Identification of Avian Erythroblastosis Virus by Precipitation with Chicken Immune Serum.* (22610)

ROBERT A. BONAR, D. GORDON SHARP, DOROTHY BEARD, AND J. W. BEARD.
Department of Surgery, Duke University School of Medicine, Durham, N. C.

Particles characteristic of avian erythroblastic leukemia(1) have been observed(2) in electron micrographs of the plasma of chicks with the disease. Morphologically, the particles were similar in shape and size to those occurring under like conditions in myeloblastosis, which is another form of avian leukemia. In the latter instance, the particles have been identified(3,4) as the viral agent of the disease. The findings already described (2) have suggested that the particles found in erythroblastosis might represent the etiologic agent. One means for establishing the identity of the virus of myeloblastosis consisted in the electron micrographic study(4) of the precipitin reaction with specific antiserums from the chicken. A similar technic has been applied in erythroblastosis. Chickens repeatedly injected with formalin-treated particles concentrated by ultracentrifugation of large volumes of plasma, develop antibodies strongly neutralizing the infectivity of the virus. Electron micrographic examination of mixtures of the antiserums with particle concentrates revealed specific clumping of the characteristic particles. The results of the precipitin studies, which establish the serological identification of the particles with the virus of erythroblastic leukosis, are here described.

Materials and methods. The disease under study is an apparently pure strain of erythroblastosis obtained(5) from Dr. Astrid Fagraeus (State Bact. Lab., Stockholm), but derived initially in the laboratory of Dr. J. Engelbreth-Holm (Dept. of Pathol. Anatomy, University of Copenhagen). It has been passed routinely in this laboratory in White Leghorn chicks and chickens by intravenous inoculations of 0.1-ml volumes of *filtered* plasma or dilutions of it. The birds were always White Leghorns of Line 15 inbred at the Regional Poultry Research Lab., East Lansing, Mich., for high susceptibility to lymphomatosis(6). The characteristics of the disease have not changed since the earlier description(5).

Particle concentrates for preparation of immunizing antigen were obtained by ultracentrifugation of plasma from diseased birds. Electron micrographic examination of many plasmas has shown that the particle content in erythroblastosis reaches a maximum of approximately 2×10^{10} per ml, in striking contrast with myeloblastosis in which particle counts reach levels of 2×10^{12} virus particles per ml. There is wide variation in the particle content in erythroblastosis, some of the plasmas containing practically none demonstrable by physical methods. Moreover, as already indicated(5), there was no means for selecting the best plasmas since the adenosine triphosphatase micro screening test(7) useful in myeloblastosis was not applicable in erythroblastosis. For these reasons, donors of the plasmas had to be chosen at random, and because of the low average particle content, large volumes of plasma were necessary to provide the mass of particles sufficient to serve as effective antigen. Fortunately, older birds are more susceptible(5,8) to this virus than the baby chicks required for myeloblastosis (9), and consequently, volumes of plasma adequate for this purpose were readily obtained. Birds of 2 to 20 weeks of age with the disease were bled from the heart shortly before death was expected. Heparin was used as anticoagulant. Cells and large particles were removed by centrifugation once at 850 x g for 15 minutes, and a second time at 2700 x g for 20 minutes. The supernatant fluids were then spun at 20,000 x g for 60 minutes

* This work was supported by a research grant to Duke University from the American Cancer Society, on recommendation of Committee on Growth; by grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; and by Dorothy Beard Research Fund.

to sediment the particles which were resuspended in Ca-free Ringer's solution at about 25 x concentration. Formalin in Ca-free Ringer's solution was added in amounts sufficient to bring the suspensions to 10-20 times concentration of particles with respect to the original volumes of plasma and 0.1% concentration of formalin. The suspensions were stored in the refrigerator for about 3 weeks. The vaccine was prepared in batches, at intervals, from a total of 2,125 ml of plasma derived from 198 chickens. This whole amount of vaccine was used for immunization of 8 roosters (Line 15 White Leghorns) of 6.5 months age at the time of the first injection. Each was given 1.0 ml vaccine intraperitoneally twice on alternate days. After 1 week, a series of 3 injections of 2.0 ml was made on each of alternate days. The third course of 2.0, 2.5 and 2.5 ml was given in the same way after an ensuing week. There was no evidence of disease in the vaccinated birds. All were bled from the heart 11 days after the last injection, and the serum was stored at —22°C.

Precipitin experiments were carried out with particle concentrates similar to those used for the immunizing antigen. After removal of the cells by centrifugation, the plasmas were stored at —78°C until used, then thawed in running tap water and chilled in an ice bath. Preparation of the particle concentrates was carried out at 2-5°C. In a typical preparation, 4 plasmas, 101 ml, were centrifuged 20 minutes at 2,100 x g, and the supernatant fluids spun 30 minutes at 29,000 x g in the Servall SS-1 rotor to sediment the particles. The small, yellowish clear pellets were resuspended and combined in a total volume of 12 ml of 0.14 NaCl containing 0.001 M sodium phosphate, pH 7.0. The cloudy suspension was stirred 2 hours and then centrifuged 20 minutes at 2,500 x g. The supernatant fluid was decanted from the white opaque pellet and centrifuged 30 minutes at 20,000 x g in an airdriven vacuum chamber centrifuge. The supernatant fluid was decanted and the pellet resuspended in 1.0 ml sodium chloride phosphate solution as before. The pellet suspension was stirred overnight and then clarified by centrifugation at 2,500 x g for 5 and then for 15 minutes.

The immune serum and the serum from a normal (uninfected) bird from the same flock were heated 30 minutes at 56°C to inactivate complement; after cooling, the serums were centrifuged 30 minutes at 47,000 x g in a small horizontal cell to eliminate particulate material, and the upper two-thirds of the supernatant taken for use. For the precipitin reaction, 1 part particle concentrate, 1 part serum and 1 part NaCl solution were mixed so that the final concentration of sodium chloride was 7.5%(10). Whether the particle concentrate and serum were first dialysed against 7.5% NaCl or simply mixed with more concentrated NaCl solution appeared not to affect the results. The mixtures were kept at 37°C for 2 hours, and then at 2°C. Samples were removed at intervals and diluted 333 fold with 0.9, 4 or 8% sodium chloride solution for electron micrography. The particles in these dilute samples were then deposited by ultracentrifugation upon an agar surface where their numbers and configuration would not be influenced during drying. They were then fixed with osmium tetroxide and pseudoreplicas prepared by the method used previously(4,11).

Results. The findings are illustrated in the electron micrographs of Fig. 1-3. As already described(2), concentrates approaching homogeneity with respect to the kind of particles suspected of being the virus are difficult to obtain. Although the predominant component may clearly represent a population of particles of fairly uniform appearance, there is always present some or much amorphous material of indeterminate characters as well as many particles smaller than those characteristic of erythroblastosis. These properties are exhibited by the concentrate of Fig. 1 which represents one of the best thus far obtained.

Treatment of such a concentrate with the immune serum as described results regularly in agglomeration of particles as seen in Fig. 2. The concentrate was distinctly opalescent before addition of the immune serum. Visible precipitation, that is, the formation of flocs and settling, did not occur when the serum was added, but the suspension was definitely

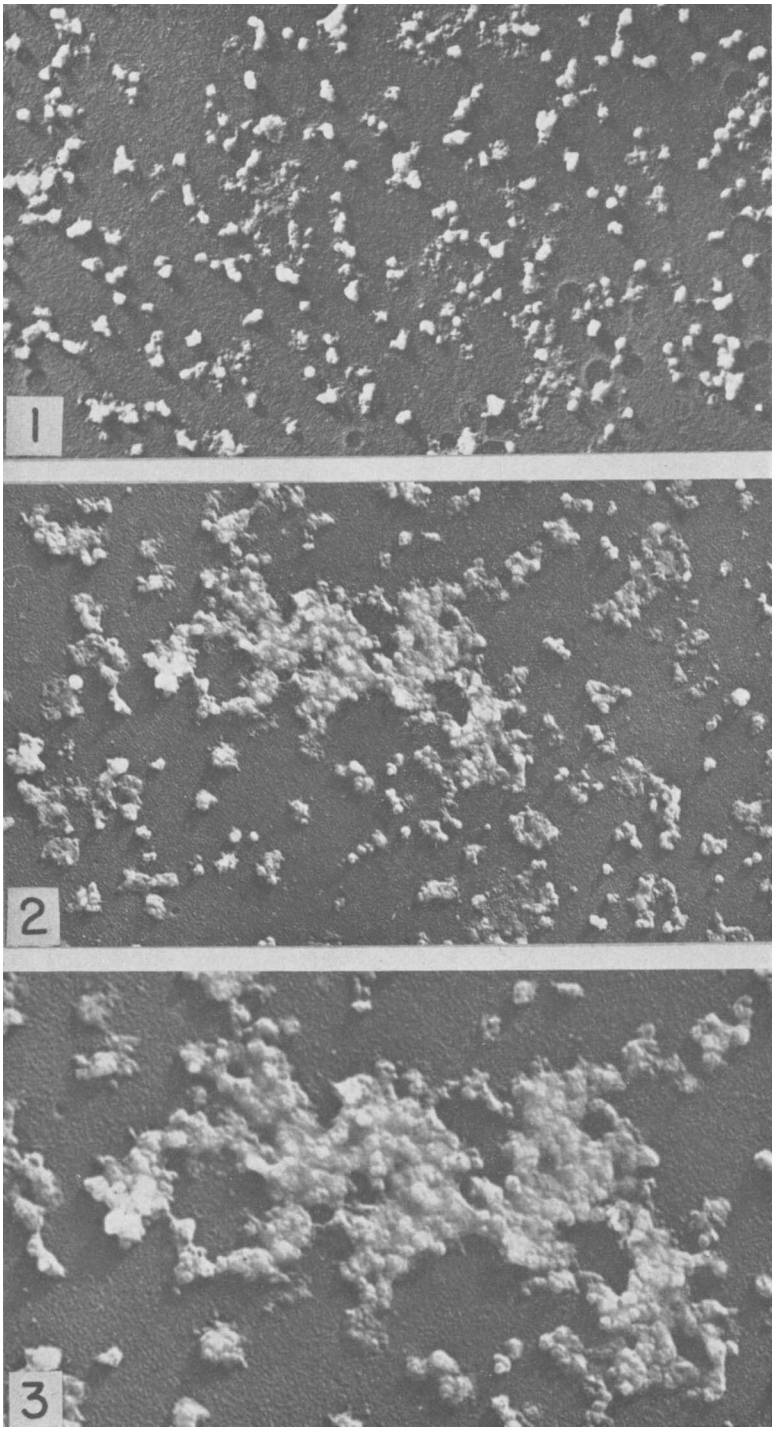

FIG. 1. Electron micrograph of concentrate of particles present in the blood plasma of chickens with avian erythroblastosis (× 15,100).

FIG. 2. Concentrate treated with immune serum for 2 hr at 37°C and for 18 hr at 2-5°C. Concentration of NaCl was 7.5% (× 15,100).

FIG. 3. A portion of Fig. 2 at higher magnification (× 25,000).

more turbid than mixtures of the same particle concentration containing serum from normal chickens. The electron micrograph of Fig. 2 reveals unequivocal agglomeration which did not occur in the untreated concentrates or in concentrates treated with normal serum. The nature of the particles included in these small flocs is shown in the picture of greater magnification in Fig. 3. It is evident that the particles involved are those of relatively uniform appearance described earlier (*2*) as characteristic of avian erythroblastosis. There remain in suspension particles of the same appearance which did not form flocs with the immune serum.

Discussion. Repeated experiments have shown, as will be described later, that the chicken immune serum employed in the present work contains antiviral immune bodies which neutralize the agent of erythroblastosis. Antibodies to this agent, which were produced by relatively small amounts of antigen inactivated with formalin, were less effective than were analogous antibodies to myeloblastosis against the homologous virus. This constitutes, probably, the principal explanation of the partial precipitation in erythroblastosis as compared with the results of similar experiments in which the virus of myeloblastosis could be precipitated quantitatively(4) with its specific avian antibodies. Incomplete precipitation is observed, also, with myeloblastosis with the use of dilute immune serum, the results being essentially identical with those found here in erythroblastosis in which there is excellent reason to suspect that the immune serum was much "weaker" than that obtained with myeloblastosis. It is possible that some of the particles had lost the capacity to react with the immune serum because of antigenic degradation either in the blood plasma or during preparation of the concentrates. This seems rather remote, however, since the agent has appeared fully as stable as the virus of myeloblastosis. In Fig. 2, the presence of individual particles in the smaller size range is notable. Possible relations of the smaller particles to the specific agent are obscure. Many are present in the plasmas of birds with erythroblastosis. Correlative sedimentation studies and particle counts, however, have shown that infectivity is associated principally with particles of approximately 120 mμ diameter. With respect to the present experiments it would appear that, insofar as the precipitin reaction with homologous antiviral immune serum from the homologous host is an acceptable criterion, it can be concluded that the particles characteristic of the disease, as described before and seen in the present pictures, are the virus of avian erythroblastosis.

The results of the present experiments are of significance in several respects. (*1*) They have demonstrated that in avian erythroblastosis specific precipitating (and neutralizing) antibodies can be produced in the homologous host with sufficient quantities of formolized antigen. (*2*) Electron micrographic study of the precipitin reaction provides a specific serological technic for the identification of particles existing in concentrations and amounts too small for adequate study by the usual physical and chemical criteria. (*3*) It is evident that the virus particles of erythroblastosis are indistinguishable by electron micrography from those of the virus of myeloblastosis. This is of critical importance since it has been shown in experiments to be reported later that the agents are distinct chemical and biological entities.

Summary. Spheroidal particles of about 100-120 mμ diameter occur characteristically in the plasma of chickens with erythroblastosis. With formalin-inactivated concentrates of these particles, immune serums have been produced in the chicken which effectively neutralize the infectivity of the agent. These antiserums also cause agglomeration of particles in concentrates identifiable by electron micrography as those found characteristically in this disease. The results have been interpreted to indicate that the characteristic particles agglomerated constitute the viral agent of avian erythroblastosis.

1. Furth, J., *Arch. Path.*, 1931, v12, 1.

2. Sharp, D. G., Beard, D., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 168.

3. Eckert, E. A., Sharp, D. G., Mommaerts, E. B., Reeve, R. H., Beard, D., and Beard, J. W., *J. Nat. Can. Inst.*, 1954, v14, 1039.

4. Eckert, E. A., Sharp, D. G., Beard, D., Green, I.,

and Beard, J. W., *ibid.*, 1955, v16, 593.

5. Eckert, E. A., Beard, D., and Beard, J. W., *ibid.*, 1956, v16, 1099.

6. Waters, N. F., *Poultry Sci.*, 1951, v30, 531-545.

7. Green, I., Beard, D., Eckert, E. A., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1954, v84, 406.

8. Beaudreau, G. S., Bonar, R. A., Beard, D., and Beard, J. W., *J. Nat. Can. Inst.*, 1956, v17, 91.

9. Eckert, E. A., Beard, D., and Beard, J. W., *ibid.*, 1955, v15, 1195-1207.

10. Goodman, M., Wolk, H. R., and Norton, S., *J. Immunol.*, 1951, v66, 225-236.

11. Sharp, D. G., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 75-79.

Received July 2, 1956. P.S.E.B.M., 1956, v92.

Comparison of Effect of 'Valmid'* and Other Hypnotics on Brain Respiration *in vitro*. (22611)

RALMOND H. TUST, ROY G. HERRMANN, AND K. K. CHEN.

The Lilly Research Laboratories, Indianapolis, Ind.

It has been reported that hypnotics in relatively low concentrations are capable of inhibiting the respiration of cerebral slices during electrical stimulation(1), stimulation due to an excess of potassium or to the addition of 2. 4-dinitrophenol(1,2), and stimulation by the removal of calcium ions(3). Ghosh and Quastel(2) interpreted their data as indicating that the inhibition of brain slice respiration by narcotics is restricted to that aspect of cortical respiration which is stimulated by potassium ions.

This study was undertaken to compare the ability of 'Valmid', a new nonbarbituric hypnotic(4,5,6), to inhibit the potassium-stimulated respiration of slices of brain cortex with that of 'Seconal Sodium'† and that of another nonbarbiturate, urethane. Included in this study was a convulsant barbiturate (sodium ethyl, 1, 3-dimethylbutyl barbiturate), (7) serial No. 09757, which has been shown to be capable of uncoupling oxidation from phosphorylation(9).

Method. All experiments were carried out with the Warburg manometric apparatus. Female albino rats of 150-200 g were sacrificed by decapitation, the brains were quickly removed, and freehand slices were prepared by the method of Deutsch(10). Slices totaling approximately 12 mg dry weight were placed into 2.4 ml Krebs-Ringer phosphate medium containing 0.02 M glucose in the main compartments of the vessel. The hypnotic agent, dissolved in 0.3 ml of 25% polyethylene glycol 200 (PEG 200) made up in Krebs-Ringer phosphate, was added to the vessel contents to give final concentrations equivalent to the anesthetic dose (AD_{50}) or 10 times the AD_{50} of each compound, and a final concentration of 2.5% PEG. The concentration of No. 09757 was based upon its convulsive dose (CD_{50}). A control flask was prepared to contain 2.5% PEG 200. PEG 200 was necessary to keep 'Valmid' in solution throughout the experimental period. The sidearm of each vessel contained 0.3 ml M KCl in Krebs-Ringer phosphate. The center wells contained 0.2 ml 10% KOH. Oxygen was used for the gas phase.

After gassing and thermal equilibration, oxygen uptake was measured for one hour to establish control values. At the end of this period the sidearm contents were tipped into the main compartments and oxygen uptake was measured for another hour. Control experiments showed no difference between first and second hour Qo_2 values. Readings were taken at 15 minute intervals throughout the experiment.

Results. All values in Table I represent the mean values of 8 experiments. Inhibitions were calculated as percentage inhibi-

* 'Valmid' (Ethinamate, Lilly) is ethinylcyclohexyl carbamic acid.

† 'Seconal Sodium' (Secobarbital Sodium, Lilly) is sodium allyl, 1-methylbutyl barbiturate.