooled only after the initial concentrating procedure) were centrifuged at 7,000 x g for 5 minutes to eliminate aggregated material. In some cases the procedures of high and low speed centrifugation were repeated for a second time. Preparations were made for electronmicrography either with a drop of undiluted concentrate dried on an agar surface, or the particles were spun down on agar(8) from suspensions diluted 1-10 to 1-100. The particles were fixed on the agar surface with osmic acid vapor and examined either after shadowing with chromium or without shadowing. Examinations were made with comparable technics of the particles directly in plasma. Counts of the particles in plasma or concentrates were made by sedimentation on agar in the usual way(8).

Results. The virus of erythroblastosis is relatively highly "virulent" in the sense that the chicks of the inbred line 15 were very susceptible to infection. With the largest doses of the agent, 0.1 ml of the best plasmas, onset of the disease, marked by the appearance of polychrome erythrocytes(3), was as early as 48 hours. Death followed frequently within 4 to 5 days. This appearance of intensity of host-virus interaction occurring in the induction and progression of the disease, as compared with the process in myeloblastosis, is emphasized by the observation of relatively low concentrations of characteristic particles which might represent virus in the plasmas.

That low particle content of the plasma is characteristic of erythroblastosis has been evident from several indications. In no case has the plasma exhibited visible turbidity ascribable to the presence of particles in high concentration. Ultracentrifugation results in no visible or very small pellets. In most instances material concentrated 3 or more times with respect to the volume of the starting plasma showed little opalescence, though occasional plasmas yielded quite turbid suspensions at

TABLE I. Approximate Characteristic Particle Content of Various Individual Plasmas from Chicks with Erythroblastosis Counted Either Directly in Plasma or Estimated from Counts with Concentrates of the Agent.

Donor	Age at bleeding (days)	Interval since inoculation (days)	Erythroblasts in blood smears†	Particles/ml plasma, $\times 10^8$
O 913*	17	12	4	257
O 911*	15	10	5	6.6
N 62	17	12	3	117
O 969	17	12	4	47
N 98	19	9	8	3.3
N 225	19	9	6	27

* Estimated from counts on concentrates.
† No. of cells/oil immersion field.

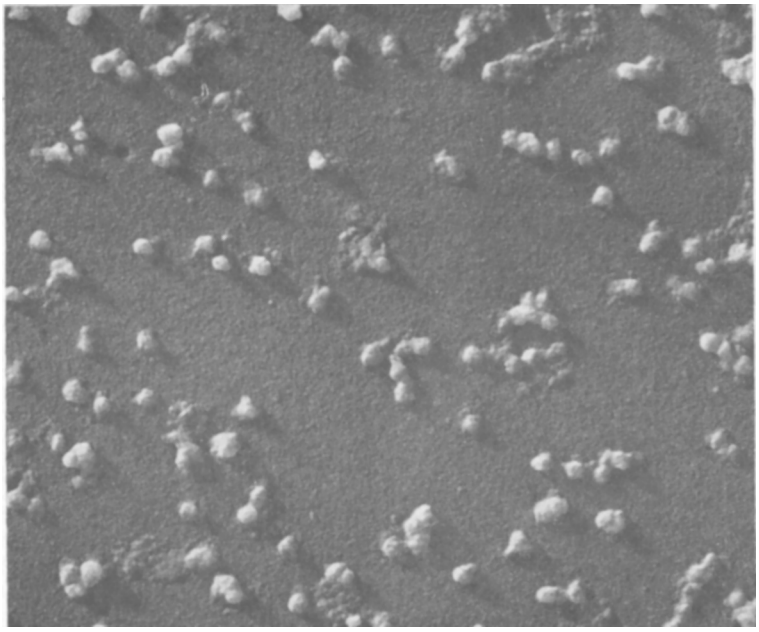

FIG. 1. Electronmicrograph of particles from the plasma of chick with avian erythroblastosis obtained by sedimenting the particles on an agar surface. Particles were shadowed with chromium. Magnification 27,600 ×.

20 times concentration. Finally, approximate estimates obtained by counting the particles in concentrates, after unmeasurable loss in the sedimentation procedure, as well as more accurate determinations made by counting the particles directly in plasma showed highly variable results ranging (Table I) from about 10^9 to 10^{10} particles per ml of plasma. The data of Table I are cited as representative of the findings. Many plasmas and concentrates were examined, but counting was excluded in most instances because of aggregation or excess amorphous material extraneous to the particles in relation to the very small numbers of particles.

Despite the technical difficulties, micrographs have been obtained clearly revealing the characters of the particles. One of the best pictures is that of Fig. 1 obtained with the concentrate of plasma O 913, Table I. The particles were sedimented from 4 ml of the plasma and taken up in 1 ml of saline. After low speed centrifugation the particle content of this suspension was 1.03×10^{11} per ml. The suspension was diluted 1-100 (equivalent to a 1-25 dilution of the original plasma), and the particles were spun onto agar.

The electronmicrograph reveals a population of particles of considerable homogeneity of kind which are of variable size and, in general, of spheroidal shape. There is present, also, amorphous material obviously different from the predominant particles and relatively few particles of a different magnitude of size from that of predominant ones. Measurements on small numbers of the particles of various sizes gave the value 102 mμ as the average diameter. It is seen that the particles are not regularly rounded but exhibit surface depressions, folds and protrusions from the main body of the particles, some as short tails or flattened membrane-like structures. The general characters of the particles are clearly shown, though the preparation contained much other material.

Shadowing with metal, though increasing the contrast and revealing 3-dimensional features of virus and other particles, obscures completely all except occasional contour evidence of internal structure. Pictures of unshadowed particles are difficult to obtain, but an informative example was available in the present work. A small drop of a suspension of twice sedimented particles was placed di-

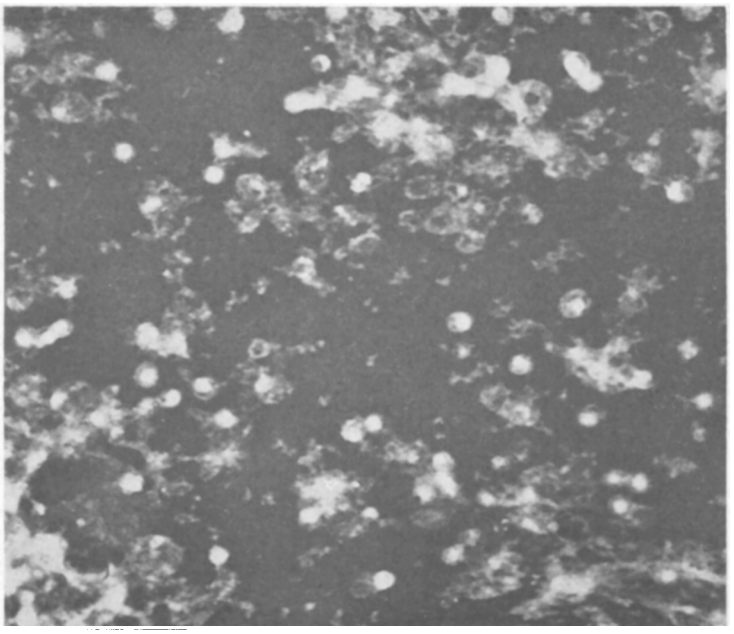

FIG. 2. Electronmicrograph of particles from the plasma of chick with avian erythroblastosis obtained by drying a drop of concentrate on an agar surface. Preparation was not shadowed. Magnification 34000 ×.

rectly on agar, dried, fixed with osmic acid and examined without shadowing. Fig. 2 shows 2 principal types of structures. One is represented by an essentially circular image with a small center of material of high electron absorbing power surrounded by a "membrane-like" substance of very low contrast. Similar images have been seen(11) with the virus of myeloblastosis, which is a particle of very low hydrated density(12) and is regarded as consisting primarily of watery, gel-like material surrounding a small region, or regions, of concentrated, relatively dense (to electrons) internal material. The images of the particles in erythroblastosis have the same appearance.

The second structures of interest are those having the appearance of empty, low contrast membranes greatly resembling particle "ghosts" consisting of the material surrounding the internal masses of the other images. In many instances there are seen empty spaces or apparent rents in the substance seeming to indicate space previously occupied by the dense material or through which it escaped. Other images have the appearance of fragments of the low-contrast material. Such images were seen not infrequently in the present studies, but the mechanism for the production of the material, that is, for the rupture of the particles and the escape of the dense constituent is not known. Although the interpretation may be faulty in detail, it seems certain that these structures of low contrast must be related to the intact particles, and do not resemble any of the usual nonspecific components of plasma either from normal birds or chicks with erythroblastosis. It is especially notable that many of the low contrast images are fairly regular in outline, are of the size of the images of the intact particles, and have the same appearance as that of the peripheral material of the specific particles.

Discussion. The present findings do not suffice to establish unequivocally the biological nature of the particles described. The component has not been observed, however, in the plasmas of normal chicks, and it occurs as a population of particles of considerable homogeneity with respect to kind only in diseased individuals. The characters of the particles and their obvious relation to the occurrence of erythroblastosis, together with considerable experience in the study of the

contents of chicken plasma in investigations of myeloblastic leukemia, constitute strong presumptive evidence that the particles represent the etiological agent of the disease.

The particles in erythroblastosis are remarkably similar to those of the virus of myeloblastosis. In the case of the latter agent, a variety of investigations has indicated that the myeloblastic leukosis virus is a spheroidal particle of low hydrated density and high water content. Most of the volume of the virus is constituted of material of low electron absorbing properties surrounding a small mass of electron-dense material, which may be regarded as nucleoprotein as in the case of analogous structures in other viruses. All of the evidence obtained in the present work is compatible with the view that the characteristic particles in erythroblastosis are essentially identical in physical structure with the agent of myeloblastosis. It would be fruitless to extend the comparison, inasmuch as sufficient amounts of the particles are accessible for analyses by other physical methods. It is desirable to note that the size value for the particles in erythroblastosis, 102 mμ, is somewhat smaller than that, 120 mμ for the agent of myeloblastosis. This difference, however, is not regarded as significant. A real difference between the two diseases is the particle content of the plasma. In erythroblastosis, the concentration of particles reaches levels of approximately 10^{10} per ml, whereas in myeloblastosis the levels(8) are as high as 2×10^{12} per ml. Another striking difference between the diseases resides in the lack of adenosine triphosphatase activity in the micro test in erythroblastosis. This does not signify a lack of enzyme activity of this virus, since the results may well have been related principally to the low concentration of the particles in the individual plasmas.

Of particular interest is the bearing of the present findings on the general problem of the etiological interrelationships among the various diseases classified(1) as members of the leukosis complex. These relationships can be determined only by intensive studies of the physical, chemical and biological properties of the agent associated with each condition. A considerable basis of information has been established with the agent of myeloblastosis(4). It would appear that the superficial physical aspects of the morphology of the particles in erythroblastosis are indistinguishable from those of the agent of myeloblastosis. Within the limits of the small number of examinations thus far made, the agent of a third form of leukosis(13), the RPL strain 12 of lymphomatosis, does not differ appreciably in morphology from the particles in erythroblastosis and myeloblastosis. This resemblance, however, may be entirely superficial, and further study may reveal significant physical and chemical differences. It would be well to emphasize the biological specificity of these 3 agents in the cell type involved; in the pathological manifestations; and in the character of host response in the induction of the respective diseases. These investigations on the agent of leukosis have been of principal value thus far in the physical demonstration of the virus entities in the plasma of birds with myeloblastosis and characteristic particles which are probably the etiological agents of erythroblastosis and lymphomatosis and of the feasibility of obtaining the particles in the state and amount needed for critical experimentation.

Summary. A particulate component, which may be the etiological virus, was isolated by ultracentrifugation of the blood plasma of chickens with erythroblastosis. The particles were of spheroidal shape and variable size, averaging approximately 102 mμ diameter. Micrographs of unshadowed preparations showed circular images of an appearance suggesting that the particles consisted predominantly of a watery, low-electron contrast, gel-like material surrounding a small relatively dense internal structure. The findings were compared with those obtained in the analogous studies of particles representing the virus of myeloblastosis and that of one form of lymphomatosis.

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Effects of Sodium Diphenylhydantoinate upon Isolated Small Intestine of the Rabbit. (21972)

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The effects of a number of drugs upon the rhythm of isolated rabbit intestine were studied. Among the drugs tested was sodium diphenyl-hydantoinate (Dilantin). This proved to have remarkable effects which, to our knowledge, have not been described previously.

Material and methods. The small intestine was rapidly removed from a freshly stunned rabbit. This was immediately placed into oxygenated Tyrode's solution. Lengths of duodenum were most generally employed, although jejunum and ileum were also used in some experiments. The unused portions were stored in cold Tyrode solution for later use. The length of the gut used was between 2 to 3 cm. One end of the gut was fixed by a silver hook which in turn was fastened to the walls of the chamber. The other end of the gut was led by a thread to a very sensitive myograph transducer. This in turn was connected to the amplifier of a Hoff-Geddes-Spencer Physiograph and the resultant recording was traced out by an ink-writing pen upon a moving strip of paper. The amplification factor for movement was about 200 at maximum gain. With the initial tension of the myograph spring overcome, a 200 mg weight produced a deflection of 7.5 mm at maximum gain.

The chamber enclosing the isolated gut had 3 openings at its base. The first of these allowed for the continuous entry of oxygen into the solution. This kept the solution well-oxygenated and also served to mix the solution and any added drugs. An opening at the lowest part of the chamber allowed for drainage at will and a third opening provided for the entry of fresh Tyrode solution of pH about 7.6. A further portal in the upper part of the chamber enabled one to add drugs to the solution. The bath was kept at any constant temperature between 35° and 38°C by means of a heat lamp which was shone upon a blackened portion of the chamber. The intensity of this heating was regulated by means of a variac.

Results. The addition of Dilantin in amounts sufficient to produce a resultant concentration of 2 to 8 mg% (0.07 to 0.29 mmol/l) in the solution resulted in a rapid decrease in the amplitude of the contractions and a fall in tone (Fig. 1, A and B). This effect was noted within 15 to 30 seconds and the contractions generally disappeared within one minute. The addition of smaller amounts of Dilantin resulted in a more gradual decrease in the tone and in the amplitude of the contractions. A concentration of 0.5 mg % (0.018 mmol/l) was sufficient to reduce considerably the amplitude of contraction of the gut (Fig. 1, C). With the smaller concentrations of Dilantin, the gut contractions would become reduced in