THE CULTIVATION OF TISSUE IN PLASMA FROM ALIEN SPECIES.*

By ROBERT A. LAMBERT, M.D., and FREDERIC M. HANES, M.D.

(From the Department of Pathology of the College of Physicians and Surgeons, Columbia University, New York.)

Plates 13 to 15.

Biological differences in the body fluids of various species of animals are well recognized. Even with species so closely related zoologically as rats and mice, there are distinguishing serological characteristics that recent studies have shown to be easily demonstrable. These differences have been interpreted as indicating the inability of a serum to supply food to alien cells. Certain phenomena in heterologous tissue transplantation have been thus explained. For example, Ribbert,1 who found that human and guinea pig skin, when inoculated subcutaneously into rabbits, did not survive more than three days, interpreted his results on the basis of cell starvation. He believed that the foreign host could supply only salts and water to the implanted cells. Furthermore, Ehrlich's theory of athreptic immunity2 as applied to the resistance manifested under certain conditions toward the transplantable rat and mouse tumors, has probably its strongest support in the behavior of a mouse sarcoma when transplanted to rats, and in the results of further transplantations to rats and mice of the nodules so produced. Ehrlich explains the phenomena by assuming the existence of a specific food stuff present only in the mouse, the exhaustion of which leads to the death of the tumor cells.

* This investigation was conducted under the George Crocker Special Research Fund. Received for publication, June 10, 1911.

A historical resumé of the development of the method of cultivating tissues in vitro was given in our article, “Characteristics of Growth of Sarcoma and Carcinoma Cultivated in Vitro,” Jour. Exper. Med., 1911, xiii, 495.

1 Ribbert, Verhandl. d. deutsch. path. Gesellsch., 1904, viii, 104.

Cultivation of Tissue in Plasma from Alien Species.

The most thorough study that has been made of the question of heterologous tissue transplantation is that of Leo Loeb upon the transplantation of guinea pig epithelium to animals of other species. Since his findings have a direct bearing upon the problems with which we have been concerned, his work will be briefly outlined.

Small pieces of guinea pig skin were inoculated subcutaneously into guinea pigs, rabbits, dogs, pigeons, and frogs. Retransplantation of the grafts from the alien hosts to guinea pigs were made at varying intervals for testing the viability of the cells, or rather for determining the extent of the injury suffered in their sojourn in the foreign species. The duration of growth of the epithelial grafts under these conditions, as indicated by the presence of mitoses, was found to be eight days in rabbits, seven days in dogs, and five days in pigeons. In frogs, the cells appeared to survive only a few hours. In homologous transplantations there were formed, after eight to ten days, minute epithelial cysts which seem to persist indefinitely.

The cultivation of tissues outside the body appeared to us to offer an excellent method for investigating the question of food specificity within the species, the toxic or cytolytic actions of foreign sera, and possibly, also, the reaction of tissue to an unfavorable but non-toxic medium; that is, the reaction of cells to an injury not severe enough to cause death. Apart from its ease and simplicity, the method offered several advantages over procedures involving the grafting of tissue in the animal body: first, the possibility of observing continuously the changes occurring in a single piece of tissue under the altered environment; and secondly, the elimination of factors, which, in investigating the conditions that influence the fate of grafts in the body, have to be taken into account. Thus Loeb showed that variations in the susceptibility of different species to bacterial infections, infiltration of the grafts by leucocytes, and the constricting action of the reactive connective tissue stroma and capsule, all undoubtedly influenced the fate of the grafts of guinea pig epithelium.

In approaching these problems, our studies have been directed chiefly toward determining the suitability of plasma from a number of species as culture media for certain mouse and rat tissues,—

mouse carcinoma, rat sarcoma, and rat spleen,—with an attempt to analyze some of the phenomena observed.

**TECHNIQUE.**

For obtaining blood from mice, rats, guinea pigs, and rabbits, we have used the method described in a former paper, that is, under anesthesia, the carotid artery is exposed, cleaned, ligated above, and clamped below. Its wall is caught by fine forceps and the vessel is severed distally. The clamp is then released and the blood allowed to spurt into paraffined tubes packed in ice. With rat blood, considerable care in avoiding contamination with tissue juices is necessary in order to prevent rapid coagulation. In this respect, the handling of mouse blood offers less difficulty; that of rabbits and guinea pigs may be handled with still greater ease. Dogs were bled from the femoral artery or vein through a needle of good caliber, previously boiled in albolene. These vessels are easily exposed by a small incision. Human blood was obtained through a needle from a superficial vein in the arm or forearm. In goats the same procedure was employed, either the jugular or the prominent superficial vein crossing the leg just above the knee being used. With pigeons, special precautions against contamination with tissue juices either by the use of a needle or by the method of direct flow from the vessel were found to be quite unnecessary. After removing the feathers and sterilizing the skin, the large wing vein was severed by a single incision through the skin and subcutaneous tissue with scissors or scalpel, and the blood allowed to drop into paraffined tubes. Plasma thus obtained remained fluid in some instances for hours when kept cool, and at no time did coagulation take place before completion of the experiments.

The remainder of the technique differs in only one or two minor points from that described by Burrows for chick embryos. Small drops of plasma are placed on cover glasses, and finely divided pieces of tissue, 0.5 to 1 mm. in diameter, are added immediately. The cover glasses are then inverted over fairly deep, hollow ground slides, previously ringed with vaseline. The slide preparations are

---

incubated at $37.5\, ^\circ\, C$. A warm, wooden microscope box is provided for subsequent observations. We have made no attempt at keeping the tissue warm during dissection, but we have studiously guarded against drying.

For determining the viability of pieces of tissue at any time during the course of an experiment, we have transferred them to homologous plasma. Since experience has shown that with proper care in the choice of tissue and in its handling, practically every piece of sarcoma, carcinoma, and spleen will grow when placed in homologous plasma, this test, besides being quick and simple, is thoroughly reliable. Animal inoculations were also made, but aside from the delay in noting the results, the procedure has other objections as a test of viability. In the first place, it is applicable only to preparations of tumors; and secondly, as we pointed out in a former paper, failures may indicate simply a decreased virulence.

CULTIVATION OF TISSUE IN ALIEN PLASMA.

In a former note, we recorded the fact that mouse and rat plasma could be interchanged as culture media for the tumors of these species, but that growth seemed to be more vigorous when homologous plasma was used. Plasma from guinea pigs, rabbits, dogs, goats, pigeons, and human beings has been employed in the course of the present investigation. In the succeeding paragraphs, the character of the growth observed in the use of the different kinds of plasma will be briefly described and frequent comparisons drawn from the growth observed in rat plasma. Three tissues,—rat sarcoma, mouse sarcoma, and rat spleen,—were used in nearly all of the experiments, but since they did not differ essentially in their behavior in vitro, description in most cases will be limited to the growth of rat sarcoma. Reference to the other tissues will be made only where the phenomena seemed of additional interest.

Guinea Pig Plasma.—As a culture medium for rat sarcoma, we have found guinea pig plasma only slightly less suitable than rat plasma, the difference consisting chiefly in the extent of the out-wandering of cells. In some preparations, the cells remained viable

---


in a single drop of plasma for twelve to fifteen days, although there was apparently little growth after the seventh day. Several specimens were transferred every seven days to fresh drops of plasma, and marked activity was observed following four such transfers; that is, after a month's sojourn in the alien medium. Such preparations were not distinguishable from those of similar age in which rat plasma was used.

This observation is of more than passing interest in that it has an important bearing on the question of food specificity within the species. According to Ribbert's conception, we should be compelled to believe that these cells could maintain life for such a period upon a nutriment of salts and water plus the insignificant amount of rat serum carried over in the first transfer; or, with Ehrlich, we should have to assume that a sufficient amount of X-stoff was retained to support life for this time. A more reasonable conception, it seems to us, is that rat cells are able to take up all necessary food from guinea pig serum, and that the failure of rat sarcoma inoculations in guinea pigs to develop tumors is dependent on obscure and probably more complex factors. Mouse carcinoma seems to grow almost as well in guinea pig plasma as in rat plasma. Mitoses were observed after eight days, and cultures nine days old produced tumors when inoculated into mice. Figure I shows a three day growth of mouse carcinoma in guinea pig plasma, in which is seen the characteristic sheet-like extension of the cells, with several groups ("alveoli") in the fibrin meshwork beyond the advancing border. Four mitotic figures can be made out.

Rabbit Plasma.—The growth of both mouse and rat tumors is much less active in rabbit plasma than in guinea pig and rat plasma. Liquefaction of the fibrin is often quite marked. With sarcoma, the growth, though relatively slow, may continue for ten to twelve days. Transfers of the pieces to rat plasma result in renewed activity. Animal inoculations of four day specimens were positive.

Dog Plasma.—Preparations of rat sarcoma in dog plasma, after one or two days, present fairly diffuse radial outgrowths of clear spindle cells, which after this time undergo rapid disintegration. About the pieces, there is often noted a clear, narrow zone due to the disappearance of the fibrin. With mouse carcinoma, liquefac-
Cultivation of Tissue in Plasma from Alien Species.

The formation of fibrin is more marked and there is little or no outwandering of cells. Transfer of pieces of sarcoma to rat plasma after two, three, four, and six days in dog plasma showed that the majority of the cells do not survive more than two days. An occasional growth was observed in the four day transfers, but none in those of longer duration.

Goat Plasma.—We have not observed any of the phenomena of growth when using goat plasma. After a few hours there is seen surrounding the pieces of tissue a wide, finely granular zone, apparently the result of cell disintegration. Liquefaction of fibrin does not occur.

In order to determine the length of life of the pieces of tissue in goat plasma, they were transferred in the usual way to rat plasma after incubation for twenty-four, forty-eight, seventy-two, and ninety-six hours. Some of the twenty-four hour specimens showed a fair growth; a smaller number of those forty-eight hours old gave evidence of life. Older preparations remained inactive. Histological sections of several pieces of tissue after two days in goat plasma revealed nothing of significance,—pyknotic nuclei and cells in various stages of disintegration.

In several experiments, a culture medium was used composed of goat plasma and rat plasma in varying proportions, with controls consisting of preparations in unmodified goat plasma and in rat plasma. The two kinds of plasma were mixed as follows: (1) goat plasma and rat plasma, equal parts; (2) goat plasma two parts, rat plasma three parts; (3) goat plasma one part, rat plasma two parts. The tissue in the goat plasma remained quite inert. The specimens in rat plasma all showed active growth. Those in mixed plasma gave rise to feeble growths of two to three days duration, the more active ones being, as a rule, those with the larger proportion of rat plasma.

The phenomena observed in the foregoing experiments suggest the presence of a substance in goat plasma toxic for rat cells. It would seem that it is not of the nature of a cytolysin, in a strict usage of the term, since there is apparently no definite cell-dissolving action, but that it is rather a body whose action on the foreign cells, if sufficiently prolonged, produces a fatal injury; that is, a cytotoxin.
A series of experiments was begun to investigate further the nature of this hypothetical substance, its resistance to heat, chemical properties, etc. Several difficulties in technique, however, were encountered, which prevented the successful pursuit of these questions.

*Pigeon Plasma.*—Although not comparable in rate and extent of growth to the cultures of rat sarcoma in rat plasma, the best preparations that we have obtained have been in pigeon plasma. The period of latency, that is, the time elapsing after incubation before the onset of active migration, is sometimes relatively long—two to three days as contrasted with eight to twenty-four hours in homologous plasma. The extension of the cells into the fibrin clot in a regular radiating fashion gives an appearance similar to that observed in guinea pig plasma. The morphology of the cells is, however, quite different. During the first three days they are large and clear, sometimes even swollen in appearance. They are ovoid to spindle in shape and each cell presents two or more processes connecting it with other cells. The nucleus is large and the protoplasm contains scattering, fine granules of fat. After four to five days, growth generally ceases and cell disintegration begins, preceded by a marked increase in the size and number of fat droplets. We have occasionally observed well stained nuclei in specimens eight and nine days old, but the protoplasm of such cells was highly granular and presented exceedingly ragged borders. Figure 2 shows a stained preparation three days old. The characteristic morphology of the cells is not very apparent. In fact, we have not been able to preserve well in the stained specimens the striking and distinctive appearance of the cells.

Attempts at prolonging the life of the cultures by the addition of fresh plasma, or by transferring the pieces of tissue to fresh plasma, were uniformly unsuccessful. This result may be contrasted with the success of subcultures in guinea pig plasma, where sarcoma cells remained active for at least thirty days. It is conceivable that in the case of pigeons, the theories of Ehrlich and Ribbert in regard to food specificity might find ready application, although it would be easy to advance some other explanation for the phenomena observed.
Cultivation of Tissue in Plasma from Alien Species.

Human Plasma.—The most striking and constant phenomenon noted in the use of human plasma is the progressive liquefaction of the fibrin, which is practically complete after six or seven days. In spite of the loss of framework, there is an active migration of cells along the cover glass. The cells often wander to the very edge of the medium. As a rule, they move out separately, but we have observed in several preparations of rat spleen radial outgrowths simulating the growth in homologous plasma where the fibrin network is usually well preserved. In these cells attached to the cover glass, the most interesting changes have been noted, especially in the cultivation of rat spleen. After four or five days cells of extraordinary size begin to appear. The rapidity of the increase in size is quite remarkable; a cell thirty to forty microns in diameter may reach 500 microns in seventy-two hours. Such a giant cell examined in the fresh state shows about its center a clear nuclear area frequently presenting knob-like prominences, surrounded by a highly granular zone, which in turn merges into a clear, filmy indefinite protoplasm with processes. In their entire extent, these cells measure 100 to 700 microns in diameter. When stained (figure 3), the knob-like prominences are seen to be nuclei with distinct nuclear membranes and prominent nucleoli. These nuclei are often seen in close apposition arranged about a light, hematoxylin staining, reticular or vacuolated area. When fresh, this portion of the cell has the appearance of a large, single nucleus. The granules, which are not different from those seen in the cells of ordinary size, react to all the stains for neutral fat. The majority of the granules are small and of fairly uniform size, but occasionally large droplets are seen. The arrangement of the granules about the nuclear area is quite striking. In several cells they seemed to radiate outward in straight lines. The greater part of the protoplasm is, as a rule, quite free from granules.

Rather large, multinucleated cells have been observed in the growth of sarcoma and carcinoma in rat plasma, but in no instance did they simulate those just described. Pieces of rat spleen or tumor, when transferred to rat plasma after five or six days in human plasma, showed marked activity. Giant cells of the type described above were sometimes noted after the transfer. In fact, our first ob-
Robert A. Lambert and Frederic M. Hanes.

The survival of such cells was made in a preparation of rat sarcoma in which the tissue had been transferred to rat plasma after five days of apparent inactivity in human plasma.

This formation of giant cells evidently represents unsuccessful attempts at cell division. With a method whereby continuous observation of any cell is practicable, the possibility of formation through a fusion of small cells is easily eliminated. It is conceivable that an injury not severe enough to kill or prevent growth may, through some derangement of the cellular mechanism, induce such a monster formation. Experimental studies with protozoa afford support for such a conception. Moreover, we have found that human sera are lytic for rat corpuscles, and, as we shall point out in a later paper, a close parallelism has been shown to exist between the hemolytic and toxic action of alien sera. Furthermore, the ragged, mossy appearance of the cells in pieces of sarcoma transferred to rat plasma after five or six days’ sojourn in human plasma is also indicative of a cell injury (figure 4).

In reviewing the above experiments, it is seen that goat plasma is the only one in which no growth whatever was observed. We see further that plasma from those species most closely related zoologically to rats, that is, guinea pigs and mice, affords the best medium for the growth of rat sarcoma. This parallelism, however, does not extend through the remaining species employed. For example, the growth of rat sarcoma is more vigorous and of longer duration in pigeon plasma than in dog plasma. Again, human plasma is distinctly less toxic than goat plasma for the tissues used. The relation of normally existing lysins in alien sera to the suitability of the corresponding plasma as a culture medium for foreign cells will be discussed in a subsequent paper.

CONCLUSIONS.

1. Rat sarcoma may be cultivated in mouse plasma and guinea pig plasma, and the growth differs only in extent from that observed in rat plasma. The cells may show active wandering in guinea pig plasma after thirty days, if transferred at proper intervals to fresh medium.

2. Rabbit plasma is less suitable than that of guinea pigs and
Cultivation of Tissue in Plasma from Alien Species.

mice for the growth of rat sarcoma; growth is slow, but it may continue for twelve days.

3. The duration of growth of rat sarcoma in dog plasma is from two to three days.

4. The duration of the growth of rat sarcoma in pigeon plasma is four to five days. Transferring the tissue to fresh pigeon plasma does not lengthen the period of activity.

5. No growth whatever is observed of mouse and rat tissues in goat plasma. Studies of the fate of the cells indicate the presence in goat serum of a substance toxic for these tissues.

6. In preparations of rat sarcoma in human plasma, liquefaction of fibrin is regularly observed. The phenomena of growth consist in an outwandering of cells along the cover glass, and, after four to six days, the formation of giant cells. Such giant cells are produced in larger number in the cultivation of rat spleen.

7. The degree of suitability of the different kinds of alien plasma used as culture media for mouse and rat tissues does not go hand in hand with the closeness of relationship of the species.

8. Rat spleen may be cultivated as readily in foreign plasma as the virulent transplantable tumors.

EXPLANATION OF PLATES.

PLATE 13.

Fig. 1. Mouse carcinoma after three days growth in guinea pig plasma. The characteristic sheet-like extension of the cells should be observed; those on the border present protoplasmic processes. At one point, the cells have grown into the fibrin meshwork in a definite alveolar fashion. Four mitotic figures are to be seen.

Fig. 2. Rat sarcoma after three days growth in pigeon plasma, showing the independent spreading of the cells regularly observed in the cultivation of sarcoma. Two mitotic figures can be seen.

PLATE 14.

Fig. 3. Cells from a five day preparation of rat spleen in human plasma, stained with hematoxylin and Sudan III. Two multinuclear giant cells are seen, and two small cells of the size ordinarily observed in cultures. In the giant cells, fat granules are numerous and of rather large size.

PLATE 15.

Fig. 4. Specimen of rat sarcoma transferred to rat plasma after six days sojourn in human plasma. The tissue was fixed on the fifth day of growth in homologous plasma. The spider-like appearance of the cells is shown.