Pathological Response of the Chicken Embryo to an Agent Which Causes Acute Leukosis (Marek's Disease)\(^1\)

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A laboratory test system specific for Marek's disease was developed by using the pathological response of the chicken embryo. Chicken epidermal scales (dander) and feather calami from infected chickens contain an agent(s) which after a 3- to 4-day incubation period caused gross or microscopic pathological changes (or both) in the embryo. A cell-free inoculum was obtained from infectious dander by 5-min sonic treatment, differential centrifugation, and membrane filtering (0.45 \(\mu\)m). Evidence for the cell-free existence of this agent(s) was obtained when membrane filtrates of dander preparations were shown to cause Marek's disease in 10-day-old chickens and in chickens inoculated at 1 day of age.

Transmission of acute leukosis (Marek's disease, MD) was first achieved experimentally by inoculation of suspensions of infective nerves into susceptible chickens (12, 14). Although clear differentiation between MD and the myeloid, erythroid, and lymphoid forms of leukosis was not made, transmission of these diseases was not consistently achieved when infective blood was passed through Seitz and Berkefeld N filters and injected into the yolk sac of 1-day-old chicks. Subsequently, blood plasma, tumor suspensions, and peripheral nerves (4) have all been successfully used in the experimental transmission of this disease.

Conflicting results concerning the filterability of the MD agent have been reported (3, 13, 16, 18). Cell-free tumor extracts, media from infected tissue cultures, and infective plasma were used in these studies.

Transmission of MD by cell-free inoculum was first achieved on a repeatable basis when infected chicken epidermal cells (dander) were subjected to 30- and 45-sec sonic treatment and injected into day-old chicks (2). Subsequently, virus antigens in the epithelium of feather follicles of MD-infected chickens have been localized by fluorescent-antibody technique (6).

In other work (9), tissue culture filtrates [filtered through 0.45- and 0.25-\(\mu\)m membrane filters (Millipore Corp., Bedford, Mass.)] induced a disease similar to MD when inoculated into chicks. This agent did not, however, resemble the virus previously described in the etiology of MD.

Chick embryo inoculations of suspensions of infected embryo tissue, filtrates of embryo tissue, and serum of leukotic chicks gave evidence of producing leukosis in the embryo (11). Transmission of MD has been achieved by inoculation of embryonated eggs with citrated blood, spleen, thymus, or tumor cells from chicks infected with MD (19). In other work (5), infective whole blood inoculated intravenously in 10- or 17-day embryos produced MD in the hatched chicks after an incubation period of the same order as that for chicks treated at 1 day of age.

Allantoic fluid collected from embryos which had been inoculated with heparinized whole blood from birds with MD was responsible for production of MD when injected intracerebrally into 4- to 10-day-old chicks (18). Lyophilization of this infective allantoic fluid destroyed its infectivity.

In the present study, attempts were made to show that the chicken embryo can be used as a laboratory test system for detection of MD, that the agent which produces the pathological effects in the embryo is filterable, and finally that this agent is present in the epidermal tissues of chickens which have MD.

MATERIALS AND METHODS

Source of infectious inoculum. Infective material consisted of epidermal cells (dander) and feather calami obtained from paralyzed birds with MD.
Infective whole blood was obtained from paralyzed birds by cardiac puncture. Control inocula consisted of the suspension components, without infectious dander or calami, and noninfected whole blood. Donors of the Athens-Canadian strain of chickens which had been inoculated intra-abdominally with infective blood at 1 day of age. Birds were kept in Horsfall-Bauer isolators throughout the experimental period.

**Preparation of the inoculum.** A flow chart of the procedure is presented in Fig. 1. After collection, dander and calami were suspended in approximately a 1:10 (v/v) dilution in Hanks balanced salt solution (HBSS) to which nystatin (1,000 units/cc), penicillin G potassium (5,000 units/cc), and streptomycin (15 mg/cc) had been added. Dander and feather calami suspensions were then sonically treated for 45 sec or 5 min (intensity setting of 8) in a Sonifier (Branson Instruments, Inc.). The suspensions were then filtered through filter paper (Whatman no. 1) to remove all large debris, and the filtrates were centrifuged for 15 min at 7,000 × g (4 C).

The supernatant from the first centrifugation cycle was equally divided; one-half was filtered through a 0.45-µm membrane filter (Millipore Corp.), and the remaining half was unfiltered. The filtrate supernatants were then centrifuged for 60 min at 200,000 × g (4 C). The pellets were then suspended in 10 cc of HBSS for injection into embryonated eggs.

**Inoculation of 10-day embryonated eggs.** Three strains of white leghorn 10-day embryonated eggs were used in the experiments. They were inoculated via the allantoic cavity with 0.2 cc of the resuspended pellets. In the blood transmission experiments, the same inoculation procedure was followed.

All inoculated eggs were checked daily for mortality and all dead embryos were examined for lesions.

**Preparation of embryo tissue.** White leghorn chickens (Athens Canadian strain) were inoculated intraperitoneally at 1 day of age with infected embryo tissue preparations. Samples of embryo tissue which were injected into day-old chickens were tested for bacterial contamination. Brain Heart Infusion slants were inoculated with 0.2 cc of homogenized liver and spleen or chorioallantoic membranes from both infected and control embryos. No bacterial growth resulted from this inoculation of the slants with any of the control or infected tissue. Chickens were then put in Horsfall-Bauer type isolators. The isolators were not opened from the time of injection until the termination of the experiment (6 weeks).

**Tissue preparation for microscopic examination.** All tissue prepared for microscopic examination was fixed in 10% buffered Formalin, embedded in paraffin, processed overnight, sectioned at 6 µm, and stained with hematoxylin eosin.

### Table 1. Embryo response to dander and feather calami preparations

| Inoculum         | Treatment                          | Total lesions cumulative | Per cent lesions |
|------------------|------------------------------------|--------------------------|------------------|
| Dander Group A   | 45-sec sonic treatment             |                          |                  |
| Control          |                                    | 0/10                     |                  |
| 7,000 × g pellet |                                    | 3/32                     | 9.4              |
| Control          |                                    | 0/10                     |                  |
| 200,000 × g pellet |                                  | 10/32*                   | 31.3             |
| Group B          | 5-min sonic treatment              |                          |                  |
| Control          |                                    | 0/40                     |                  |
| 7,000 × g pellet |                                    | 65/124*                  | 52.4             |
| Control          |                                    | 0/70                     |                  |
| 200,000 × g pellet |                                  | 108/195*                 | 55.3             |
| Membrane filtered; 200,000 × g pellet | | 52/123* | 42.2 |
| Feather calami   | 5-min sonic treatment              |                          |                  |
| Control          |                                    | 0/20                     |                  |
| 200,000 × g pellet |                                  | 27/57*                   | 47.3             |

* Significantly different from control at P ≤ 0.05.
RESULTS

Embryo response. Chicken epidermal scales (dander) subjected to sonic oscillation for 5 min followed by subsequent differential centrifugation (7,000 and 200,000 × g) resulted in the preparation of an infectious MD agent as observed by intra-allantoic inoculation into 10-day embryonated eggs (Table 1). Additional purification of this material by filtration [0.45-μm membrane filter (Millipore Corp.)] resulted in a slight but insignificant loss in infectivity as assayed by the embryo response (52 to 55 % lesions, compared to 42% for the membrane-filtered inoculum). When infective whole blood from paralyzed chickens was injected into embryos and when infective embryo tissue was serially passed in the embryo (Table 2), a similar pathological response was observed in the embryo. One group of embryos inoculated with infective blood was allowed to hatch in Horsfall-Bauer type isolators with resultant development of MD (appearance of gross tumors) after approximately 6 weeks.

Gross lesions. The gross pathological responses of the chicken embryo to the MD agent were typically characterized by the appearance of small foci to gradually converging perilobular lesions with hepatomegaly (Fig. 2), small granular appearing white foci on the surface of the spleen with splenomegaly and liver discolorations. Occurring less frequently were diffuse, white, granular regions on the surface of the kidney. Other typical lesions were thickenings of the chorioallantoic membrane with the appearance of small, white, granular clumps on the membrane surface.

Microscopic appearance. Areas of focal necrosis occurred in the livers of many infected embryos.

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Table 2. Embryo response to infective whole blood and embryo tissue inoculum

| Inoculum                        | Total lesions | Per cent lesions |
|---------------------------------|---------------|------------------|
| Whole blood                     |               |                  |
| From control chickens           | 0/34          |                  |
| From paralyzed chickens         | 54/84<sup>a</sup> | 64.2             |
| Embryo tissue                   |               |                  |
| Control (allantoic fluid)       | 0/15          |                  |
| First passage, allantoic fluid  | 30/35<sup>a</sup> | 85.7             |
| Control (tissue extracts)       | 0/45          |                  |
| Second passage, liver and spleen extracts | 56/122<sup>a</sup> | 45.9            |

<sup>a</sup> Significantly different from control at P ≤ 0.05.

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FIG. 2. Typical appearance of infected embryo (left) compared with control. Most prominent features are decreased size and perilobular liver lesions of infected embryo.
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Although no inflammatory reactions were associated with these areas, in some instances proliferation of lymphocytes and granulocytes occurred in the periportal areas. Splenic enlargement due to reticuloendothelial cell hyperplasia was observed.

Chorioallantoic membranes were edematous and thickened and contained areas of focal hematopoiesis (Fig. 3). These foci were composed of lymphocytes, granulocytes, and red blood cell precursors. Many cells were in the process of mitosis. Occasional proliferation of membrane epithelium was also noted.

Size differential of infected and control embryos. Many infected embryos were considerably smaller in size than the controls (Fig. 4). This size differential was manifested by a significant decrease ($P = 0.001$) in head-to-tail length of infected embryos as compared to controls.

Transmission to 1-day-old chicks. Preparation of tissue from the embryos which were inoculated with the MD agent (in dander) were infective for 1-day-old chickens (Table 3). Positive confirmation for the presence of the MD agent in the infected embryos was obtained when some groups of the isolator birds developed the clinical signs of MD after approximately 6 weeks. In experiment I, only birds which had been injected with embryo tissue from the $7,000 \times g$ pelleted dander had gross lesions. In experiment II, birds which were injected with embryo tissue and membranes from the twice-centrifuged, membrane-filtered groups developed MD.
**Table 3. Transmission of Marek's disease by inoculation of day-old chicks with materials from experimentally infected embryos**

| Exp | Treatment              | Mortality | Gross lesions |
|-----|------------------------|-----------|---------------|
| I   | Control                | 0/29      | 0/29          |
| I   | 7,000 × g              | 1/14      | 5/14          |
| I   | 200,000 × g            | 0/28      | 0/28          |
| I   | 200,000 × g (membrane) | 0/30      | 0/30          |
| II  | Control                | 1/12      | 0/12          |
| II  | 7,000 × g              | 0/19      | 4/19          |
| II  | 200,000 × g            | 0/23      | 0/23          |
| II  | 200,000 × g (membrane) | 0/29      | 19/29         |

**DISCUSSION**

Useful laboratory diagnostic techniques for the detection of MD have not been adequately developed. Measurements of antibody activity against various MD antigens have provided inconsistent evaluations of the disease state. Indirect hemagglutination of antigen prepared from duck embryo fibroblast tissue cultures with tanned horse erythrocytes (10) has suggested a relationship between antibody titer and recovery of birds infected with MD. Others (7) have indicated that chickens infected with MD have antibodies with specific precipitins to tissue culture-produced antigens. According to one study, the presence of maternal antibodies as assayed by reaction with tissue culture antigens was associated with decreased morbidity and mortality (8). Passive transfer of immune serum resulted in only a small decrease in mortality.

The specificity of these tests for the agent causing MD has not been demonstrated. At present, injection of day-old chickens with infectious material and subsequent postmortem examination for gross tumors after a 4- to 6-week incubation period provide the most accurate means of detection of the agent.

Previous studies (5, 11, 18) have shown that infective whole blood and embryo tissue could be used to infect embryos with MD. A yolk sac test (19) was developed in which 4-day embryonated eggs were inoculated with infectious material via the yolk sac, and pock-like foci were observed from the 11th day after inoculation. Attempts to transmit the infection to 1-day-old chicks from the infected embryos were successful. The reactions in the yolk sac test were dependent on the source of the embryonated eggs and the age of the embryos inoculated. In the present study, the pathological response of the embryo to the infectious agent occurred after an incubation period of 3 to 4 days postinoculation. This response was independent of breed (no graft versus host reaction); because the results of infectivity could be scored after a short latent period, the requirement of allowing the infected embryo to hatch and subsequently develop the MD after a 6-week incubation period was abolished.

The chicken embryo has been more routinely used as a growth medium for many viruses (1, 15, 17) than as a test system for oncogenic viruses. The changes, however, elicited by this MD agent provided several criteria by which to judge the reliability of this technique.

Macroscopically, the combination of reduced embryo size, thickenings of the chorioallantoic membrane, and liver and spleen lesions along with the microscopic appearance of these tissues provided a repeatable means of MD diagnosis. Demonstration that these infected embryo tissues contained the MD agent was provided by passage of this tissue to day-old chickens which subsequently developed MD.

Certain physical characteristics of the MD agent were determined in the process of preparing various infectious inocula. The agent demonstrated high resistance to extended periods of sonic oscillation. Five-minute sonic treatment released a significantly greater quantity of infectious material from dander, as assayed by the embryo response, than did 45-sec sonic treatment. Evidence for the cell-free existence of this agent was obtained when membrane filtrates of dander preparations were shown to cause MD in embryos and in 6-week-old chickens.

The pathological responses of the chicken embryo to other agents such as Newcastle disease virus and encephalomyelitis virus (18), reticuloendotheliosis virus (T-virus) (16), mycoplasma (PPLO), and various bacteria have demonstrated only a small degree of specificity, none of which could be confused with those lesions produced by MD. The ribonucleic acid-containing leukemia viruses are normally found in the embryo (maternal transfer; reference 16) but cause no gross or microscopic lesions when experimentally passed in the embryo.

Compared with these agents, the response of the embryo to MD is specific when response parameters are evaluated in terms of both gross and microscopic lesions. Also, the embryos could be examined as soon as 3 to 4 days postinoculation for these changes.

Thus, utilizing the pathological response of the chicken embryo as a model for determination of the presence of the MD agent, it has been shown that chicken epidermal scales (dander) contain a agent which cause MD. These scales are constantly being shed by the chicken and represent a
potentially important means of natural transmission of the disease. It has been shown previously that sonically treated infectious dander produces MD when injected into day-old chicks (2) and that virus-related antigens are present in feather follicles of infected chickens (6).

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LITERATURE CITED

1. Ackermann, N. W., and T. Francis. 1950. Experimental transmission of avian leucosis. Proc. Soc. Exp. Biol. Med. 74:123-126.
2. Beasley, J. N., L. T. Patterson, and D. H. McWade. 1970. Transmission of Marek's disease by poultry house dust and chicken dander. Amer. J. Vet. Res. 31:339-344.
3. Biggs, P. M. 1968. Marek's disease—current state of knowledge. Curr. Top. Microbiol. 43:110-125.
4. Biggs, P. M., and L. N. Payne. 1967. Studies on Marek's disease. I. Experimental transmission. J. Nat. Cancer Inst. 39:237-280.
5. Biggs, P. M., and L. N. Payne. 1967. Studies on Marek's disease. II. Pathogenesis. J. Nat. Cancer Inst. 39:281-302.
6. Calnek, B. W., and S. B. Hitchner. 1969. Localization of viral antigen in chickens infected with Marek's disease herpesvirus. J. Nat. Cancer Inst. 44:935-949.
7. Chubb, R. C., and A. E. Churchill. 1968. Precipitating antibodies associated with Marek's disease. Vet. Rec. 93:4-7.
8. Chubb, R. C., and A. E. Churchill. 1969. Effect of maternal antibody on Marek's disease. Vet. Rec. 92:303-305.
9. Cook, M. K. 1969. Cultivation of a filterable agent associated with Marek's disease. J. Nat. Cancer Inst. 43:203-212.
10. Eison, C. S., and S. C. Schmitte. 1969. Studies on acute Marek's disease. XII. Detection of antibodies with a tannic acid indirect hemagglutination test. Avian Dis. 13:774-782.
11. Hall, W. J., C. W. Bean, and M. Pollard. 1941. Transmission of fowl leucosis through chick embryos and young chicks. Amer. J. Vet. Res. 1:2272-279.
12. Johnson, E. P. 1934. The etiology and histogenesis of leucosis and lymphomatosis of fowls. Vir. Agr. Exp. Sta. Tech. Bull. 56:3-32.
13. Nazerian, K., J. J. Solomon, R. L. Witter, and B. R. Burmes-ter. 1968. Studies on the etiology of Marek's disease. II. Finding a herpes-virus in cell culture. Proc. Soc. Exp. Biol. Med. 127:177-182.
14. Pappenheimer, A. M., L. C. Dunn, and V. Cone. 1926. A study of fowl paralysis (neurolymphomatosis gallinorum). Storrs Agr. Exp. Sta. Bull. 143:186-290.
15. Ross, P., and J. B. Murphy. 1911. Tumor transplantation in the developing embryo. Experiments with a transmissible sarcoma of the fowl. J. Amer. Med. Ass. 56:741-742.
16. Sevolan, M., D. M. Chamberlain, and F. Counter. 1962. Avian lymphomatosis, experimental reproduction of the neural and visceral forms. Vet. Med. 57:500-508.
17. Sevolan, M., R. N. Larose, and D. M. Chamberlain. 1964. Avian lymphomatosis. VIII. Pathological response of the chicken embryo to TV virus. Nat. Cancer Inst. Mono. 17:99-119.
18. Vindel, J. A. 1964. Cytochemistry of neurolymphomatosis virus reproduction in vitro. Nat. Cancer Inst. Mono. 17:147-157.
19. Von Bulow, V. 1969. Mareksche huhnerlahmung: reaktionen im experimentell infizierten embryonierten ei. Zentralbl. Veterinarmed. Reihe B 16:97-114.