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GROWTH OF MACROPHAGES OBTAINED FROM VARIOUS SOURCES

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In the past, studies on the growth of *Mycobacterium leprae* in cultures of macrophages have been unsuccessful. The macrophages used were generally obtained from mouse perionteal exudate or human blood leukocytes, which showed little multiplication in the cultures. Recently, techniques were developed in this laboratory to grow macrophages obtained from various sources of tissues of the mouse. These macrophages showed slow but regular multiplication in cultures. This is desirable in order to keep the viable cells for a long period of time. It is hoped that these techniques could be adapted to grow the macrophages of humans or armadillos from tissue specimens obtained by surgery, biopsy or necropsy. The methods for growing macrophages from various sources of the mouse as well as the human blood leukocytes are reported here.

Materials and Methods

The general purpose strain of NIH Swiss white mice was used. Large animals, preferably the ex-breeders were used to obtain macrophages from the blood, peritoneal exudate, alveolar lavage and subcutaneous cover slip implantation. Young mice over 4 weeks old were used to obtain macrophages from other sources.

The culture medium consisted of horse serum 40%; medium NCTC 135, 50% (both from Flow Laboratories, Rockville, Md.); bovine embryo extract (Grand Island Biological Co., Grand Island, N. J.), 1:5 dilution in medium NCTC 135, 10%; penicillin, 1 unit/ml; and heparin, 1:20,000.

Cell suspensions were added in 1 ml aliquots to either Leighton tubes (Bellco Glass, Inc., Vineland, N. J.) or the Sight culture chamber (Sight Instruments, Long Beach, Calif.). Renewal of medium (omitting heparin) was made twice a week.

Motion picture study of macrophage activity was made using the Sight culture chamber which contained 1 ml of cell suspension and 3 ml of air. A #19 gauge needle was permanently inserted into the chamber to allow adequate exchange of CO_2 and oxygen between the culture medium and the

atmosphere in the microscope incubator. This arrangement permitted continuous cinemicrography for long periods of time with only a limited amount of effort in handling.

Macrophage suspensions were placed either directly into the culture chamber or first grown on cover slips in Leighton tubes then placed in the chamber. Cultures were kept at 37° C in a humidified incubator with a constant flow of 5% CO_2-air mixture. Multiple culture chambers containing macrophages obtained from various sources were kept in the incubator. Motion pictures of macrophage activities were taken either by filming a single chamber for a long period of time or by filming individual chambers each for a short duration. Details of the techniques for cinemicrography have been reported elsewhere (1).

Macrophages Obtained from the Mouse.

Peritoneal lavage. Female ex-breeders with large abdominal cavities were used. The unstimulated peritoneal exudate was lavaged with 6 ml culture medium. The cell suspension was placed into the culture chamber. The non-adherent cells were washed out next day and replaced with 1 ml of fresh medium without heparin (2).

Bone marrow. Using a 3 ml syringe and 25 gauge needle, the marrow of tibia and febula was flushed with 5 ml of culture medium. Single cell suspension was made by several aspirations of the cell suspension through the needle (3).

Alveolar macrophages. A piece of polyethylene tubing (I.D., .034" X O.D., .06", Clay Adams, Parsippany, N. J.) was attached to a 22 gauge needle and a 3 ml syringe. The tubing was introduced into the trachea. 2 ml of culture medium was introduced into the trachea and about 1.5 ml of cell suspension was withdrawn.

Spleen, thymus, lymph nodes (mesenteric), lungs and liver. The organs were minced by cutting the tissues with two #11 blades crossing each other in a scissor-like motion. The cells were suspended in culture medium and single cell suspension was made by repeated aspiration through a 25 gauge needle.

Blood. The animal was anesthetized by intraperitoneal injection of sodium pentobarbitol, 0.7 mg per 10 g body weight. Blood was collected from the axillary blood vessels. Haparin was added to prevent coagulation. 3 ml of 6% dextran (M.W. 234,000, Sigma Chemical Co., St. Louis, Mo.) in medium NCTC 135 was added to each 5 ml blood. The mixture was placed in

an inverted syringe with a 22 gauge needle attached to a piece of polyethylene tubing. After the sedimentation of red blood cells was completed (at room temperature), the plasma was forced out of the syringe by pulling down the barrel of the syringe and collecting the plasma in a test tube. The plasma was centrifuged (International Centrifuge, Size II) at 1,000 RPM for 5 minutes. The supernatant was discarded and the pellet was washed once with medium NCTC 135 containing heparin. The pellet was then suspended in culture medium and dispersed into Leighton tubes or the Sight chamber.

Thyroid, kidney and heart muscle. Organ culture technique (4) was used for these sources. The tissues were cut into 1 mm^3 pieces and washed 3 times with culture medium. 3 or 4 pieces were placed directly on the grid of organ culture dishes (Falcon Plastics, Los Angeles, Calif.) containing an 8 x 11 mm cover slip. The center well was filled to the level of the grid with culture medium. The absorbent disk in the outer wall was filled with distilled water to prevent medium evaporation.

Subcutaneous cover glass implantation. An 8 x 11 mm cover glass was implanted subcutaneously on the back of mouse under pentobarbitol anesthesia. After 7 days the cover glass was removed and cultivated in Leighton tubes or in the Sight chamber.

Macrophages obtained from human blood. 50 ml of heparinized blood was obtained by venopuncture. The blood was placed in an inverted 50 ml plastic syringe and sedimented at room temperature for about 3 hours. The syringe was attached to a needle and a piece of polyethylene tubing. After the sedimentation of red blood cells was completed the plasma was collected in test tubes, as before. Without further treatment the plasma was placed, 1 ml each, in Leighton tubes or the Sight chamber.

The culture medium consisted of pooled human serum, 30%; McCoy's 5A medium with supplements (5), 60%; bovine embryo extract, 1:5, 10%; and penicillin, 1 unit/ml. Renewal of medium was made twice weekly.

Aseptic technique was used for all the above schedules.

Results

Macrophages obtained from the mouse. Peritoneal macrophages were the easiest cells to maintain in the cultures. Monolayers of macrophages were generally observed on the next day of cultivation. They remained in good condition for many weeks in cultures (2).

Bone marrow and spleen. The number of macrophages were sparse at the beginning. They increased markedly during the first few days of cultivation and covered the entire culture surface in 5 to 7 days. Growth of granulocytic cells was observed in both of these cultures during the first 2 or 3 weeks. Megakaryocytes were present in bone marrow cultures for about 2 weeks (3).

Blood macrophages were observed first as scattered, small, slender cells a few days after cultivation. The number of macrophages increased steadily, and the cultures were full of mature macrophages in 2 to 3 weeks. Occasionally colonies of granulocytes and lymphocytes were observed during the first 2 or 3 weeks.

Macrophages of the thymus, lungs and lymph nodes began to appear after a few days cultivation. Good growth of macrophages was observed later on continued cultivation. Alveolar lavage gave various numbers of cells in the washings. Good growth of macrophages was observed in harvests having a large number of alveolar cells. Liver macrophages were very scanty at beginning, but gradually filled up the culture.

In organ cultures, cells shed from the pieces on the grid consisted of macrophages, lymphocytes, fibroblasts and the cell type of the particular organ. Shedding of macrophages began at the beginning and continued for many days in the cultures. Cover slips covered with many macrophages were selected for motion picture studies. Good growth of macrophages was observed in all the 3 organs studied, i.e., thyroid, kidney and cardiac muscle.

Cover glasses implanted under the skin usually revealed patches of macrophages when the slips were transferred to the Leighton tubes or the Sight chamber. Good growth of macrophages was observed on continued cultivation.

Good growth of macrophages was observed in motion pictures filmed from all the sources mentioned above. The time lengths of sustained good growth for each source of macrophages is shown in Table 1.

Increase in the number of macrophages was observed in all the cultures studied with the exception of the peritoneal exudate cells. In most cultures macrophages were usually sparse at the beginning. The number increased steadily on continued cultivation and a monolayer of macrophages eventually filled up the whole culture. The pattern of peritoneal macrophages was different. A good monolayer of macrophages

was always obtained at the beginning. When the cells became larger on cultivation many of them were crowded out on account of lack of space, and the cell population showed a false decrease.

Cell division was not observed in the peritoneal macrophages even in a continued cinemicrography which lasted for more than 50 days. Mitosis, however, were recorded in the motion pictures for many sources of mouse cell cultures, i.e., bone marrow, blood leucocytes, alveolar lavage, subcutaneous implants, spleen, liver, thymus and lungs. The absence of cell division in the cultures of other sources, i.e., lymph nodes, thyroid, kidney, heart muscle of the mouse and the blood leucocytes of human (Table 1) was probably due to insufficient footage of film for these cultures in the present study, since an increase in the number of macrophages was observed in all these cultures on continued cultivation.

There were two types of macrophages: the regular type which showed frequent cell division, and a very large type, measuring up to 500 μm in diameter, which showed cyclic accumulation of large fluid droplets followed by sudden bursting. Many features of macrophages were observed in the motion pictures, such as membrane ruffling, cell division, cell fusion, cell death, removal of debris, phagocytosis of dead cells, clasmatosis, mosaic and bleb formation and the rare worm-like configuration. Many of these features have been reported elsewhere (6).

Pure culture of macrophages was observed in cultures of mouse blood leucocytes. A few lymphocytes and granulocytes were observed at the beginning but disappeared in 2 or 3 weeks. Then the pure culture remained in good condition for many weeks.

An apparently pure culture of macrophages was obtained from peritoneal exudate cells. Although a few lymphocytes and mast cells appeared at the beginning they disappeared after a few days cultivation. Generally, fibroblasts did not show up whenever the macrophages occupied the whole culture space. A few scattered small patches of fibroblasts might appear on continued cultivation. They seldom spread to large areas in the cultures.

Marked growth of fibroblasts was observed, however, in cultures in which the number of macrophages was sparse at beginning. Patches of fibroblasts appeared over large areas in many cultures. Several methods for eliminating the fibroblasts were employed: (i) washing with cold medium NCTC 135; (ii) using sodium arsenite to kill fibroblasts selectively (7); (iii) selecting pure macrophage cultures among a number of Leighton

Source of macrophages	Method used	Time of sustained good growth filmed. days	Cell division observed in motion picture
Mouse source:			
Peritoneal exudate	Suspension	>100	0
Bone marrow	Suspension	80	+
Blodd	Suspension	>100	+
Alveolar lavage	Suspension	28	+
Subcutaneous implant	Implantation	68	+
Spleen	Mince	59	+
Liver	Mince	36	+
Thymus	Mince	51	+
Lungs	Mince	84	+
Lymph nodes	Mince	24	0
Thyroid	Organ	34	0
Kidney	Organ	34	0
Cardiac muscle	Organ	30	0
Human source:			
Blood	Suspension	35	0

Table 1. Time lapse photomicrographic studies on the growth of macrophages obtained from various sources.

tubes; and (iv) picking up the cover slips of the grid organ cultures after the period of fibroblast shedding was over. Each technique gave successful results in some instances, but not all.

Human blood macrophages. A few scattered cells appeared a few days after cultivation. The number of cells increased stadily and the whole culture was filled up in a period of about 10 days. The cells were small, slender and somewhat transparent at the beginning, becoming dark and active later on cultivation. One particular feature of human blood macrophages was the rapid extension and retraction of their processes in a "star-like" or "crab-like" manner, which lasted for many hours in the cultures.

Addition of hydrocortisone to the culture seemed to maintain the cells in good condition for a longer period of time. In control cultures, macrophages began to show deterioration after 3 or 4 weeks cultivation. With hydrocortisone, good growth was observed for at least 50 days in cultures (6).

Discussion

Success in the long-term cultivation of macrophages depended on two factors. The first was the rich culture medium which contained bovine embryo extract and a high content of horse serum. There are two commercial sources of bovine embryo extract; the DIFCO 2 ml vials and the Grand Island 10 ml bottles. Both are lyophilized, 50% extracts. Both were satisfactory for the growth of macrophages except that a short supply was often claimed by DIFCO. The horse serum from Microbiological Associates, Bethesda, Md. used to give satisfactory growth of macrophages until about 3 years ago. The mycoplasma and bacteriophage free horse serum of Flow Laboratories has been found highly satisfactory at the present time.

The second factor was the minimal amount of handling of the cell suspension. Cells were suspended directly in the culture medium. The cell suspension was immediately placed into culture vessels without the usual centrifugation and washing employed by most investigators. It has been known that leakage of cell contents has occurred when cells are exposed to the balanced salt solution or the simple synthetic media.

Our knowledge concerning the function of macrophages of various origins is limited. It is known that the lysozyme content of alveolar macrophages is much higher than that of the peritoneal macrophage. The large macrophages of hemopoietic organs have an intimate relationship

with the growth of various hemopoietic precursors (7). Whether there are other major differences among the macrophages of various organs is of paramount interest for the many disciplines of investigators at the present time.

Our knowledge of human macrophages is even more limited. The sources of human macrophages used for investigations are generally limited to blood leucocytes or skin windows. Our present study offers an opportunity to broaden the spectrum by adapting the techniques used in animals to obtain macrophages from various human tissues. The sources of materials are numerous, such as specimens obtained from biopsies, necropsies, and surgical operations of normal and pathological tissues.

Summary

Techniques to obtain macrophages from various sources of the mouse were reported. The following sources were included: peritoneal exudate, alveolar lavage, blood leucocytes, bone marrow, spleen, liver, lungs, lymph nodes, thymus, thyroid, heart muscle, kidney, and subcutaneous cover glass implants. Human blood macrophages were also included. Long-term cinemicrographic studies revealed sustained good growth of these macrophages. Cell multiplication was detected in all of these cultures except thos obtained from the peritoneal exudate. Pure cultures of macrophages were obtained from blood of the mouse and human. Macrophages obtained from other sources were accompanied by some growth of fibroblasts. Methods to eliminate the fibroblasts in cultures were discussed.

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