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## Studies on the Etiology of Marek's Disease. I. Propagation of the Agent in Cell Culture (32649)

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Marek's disease (MD) is a highly contagious, lymphoproliferative disease causing severe mortality and economic loss in commercial chicken flocks. Affected birds often have lymphoid tumors in the nerves, skin, muscle, or visceral organs. The etiological agent is highly infectious but its nature is uncertain. Although the agent may be detected by inoculation of susceptible chicks, this technic is difficult and time consuming. A rapid and sensitive *in vitro* assay would facilitate research on many aspects of this disease.

Considerable effort has been directed toward the development of methods for *in vitro* propagation and assay. Biggs and Payne (1) failed to detect infectivity in chick embryo fibroblast cultures following inoculation with the B14 strain of MD. Vindel (2) isolated a filtrable, cytopathogenic agent from chickens with neurolymphomatosis but its relationship to the MD agent was not established. In recent studies (Witter *et al.* (3); B. W. Calnek, personal communication), monolayer cultures prepared from normal chick embryo bone marrow and other tissues were infectious for several weeks following treatment with cellular MD inocula. However, evidence for replication of agent in these *in vitro* systems was inconclusive and no morphological alterations were observed in infected cultures.

Kottaridis and Luginbuhl (personal communication) have observed a cytopathic effect (CPE) in chick embryo fibroblast cultures inoculated with bone marrow from birds with MD and these cultures reproduced MD when inoculated into chickens. We have just learned that Churchill and Biggs (4) have isolated a herpes-like virus from chick kidney cells inoculated with MD tumor cell suspensions that produced a CPE characterized by the presence of Cowdry Type A intranuclear inclusion bodies. Chicks inoculated with cultures showing typical CPE developed MD.

This report describes the propagation in duck embryo fibroblast (DEF) cultures of a cell associated, cytopathogenic agent derived from the JM strain of MD. Evidence presented here and in another report (5) suggests that this agent is the etiological agent of Marek's disease.

*Materials and Methods. Marek's disease agent.* Blood collected in heparin (20 units per ml) from chickens inoculated with the JM strain (6) of MD was used for inoculation of cell cultures. Propagation of the JM strain in chickens at this laboratory has been described previously (7).

*Duck embryo fibroblast cultures.* Fibroblasts were prepared by the trypsinization of decapitated 12 to 13-day-old duck embryos derived from flocks in Michigan and New York. Cells were diluted in growth medium, transferred to 100-mm petri dishes and incubated at 38–40°C in a humidified atmosphere containing 3–5% carbon dioxide. The growth medium consisted of medium 199[2] and nutrient medium F10[2] in a ratio of 4:5 plus 5% tryptose phosphate broth, 0.084% sodium bicarbonate and 4–6% calf or bovine fetal se-

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rum. Penicillin, streptomycin, and mycostatin at 100 units, 100 $\mu$g, and 25 units, respectively, per ml were used. Primary cells were subcultured by dispersment with 0.05% trypsin in phosphate buffered saline, centrifugation at about 400*g* for 5 min, resuspension of cells with growth medium and plating at 0.5 to 3.0 $\times$ $10^6$ cells per 100-mm petri dish. Cultures were inoculated with whole blood or supernatant fluid at least 1 day after subculture to permit attachment of the cells. Volume of inoculum per dish varied from 0.01 to 1.0 ml for blood and from 3.0 to 10.0 ml for fluids. After 18–24 hours, the cultures were washed with growth medium to remove excess blood cells. Cells were subcultured at 3–5 day intervals by the method described for primary cultures above. Morphology of unstained cultures was noted at each passage and coverslip preparations obtained from selected passages were stained with Giemsa.

*Marek's disease assay.* Cell cultures were assayed for MD agent by the intraabdominal inoculation of day-old 15 $\times$ 7 chicks. These chicks were progeny of Regional Poultry Laboratory inbred line 15 males and line 7 females and were highly susceptible to MD (3). Following trypsinization the cells were suspended in growth medium and the concentration was adjusted to give 5 $\times$ $10^5$ to $10^6$ cells per chick dose of 0.2 ml. Each lot of inoculated chicks plus appropriate controls was held in separate Horsfall-Bauer isolators for 4–6 weeks. Chickens dying within this period or killed at termination were necropsied and examined for gross lesions of MD.

*Results.* Certain DEF cultures inoculated with JM blood developed discrete areas of altered cells after several passages (Fig. 1). These areas consisted of rounded or shrunken, spindle cells which were deeply basophilic and were piled up to varying degrees (Figs. 2 and 3). The affected cells appeared degenerate and were considered to represent a cytopathic effect, although extensive cellular destruction was not observed. Occasionally in older cultures, the CPE became more diffuse (Fig. 4). No inclusions were seen, but the dense staining of the altered cells with Giemsa precluded detailed cytologic examination.

This CPE was first observed 11–25 days following inoculation with JM blood. Although similar changes were present in further subcultures, the number of focal alterations per culture remained constant or increased only slightly. The CPE was not observed in every inoculated culture. In trials involving 17 different JM blood inocula and 30 recipient cultures, 7 inocula induced CPE in a total of 9 cultures. The CPE was not observed in DEF cultures inoculated with normal blood in one trial with 4 replicate cultures or in 12 uninoculated control cultures observed through periods of 44–79 days.

Cultures with CPE consistently reproduced lesions of MD when inoculated into chicks. Each of 10 inoculated DEF cultures with CPE produced MD characterized by a rapid onset of mortality and a high incidence of gross lesions (Table I). The CPE was not observed in 8 inoculated and 8 uninoculated control cultures during periods that for 15 of these cultures exceeded 43 days. None of these morphologically normal cultures produced lesions of MD when inoculated into chicks. The lesion response of chicks inoculated with cultures showing CPE was greater or equal to the response obtained with undiluted JM blood in 15 $\times$ 7 chicks in other trials (3).

Supernatant fluids or cell extract from inoculated cultures with CPE induced a similar CPE when transferred to normal DEF cultures. Supernatant fluids (centrifuged twice at 1600*g* for 3 min) induced CPE in 5 of 7 trials while only 1 of 4 cultures receiving cell extract prepared by 2 cycles of freezing and thawing developed CPE. However, CPE was not induced with cell extract prepared by 3 cycles of freezing and thawing in 2 trials or with supernatant fluid passed through a 0.45 $\mu$ millipore filter in 2 other trials. Culture fluids in one trial or extracts of cultured cells with CPE in 2 trials were not infectious for chicks.

The CPE and MD infectivity have been maintained for long periods in DEF cultures. One culture maintained the CPE through 137 days (32 subcultures) and cells on the 83rd day (20th subculture) induced lesions of MD in 3 of 9 recipient chicks. Another culture was maintained by a combination of subculture and, on 3 occasions, transfer of fluids or cell extract to new DEF cultures. The CPE was present through a total of 197 days (53 sub-

cultures or passages) and cells obtained at the 182nd day (50th subculture) produced lesions of MD in each of 5 recipient chicks.

*Discussion.* The maintenance of MD agent in high concentration for up to 182 days in DEF cultures strongly suggests that the agent replicates *in vitro*. The MD agent in inoculated DEF cultures appeared cell associated since cells were infectious while fluids and cell extracts from such cultures produced

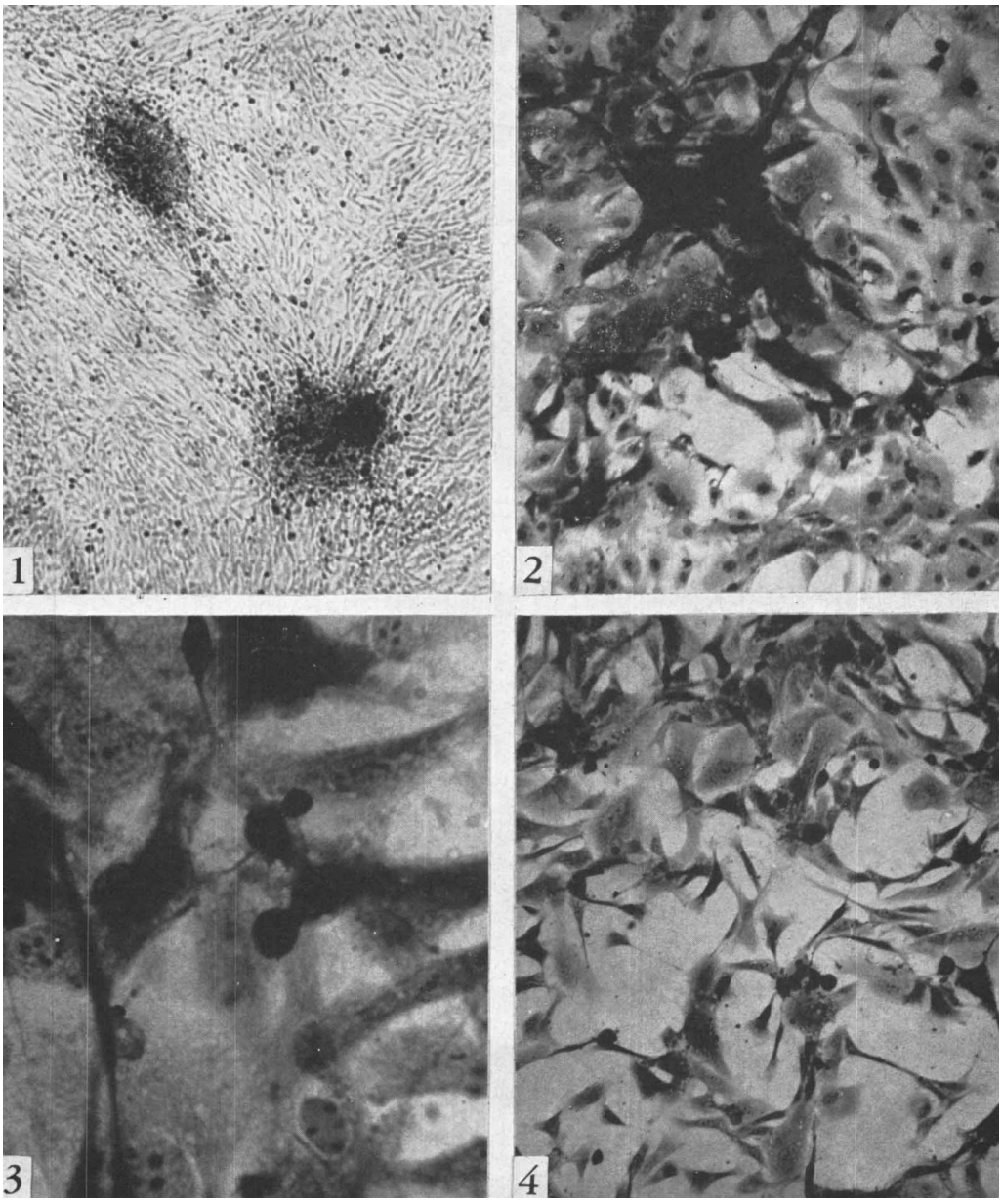


FIG. 1. Two focal areas of CPE in a DEF monolayer culture inoculated with JM blood. Unstained. ×42. Fig. 2. Focal area of CPE showing marked basophilia of affected cells and some piling up. Giemsa; ×110. Fig. 3. Area of CPE showing rounded, pyknotic cells and shrunken, spindle-shaped cells lying above normal appearing fibroblasts. Giemsa; ×495. Fig. 4. Diffuse CPE typical of that occasionally seen in older cultures. Giemsa; ×110.

no MD in inoculated chicks. These findings are consistent with reports (8, 9) in which the close association of the MD agent with intact tumor and blood cells has been well documented.

The DEF cultures which were infected with MD agent also developed CPE. This effect could be transferred to normal cultures with fluid from altered cultures and could be maintained through many subcultures. The factor responsible for the induction of CPE also appeared to be cell associated since attempts to transmit the CPE with cell-free filtrates of fluids from altered cultures were unsuccessful. Centrifuged fluids and cell extracts induced CPE in a variable proportion of recipient cultures but these materials could not be considered cell free.

The CPE could be due either to the infection of cells with the MD agent or to a latent virus activated by the culture treatment. Although it is impossible to rule out the latter hypothesis, this appears unlikely since CPE was never observed either in cultures in-

TABLE I. Correlation of CPE in Inoculated and Control Duck Embryo Fibroblast Cultures with Induction of MD in Chicks.

| Trial and culture no. | Culture inoculum | | Culture days postinoculation | | MD assay (chick response) | |
|---|---|---|---|---|---|---|
| | Material | Dose (ml/plate) | CPE | MD assay[a] | Gross lesions | Median days to death[b] |
| 1–1 | JM blood | 0.1 | + (25) | 42 | 10/10[d] | 32 (7) |
| 1–2 | None | | — | | 0/10 | |
| 2–1 | JM blood (A)[c] | 0.02 | — | 14 | 0/6 | |
| 2–2 | JM blood (A) | 0.02 | — | 14 | 0/6 | |
| 2–3 | DEF extract (B) | 0.3 | — | 14 | 0/11 | |
| 2–4 | DEF extract (B) | 0.3 | — | 14 | 0/11 | |
| 2–5 | None | | — | | 0/6 | |
| 2–6 | JM blood | 0.02 | + (22) | 56 | 6/6 | 27 (4) |
| 2–7 | None | | — | | 0/6 | |
| 3–1 | DEF supernate (C) | 3.0 | + (14) | 34 | 6/6 | 31 (5) |
| 3–2 | DEF supernate (C) | 3.0 | — | 34 | 0/10 | |
| 3–3 | DEF extract | 0.5 | + (25) | 34 | 10/10 | 24 (9) |
| 3–4 | JM blood | 0.1 | — | 37 | 0/10 | |
| 3–5 | None | | — | | 0/10 | |
| 4–1 | DEF supernate | 5.0 | + (11) | 66 | 5/5 | 27 (3) |
| 4–2 | None | | — | | 0/8 | |
| 5–1 | JM blood | 0.01 | — | 31 | 0/8 | |
| 5–2 | JM blood | 0.01 | — | 31 | 0/8 | |
| 5–3 | None | | — | | 0/8 | |
| 6–1 | JM blood | 1.0 | + (21) | 41 | 8/8 | 24 (4) |
| 6–2 | JM blood | 1.0 | + (11) | 14 | 8/8 | 27 (4) |
| 6–3 | None | | — | | 0/7 | |
| 7–1 | JM blood | 1.0 | + (11) | 14 | 8/8 | 27 (6) |
| 7–2 | JM blood | 1.0 | + (11) | 14 | 8/8 | 27 (3) |
| 7–3 | DEF supernate | 10.0 | + (6) | 10 | 8/8 | 27 (1) |
| 7–4 | None | | — | | 0/7 | |

[a] Age of uninoculated control cultures at assay was identical to that of inoculated cultures in the same trial.

[b] Number of deaths in parenthesis; experimental period was 41 days (Trials 1–5) or 27 days (Trials 6 and 7).

[c] Inocula with similar parenthetical designations are identical.

[d] Number of chicks with gross lesions/experimental number (starting number less nonspecific deaths).

oculated with normal blood or in every culture treated with JM blood. The former hypothesis is supported circumstantially by (a) the perfect correlation between CPE and MD infectivity of inoculated DEF cultures and (b) the cell association of both the MD agent and the factor responsible for the induction of CPE. This evidence strongly suggests that the CPE is the direct result of infection of DEF with the MD agent.

The induction of CPE by MD agent in DEF cultures might be utilized for the *in vitro* assay of the agent. However, the inconsistent induction of infection in cultures with JM blood would appear to limit the usefulness of this system at present. Greater consistency was obtained in recent trials when cultures were inoculated with 1.0 ml of blood. However, the dose dependency of this response as well as many other important parameters have yet to be determined. Further studies are in progress to determine the potential usefulness of this system for the *in vitro* assay of the MD agent.

Of potentially greater importance, however, were investigations in which infected tissue cultures were employed in an attempt to elucidate the fundamental nature of the MD agent. Preliminary studies by Nazerian *et al.* (5) have established through electron microscopy the presence of a herpesvirus in JM inoculated DEF cultures with CPE and have circumstantially implicated this virus in the etiology of MD.

*Summary.* A focal cytopathic effect (CPE) was observed in duck embryo fibroblast (DEF) cultures 11–25 days postinoculation with blood from the JM strain of Marek's disease (MD). All cell suspensions from such cultures reproduced MD when inoculated into chicks, while all of the morphologically normal DEF cultures were noninfectious. The CPE and infectivity were maintained in DEF cultures for 182 days. Both the induction of CPE in cell cultures and MD in chickens required intact cells in the inoculum. These data indicate that the JM strain of MD was successfully propagated in DEF cultures and produced a characteristic CPE.

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## Studies on the Etiology of Marek's Disease. II. Finding of a Herpesvirus in Cell Culture (32650)

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The etiological agent of Marek's disease (MD) is easily transmitted from infected to susceptible chickens by direct or indirect contact (1, 2). Although the agent apparently passed a 0.3 $\mu$ filter in one trial (3), this observation could not be confirmed (Biggs and Payne (4); H.G. Purchase, personal communication; R.L. Witter, unpublished data). Infectivity of cell suspensions varied directly with the number of viable cells, thereby suggesting a direct association of the agent with the cell (4). However, chromosome studies in-

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