Cellular Activities in Tissue Cultures of Leukemic Human Bone Marrow

By JOSEPH G. SINKOVICS, CLIFTON D. HOWE AND C. C. SHULLENBERGER

HEMATOPOIETIC CELLS maintained in vitro may reveal features that are undetectable in vivo. The purpose of this report is to describe some of the cellular activities observed with the cultural technic outlined below.

METHODS

Bone marrow specimens were obtained by aspiration from the sternum. Two to 3 ml. of aspirate were injected into a test tube containing 9 ml. of tissue culture medium and 1 ml. of heparin (Liquaemin heparin, Organon). Bone marrow particles were collected with a capillary pipette from the sediment and from the fatty surface layer. The bone marrow particles were transferred into a second and then into a third (and sometimes into a fourth) tube containing 5 ml. of medium each. The particles were then distributed on the coverslips in Leighton-tubes, or in Sykes-Moore chambers.1 Medium was added and the cultures were incubated at 37 C. The medium was first changed 24 hours later, and then at approximately 5 to 7 day intervals. Five to 12 Leighton-tubes were set up from one bone marrow specimen, each tube carrying 3 to 8 bone marrow particles. Coverslips were periodically removed, rapidly dried and stained with Wright stain.

The tissue culture medium consisted of Hanks balanced salt solution containing amino acids and vitamins (Eagle basal medium),2 excess glutamine (10 ml. 200 mM per 1000 ml. medium), and 0.5 per cent lactalbumine hydrolysate; 20 per cent calf serum was added. The medium contained 100 units or penicillin and 0.01 mg. streptomycin per ml. The pH preferred for Leighton-tube cultures was 7.4; for chamber cultures, 7.0.

Observations on living cultures maintained in Sykes-Moore chambers were made by high power magnification phase contrast microscopy. The bone marrow particles were grown between the coverslip and a perforated cellophane membrane. Certain cultures were grown also in T flasks. Morphological observations with approximately 200X magnification were possible. Bacteriological sterility was regularly checked and a few contaminated cultures were immediately discontinued.

RESULTS

Material Examined and Occurrence of Certain Morphologic and Biological Phenomena

Table 1 shows the number of bone marrow specimens grown in tissue culture, grouped according to the diagnosis of the patients. Certain patients contributed two specimens. The age of the patients ranged from adolescent to elderly. Except in a very few instances, the patients were receiving no chemotherapy at the time of bone marrow aspiration. “No hematologic disease” represents a group of patients with carcinoma of the breast, broncho-
Table 1.—Material Examined and Main Observations

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Observations</th>
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<tbody>
<tr>
<td></td>
<td>Number of</td>
</tr>
<tr>
<td></td>
<td>Specimens</td>
</tr>
<tr>
<td>Acute L (blastic)</td>
<td>6</td>
</tr>
<tr>
<td>Chronic lymphocytic L</td>
<td>14</td>
</tr>
<tr>
<td>Acute and chronic myelogenous L</td>
<td>5</td>
</tr>
<tr>
<td>Acute monocytic L</td>
<td>5</td>
</tr>
<tr>
<td>Acute myelomonocytic L</td>
<td>1</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>2</td>
</tr>
<tr>
<td>Malignant histiocytosis</td>
<td>2</td>
</tr>
<tr>
<td>Hodgkin's disease (generalized)</td>
<td>13</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>1</td>
</tr>
<tr>
<td>No hematological disease</td>
<td>7</td>
</tr>
<tr>
<td>Leukemoid reaction due to invasive carcinoma</td>
<td>1</td>
</tr>
</tbody>
</table>

L = leukemia.

*Round cells resembling small and large lymphocytes.

†Both types: see Discussion.

Genic carcinoma and carcinoma of the kidney without evidence of bone marrow invasion. All specimens listed yielded growth of fibroblast-like cells. Cytoplasmic inclusions and enlarged or deformed perinuclear zone were included under the heading "Observations" because these changes frequently were seen previously in tissue cultures of lymph node biopsy material obtained from patients with lymphoma and leukemia. The term "polycytes" is utilized for giant cells with more than four nuclei. "Persistence" means the survival of various identifiable immature hematopoietic cells in small groups or colonies in cultures older than 6 weeks. "Re-conversion" is used here to designate those exceptional cultures which at one advanced phase of growth appeared to be almost entirely composed of fibroblast-like cells, but in which certain cell groups later assumed the morphologic characteristics of undifferentiated hematopoietic cells.

General Characteristics of Human Bone Marrow Tissue Cultures

The growth pattern most commonly seen with the technic described can be visualized as follows. The bone marrow particle, herein referred to as explant, first attaches itself to the glass. Within hours a massive emigration of various immature hematopoietic elements takes place. These cellular elements surround the explant. At about 24 to 48 hours after explantation a large number of granulocytes may be seen at the outer margin of the emigrating cell mass. Closer to the explant, a large number of large lymphocytes and a few nests of erythropoietic cells may be seen. Megakaryocytes and platelets have not been identified. Different bone marrow particles, even from the same patient, released varying numbers of cells. Certain leukemic explants may release an enormous number of leukocytes (figs. 1, 2). Mitotic and amitotic divisions of the emigrating cells were seen in cultures from myelogenous leukemia and multiple myeloma. The erythroblasts disappeared from the cultures first; the granulocytic elements next. Presumably, as they
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age, they float away from the glass. Round (lymphoid) cells persisted much longer. The formation of elongated, fibroblast-like cells began between the third and seventh day. These cells originated from (1) the explant, and (2) from emigrating cells, described above as round (lymphoid) cells. The fibroblast-like cells often underwent mitosis. Cultures older than 4 weeks usually formed a sheet of fibroblast-like cells. The emigration of hematopoietic cells from the explant usually ceased by the second week of culturing; however, small numbers of lymphoid cells may be released from the explant for several weeks. Exceptionally, in old cultures, such lymphoid cells may exist between and inside fibroblast-like cells.

Examples of the Observations

Cytoplasmic inclusions, nuclear and nucleolar abnormalities, and enlarged or deformed perinuclear zone were seen less frequently in these human leukemic bone marrow tissue cultures than in tissue cultures of lymph node biopsy specimens from human leukemia and lymphoma.\textsuperscript{5} Certain fibroblast-like cells appeared to be invaded by groups of lymphocytes in bone marrow tissue cultures from chronic lymphocytic leukemia. Both small and large lymphocytes were seen inside other cells (figs. 3-5). Mononuclear cells were seen to lodge inside large fibroblast-like cells in cultures from acute monocytic leukemia (fig. 6). Immature granulocytes and plasma cells were not seen, as a rule, to enter other cells. The association between lymphoid cells and fibroblast-like cells eventuated in several ways:

1. A few (one to four) lymphocytes persisted inside fibroblast-like cells for 6 days without any morphologic evidences of injury either to the lymphocytes or to the invaded cell.

2. A few (one to four) lymphocytes appeared to be eliminated from the cytoplasm of a fibroblast-like cell, or converted into amorphous cytoplasmic inclusions, as seen by phase contrast microscopy.

3. Occasionally, a single lymphocyte deeply indented or entered the nucleus of a fibroblast-like cell. Such a lymphocyte was observed by phase contrast microscopy, with Dr. D. A. Dreyer, over a period of 8 hours (fig. 7). It is believed (but not as yet proved by cinematography) that intranuclear inclusions may result from such an association (fig. 8).

4. Occasionally, a single lymphoid cell was seen to undergo division inside the cytoplasm of a fibroblast-like cell (fig. 9).

5. Large clusters of lymphocytes were seen around and overlying certain fibroblast-like cells; it could not be determined whether the lymphocytes actually entered the cytoplasm of the fibroblast-like cells (fig. 10). Subsequent to the appearance of this phenomenon, the fibroblast-like cells were not seen to regain their morphological and functional integrity.

6. A large number (10 to 100) of lymphocytes at times occupied the cytoplasm of a fibroblast-like cell; the cytoplasm appeared to burst releasing the lymphocytes; the structure and tinctorial characteristics of the lymphocytes remained intact (figs. 11, 12).

7. A few (one to four) lymphocytes attached to or penetrated the cytoplasm of a fibroblast-like cell, which showed vacuolization at the points
Fig. 1.—Emigration of monocytes and lymphocytes from a bone marrow explant on the fourth day in vitro. Culture obtained from a patient with acute monocyctic leukemia. (Wright) X 150.

Fig. 2.—Emigration of immature granulocytes from a bone marrow explant on the second day in vitro. Culture obtained from a patient with acute myelogenous leukemia. Phase contrast, X 580.

Fig. 3.—Small and large lymphoid cells are seen around and inside the cytoplasm of two fibroblast-like cells in a 2 week old culture obtained from a patient with chronic lymphocytic leukemia. (Wright) X 290.
of attachment of lymphocytes and at other points distant from the attachment of lymphocytes (figs. 9, 13).

8. A large number (10 to 20) of lymphocytes entered the cytoplasm of a fibroblast-like cell which disintegrated; concurrently, some of the intracellular lymphocytes exhibited morphologic evidence of degeneration, both cytoplasmic and nuclear (figs. 14, 15).

In general, lymphocytic activity appeared to be directed toward certain fibroblast-like cells, while other fibroblast-like cells of the same culture were spared.

Cells with two or four nuclei were seen in almost every culture. They commonly occurred in tissue cultures from patients with multiple myeloma (fig. 16). Polyskaryocytes with more than 10 nuclei or true syncytia seldom developed. In one type of multinucleated giant cell, numerous nuclei of equal size were arranged at the periphery of the cytoplasm, while the center contained an amorphous eosinophilic inclusion (fig. 17); no intranuclear inclusions were seen. Syncytium formation was seen in a culture from monocytic leukemia (fig. 18); this culture did not show hemadsorption when washed human type O red blood cells were added (see Discussion). In another type of multinucleated giant cell, the nuclei remained in the center of the cell often forming a large nuclear mass. The formation of this type of giant cell was preceded by nuclear abnormalities such as budding and polyploid division. In one case, lymphocytes surrounded such a nuclear mass (fig. 19).

The fibroblast-like cells frequently were seen to undergo mitosis in cultures older than 2 weeks. Lymphocytes and lymphoid cells sometimes persisted in such cultures, forming small colony-like accumulations; however, they were not seen to divide. Observations suggest three possible derivations of these lymphocytes: (1) from large fibroblast-like cells, inside the cytoplasm of which they may have survived for weeks; (2) from the original explant which, acting as an "organ culture," continued producing lymphoid cells for several weeks; and (3) from the re-conversion of certain fibroblast-like cells into immature lymphoid cells. In the material presented in this study, two cultures were encountered which may be regarded as supporting the third possibility (fig. 20); no cinematographic evidence of the re-conversion can be presented.

**DISCUSSION**

A fibroblastic conversion of human bone marrow tissue cultures was described earlier by several authors. Special cultural technics, however,
Fig. 6.—Various mononuclear cells parasitize the cytoplasm of fibroblast-like cells in a 2 week old culture obtained from a patient with acute monocytic leukemia. (Wright) X 150.

Fig. 7.—A lymphocyte (arrow) seen inside the nucleus, close to the sausage-shaped nucleolus, of a fibroblast-like cell in a 4 week old culture obtained from a patient with chronic lymphocytic leukemia. Phase contrast, X 920.

Fig. 8.—Intranuclear structure resembling inclusion body. Culture is the same shown in figure 7. Phase contrast, X 615.
may result in the growth of leukemic cells for a prolonged period; however, fibroblastic growth may occur even in such cultures. The conversion of all mesenchymal cells to fibroblasts in vitro has been described as a generally occurring phenomenon and has been termed "nomicoplasia." Certain types of fibroblasts may exhibit or retain malignant potentialities. A highly malignant anaerobic mouse fibroblast culture has been described. A fibroblastic appearance in certain human leukemic reticuloendothelioses has recently been reported. Furthermore, a possible transformation of fibroblastic tissue cultures obtained from the bone marrow of leukemic children into a culture of undifferentiated lymphoblastoid cells has recently been suggested.

The intracellular existence of lymphocytes in a variety of tissue cultures has become well known in the last few years. The term "emperipolesis" has been used to designate intracellular lymphocytic activities in tissue cultures. When one of us first encountered this phenomenon in a bone marrow tissue culture from a patient with chronic lymphocytic leukemia and positive Coombs test, the idea that an autoimmune cellular aggression had been observed in vitro was suggested. Further experience indicates, however, that lymphocytes entering other cells in vitro may be guided by a variety of mechanisms other than autoimmune. First, lymphocytes may become phagocytised and end up as amorphous cytoplasmic inclusions in the large cells. Second, lymphocytes, according to a widely held view, do not grow readily in vitro. Their invasion of other cells in vitro may serve a nutritional need and may be regarded as a feeder layer mechanism. In the direct bone marrow smears of the patients presented in this study, no intracellular lymphocytes were seen. Finally, autoaggressive lymphoid cells may operate not only in vivo but also in vitro. It has been suggested that an "allergic cell death phenomenon" occurs when immunologically competent presensitized cells make contact with their cellular target; both the immunologically aggressive cell and the target cell succumb and disintegrate upon contact. It has recently been shown that this phenomenon takes place when macrophages from mice immunized with mouse sarcoma cells, are brought together with mouse sarcoma cells in the peritoneal cavity. A similar phenomenon was shown to occur between presensitized mouse lymphocytes and homologous liver cells grown in vitro. In splenic tissue cultures from a prolonged form of Rauscher's viral mouse leukemia, which was produced in mice with chronic runt disease and hematopoietic chimerism, a somewhat similar lymphocytic activity was seen. Recently, lymphocytes have been shown to adhere to multinucleated giant cells in tissue cultures prepared from lymph nodes from bovine leukemia; an autoimmune mecha-

Fig. 9.—Dividing lymphoid cell inside the cytoplasm of a fibroblast-like cell. Another fibroblast shows wide-spread vacuolization of the cytoplasm and contains two lymphocytes. Culture from a patient with chronic lymphocytic leukemia. (Wright) X 370.

Fig. 10.—A very large number of lymphocytes densely surround fibroblast-like cells in a 2 week old culture from a patient with chronic lymphocytic leukemia with positive Coombs test. (Wright) X 170.
Fig. 11.—The cytoplasm of a fibroblast-like cell is heavily packed by lymphocytes in a 5 week old culture from a patient with chronic lymphatic leukemia; arrow points to a lymphocyte in division. Phase contrast, X 350.

Fig. 12.—A massive invasion of fibroblast-like cells by lymphocytes in a 3 week old culture obtained from a patient with chronic lymphocytic leukemia with positive Coombs test. (Wright) X 320.
nism has been suggested. Thus, when small lymphocytes are seen to adhere to or penetrate other cells in tissue cultures of leukemic human bone marrow, an autoimmune attack may be conjectured, particularly when the marked degree of damage done to the attacked cell appears disproportionate to the small number of lymphocytes involved. In seeking further understanding of this phenomenon, it is suggested that lymphocytic activities in bone marrow tissue cultures from lupus erythematosus be studied.

The formation of polykaryocytes in tissue cultures from human tumors has recently been discussed. Multinucleated giant cells and syncytium formation in young cultures may be the result of a virus infection. Most of the viruses which elicit the formation of these structures, but without the formation of intranuclear inclusions, belong to the Myxovirus group. The polykaryocytes and syncytia are formed by coalescing individual cells and the size and shape of the nuclei are fairly uniform. In our material, polykaryocytes and syncytia of this type occurred in 3 to 4 week old cultures of marrow from monocytic leukemia; these cultures did not show hemadsorption. Syncytia in which common (respiratory) Myxoviruses are grown, readily show hemadsorption. The mouse and hypothetical human leukemia viruses are believed to be of Myxovirus structure but deprived of the faculty of hemagglutination or hemadsorption when present (perhaps in very small amounts) in tissue cultures. This point gives ground for speculations as to the virological significance of syncytia and polykaryocytes formed in certain leukemic bone marrow tissue cultures. Another type of multinucleated cell appeared in cultures from patients with malignant histiocytosis, multiple myeloma and lymphocytic leukemia; it also may occur in any aging tissue culture. In this type, numerous nuclei develop in the middle of the cell by buddings and polyploid reduplications of the nucleus. This type of giant cell commonly occurs also in vivo in various malignancies, and is seldom seen associated with common viral diseases.

The reconversion of an established fibroblastic culture, or large areas of such a culture, into a cell population consisting of undifferentiated round cells, thus resembling the original leukemic culture from which the fibroblastic culture derived, is difficult to document. Further observations, preferably carried out by cinematography, are obviously required.

Fig. 13.—The adherence of two small lymphocytes to a fibroblast-like cell is accompanied by widespread cytoplasmic vacuolization. Culture obtained from a patient with chronic lymphocytic leukemia. (Wright) X 450.

Fig. 14.—One disintegrating fibroblast-like cell is heavily packed by lymphoid cells; some of the lymphocytes appear to be damaged. The cytoplasm of another fibroblast-like cell disintegrated and the denuded nucleus (arrow) is surrounded by lymphocytes with intact structure. One pole of a third fibroblast-like cell is surrounded by lymphocytes. Culture obtained from a patient with chronic lymphocytic leukemia. (Wright) X 250.

Fig. 15.—A severely damaged fibroblast-like cell contains remnants of lymphoid cells in a 2 week old culture obtained from a patient with chronic lymphocytic leukemia. (Wright) X 310.

Fig. 16.—Plasma cells and a cell with four nuclei seen in a 10 day old culture obtained from a patient with multiple myeloma. (Wright) X 400.
Summary

1) The growth from human leukemic bone marrow particles begins with the emigration of cells having the morphologic characteristics of erythrocytic, granulocytic, monocytic and lymphocytic elements. Proliﬁcation of ﬁbroblastic-like cells replaces these elements within 3 to 4 weeks. Various types of immature leukocytes occur in larger numbers, and persist longer, in tis-
sue cultures of human leukemic bone marrow than in bone marrow cultures from patients with no hematological disease.

2) Lymphocytes from either leukemic or non-leukemic bone marrow cultures often appear to enter the cytoplasm of fibroblast-like cells, but those derived from cultures of chronic lymphocytic leukemic bone marrow appeared intracellularly in much larger numbers and persisted there longer. One of the mechanisms of this phenomenon may be the feeder layer principle. Extensive injury of certain fibroblast-like cells in relation to relatively few small lymphocytes suggests the possibility of an autoimmune attack. It is possible that both the lymphocytes and the "target cells" may disintegrate.

3) Two types of multinucleated giant cells have been observed. One type is syncytial in structure, suggesting a viral mechanism of initiation. Another type resembles the polyploid giant cells which are commonly seen in old tissue cultures of whatever origin, and in various malignant diseases in vivo as well as in vitro.

4) At times, poorly differentiated round cells may persist in old fibroblastic cultures. These cells may be derived from the original explant which continued to produce them, or they may originate from fibroblast-like cells reconverted into a morphologically undifferentiated mesenchymal cell type.

**Summario in Interlingua**

1. Le crescentia al partículas de leucemic medulla ossee human comencia con le emigration de cellulas con le características morphologic de elementos erythrocytic, granulocytic, monocytic, e lymphocytic. Proliferation de cellulas fibroblastoide reimplacia iste elementos intra 3 a 4 septimanas. Varie typos de immatur leucocytos occurre in plus grande numerose persiste plus longemente in histoculturas de leucemic medulla ossee human que in histoculturas de medulla ossee ab subjectos human sin morbo hematologic.

2. Lymphocytos de leucemic e non-leucemic culturas de medulla ossee frequentemente pare entrar in le cytoplasma de cellulas fibroblastoide, sed illos derive ab culturas de medulla ossee in chronic leucemia lymphocytic esseva notate in sito intracellular in mucho plus grande numerose e illos persisteva illac plus longemente. Un del mechanismos in iste phenomeno es possibilemente le principio del strato alimentatorii. Le extense vulneration de certe cellulas de character fibroblastoide in relation a relativemente rar lymphocytos miere suggere le possibilitate de un attacco autoimmun. Es possibile que tanto le lymphocytos como etiam le cellulas a "oculo de ave" se disintegra.

3. Duo typos de multinucleate cellulas gigante esseva observate. Un del typos es synctial in structura, lo que suggere un mechismo viral de initiation. Le altere typo resimila le polyploide cellulas gigante que es commummente incontrate in ancian histoculturas de non importa qual origine e in varie morbos maligne in vivo e etiam in vitro.

4. A vices, mal differentiate cellulas ronde persiste in ancian culturas fibroblastic. Iste cellulas es possibilemente derive ab le explanta original (que ha continue producir los), sed il es etiam possibile que illos ha lor origine in cellulas fibroblastoide reconvertite in morphologicamente non-differentiate cellulas de typo mesenchymal.
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