Transformation of Lymphocytes in Cultures of Human Peripheral Blood

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In a previous study from this laboratory, evidence was presented that the proliferating cells in cultures of human peripheral blood originate from small lymphocytes. Additional support for this view has come from the demonstration that separated lymphocytes have the same growth potential as whole blood and that the cultured cells share a number of histochemical reactions with lymphocytes. The present study was undertaken to delineate the fine structure of the cells in culture by phase and electron microscopy.

Materials and Methods

Blood was obtained from normal human adults and cultured by a modification of the method of Hungerford et al. Normal human venous blood was withdrawn into syringes previously treated with phenol-free heparin. The heparinized blood was transferred to a collection flask to which had been added 0.2 ml. Phytohemagglutinin M (Difco) and 0.02 ml. Phytohemagglutinin P (Difco) for every 10 ml. of blood. The collection flask and its contents were allowed to remain in an ice bath for 55–60 minutes and then centrifuged in an International Centrifuge at 400 rpm at 5°C for 4 minutes, allowing an additional 2 minutes each for acceleration and deceleration. The leukocyte-rich plasma was withdrawn and 4 ml. aliquots were transferred to sterile culture bottles to which 16 ml. T.C. 199 with penicillin and streptomycin had been added. The cultures were incubated at 37°C in room air. Cultured materials were examined at 2 hours, 24 hours, 48 hours, and 72 hours. Three such sets of cultures from different individuals were examined with both the phase and electron microscopes, and an additional 12 cultures with phase microscopy only. The cultured cells were collected by 3–5 minute centrifugation periods at 5–800 rpm. Wet preparations were examined under the phase microscope. For electron microscopy, cells were fixed for 30 minutes at 4°C in 2 per cent osmic acid with veronal acetate. Embedding was carried out using Epon epoxy resin after dehydration in graded ethyl alcohol. The Epon epoxy was prepared by mixing 10 ml. of Epon 812 with 25 ml. of dodecenyl succinic anhydride (DDSA) and 0.6 ml. of 2,4,6-dimethylaminomethyl phenol (DMP 30). Thin sections were made on a Porter-Blum Microtome. Sections were stained with 1 per cent lead monoxide for approximately 10 minutes. An RCA EMU-2C microscope was used. Phagocytosis of surviving and growing cells was tested by adding to the culture a dilute suspension of killed and washed staphylococci or starch granules. After 15 minutes incubation at 37°C, cultures were spun and spread on slides, fixed with methanol and stained with Giemsa.

Results

At 2 hours, the cells of the peripheral blood were largely unchanged in appearance and lymphocytes, monocytes, neutrophils, and eosinophils were readily identified as such. A variable number of cells, usually less than 10

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per cent and for the most part granulocytes, were dead or dying as evidenced by loss of motility, bizarre pseudopodia, nuclear blebs, swollen cytoplasm with Brownian movement, and other characteristics of damaged cells described in detail by Bessis. In general, the percentage of mononuclear cells was increased compared with their proportion in whole blood, and occasionally small lymphocytes entirely dominated the picture. When fixed for electron microscopy or when first placed on a slide, these cells were round or oval, but on continuous observation under the phase microscope some of them were seen to have very slow motility and a tendency to stretch out as illustrated in figure 1. Occasionally the outline of these cells simulated the familiar appearance of moving polymorphonuclear cells in normal peripheral blood (fig. 4). The actual rate of movement, however, was far too slow to be appreciated without time lag photography. Numerous platelets were always present, both singly and in large clumps. In 24-hour cultures, lymphocytes always predominated. On occasion monocytes with dust-like mitochondria and typical perinuclear “rosettes” were present (fig. 2). In some cultures, macrophages were seen, occasionally in groups of three or four laden with platelets and debris from platelets or other sources (fig. 3). An increased percentage of lymphocytes had the same motility already described in 2-hour specimens (fig. 4). In addition, many lymphocytes were larger with both increased size of nuclei and more ample cytoplasm (fig. 2). In electron micrographs the cells were usually round or oval and measured between 11.5 and 12.0 μ in their larger diameter and 5–10.0 μ in their shorter diameter. The corresponding measurements of the cell nuclei were 6–9 μ and 4.5–7.5 μ for the two diameters. These measurements were based on examination of 80 cells in which the presence of either a nucleolus or a Golgi apparatus or both, as well as the constant nuclear cytoplasmic ratio, suggested that the section had transversed the cell near a central plane. At times larger cells up to 20 μ in diameter and of irregular, spindle shape or bizarre outline were seen, presumably reflecting their motility seen under phase microscopy. The predominant cell type, though closely resembling lymphocytes of the peripheral blood, had a slightly less homogeneous nucleoplasm (fig. 9). The nucleolus was always conspicuous. The nucleus was usually located centrally within the cell and surrounded by a narrow rim of cytoplasm which contained a few large mitochondria, abundant ribosomes, a poorly developed Golgi apparatus, and sparse endoplasmic reticulum (fig. 10). Cytoplasmic granules were occasionally present. The compound vesicular bodies which were common in later cultures and will be described below, were noted in a few cells. Most of the granulocytes present were in various stages of degeneration and dissolution. Monocytes were rarely seen in either the phase or electron microscopy. They were easily distinguished from the lymphocytic cells by their more numerous small mitochondria, fewer ribosomes and active phagocytosis (fig. 18). It appeared that much of the ingested material was derived from platelets. Occasionally lymphocytes were also present within macrophages.

In 48-hour cultures, small lymphocytes persisted in varying numbers, but most cells were considerably larger than at 24 hours (figs. 5 and 6). Both nuclear size and amount of cytoplasm was increased. Nucleoli were large or
Figs. 1–4.—Phase microscopy of peripheral blood cultures at 2 hours (fig. 1) and 24 hours (figs. 2–4). X1100. See legends, facing page.
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multiple and occasionally of irregular or bizarre shape. Mitotic figures were present. Their number varied in different cultures, but generally they were rare. Monocytes were present in similar numbers as at 24 hours and distended with ingested material, including platelets, whole nuclei and unidentifiable debris. The electron microscopic appearance of the larger cells will be described for the 72-hour culture in which the changes in cellular morphology were similar but more advanced than at 48 hours.

In 72-hour cultures, typical small lymphocytes usually represented less than 10 per cent of the total cells. The predominant cell type had only a remote resemblance to normal small lymphocytes in Giemsa stained smears or in supravital preparation under phase microscopy. The cells were even larger than at 48 hours, the nuclei contained multiple and often bizarre nucleoli, the ample cytoplasm frequently contained vacuoles (figs. 7 and 8). The extent of vacuolization was quite variable both within a culture and from culture to culture. Mitoses were readily seen and appeared to occur both in the very large and medium sized cells (fig. 7). The larger cells seldom showed distinct motility, but the medium sized cells which closely resembled the large cells shared the tendency to stretch out with the small typically lymphocytic cells. Granulocytes were present only rarely, and those seen were usually eosinophils. Under the electron microscope the same variation in cell types was seen. There were relatively small numbers of typical lymphocytes, many large bizarre cells and some intermediate forms of medium size (figs. 11 and 12). Mitotic figures were seen most commonly in cells of medium size. Measurement of 85 cells gave the following results: the larger diameters varied from 9-17 μ, the smaller from 7-13 μ. The diameters of nuclei were 5-12 μ and 3-9 μ. Apparently fixation considerably shrunk the large cells, since the increase in size compared with 24-hour cultures was less in electron micrographs than in supravital preparations (figs. 1-8). Many of the cells had pseudopodia (fig. 11). The nucleoplasm was coarser than at 24 hours. Nucleoli were always prominent and multiple nucleoli occurred commonly in the larger cells. The cytoplasm contained abundant ribosomes, mitochondria had increased in number when compared with the 24-hour specimen and many were swollen without increase in the number of cristae (fig. 11). Intramitochondrial granules were not seen. Endoplasmic reticulum was frequent, often in the form of small vesicles, but occasionally elongated lamellar sacs were present. The Golgi apparatus was usually moderately well developed, especially in its vesicular component (figs. 13-15). Many small vacuoles containing amorphous material of moderate to high opacity and measuring 0.1 to 0.3 μ in diameter, of round or polygonal shape, were present both near the Golgi apparatus and elsewhere in the cytoplasm.

Fig. 1.—Small lymphocytes. Note stretched out cells suggesting motility. X1100.
Fig. 2.—Note typical small lymphs, some larger cells, and two monocytes with rosette-like arrangement of mitochondria and small vacuoles. X1100.
Fig. 3.—Note macrophages laden with platelets and debris. X1100.
Fig. 4.—Motile lymphocyte simulating outline of moving polymorphonuclear cells. X1100.
Figs. 5-8.—Phase microscopy of peripheral blood cultures at 48 hours (figs. 5 and 6) and 72 hours (figs. 7, 8a and 8b). X1100. See legends, facing page.
Groups of vesicles near the cell membrane suggest active micropinocytosis. Compound vesicles measuring 40–100 μ were frequently present (fig. 15). Lipid material, presumably corresponding to the vacuoles seen in the phase microscope, were noted in many of the larger cells (fig. 11). The outline of these structures measured about 2.0 μ in diameter, with quite irregular outlines. They were bordered by a single clearly defined membrane which sometimes communicated with the endoplasmic reticulum. On rare occasions, fibrillar formations were seen in cells both at 24 and 72 hours (figs. 16 and 17). The individual fibrils measured 50–70 A and had a beaded appearance. They appeared identical with the fibrils described in mononuclear phagocytes by Petris. In one of the three cultures, many of the larger cells seen at 72 hours had a slightly different appearance even though they shared the general characteristic just described. The points of difference were that nucleoli were rarely seen in these cells and that mitochondria were not only larger than at 24 hours but also had an increased number of cristae (fig. 13).

In phagocytosis experiments, ingestion of bacteria was always restricted to the surviving granulocytes, monocytes, and a few macrophages the origin of which could not be determined. At no time did we observe phagocytosis in predominant cell types pictured in figures 1, 2, 4, and 5–17.

**DISCUSSION**

The detailed morphologic study by phase and electron microscopy of human peripheral blood cells in culture illustrates the difficulty of tracing the origin of cells by morphologic means such as "transitional forms." In describing the appearance of cells at various intervals of culture one may stress the persistance of small cells with typical features of lymphocytes and a resemblance of some of the larger cells to lymphocytes. Conversely, one may point with equal justification to the peculiarities of the large and bizarre cells in 72-hour cultures which could not be identified individually. Fortunately, we need no longer rely on the presence of "transitional forms" since isotopic studies have clearly indicated the origin of the proliferating large cells from small lymphocytes. It will suffice to note that the morphologic observations are in keeping with the lymphocytic origin of these cells.

Two points, however, require discussion. One concerns the possible significance of the morphologic changes in the cultured cells observed by electron microscopy. The increased number of ribosomes correlates well with the increased basophilia noted in the Giemsa-stained smears and with the isotopic evidence of increased RNA synthesis which precedes DNA synthesis and division. The bizarre shape of the cells, their large size, the presence of vacuoles,

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Fig. 5.—Clump of cells in centrifuged specimen. X475.

Fig. 6.—Note increasing size of cell and prominence of nucleoli, and cytoplasmic vacuoles. Compare with 24-hour culture in figs. 2–4. X1100.

Fig. 7.—Further increase in cell size. Note three mitoses. X475.

Fig. 8a.—Note vacuoles in large cells (top left) and persistence of same small lymphocytes. X1100.

Fig. 8b.—Giemsa-stained cells from same culture. X1100.
Fig. 9.—Predominant cell type at 24 hours. Note typical lymphocytic structure. X9500.

Fig. 10.—Typical cell in 24-hour culture. Detail showing a centriole and poorly developed Golgi apparatus. X30,000.

Fig. 11.—Most common cell type at 72 hours. Mitochondria increased in number and swollen. X7500.
Figs. 12 (above) and 13 (below)—Cells from 72-hour cultures.

Fig. 12.—Another frequent cell type closely resembling lymphocytic cells seen at 24 hours, but larger. X6000.

Fig. 13.—Note moderately well developed Golgi apparatus and large mitochondria with increased number of cristae. X10,000.
Figs. 14 (above) and 15 (below)—Cells from 72-hour cultures.

Fig. 14.—Note cytoplasmic granules and small vacuoles in predominant cell type. X25,000.

Fig. 15.—Similar cell. Note Golgi zone with centrosome, granules, and multi-vesicular bodies. X30,000.
Figs. 16–18.—Cells from 72-hour cultures.

Fig. 16.—Cell similar to fig. 14. Note fibril formation (arrow). X6500.

Fig. 17.—Detail of fig. 16. X51,000.

Fig. 18.—Monocyte with ingested debris and vacuoles. X6500.
and prominent nucleoli give the cells an appearance reminiscent of acute leukemias. In trying to assess the significance of this resemblance, it should be recalled that there are only two features which can be considered in themselves diagnostic of leukemic cells: Auer bodies and crescentic or circular fibrillar formations. Neither was present in the cultures. The cytoplasmic inclusions, namely the multivesicular bodies, lipid vacuoles and fibrillar formations, which were found in the cultured cells, have all been observed in normal cells. Multivesicular bodies and lipid vacuoles are known to occur with increased frequency in cultured cells. However, it should also be noted that they were present in vivo in pyroninophilic cells produced in response to antigenic stimulation.

The second point requiring discussion concerns the relevance of the observations on cultures for the physiology of lymphocytes in the intact organism. The transformation of small lymphocytes into larger cells capable of division in vitro strikingly parallels the observation in the living rat by Gowans. In his experiments, the small lymphocytes have been conclusively shown to transform into a large pyroninophilic cell capable of division. The observations by Porter and Cooper furnish additional evidence that the small lymphocyte is capable of division when antigenically stimulated in vivo. Phytohemagglutinin apparently induces a similar transformation in vitro. Elves, Roath, and Israels have recently reported that antigenic stimulation can also produce the transformation in vitro. Nowell has shown that this transformation produced by phytohemagglutinin can be inhibited by prednisolone which has little influence on subsequent cell division once transformation has taken place. In keeping with this finding are his observations that delay of the addition of phytohemagglutinin to a culture for 1 or 2 days will delay appearance of mitoses for the same periods. Berman and Stulberg produced cultures of human peripheral blood in which after 15-25 days two distinct populations survived with virtual absence of mitoses: small lymphocytes and large macrophages. Addition of phytohemagglutinin at that time led to degeneration of the macrophages and to marked mitotic activity of large cells apparently derived from the small lymphocytes. Thus, the potential of the small lymphocyte for division appears to manifest itself only after specific stimulation accompanied by morphologic transformation.

The view originally put forward by Maximow, and at present carried forward by Yoffey, that the small lymphocyte is not an “end cell,” is clearly substantiated by these observations. The potential for transformation and division, however, does not establish the lymphocyte as a totipotential hemopoietic stem cell. The pyroninophilic cells of Gowans appear to produce small lymphocytes only. In tissue cultures, the dividing cells appear to reproduce their own kind of medium and large cell. No differentiation was observed in the present short-term culture, nor was phagocytosis by the newly formed cells observed. However, in diffusion chambers introduced into the abdominal cavity of rabbits, Holub observed transformation of small lymphocytes into plasma cells and other antibody-producing cells resembling those in Gowans’s experiments. Conversely, plasma cells produced in response to antigenic stimulation have been shown to be derived from cells that had recently under-
gone division, presumably lymphocytes. Both small and large lymphocytes have been implicated. There is also an impressive body of radiobiologic evidence that lymphocytes cannot function as precursors of bone marrow cells. The crucial experiments deal with the repopulation of marrow and lymphoid organs made aplastic by high doses of whole body irradiation. Accelerated repopulation of the marrow and nodes can be accomplished by transplanted marrow cells and in mice also by cells of the spleen, which is the site of extensive extramedullary hemopoiesis in this species. Cells from the thoracic duct or nodes, however, accelerate recovery of lymphoid organs only. A single contrary observation has not been confirmed. The objection that pluripotential lymphoid cells may have been damaged by the transplantation procedure has been made by the demonstration that shielding of a single Peyer’s patch during whole body irradiation assures rapid repopulation of lymphoid tissues, but not of the marrow.

Indirect evidence for a separate origin of lymphocytes and bone marrow cells has come from observations on chronic myelocytic leukemia in which dividing erythroid, granulocytic, and probably megakaryocytic cells shared the Philadelphia chromosome as a marker, while cultures of peripheral blood during remission produced cells of normal karyotype, presumably because lymphocytes do not contain the marker.

The problem of the potentialities of the lymphocyte is complicated by the presence in the peripheral blood of cells which can act as precursors of marrow cells. This has been deduced from experiments in which post-radiation parabiosis lead to accelerated bone marrow repopulation of the irradiated partner. More recently, infusion of a suspension of nucleated cells of the peripheral blood when given in sufficiently high concentration has been shown to lead to bone marrow repopulation of heavily irradiated mice. Since granulocytes are incapable of division and since lymphocytes derived from thoracic duct or node are incapable of repopulating the marrow, the stem cells in the peripheral blood must travel under the guise of some mononuclear cell which is not recognized morphologically as a separate entity. It is tempting to speculate that some of the precursor cells may be identical with the 0.1-0.5 per cent of mononuclear cells of the blood capable of immediate DNA synthesis when incubated in vitro. When these cells are studied in radioautographs, it becomes apparent that they do not belong to a single well-characterized group. Only some of the cells in DNA synthesis have the deep blue cytoplasm or lacy chromatin structure usually associated with “youthfulness” of blood cells. None have nucleoli. Most would probably be classified as large lymphocytes or monocytes in routine smears, a few as young lymphocytes or plasma cells. Some of them are undoubtedly cells from the thoracic duct which always carries a percentage of large lymphocytes capable of incorporating thymidine in vitro. None of the cells capable of DNA synthesis immediately upon removal from the body appear to proliferate in vitro, presumably because the conditions of culture used are unfavorable to division of these cells. It may be suggested from these data that both small lymphocytes and some other “mature” mononuclear cells of the peripheral blood are potential “stem cells” capable of division under suitable stimulation.
progeny of lymphocytes, however, would be restricted to lymphocytes and plasma cells, while the somewhat diverse group of mononuclear cells capable of immediate DNA synthesis could serve as precursor of the three cell lines of the marrow.

While the bulk of the available evidence is compatible with the view presented, certain experiments strongly suggest that a single cell may occasionally serve as a common precursor for all hemopoietic tissues. The most striking piece of evidence comes from chromosome studies in heavily irradiated mice which were made to survive by homologous marrow transplants. Subsequently, host cells replaced the transplant and on occasion the host's own precursor cells, having been heavily irradiated, fortuitously carried a chromosome marker. In one such instance 95 per cent of mitoses in the marrow, 55 per cent in the spleen, 80 per cent in the thymus, and 67 per cent in the nodes carried the identical anomalous karyotype. Since the same chromosome abnormality can hardly have arisen in more than one cell, a common stem cell to both lymphoid and marrow must have been involved.\textsuperscript{42} A recent study by Whang, Tjio et al. may also be germane here. A patient with lymphosarcoma had a chromosome abnormality in the cells of the lymphoma as well as in all bone marrow cells, while culture of the peripheral blood contained only cells of normal karyotype, presumably derived from normal circulating small lymphocytes.\textsuperscript{43} The probable implication is that the change in karyotype involved a common precursor, although it did not replace all of the normal lymphoid tissue, thus accounting for normal karyotypes in cultures of peripheral blood.

Finally, circulating blood cells may transform into histiocytes and fibroblasts as previously discussed.\textsuperscript{1} Again a mononuclear cell is implicated as the cell of origin, but its identity is still controversial.

**Summary**

Previous tracer studies have demonstrated the lymphocytic origin of proliferating cells in blood cultures. Detailed morphologic observations are reported on the transformation of small lymphocytes into larger cells capable of division in tissue culture of human peripheral blood. The large cells have ample cytoplasm with multivesicular bodies, well-developed Golgi apparatus, scanty endoplasmic reticulum, ample ribosomes, and variable fat-laden vacuoles as well as peculiar granular inclusions, large and occasionally bizarre nuclei with prominent nucleoli. Under the condition of culture no further differentiation of these cells has occurred.

The potentialities of the small lymphocyte are discussed in the light of recent radiobiologic and cytogenetic investigations.

**Summario in Interlingua**

Previe studios con traciantes ha demonstrate le origine lymphocytic de proliferante cellulas in cultivas de sanguine. Detaliate observationes morphologic es reportate in re le transformation de micre lymphocytes ad in plus grande cellulas cepabile de divider se in histoculturas de human sanguine peripheric. Le grande cellulas ha ample cytoplasma con corpores multivesicu-
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lar, un ben disveloppate apparato de Golgi, sparse reticulo endoplasmatic, ample ribosomas, e variabile vacuolos a cargation de grassia e etiam peculiar inclusiones granulari e grande e occasionalmente bizarre nucleos con prominente nucleolos. Sub le conditiones del cultivation, nulle differentiation additional de iste cellulas ha occurrite.

Le potentialitates del micre lymphocytos es discutite in le lumine de recente investigationes radiobiologic e cytogenetic.

REFERENCES


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