TECHNIQUE OF CULTIVATING HUMAN TISSUES IN VITRO.

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In 1910 Carrel and Burrows[1] reported the successful cultivation *in vitro* of a human sarcoma. They used as a culture medium plasma from the patient from whom the tumor was removed, and observed an active migration of cells during several days' incubation. No subcultures were made. In subsequent attempts to cultivate human tissue Carrel[2] experienced considerable difficulty owing apparently to the liquefaction of the clotted plasma medium. He noted, as did also Lambert and Hanes,[3] Maccabruni,[4] and others, that within 24 hours a clear liquefaction zone appears around each tissue fragment, and that after a few days the entire fibrin clot becomes liquefied. Unless the cells wander out early they find no framework upon which to grow.

Losee and Ebeling[5, 6] attempted various modifications of human plasma with the object of preventing liquefaction, but were unsuccessful. However, by diluting the plasma with Ringer's solution, which seemed to delay digestion, and by making transfers of the tissue fragment to fresh plasma every 24 to 48 hours, they were able in a few instances to propagate human connective tissue cells obtained from fetuses through a number of subcultures, in one case as long as 60 days. They attributed their success in part to the addition of tissue extracts to the medium, as recommended by Carrel.[7] They emphasized the shortcomings of

[1] Carrel, A., and Burrows, M. T., Human Sarcoma Cultivated outside of the Body, *J. Am. Med. Assn.*, 1910, lv, 1732.

[2] Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

[3] Lambert, R. A., and Hanes, F. M., The Cultivation of Tissue in Plasma from Alien Species, *J. Exp. Med.*, 1911, xiv, 129.

[4] Maccabruni, F., Esperienzi di coltivazioni "in vitro" del cancro uterino umano, *Ann. ostet. e ginecol.*, 1914, xxxvi, pt. i, 57.

[5] Losee, J. R., and Ebeling, A. H., The Cultivation of Human Tissue *in Vitro*, *J. Exp. Med.*, 1914, xix, 593.

[6] Losee and Ebeling, The Cultivation of Human Sarcomatous Tissue *in Vitro*, *J. Exp. Med.*, 1914, xx, 140.

[7] Carrel, Artificial Activation of the Growth *in Vitro* of Connective Tissue, *J. Exp. Med.*, 1913, xvii, 14.

their technique and stated that they experienced many failures, for which the early rapid liquefaction of the fibrin was held responsible.

In our first efforts to cultivate human tissues in which unmodified human plasma was used as a culture medium, the results were practically the same as those of Carrel, and Losee and Ebeling. In only exceptional cases were we able to obtain satisfactory growths. There was regularly rapid digestion of the fibrin about the pieces of tissue, thus completely blocking cell migration. We therefore undertook to overcome this difficulty. It may be noted here that it is not the ability of human tissue to digest fibrin, but rather the susceptibility of human fibrin to digestion which is the basis of the trouble, for we found that human tissue did not liquefy the fibrin of foreign plasmas, while it was observed that human fibrin was readily digested by the ferments of practically every foreign tissue.

In the cultivation of the tissues of a number of lower animals,—rats, mice, guinea pigs, rabbits, dogs, cats, fowls, and pigeons,—using the plasma from these and other species, liquefaction was not infrequently observed, though never so regularly or extensively as in cultures containing human plasma. It was noted, moreover, that fowl and pigeon plasma were never liquefied, except in the presence of particular types of bacteria. Since earlier experiments had shown that tissues from certain of the lower animals could be cultivated in plasma from foreign species,[3] it occurred to us that this property of fowl and pigeon plasma to resist digestion might be utilized in growing human tissues. It seemed possible that chick fibrin might be made to serve as the framework of the culture medium with human serum or plasma added to supply the necessary nutritive substances. A medium was therefore prepared by mixing a small quantity of chick plasma with a considerably larger quantity of human serum. Various human tissues (lymph gland, spleen, skin, etc.) obtained at operation were put up in this mixed medium. No liquefaction was observed, and active progressive growth with numerous mitotic figures was seen in the majority of the preparations where soft friable tissue was used. Tissues removed at autopsy several hours after death were cultivated, the preparations showing in some instances a very active growth of connective tissue cells. It was not necessary

to make transfers to fresh plasma oftener than every 5 days. Continued propagation through subcultures was carried on for several months with as little difficulty as in the cultivation of similar tissues from lower animals. It may be noted that human tissues will grow in pure fowl plasma, but growth is not so active or so prolonged as in the modified medium containing human serum. Various proportions of fowl plasma and human serum were tested with the object of working out an optimum medium. A mixture of chick plasma, one part, and human serum, four parts, seemed to yield the best growth. The use of a relatively small amount of plasma has also an economic advantage in that human serum may obviously be obtained more easily and in larger quantity than fowl plasma. Furthermore, serum is easily preserved, whereas plasma sometimes clots after a few hours, even when kept cold in paraffined receptacles. It may be suggested that a still smaller proportion of plasma can be used since one part of plasma to twenty of serum will form a fairly firm clot.

The human serum used in these preliminary experiments came from a single individual. Autogenous plasma was not used since studies upon the cultivation of the tissues of lower animals had shown that plasma from the animal supplying the tissue was no better as a culture medium than plasma obtained from other members of the same species. For example, it has been found that the cells of transplantable mouse and rat tumors would grow quite as well in the plasma of rats and mice artificially or naturally immune to these tumors as in the plasma of tumor-bearing animals.[8] It seemed conceivable, however, that human tissues might prove more sensitive to individual variations in the culture medium. While studying this question it occurred to us that the influence of naturally existing iso-antibodies (agglutinins and hemolysins) upon growth *in vitro* might be investigated at the same time.

The interagglutinating reactions of human bloods, due, as Landsteiner and Leiner[9] and others have shown, to the existence of two

[8] Lambert and Hanes, A Study of Cancer Immunity by the Method of Cultivating Tissues outside the Body, *J. Exp. Med.*, 1911, xiii, 505.

[9] Landsteiner, K., and Leiner, K., Ueber Isolysine und Isoagglutinine im menschlichen Blut, *Centr. Bakteriol., Ite Abt., Orig.*, 1905, xxxviii, 548.

agglutinins and two agglutinogens in four different combinations[10] are well known. By simple agglutination tests any individual is found to fall into one of four groups depending on the power of his serum to agglutinate the blood of certain other persons, and on the susceptibility of his blood to agglutination; that is, on the presence or absence of one or both of the two agglutinins and agglutinogens.

For our experiments serum was obtained from individuals belonging to each of the four groups, and from the patients whose tissues were used for cultivation. The following protocol is illustrative of the results obtained.

Experiment 210.—An axillary lymph gland, removed at operation and showing microscopically changes characteristic of Hodgkin's disease, was placed in a sterile dish of cold salt solution. 1 hour later tissue cultures were put up in each of the following sera, to which chick plasma was added in the proportion of one part of plasma to four of serum: Group I, Group II, Group III, Group IV, and patient's serum (Group II). Ten cultures of each series were prepared and examined daily during the following week of incubation. Within 24 hours active migration of large mononuclear wandering cells and leukocytes was noted in practically every culture. On the 2nd and 3rd days a beginning outgrowth of connective tissue cells was observed, reaching a maximum activity about the 5th day. At no time could any difference in the five series be detected. The usual variations in individual cultures were seen, but the average extent of outgrowth was practically the same for each series. There was likewise no difference in the frequency of mitotic figures, which can be readily recognized by the practised observer in the living unstained preparations.

Other experiments in which tissues from Group I and Group III individuals were used gave the same result. While we were unable to secure any tissue from a person belonging to Group IV, as such

[10] It is assumed that there exist in human sera two agglutinins, *a* and *b*, and two corresponding agglutinogens, *A* and *B*. The blood of Group I possesses both agglutinins but no agglutinogens. It therefore agglutinates the blood of all other groups, but is not agglutinated by any serum. Group II possesses agglutinin *a* and agglutinogen *B*. It agglutinates blood of Group III and Group IV, and is agglutinated by members of Group I and Group III. Group III, the reciprocal of Group II, possessing agglutinin *b* and agglutinogen *A*, is agglutinated by Group I and Group II sera, and agglutinates the corpuscles of the third and fourth groups. Group IV contains no agglutinin but has both agglutinogens. Its serum therefore has no agglutinating power, but its corpuscles are agglutinated by the sera of all other groups.

people are rare, we feel justified in concluding that normally existing iso-antibodies in human blood do not influence in any way the growth of human tissues *in vitro.*

In addition to the question of culture medium we have been concerned with another problem in connection with the technique of cultivating human tissues *in vitro;* namely, the preservation of tissues after removal from the body. It is often not convenient to prepare cultures immediately upon the receipt of the tissue from the operating room or autopsy table. It is also desirable in many cases to carry out a number of experiments with the same tissue, not possible in a few hours' time. Furthermore, the duration of life of tissues in cold storage is a question of biological interest as well as of practical importance. In order to determine the period of survival under conditions of ordinary ice box preservation, cultures of the tissue were made on the day of removal, and at 2 day intervals thereafter, up to 12 days. A type experiment is described in the following protocol.

Experiment 320.—A piece of small tumor removed surgically from the region of the parotid gland (microscopically, a typical mixed tumor) was placed immediately in the ice box in a dish of physiological salt solution (0.8 per cent). Half an hour later the tumor, which was composed of soft friable tissue, rather gelatinous in places, was cut into small pieces. From some of these, cultures were made immediately. The remainder, covered with salt solution in a Petri dish, were returned to the ice box, an ordinary, small wooden structure with a lid, the temperature of which fluctuated between 10° and 15°C. The ten cultures put up on the day of operation grew well, showing after 2 to 3 days' incubation numerous large cells of irregular shape with frequent mitotic figures. Cultures made on the 2nd and 4th days of preservation, showed similar activity, though in the case of the tissue preserved for the longer time there was a longer latent period before migration was observed. Tissue kept for 6 days showed growth in the majority of preparations, though not so good as those put up on the 4th day. In the 8 day series only a few active cells were seen. Preparations of tissue kept for 10 and 12 days were all negative.

In another experiment in which a piece of lymph gland was used, no cells survived longer than 6 days. A glioma showed some growth after 8 days' preservation, but none after 10 days.

These experiments show that under very simple conditions of preservation human connective tissue cells and the cells of certain benign

tumors remain alive in the ice box for 6 to 8 days at least. 10 days probably represents the limit of survival. The period of survival is approximately the same as that determined for normal rat connective tissue, though somewhat shorter than that for embryonic chick tissues, which previous experiments have shown live 8 to 16 days in storage.[11] The optimum temperature for chick tissues was found to be about 6°C. The optimum temperature for preserving human tissues was not determined.

CONCLUSIONS.

1. Unmodified human plasma is not a satisfactory culture medium for human tissues owing to the susceptibility of human fibrin to digestion by tissue ferments. The necessary framework is thus destroyed before the cells begin to migrate. The difficulty can be overcome by adding to human plasma or serum a small quantity of fowl or pigeon plasma, the fibrin of which is highly resistant to digestion. Human tissues have been propagated in this medium for several months through subcultures, and growth *in vitro* can probably be maintained indefinitely.

2. Human tissues show no greater sensitiveness to changes in temperature and mechanical injury associated with preparation of cultures than those of lower animals. They may be preserved in an ordinary ice box at 10–15°C. as long as 6 or 8 days. Tissues obtained at operation give best results, but pieces of organs removed at autopsy 1 to 4 hours after death sometimes show active growth.

3. The presence of normally existing iso-antibodies (agglutinins and hemolysins) in human serum is without influence on the growth of human tissues *in vitro*. In other words, autogenous serum has no advantage in tissue cultures over homologous serum.

For the agglutination tests carried out in connection with the experiments reported, I am indebted to Miss M. P. Olmstead of the bacteriological laboratory of the Presbyterian Hospital.

[11] Lambert, R. A., The Influence of Temperature and Fluid Medium on the Survival of Embryonic Tissues *in Vitro*, *J. Exp. Med.*, 1913, xviii, 406.