The Cultivation of Mouse Bone Marrow in Vivo

By IRWIN BERMAN AND HENRY S. KAPLAN

ALTHOUGH BONE MARROW CELLS have been cultivated by traditional tissue culture technics, they tend to lose their morphologic identity and to cease differentiating in cultures maintained for long periods of time. The morphologic changes which cultures of blood and bone marrow cells undergo when cultivated on glass substrates have been reviewed by Bloom and most recently described for human bone marrow by L. Berman and Woodliff.

Another approach to the cultivation of mammalian tissues has been the system devised by Algire and his co-workers, which combines both in vitro and in vivo conditions. The method described by Algire et al. entails the placement of the tissue into a diffusion chamber which is then implanted into the peritoneal cavity of a host animal. Several mammalian tissues and various cellular types have been shown to maintain both their growth and functional characteristics when cultivated in this fashion. To our knowledge, however, no reports have yet appeared indicating the feasibility of using the diffusion chamber technic for the cultivation of hemopoietic cells. It was for this reason that the present study using diffusion chambers was undertaken.

Since no attempt was made to quantitate the growth of cells in these experiments, the material presented in this paper is concerned mainly with the technics used for the cultivation of normal mouse bone marrow in diffusion chambers, and with the morphologic appearance of the marrow cells at serial intervals after implantation. The quantitation of cellular growth within the diffusion chamber and the ability of mouse bone marrow to function as bone marrow after cultivation in vivo will be the subject of a future report.

METHODS

A slight modification of the diffusion chamber described by Algire was used in these experiments. Chambers were constructed of Plexiglas rings (0.75 inch outside diameter, 0.5 inch inside diameter, 1 16 inch thick) which served as supports for millipore filter membranes. The membranes (150 microns thick, average pore diameter 0.45 microns) were sealed to the rings by a suspension of approximately 1 per cent lucite in acetone.

Femoral bone marrow obtained from male and female donor mice of strain C57BL...
was suspended in Locke’s solution and concentrated to provide an inoculum of approximately 3 to 5 × 10⁴ cells per chamber. Host mice (male or female, corresponding to the sex of the donor animal) were anesthetized with ether. The chambers were placed in the peritoneal cavity through a small incision on the midventral surface of the animal. At various times after implantation, the chambers were removed and their outer surfaces cleared of adherent host cells. The membranes were then cut from the rings, fixed in Bouin’s fluid or Zenker’s formol and stained with hematoxylin and eosin (H & E). In one experiment imprints of membranes were made, fixed in absolute methyl alcohol and stained with May-Grunwald-Giemsa stain. Occasionally membranes were treated with 0.25 per cent trypsin or 0.1 ml. hyaluronidase* at room temperature for 15 minutes prior to staining. This treatment aids in the dissolution of a clot-like structure which frequently is found in early cultures and clears the membrane of intercellular substances which tend to obscure the stained cells. The use of the enzymes does not alter either the morphology or susceptibility to staining of the cells on the membrane.

Observations

In general, few changes in the morphology of the implanted bone marrow cells could be detected in chambers removed after 3 days in the peritoneal cavity (fig. 1). All cell types known to occur in normal bone marrow were found, although the more mature leukocytic (myeloid) elements appeared to predominate. Scattered throughout the membrane were many small round pyknotic nuclei typical of orthochromatic normoblasts. Lymphocytes, monocytes and megakaryocytes could be readily identified. Considerable numbers of blast cells were observed, but these could not be further identified, owing to the lack of differentiation with the H & E stain. Numerous mitotic figures could be observed.

The majority of normal bone marrow elements observed at three days were still present in chambers removed seven days after implantation (figs. 2 and 3). Mitotic activity as well as differentiation to mature cellular forms was strikingly evident (fig. 2). Although the H & E stain made it difficult to determine in which direction the blast cells were differentiating, the group of cells shown in figure 2 appear to be of the myeloid type. Many mature myeloid cells were observed, as were cells which appeared to be lymphocytes. An occasional megakaryocyte was found, but precise identification of erythroid precursors was difficult. Considerable numbers of cells resembling histiocytes could be identified in 7 day cultures.

To circumvent the limitations imposed by the use of H & E stains, an imprint of a membrane, removed 20 days after cultivation, was stained by the May-Grunwald-Giemsa method (fig. 4). Using this technic, cells of the erythroid series were readily identified. Proerythroblasts, as well as intermediate and late normoblasts, were found in 20 day old cultures (fig. 4). Also present at 20 days were lymphocytes, eosinophils and many primitive cell forms. All states of myelopoiesis could be seen, as well as mitotic figures in cells of both the erythroid and myeloid series. Although the imprint method permitted better identification of specific cell types, it did not give an accurate picture of the relative abundance of various cell types growing on the membranes. On other chambers removed 20 days after implantation there

*Varidase (Wyeth)—300 units per ml.
appeared to be an increase in the numbers of histiocytic elements. Preliminary evidence indicates that the majority of these cells are capable of phagocytosis of trypan blue. Although lymphoid and erythroid elements were present in 30 day cultures, the predominant cell type was the myeloid. These cells ranged in maturity from promyelocytic to segmented forms, indicating that myeloid differentiation is still taking place at 20 to 25 days (fig. 5). Megakaryocytes have not been observed in 20 day old cultures.
Fig. 5 (top, left).—Bone marrow 25 days after cultivation in vivo. Persistence of immature myeloid cells. Mononuclear cells in background are histiocytes.

Fig. 6 (top, right).—Bone marrow 43 days after cultivation in vivo. Predominant myeloid cell type is the mature segmented form.

Fig. 7 (bottom, left).—A sinusoidal-like network of cells which was observed on a membrane removed 43 days after cultivation in vivo.

Fig. 8 (bottom, right).—Bone marrow cells 76 days after cultivation in vivo. Mature myeloid cells can be observed. The predominant cell type is the histiocyte, two of which appear to be in the prophase stage of mitosis.

By 43 days, however, the character of the cultures had changed appreciably (fig. 6). The most obvious change was the relative increase in numbers of mature myeloid cells as compared to their precursors. Few promyelocytes and myelocytes were evident, and more immature forms were rarely encountered. There also appeared to be a relative increase of the histiocytes. Erythroid and lymphoid cells were found infrequently. Occasionally a cellular network has been noted which appears sinusoidal in character. Figure 7 shows an example of such a network found in a chamber removed 43 days after implantation. The cells which make up this network are morphologically distinct from the histiocytes, and resemble those cells which comprise the endothelial lining of blood vessels. Whether these are true endothelial cells derived from the implanted bone marrow or a metaplastic derivative of other cell forms is at present unknown. However, within the spaces of the sinusoidal-like structures are found various cell types characteristic of 43 day old cultures.
Fig. 9 (left).—Bone marrow cells 215 days after cultivation in vivo. Mature myeloid cells are present, and two forms of fibroblast-like cells are also shown.

Fig. 10 (right).—An area of fat cells found on a membrane removed 215 days after cultivation in vivo.

Cells typical of 76 day old cultures are shown in figure 8. The majority of cells found at this time are of the histiocytic series. While mature (segmented) myeloid cells are present, their precursors are rarely encountered. Lymphocyte-like and erythroid elements are still found at 76 days, but not as readily as in earlier cultures. In these cultures, and in those removed at later times, numerous prophase mitotic figures are observed in what appear to be cells of the histiocytic type. Metaphase, anaphase or telophase stages have rarely been observed.

While the majority of cells found on membranes removed 215 days after implantation are of the histiocytic type, other cell forms are still present. Figure 9 shows an area of a membrane in which segmented myeloid cells were observed, and figure 10 an area of the same membrane in which a heavy growth of fat cells was found.

**DISCUSSION**

The use of the diffusion chamber technic as a means of cultivating normal bone marrow cells appears to offer some biologic advantages over traditional tissue culture methods. Mitotic proliferation and differentiation into mature forms persist in at least two major hemopoietic cell lines for a considerable period of time. All stages of erythroid maturation have been observed after as long as 20 days' cultivation in the peritoneal cavity, and maturing myeloid cells may still be found in chambers at much later times.

These results are in contrast to those of L. Berman and Woodliff, who were unable to observe the actual differentiation of myeloid or erythroid elements of normal and neoplastic human marrow cells when cultivated in a strictly in vitro system. The persistence of distinct monocytes in chambers removed 20 days after implantation, as well as the paucity of giant cell formation in all chambers, is also in contrast to the results of Goldstein, and
others who have observed the transformation of monocytes to giant cells within a short time in cellophane cultures of human buffy coat material. The fact that lymphocytes can be demonstrated in chambers removed after 20 days in the peritoneal cavity suggests that the diffusion chamber technic may be suitable for at least the short-term cultivation of these elements. Work now in progress in which imprints are being made from chambers cultivated for more than 20 days should give a more accurate estimate of the length of time that lymphocytes and erythroid forms persist in the chambers.

Why all cell forms of the marrow do not persist indefinitely in the chambers is at present unknown. All chambers upon removal from the peritoneal cavity have a coating of host cells on the outer surfaces of the membranes. With time this coating becomes more than unicellular in thickness. In many instances chambers which have been in the peritoneal cavity for long periods have been found to be surrounded by a heavy coating of tissue reminiscent of an inflammatory reaction or infection. All such chambers have been found to contain no cellular growth. Sterilization of the membranes and various components of the chambers has not prevented the adherence of host cells or the formation of this thick coating. The growth of the host's cells on the outer surfaces of the chambers may restrict nutrient diffusion across the membranes. Under such conditions the growth within the chamber of a specific cell type whose nutrient requirements are less than other cell types would be selectively favored. This may account for the appearance of increasing numbers of histiocytic elements in older cultures. That the histiocytes may represent a transformation from other cellular forms present in the chambers at the time of implantation is also a possibility. It is interesting to note, however, that chambers removed 215 days after cultivation are still somewhat pleomorphic in nature and contain mitotically active elements. To date no chamber has contained a single morphologic form to the complete exclusion of other cell types. Furthermore, no evidence has been obtained indicating that a transformation of the implanted bone marrow cells to "fibroblast-like" or "epithelial-like" cell types takes place in the chambers, as has been reported for human marrow when cultivated on a glass substrate.

**Summary**

The cultivation of normal mouse bone marrow cells in diffusion chambers implanted into the peritoneal cavity of mice has been described. Mouse bone marrow cells cultivated by this method continue to undergo differentiation and maintain their morphologic identity for a considerable time.

**Summario in Interlingua**

Es describite le cultivation de normal cellulæs de medulla ossee murin in camaras de diffusion implantate in le cavitate peritonee de muses hospite. Cellulas de medulla ossee murin cultivate per iste metodo continua differentiare se e mantenir lor identitate morphologic durante un intervallo considerabile.
REFERENCES

The Cultivation of Mouse Bone Marrow in Vivo

IRWIN BERMAN and HENRY S. KAPLAN