Establishment of Lines from Normal Human Blood Leukocytes by Co-cultivation with a Leukocyte Line Derived from a Leukemic Child[1] (34189)

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When white cells from the peripheral blood of patients with certain diseases such as leukemia (1) and infectious mononucleosis (2) are cultured *in vitro*, they may become established as cell lines. In contrast, peripheral blood leukocytes from healthy individuals do not usually survive *in vitro* for longer than several weeks, even when stimulated by blastogenic agents, unless cultures are initiated with very large numbers of leukocytes (3). We sought to enhance the growth of normal blood leukocytes by exposing them to a cell line which was developed in our laboratory from a leukemic patient with the idea that such a system, if successful, might be useful both for understanding factors which control normal white cell division *in vitro* and in investigating the possibility that factors associated with leukemic cells might be transferable to normal cells.

Similar experiments were reported by Henle and his co-workers (4) who showed that contact with Burkitt lymphoma cells induced proliferation of normal white blood cells. In Henle's studies Epstein-Barr virus (EBV) present in the inducing line was invariably transferred to the normal cells. Furthermore, a Burkitt lymphoma cell line without EBV antigen was incapable of promoting the growth of normal leukocytes. Henle's data suggested that EBV was the determinant in the stimulating effect. Accordingly we also examined our line of leukemic cells for the presence of this agent.

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Methods. Leukemic cell line. A continuously growing line of leukocytes (LS-M) was derived from the bone marrow of a 3-year-old girl with acute leukemia in remission. The white cell cultures were initiated 1 week after the patient was suspected clinically to be affected by viral hepatitis. To establish the LS-M line, the cellular elements of a heparinized specimen of bone marrow were washed twice in Puck's saline A and suspended in two 8-oz glass bottles at a concentration of approximately 2×10^6 white cells/ml of RPMI 1640 medium containing 20% fetal bovine serum (FBS). After 1 week, the cultures consisted of a monolayer of fibroblastic cells attached to glass and round cells. Some of the latter were loosely attached to the monolayer and others were floating in the medium. Medium was changed weekly and the suspended cells were replaced after slow sedimentation. At the end of 3 weeks the cells in suspension were pooled and cultivated separately. After approximately 6 weeks these cells showed definite evidence of multiplication, marked by clumping and acid production. Giemsa stained smears showed individual cells with "whisker-like" cytoplasmic processes, a large nucleus and often one or more nucleoli (Fig. 1). Degenerated cells were frequently present in the cultures. Suspension cultures of cells hovering in the medium were maintained without agitation, and the cultures were split every 5–7 days. No mycoplasma was cultured from the line in tests performed by Dr. Yolanda Low.

Primary normal leukocyte cultures. Peripheral blood leukocytes (WBC) from 20 ml of blood from a healthy adult male were harvested, washed, and cultured as previously

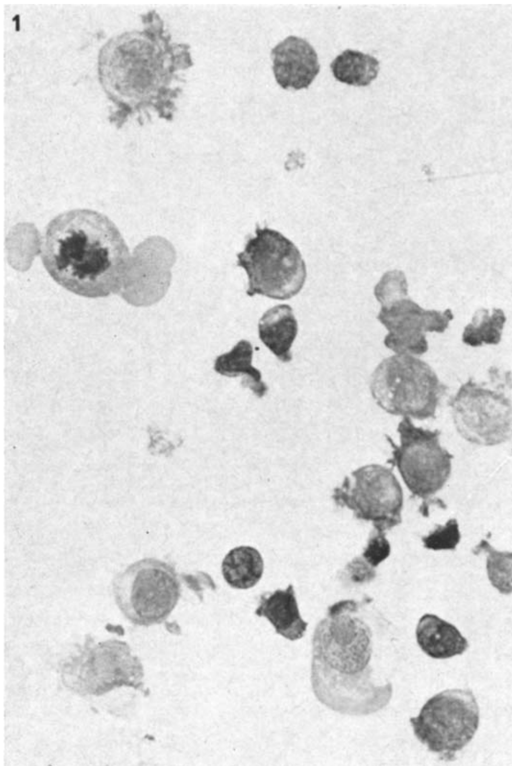

FIG. 1. Giemsa stained smear of cells from LS-M line; ×1600.

described (5) with the exception that the medium was RPMI 1640 with 20% FBS. The WBC were treated initially with 200 μg/ml of phytohemagglutinin-M (PHA)[2] and, after 3–4 days in culture, they were used in the co-cultivation experiments. EBV antibodies were demonstrated in the serum of the donor, who never had infectious mononucleosis.

Co-cultivation of X-irradiated LS-M Cells and Normal WBC. In co-cultivation experiments 3 systems were studied: PHA-treated WBC, X-irradiated LS-M cells, and a combination of PHA treated WBC and X-irradiated LS-M cells. To one half the cultures in each group beta-propriolactone-inactivated Sendai virus was added to induce cell fusion (6).

For irradiation a 20-ml aliquot of LS-M cell suspension (10^6 cells/ml) was placed in a glass petri dish (10 cm in diam) which was then covered with Saranwrap. The X-ray dose was about 4500 rads. The radiation factors were: 250 Kvcp, HVL 0.35 mmCy, dose rate 320 rads/min. One-ml aliquots of X-irradiated LS-M cells or WBC at a concentration of 1–2 $\times$ 10^6/ml in Hanks' salt solution were distributed in Wasserman tubes. When combined, 0.5 ml of each kind of cell was placed in a tube. Cells were held at 4° for 15 min and then shaken at 37° for 2 hr at about 100 excursions/min. Cells were centrifuged and resuspended in 2 ml of RPMI 1640 with 20% FBS. PHA was again added to suspensions containing normal WBC. Cell suspensions were added to monolayer cultures of nonirradiated fibroblastoid cells from a human placenta (PC). Medium in all cultures was changed weekly. In Expt. 2 a set of leukocyte cultures of each group without the PC monolayer was included. Leukocyte lines emerging from co-cultivation experiments are referred to as transformed normal leukocytes (TNL).

Demonstration of EBV by immunofluorescence. Acetone-fixed smears of leukocytes were examined for EBV antigen by the indirect method (7). Smears were treated either with a 1:10 dilution of human serum known to contain EBV antibodies, or, as a control, with a 1:10 dilution of a human serum in which EBV antibodies could not be demonstrated. After being washed the smears were overlayed with a 1:10 dilution of rabbit antihuman gamma-globulin serum conjugated with fluorescein-isothiocyanate.[3] The presence or absence of EBV antigen was evaluated in coded slides. Specific fluorescence was considered to consist of bright green staining material associated with both nucleus and cytoplasm. Such positive cells frequently showed a diffused fluorescent glow around the cell.

Examination of chromosomes. For cytogenetic studies, colchicine in a concentration of 10^{-7} *M* was added to the cultures for 2 hrs prior to harvesting and preparation of cells by conventional methods (8). The sex, number, and morphology of chromosomes was determined for 30 cells in each instance.

[2] Difco.

[3] Antibodies, Inc.

TABLE I. Co-cultivation of X-Rayed LS-M Cells with Normal Blood Leukocytes on a Feeder Layer of Placental Cells.

Expt.	No. of continuous lines established/no. of tubes attempted					
	Normal WBC	Normal WBC + Sendai	X-rayed LS-M	X-rayed LS-M + Sendai	Co-cult.	Co-cult. + Sendai
1[a]	0/2	0/2	0/2	0/2	0/2	1/2
2[b]	0/4	0/4	0/4	0/4	4/4	4/4

[a] Medium changed to Eagles (BME) + 10% FBS after 20 days.
[b] Medium was RPMI-1640 + 20% FBS throughout experiment. LS-M cells used which had been stored at —70° for 3 months.

Results. Co-cultivation Expt. 1. In the first experiment, (see Table I), some of the placental cells were destroyed within 24–48 hr after contact with the rapidly growing normal WBC. Contact with X-rayed LS-M cells, or with unirradiated LS-M cells did not markedly affect the underlying PC. After 2 weeks, growth of the normal WBC gradually declined and the PC repopulated the tubes.

In Expt. 1 after the third week, cells suspended in medium were pooled and placed in separate cultures without a feeder monolayer. These suspended cells never established a growing culture.

After approximately 4 weeks, the first sign of an increase in number of lymphoblastoid cells was seen in PC cultures with cocultivated irradiated LS-M and WBC. In one of 4 tubes these small round cells persisted in focal areas for 3 months before they began to rapidly increase in numbers and size. At this time they were successfully subcultured without the PC feeder layer, or LS-M cells. The single leukocyte line obtained in Expt. 1 has so far been maintained for 6 months. Leukocyte lines did not develop from tubes inoculated with normal WBC or X-rayed LS-M alone.

Expt. 2. In the second co-cultivation experiment, two technical modifications were made. First, cells floating in the medium were not removed to separate cultures but were centrifuged and replaced. Second, RPMI 1640 medium with 20% FBS was used throughout the experiment. The results shown in Table I indicate that independently growing leukocyte cultures were established in all 8 of the co-cultivated tubes after 6 weeks. The Sendai cell fusion factor was not essential for the establishment of the lines. No cell lines developed in cultures maintained without PC monolayers.

Morphologic appearance of the cultures. After 6 weeks, only a few viable leukocytes in the tubes containing only normal WBC remained together with scattered pyknotic leukocyte remnants (Fig. 2b). In the tubes containing x-rayed LS-M cells, some clusters of intact lymphoblasts persisted for about 3 weeks and thereafter gradually disappeared. At 6 weeks there remained only large single refractile bodies (Fig. 2c), which appeared to be large eosinophilic degenerating cells when stained with hematoxylin and eosin. By contrast, in the co-cultivated cultures, lymphoid cells increased in number beginning about 5–6 weeks (Fig. 2d). The underlying PC gradually degenerated. No indication of a virus specific cytopathic effect was noted in stained preparations of the PC.

Cytogenetic findings. The LS-M line showed female sex chromosomes on several examinations. In the co-cultivation experiments the TNL line in Expt. 1 and a pool of

TABLE II. Sex Chromosomes and EBV Antigen in LS-M Cells and in Cell Lines Resulting from Co-cultivation Experiments.

Expt.	LS-M cells		TNL cells		
	Sex	EBV antigen	Sex	EBV antigen[a] +	EBV antigen[a] —
1	F	+	M	0	1
2	F	+	M	7	1

[a] No. of TNL lines demonstrating presence or absence of EBV antigen.

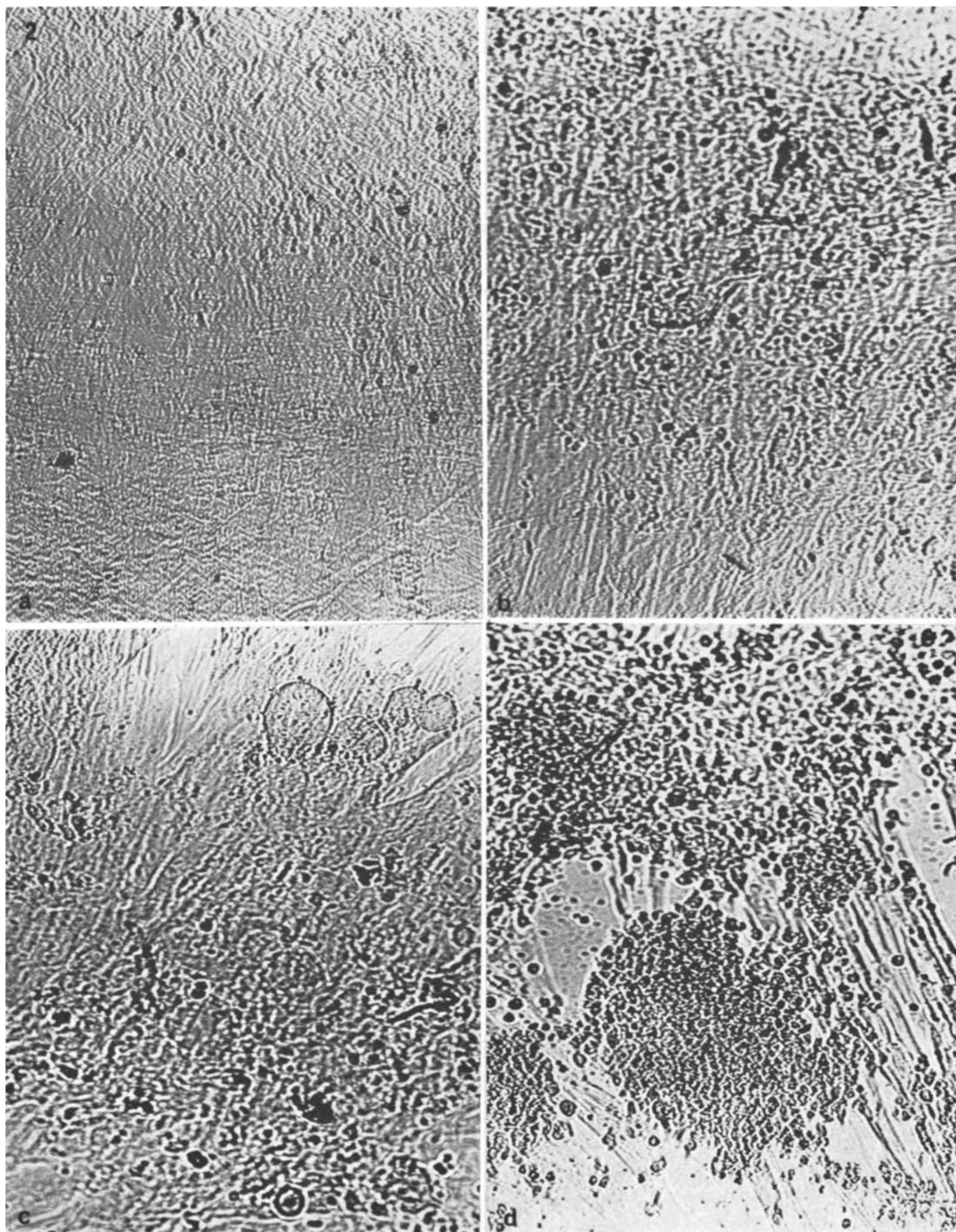

FIG. 2. Morphologic appearance of cultures in Expt. 2 after 6 weeks. (unstained) ×400: (a), control placental cells; (b), placental cells inoculated with normal PHA-treated WBC; (c), placental cells inoculated with X-irradiated LS-M cells; (d), placental cells inoculated with a combination of PHA-treated WBC and X-irradiated LS-M cells.

4 of the 8 TNL lines in Expt. 2 showed male karyotypes. The cells of the LS-M and TNL lines were generally euploid. For example, in Expt. 2, 29 of 30 LS-M cells and 26 of 30 pooled TNL cells contained 46 chromosomes. Apart from a few chromatid breaks no gross chromosome abnormalities were seen in the small number of cells examined so far.

Tests for EBV antigen. EBV antigen was not seen in 4-day or 7-day cultures of PHA-treated leukocytes from the normal donor. The LS-M cell line consistently demonstrated EBV antigen in 0.5–2.0% of cells during a period of 8 months over which it was observed. The TNL line in Expt. 1 has not revealed EBV antigen in repeated examinations with several different positive anti-EB sera over a period of 4 months. EBV antigen was detected in 7 of the 8 TNL lines in Expt. 2 (see Table II). The eighth line was EBV negative in several examinations using "double-blind" procedures.

Discussion. These experiments demonstrated that in the presence of an X-irradiated line of leukocytes from a leukemic child and a feeder layer of human placental cells normal adult PHA-treated peripheral blood leukocytes grew continuously in culture. In the absence of the irradiated cells normal leukocytes failed to grow for more than 3 or 4 weeks under conditions that were otherwise the same. These results with a leukemic leukocytic cell line harboring herpes-like virus antigen, are similar to those of Henle and his co-workers who used X-irradiated Burkitt lymphoma cells as the source of transforming factor. A recent report from Australia (9), made after our experiments were initiated showed that continuous growth of human fetal leukocytes could be induced by filtrates of a leukemic cell line.

Additional unpublished experiments using the techniques of Expt. 2 demonstrated that this method for inducing cell lines from small numbers of normal adult WBC yields reproducible results. Perhaps these consistent findings are attributable, in part, to the use of PHA-stimulated blasts as the recipient cells. PHA was employed with the thought that a transforming virus might have enhanced capacity to replicate in blast cells as compared with unstimulated leukocytes (5). This possibility has not yet been tested experimentally.

One variable in the experimental system which requires more systematic study is the PC monolayer which, so far, appears necessary. The role played by these PC is unknown. They may "condition" the medium to allow growth of a small number of leukocytes, they may provide a substrate suitable for interaction between X-rayed LS-M cells and normal WBC, or they may induce infectious virus production by the LS-M cells. It is likely that the PC could be replaced with monolayer cultures of other cell types. The PC strain was used because it never showed signs of growth in suspension, and thus was easily distinguished from the leukocytes and because it was more resistant to the destructive effects of normal WBC than cultures of human lung cells which had been seen to undergo widespread degeneration.

One interesting departure of our experimental results from those previously reported by others was the finding that TNL lines did not invariably demonstrate EBV antigen. The failure of 2 TNL lines to demonstrate EBV antigen cannot be explained until more is known of the nature of cell–virus relationships in leukocyte lines containing EBV. One possibility is that EB viral genome is present in EBV negative cells, even though viral antigen is not produced in quantities detectable by immunofluorescence.

Although EBV was not detected in the 2 lines just discussed, it is possible that the establishment of TNL lines depends upon transformation by EBV present in the LS-M line. However, in the absence of definitive evidence in the form of neutralization by specific antiserum or of transformation by cell-free purified virus, several alternatives should be considered. It is possible that other agents capable of inducing leukocyte growth are present in the LS-M line and are transferred to normal cells. Theoretically, host cell genetic material might also be released following X-ray and influence the behavior of normal WBC. Finally, it might be suggested that some factor, possibly derived from the

cell membranes of the LS-M cells, interacts with the cell membranes of normal WBC and provides the stimulus for continued growth. The initial evolution from small lymphocyte to blast can be triggered by agents such as antilymphocyte serum which presumably affect the lymphocytic membrane (10). The next stage, induction of continuous growth, might be brought about by contact with the membranes of cells already transformed. Experiments in progress are designed to determine whether the transformation of normal WBC into lines in the presence of irradiated lines containing EBV is effected by EBV alone or by other associated cellular or viral factors.

Summary. White blood cells from a normal adult male regularly became established as continuous lines when cultured in the presence of X-irradiated cells of an EBV-infected line of leukocytes derived from a leukemic girl and a monolayer of human placental cells. Seven of the 9 lines thus established became infected with EBV. The possible role of the various components of the culture system in the observed transformation is discussed.

Note added in proof: The TNL line in Expt. 1, which did not demonstrate EBV antigen at the time the manuscript was submitted, showed approximately 0.4% cells with antigen when it was subsequently examined.

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