Epithelioid Cell Strains Derived from Rabbit Peripheral Blood* (32695)

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Numerous reports in the literature have described the growth and morphological alteration of leucocytes *in vitro* (1–3). Berman has described the emergence of epithelioid cell populations from human peripheral blood cultures (4–6). These populations initially grew as monolayers, underwent heteroploid transformation, and could be subcultured with infinite life expectancy as established cell lines (7,8). The work of Berman was particularly significant, since it suggested that cell strains and lines could be initiated simply by culturing peripheral blood. Such a method would constitute an important contribution to the general field of somatic genetics, as well as affording a system suited to the study of cell differentiation. We have observed a similar phenomenon in experiments relating to the explantation of peripheral blood cells *in vitro*. Our experience, however, has led us to quite different conclusions than those of Berman. On the basis of our experiments, we conclude that cell populations emerging from rabbit peripheral blood cultures in all likelihood arise from contaminating somatic cells which are unrelated to peripheral blood cells.

Materials and Methods. The methods employed for the isolation and cultivation of rabbit peripheral blood leucocytes have been described in detail elsewhere (9), and only the essential steps will be outlined here. Male New Zealand rabbits were employed. Blood was removed by cardiac puncture, and the erythrocytes were separated by gravity sedimentation, potentiated by the addition of gelatin at a final concentration of 0.8–0.9%. The leucocytes were then filtered through nylon (Fenwall Filters) at 37°C. This substantially removed platelets and reduced the number of granulocytes. The cells were washed by centrifugation three times in complete nutrient medium 199 plus 20% Fetal Bovine Serum (Gibco). This further removed platelets and residual erythrocytes. No attempt was made to isolate pure populations of mononuclear cells; however, this cell type greatly predominated at a level of approximately 80–90% as determined by stained smears (Fig. 1). The washed cell population

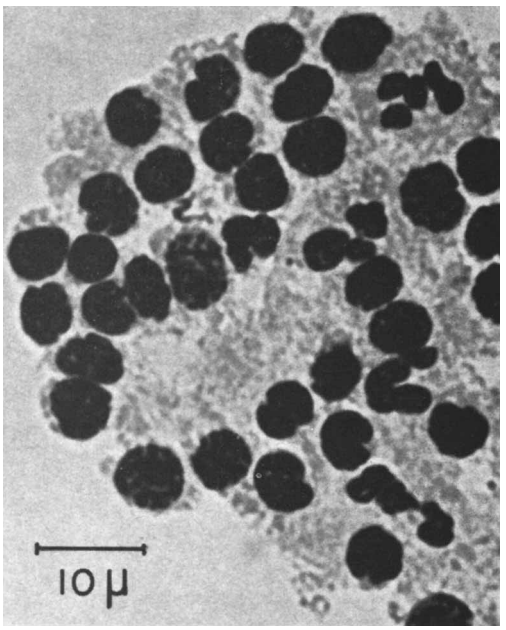

FIG. 1. Aggregation of leucocytes after 1 hour *in vitro*. Medium with precolostrum serum. Cells have been fixed with acetic acid, stained with orcein, and flattened by squashing. The cytoplasm has been disrupted by the preparative procedure. Mononuclear cells predominate but several granulocytes are present.

was counted by means of a Coulter Electronic Cell Counter equipped with a 50 μ pore. The platelet, erythrocyte, and leucocyte components of the population could be distinguished on the basis of their mean cell volumes. Counts were made only on the leucocyte component. Cultures were established at cell concentrations of 1×10^6 leucocytes/ml of complete nutrient medium. Glutamine was added prior to culture at a final level of 2 mM. Cultures were set-up in either 10 ml

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screw-cap tubes on their sides, or in 1-ounce medicine bottles (Armstrong) fitted with silicone stoppers. Culture volume was 5 ml. The cultures were gassed once with 5% CO_2 in air just prior to incubation at 37°C. The results to be reported are based on long-term observations of ten independently initiated cultures. Each of these ten experiments was also subdivided into internal subexperiments with respect to such variables as serum, nutrient medium, and phytohemagglutinin. Observations have also been carried out on innumerable independently initiated short-term cultures from the rabbit.

Experiments and Results. The time course of emergence of epithelioid cells from peripheral blood cultures was as follows. During the first 24 hours of culture the leucocytes first adhered to the glass substrate. Progressively, duing this early period, most of the cells detached from the glass and adhered to each other forming free-floating, round aggregates of cells. During the first week of cultivation, many of the cells in the aggregates increased their volume slightly (about 2-fold), and some cells underwent degeneration. At the end of the first week very large vacuolated cells appeared in the cell aggregates and in isolation apart from the aggregates. These large cells (Fig. 2), which had adhered to the glass substrate, increased in volume by at least tenfold and only on rare occasion were observed to undergo mitosis. As judged by their ability to ingest iron filings (Fig. 3), these cells had a pronounced phagocytic capacity. From the moment of their first appearance these cells increased rapidly in number, and, at the same time there was a progressive concomitant decrease in other cell types. This observation suggests that the macrophages did not increase appreciably by proliferation, but by transformation of a precursor cell. The precursor cell is not definitely known, but the monocyte is a likely candidate (10,11). After the increase in large cells there was an appearance and subsequent increase in fusiform cells and a concomitant decrease in macrophages. The relative decrease of macrophages and increase in fusiform cells was abrupt. Round and fusiform cells were observed together only for a short time (Fig. 4). After approximately 2 weeks epithelioid cells emerged. Cells of this type were

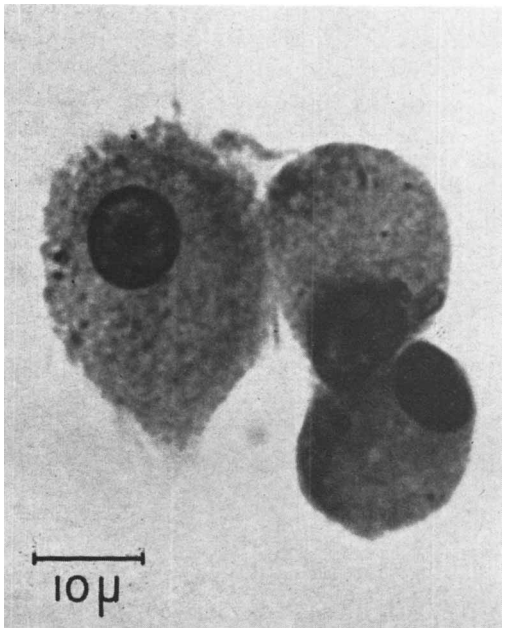

FIG. 2. Large round cells adhering to substrate. Twelve days *in vitro*. Note greatly increased size. Methanol fixation and stained in May Grünwald Giemsa.

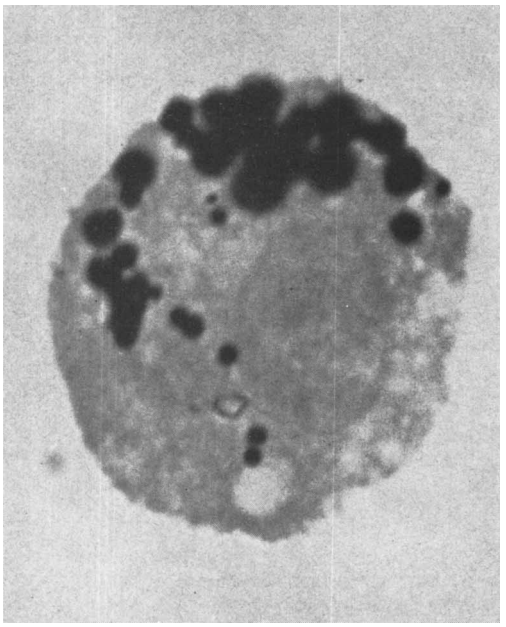

FIG. 3. Phagocytic cell with injested iron filings. Phase-contrast photograph of living cell.

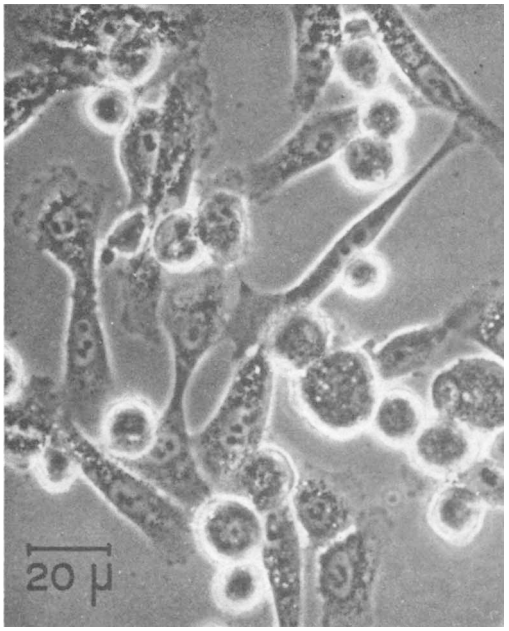

FIG. 4. Large round and spindleloid cells adhering to substrate. Eighteen days *in vitro*. Phase-contrast photograph of living cells.

typically found where high cell densities prevailed. At the periphery of epithelioid cell monolayers where the density was low, fusiform cells were frequently observed. This suggests that one basic cell type is present that possesses a density-dependent morphologic pleiomorphism. In summary, these observations suggest a transformation of a leucocytic precursor to a large phagocytic cell which does not further proliferate or differentiate. An epithelioid type of cell emerges about 2 weeks after the cultures have been established. The experiments outlined below indicate that this cell lineage derives not from the blood but from contaminating somatic cells of nonblood origin.

Two types of experiments were performed. In the first, rabbits were bled by cardiac puncture through the body wall and at the same time from an exposed femoral artery. Blood was drawn from the femoral artery to reduce the possibility of introducing foreign cells. After the overlying skin was anesthetized with lidocaine, the artery was dissected as cleanly as possible. Blood was drawn from the artery into a syringe. The first 6–10 ml of blood drawn was discarded by changing the syringe to further obviate contamination. Blood drawn in this manner was prepared for culture in exactly the same way as cardiac blood. Two independent experiments performed on two separate rabbits were carried out. In each experiment ten cultures were initiated from the femoral and cardiac bloods of the same rabbit.

The results of the experiment were as follows. The cardiac blood cultures yielded macrophages and eventually epithelioid cell lines. The femoral blood cultures yielded only macrophages, and in no instance were fusiform or epithelioid cells seen. These results suggest that foreign cells picked up inadvertently by passage of the needle through the body wall and structures in the chest in the case of cardiac puncture were the origin of the fusiform and epithelioid cells. The second set of experiments support this supposition.

In the second experiment, simulated cardiac punctures were made. The needle was passed through the thoracic body wall in the direction of the heart, but the heart and the large blood vessels were carefully avoided. The syringe was aspirated gently and then removed, and the contents were flushed out with medium 199, 20% fetal bovine serum. The medium which had been used to wash the syringe was then placed in culture bottles and incubated. Six separate preparations of this type were made. Every culture gave rise to a proliferating cell population similar to those derived from cardiac blood (Fig. 6).

The epithelioid cells have been cultured as monolayers, and subcultured by means of trypsin-versene digestion through as many as 10 culture generations. The cells filled the surfaces of the culture flask after subculture, and appeared to be contact-inhibited, since there was no vertical piling to form multiple layers of cells. One growth study on an epithelioid population at the fourth passage showed a mean generation time of 40 hours. Chromosome studies indicated that rabbit cells with a chromosome number of $2N = 44$ predominated. This finding rules out the possibility of contamination by other cell populations, since no other rabbit cell cultures are maintained in this laboratory. There has

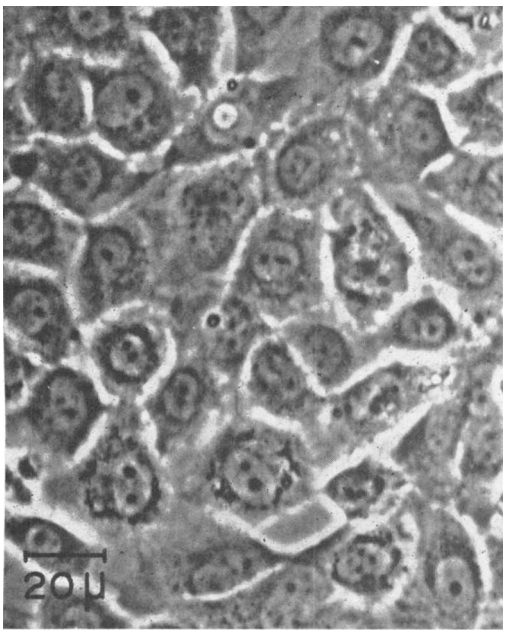

FIG. 5. Epithelioid cells monolayer adhering to substrate. Sixth passage. Phase-contrast photograph of living cells.

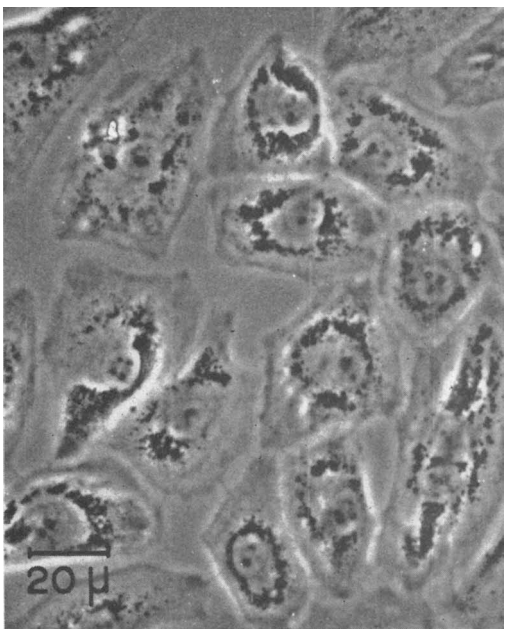

FIG. 6. Epithelioid cell monolayer adhering to substrate. Initial outgrowth. Phase-contrast photograph of living cells.

been no appreciable increase in cells having a tetraploid or subtetraploid (heteroploid) chromosome number in the various epithelioid cell cultures, nor has there been any tendency for the establishment of aneuploid subpopulations at the near diploid level. We have also been able to freeze these cells at liquid nitrogen temperatures according to standard procedures (12) and to recover viable, unaltered cells with high efficiency. Observations to date thus suggest the reproducible outgrowth of stable diploid cell strains through at least 10 culture generations.

Discussion. It is our conclusion that long-term cell populations derived from cardiac blood in all likelihood have arisen from needle biopsy contaminants. These results and conclusions again reopen the question as to the origin of cells which reputedly have been cultivated from peripheral blood (5,13). It should be pointed out, however, that our own evidence is to a great extent circumstantial. It is possible that femoral and cardiac bloods differ in their cellular composition. While this alternative explanation is possible, we regard it as being highly unlikely.

The finding that cell populations can be derived reproducibly from needle biopsy in the rabbit recommends this procedure as a simple and effective way to initiate cell cultures from this species. It would be of interest to adapt this technique to other animals and man.

Summary. Mononuclear leukocytes obtained from rabbits by cardiac puncture, and purified of erythrocytes by gravity sedimentation in a gelatin solution and of platelets and polymorphonuclear leukocytes by filtration through nylon columns, gave rise to cell strains when cultured for 2 weeks. During serial passage, the emergent strains retained a diploid number of chromosomes and displayed a morphology ranging from the fusiform to the epithelioid. Similar cell strains were also initiated by culture of cells obtained by needle biopsy simulating cardiac puncture in the thoracic region. Contrariwise, cell populations could not be established from blood drawn from the exposed femoral artery. These observations cast doubt on previous reports which suggest that stable tissue culture cell populations were derived from cells of the blood. The use of aspiration material from

the chest provides a simple and reproducible method of initiating primary cell strains from rabbits.

Authors' note. While this article was in press we became aware of two articles which supported the possibility that peripheral blood long-term cell strains arise by needle biopsy contamination. These reports were based on experiments with guinea pigs (Ross, R., and Lillywhite, J. W., *Lab. Invest.* **14**, 1568 (1965)) and chickens (Rangan, S. R. S., *Exp. Cell Res.* **46**, 477 (1967)), and thus support and extend our own observations.

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Reaction of Infectious Mononucleosis Sera with Cell Cultures Infected by Newcastle Disease Virus* (32696)

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It has been shown (1–3) that human red blood cells (RBC's) treated by Newcastle disease virus (NDV) are agglutinated by sera of patients suffering from infectious mononucleosis (IM). It was decided to investigate whether alteration of cells other than RBC by NDV would result in their reaction with IM sera. Cell cultures infected with NDV were selected for this investigation. To detect the reaction of IM sera with the surface of the cell cultures, altered as a result of infection by NDV, the mixed agglutination (MA) procedure was used (4).

Materials and Methods. Viruses. The VIC strain of NDV was obtained from Dr. D. T. Karzon, Virology Laboratory, Children's Hospital, Buffalo. The B1 strain of NDV was received from the American Type Culture Collection. Viruses were propagated in the allantoic cavity of the chick embryo. For mixed agglutination experiments, VIC strain was passaged in human cell cultures of HEp-2 line.

Cell cultures. The HEp-2 line passaged routinely in this laboratory was purchased originally from Flow Laboratories, Rockville, Maryland. The growth medium consisted of Eagle's basal medium (BME), and 10% newborn calf serum (Grand Island Biological Company) in a base of Hanks' balanced salt solution (BSS). When cultures were inoculated with virus, the growth medium was replaced with maintenance medium composed of Eagle's minimal essential medium (MEM), and 3% calf serum in a base of Earle's salt solution. Primary cell cultures of Rhesus monkey kidney were grown in a medium consisting of 0.5% lactalbumin hydrolysate (Nutri-

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