medium at 25 °C. At the ages of 60, 72, 82, 86, 93, 96, and 104 h, groups of *Ore-R* and *tu^{w}rc* larvae/pupae were fixed in buffered formadehyde followed by osmic acid[11]. The specimens were dehydrated in a graded series of ethanol and transferred to amyl acetate. Liquid CO_2 was used to replace the amyl acetate in the critical point drying method essentially as described by ANDERSON[12], and the specimens were attached to cover glasses mounted on metal stubs. The specimens were then coated with gold and examined with a JEOL (Model JSM-U3) scanning electron microscope.

The topology of the caudal and anterior adipose tissues of the tumor and normal strains do not differ during the early 3rd instar. Adipose tissue is covered with basement membrane and its topography is marked by crater-like depressions (Figure 1, A). Fat body cells at this stage of development contain large lipid droplets[13] and loss of lipid contents during the fixation and dehydration procedures might account for the circular depressions overlying locations of lipid droplets. Mechanical brushing and peeling during mounting of the specimens illustrates the relationship between cytoplasmic content and the cell surface (Figure 1, B). The topography of the fat body changes slightly as development proceeds and at 82 h the depressions over the surface of the fat body cells begin to take on the appearance of folds and gullies. During this period (72–82 h) the surface of the *tu^w* caudal fat masses differs distinctly from *Ore-R* caudal fat masses. The basement membrane overlying the *tu^w* fat cells is lost and individual fat cells or groups of cells begin to separate from one another (Figure 1, C). Small droplets of material not seen in younger *tu^w* larvae or normal larvae appear between and around the dissociating cells. These droplets resemble inclusions found in the fat body cells and they may represent seepage from cells which have lost their surrounding basement membrane (Figure 1, D). Blood cells invade the affected area (Figures 1, C; 2, A and B). As reported previously[4, 10], spherical hemocytes (plasmatocytes and podocytes) undergo a morphological transformation to form extremely flattened cells that have been designated lamellocytes. A hemocyte in the process of cellular transformation is pictured in Figure 2A and enlarged in Figure 2B where the foldings of the cell's surface are illustrated as well as the manner in which the margin is extended in extremely flattened sheets (upper left corner of the photograph B). The process of cell transformation continues until the entire cell becomes a flat sheet spread over the surface of the underlying substrate. This layering of lamellocytes binds the fat cells into a relatively smooth, compact mass. These masses become melanized at approximately 93–96 h of age and the melanotic masses are retained throughout the life of the individual. Figure 2C is a *tu^w* melanotic mass which has been positioned for comparison with this same region of a normal caudal fat mass from an *Ore-R* larva (Figure 2, D). Basement membrane surrounding *Ore-R* fat body remains intact during larval development and anterior fat body of *tu^w* larvae is indistinguishable from *Ore-R* anterior fat body at 93 h of age. Only after pupation do changes in the basement membrane of *Ore-R* fat body cells become noticeable when dissociation of the adipose cells from each other sets in.

The origin of the basement membrane in insects has not been established, although a number of observations suggest epithelial cells as its source. WIGGLESWORTH[14] has recently reviewed the reported variety of sources of basement membrane material and presented evidence that the hemocytes contribute to basement membrane formation in *Rhodnius*. In *Drosophila* the nature of the glycoproteins in the basement membrane is unknown, and it is not clear whether the acellular membrane is a product of the cells underlying a given tissue or a product elaborated by the hemocytes. This distribution is relevant to an analysis of 'melanotic tumor' formation in *Drosophila*; if the hemocytes contribute to basement membrane formation, their neutrality to surfaces covered by products of their own metabolic activity might provide the factor for recognition of 'self' as opposed to foreign entities, and this mechanism would apply to the organism's own tissues denuded of their membranous cover as in *tu^w*. On the other hand, the appearance of specific intercellular fat body contents in the hemocoel following loss of the overlying basement membrane may also serve as a stimulus for hemocyte aggregation about this area to contain the affected cells within an enclosed capsule. The latter stimulus, however, must be considered a consequence of changes at the cell surface supporting our conclusion that the etiology of 'melanotic tumor' formation in *Drosophila* includes a hereditary factor affecting the integrity of basement membrane.

Zusammenfassung. In *Tumor^w*-Mutanten von *Drosophila melanogaster* kapseln Hämocyten den hinteren Teil des Fettkörpers ein und bilden melanotische Tumoren. Mit Hilfe des Rasterelektronenmikroskops wurde nachgewiesen, dass mit dem Beginn der Tumorbildung ein Zerfall der Basalmembran, und eine Auflösung des hinteren Fettkörpers in Einzelzellen gekoppelt sind.

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12 December 1973.

[11] T. M. RIZKI, J. Cell Biol. *32*, 531 (1967).
[12] T. F. ANDERSON, Trans. N.Y. Acad. Sci. II, *13*, 130 (1951).
[13] F. M. BUTTERWORTH, D. BODENSTEIN and R. C. KING, J. exp. Zool. *158*, 141 (1965).
[14] V. B. WIGGLESWORTH, J. Insect Physiol. *19*, 831 (1973).
[15] Supported in part by Grant No. NIH CA12600 and NIH Biomedical Institutional Grant No. 011295. We would like to acknowledge help of the staff of the Electron Probe Laboratory: L. ALLARD, P. HOLLINGSWORTH, and particularly J. MARDINLY for the use of his critical point drying apparatus. We also pay special tribute to Professor W. BIGELOW for his foresight and effort in establishing this laboratory, and thank A. TOMPA for his friendly persuasion to work with the SEM.

Development of an Established Cell Line Derived from *Dasypus novemcinctus* (Armadillo), a Laboratory Animal Susceptible to Infection by *Mycobacterium leprae*

The search for a classical bacteriological medium applicable to the rapid and reproducible laboratory growth of *Mycobacterium leprae* has not been successful[1]. The problems encountered tend to support the possibility that the human leprosy bacillus is an obligate intracellular parasite. If such is the case, the need for a viable, susceptible eukaryotic cell system is quite evident. Tissue culture systems long exploited in the study of

viruses could provide a reproducible, relatively inexpensive model for leprosy research. Specifically such a system would: 1. Provide a source of the leprosy bacillus free from the effects of therapeutic antibiotics. 2. Provide a model for the study of the mechanism of pathenogenicity of the human leprosy bacillus. 3. Provide a model for assessing antileprosy drugs.

Two experimental animals are presently being used in leprosy research. In 1960 it was discovered that limited growth of *M. leprae* could be sustained in the foot pads of mice, but in the absence of total body irradiation this model failed to reproduce the human disease[2]. Tissue culture systems subsequently developed from the mouse have not supported the growth of the human leprosy bacillus, but the same tissue culture systems have been successfully used in the laboratory growth of *M. lepramurium*, the rat leprosy bacillus[3].

The induction of a disease which apparently duplicates human lepromatous leprosy in the nine-banded armadillo[4] has not only provided a second animal model for the study of human leprosy, but also suggests that a tissue culture system derived from the same animal might be applicable to future studies on the disease. This report describes the initiation and characterization of monolayer cultures derived from the nine-banded armadillo.

Materials and methods. Unless otherwise indicated Eagles minimal essential medium (MEM) supplemented with 15% fetal calf serum, 0.1 *M* Hepes buffer, 50 units/ml penicillin and 50 µg/ml dihydrostreptomycin was used. Armadillos were collected from their local habitat. Based on size comparisons with armadillos in a breeding colony under development at the Public Health Service Hospital, Carville, La., we estimate the age of the test animals to be between 3 and 6 months. Armadillos were sacrificed by etherization, the ventral surfaces were washed with 70% ethanol and tissue samples including kidney, liver, heart, spleen, abdominal muscle and skin were set up as explant cultures at 37 °C in 75 cm² plastic tissue culture flasks. After 3 weeks the individual cellular outgrowths were trypsinized with 0.5% trypsin (Difco 1:250) prepared in pH 7.2 phosphate buffered saline and the cells were transferred (1:1) to another sterile 75 cm² tissue culture flask. At confluency the cells were trypsinized and subcultured using a split ratio of 1:3. Flasks were incubated at 37, 33 and 31 °C and subsequent serial subpassages were then made at each temperature for each tissue. Cell counts were determined on triplicate 25 cm² flasks with a Coulter Electronic Counter Model B. Fixing, embedding, and sectioning for electron microscopy were described previously[5]. Changes in pH were determined by color comparison with prepared standards.

Results and discussion. The apparent transmission of lepromatous leprosy to the armadillo indicates that man is not the sole susceptible host for *M. leprae*. This had also been suggested in one of the few but unconfirmed reports of the multiplication of *M. leprae* in both human

[1] T. Murohashi and K. Yoshida, Bull. World Health Org. *47*, 195 (1972).
[2] R. J. W. Rees and A. G. M. Weddell, Ann. N. Y. Acad. Sci. *154*, 214 (1968).
[3] Y. Matsuo, Jap. J. Microsc. *14*, 233 (1970).
[4] W. F. Kirchheimer and W. F. Storrs, Int. J. Lepr. *39*, 693 (1971).
[5] R. F. Silva, R. L. Amborski, M. R. White and G. F. Amborski, J. comp. Path. *83*, 161 (1973).

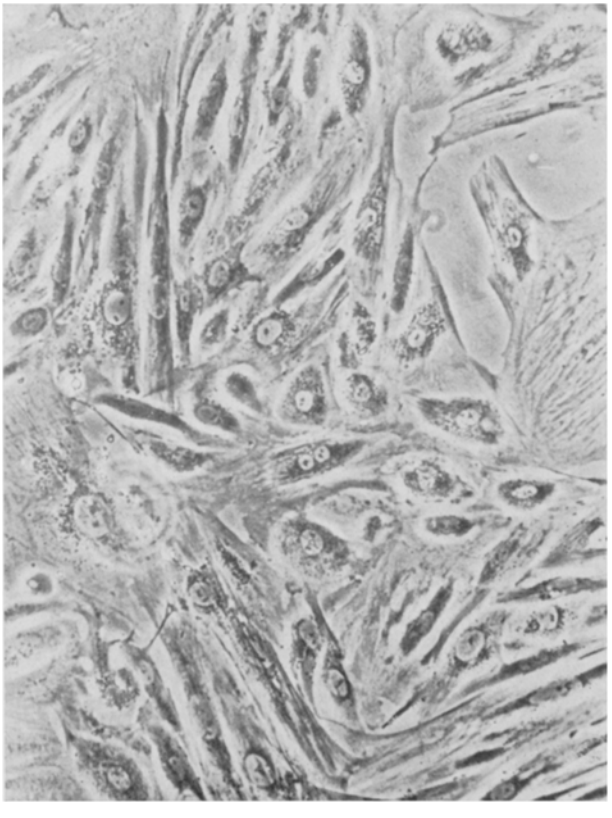

Fig. 1. Phase contrast micrograph of kidney derived cells at subpassage No. 60. ×675.

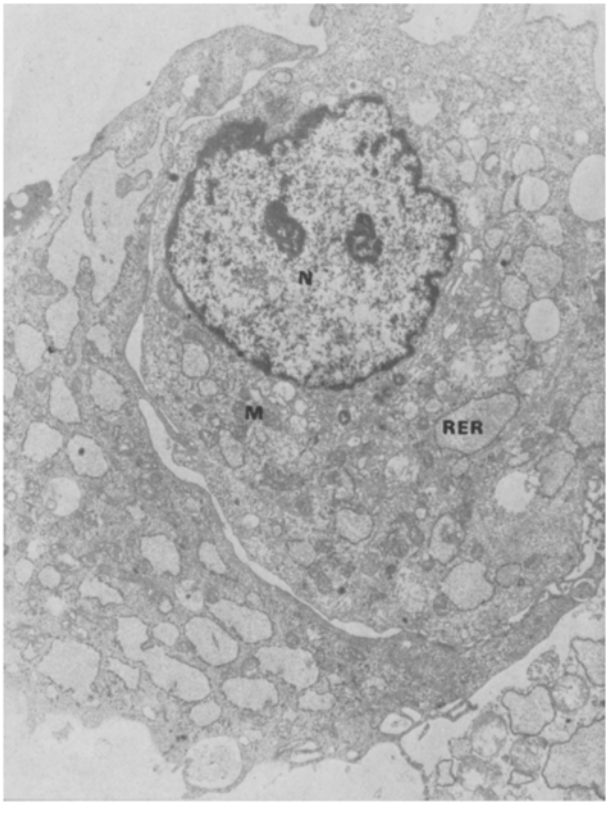

Fig. 2. Electron micrograph of kidney derived cells at subpassage No. 60. N-nucleus, RER-dilated rough endoplasmic reticulum, M-mitochondria. ×28,000.

and rat tissue culture systems[6]. This same report further suggested that cells derived from a number of different tissues might serve equally well in tissue culture studies with *M. leprae*, and this consideration served as the basis of our attempt to develop cell culture systems from a variety of tissues. Of the indicated armadillo tissues processed, cells derived from the liver and spleen did not survive the first serial subpassage whereas cells derived from the heart and skin survived 8 and 10 serial subpassages respectively. Cells derived from abdominal muscle survived 45 serial subpassages, and kidney derived cells have now been in culture for 70 serial subpassages, representing approximately 14 months of successful culture.

The fibroblastic nature of the kidney derived cells is demonstrated in Figure 1. The morphological appearance of the kidney derived cells varied according to pH changes within the medium. The kidney derived cells tended to flatten out at pH 7.3, but after 5 days of culture at 37 °C as the density of the culture increased and the pH approached 6.8, the cells assumed a more spindle shape. This effect was especially pronounced in those cultures incubated at 33 °C and 31 °C. Under these conditions the pH of the growth medium did not drop below 7.0 until after 30 days of culture. However, cells maintained for such extended periods at 31 °C tended to form extremely large flat cells which could not be subcultured. The fibroblastic nature of the kidney derived cells was further emphasized by electron microscopy as demonstrated in Figure 2. No unusual ultrastructural features were observed, and at an increased magnification the apparent vessicles were identified as dilated rough endoplasmic reticulum in close association with the mitochondria of the cell.

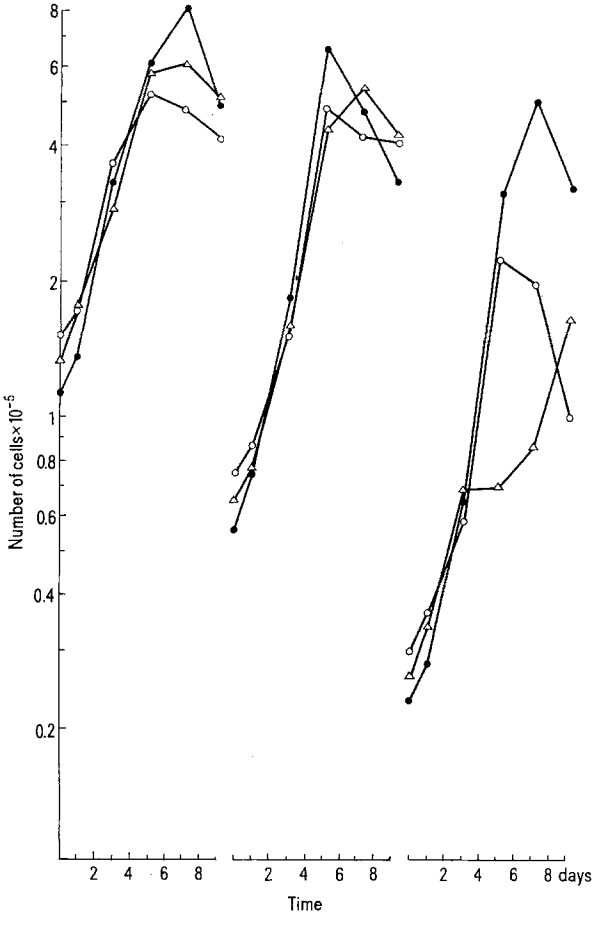

Fig. 3. Growth responses of kidney derived cells at subpassage No. 60. Growth determinations were made at the indicated temperatures using cells subcultured at that same temperature. Initial inocula were as indicated. ●—●, 37 °C; ○—○, 33 °C; △—△, 31 °C.

SHEPARD[7] demonstrated that the temperature of human skin sites most severely involved in leprosy averaged 25 to 33 °C, and that of the skin sites which escape leprosy averaged 35 to 36 °C. Similar peripheral sites of heat loss average about 30 °C in the mouse[8], and the skin and body temperature of the armadillo is approximately 32 °C[4]. Thus it might be expected that tissue culture systems applicable to leprosy research should be physiologically active at these reduced temperatures. Figure 3 shows the effect of temperature and the concentration of initial cell inoculum on the growth response of the kidney derived cells. With the exception of the cells grown at 37 °C, cultures initiated at a concentration of approximately 5000 cells/ml or a density of approximately 300 cells cm² gave erratic and inconsistent results from experiment to experiment. The use of a 20% lower cell inoculum resulted in an almost complete lack of growth whereas an approximate 3-fold increase in cell inoculum overcame the population effects. Furthermore the data indicates that at the two higher initial cell inocula, the growth rate at each cell concentration was unaffected by the variation in temperature. However, as one might predict and as discussed previously, growth at 37 °C was accompanied by excess acid production as compared with growth at 33 °C. Recent reports have emphasized the importance of pH control in cell culture systems[9]. Growth rate, contact inhibition of growth, cell mobility and catabolism of sulfated mucopolysaccharides are all strongly influenced by the pH of the culture medium. What if any effect these problems would have on the interaction between the leprosy bacillus and cultured armadillo cells remains to be determined. The nature of the serum supplement was apparently not critical as equine, bovine, fetal calf, chicken, human, and porcine sera tested at 5, 10, 15, and 20% concentrations resulted in no gross changes in either the morphological or quantitative responses of the cultures. Attempts to initiate or maintain the cultures in MEM supplemented with Difco-Peptone, Proteose Peptone or Tryptone at 0.5, 1.0, and 2.0% concentrations were unsuccessful.

Résumé. Cette note concerne l'isolement d'une préparation linéaire de cellules du *Dasypus novemcinctus* (l'armadillo è neuf bandes), susceptible d'être infecté par le *Mycobacterium leprae*.

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[6] E. W. GARBUTT, Int. J. Lepr. *33*, 578 (1965).
[7] C. C. SHEPARD, Int. J. Lepr. *33*, 541 (1965).
[8] C. C. SHEPARD, J. Bact. *90*, 1271 (1965).
[9] S. O. LIE, V. A. MCKUSICK and E. F. NEUFELD, Proc. natn. Acad. Sci. *69*, 2361 (1972).