Establishment of Cell Lines from Normal Adult Human Blood Leukocytes by Exposure to Epstein-Barr Virus and Neutralization by Human Sera with Epstein-Barr Virus Antibody[1] (35810)

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(Introduced by J. F. Enders)

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In a preliminary report, we showed (1) that continuous cell lines were engendered when phytohemagglutinin-treated normal adult human peripheral blood leukocytes were cultured in the presence of a human placental cell (HPC) feeder layer and lethally X-irradiated cells of a line (LS-M) derived originally from a leukemic child. In the absence of LS-M cells, but under otherwise identical cultural conditions, normal blood leukocytes failed to grow as cell lines. Epstein-Barr viral (EBV) antigen present in the LS-M line was also observed by immunofluorescent assay in 7 of 9 lines of transformed normal leukocytes (TNL) on initial examination. The hypothesis considered most likely was that formation of TNL was due to EBV. Other possible explanations were that the transforming factor was another microorganism which coexisted with EBV, that the transforming factor was cellular or viral DNA, or that it was on or part of the surface of the X-irradiated LS-M cells.

The present experiments were undertaken to provide evidence on whether induction of TNL is due to EBV or some other factor by exploring 2 questions: (i) Does the capacity of various strains of human lymphoblastoid cell lines (HLCL) to transform leukocytes correlate with the presence of detectable EBV antigen in the HLCL? (ii) Do human sera, with and without detectable EBV antibodies, affect the formation of TNL? The results presented summarize a series of experiments bearing on these points.

Methods. Human lymphoblastoid cell Lines (HLCL). Of 11 different leukocytic lines employed, 7 originated in our laboratory either directly from the blood or bone marrow of a patient or from normal leukocytes transformed by the LS-M line as described previously. The CCRF-CEM line (2), reported to be free of herpes-like virus particles or other virus particles was obtained through the generosity of the Children's Cancer Research Foundation, Boston. EB_3, from a Burkitt lymphoma (3) was obtained from Dr. A. S. Evans. NC37 and F265, lines established from peripheral blood of healthy individuals and apparently free of EBV particles, were gifts from Dr. Paul Gerber and Dr. F. E. Durr, respectively. Stock cultures of all HLCL, maintained in medium RPMI1640 (Grand Island Biological Co., Grand Island, N.Y.) with 20% fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B, were subdivided 2:1 weekly.

Primary leukocyte cultures. Peripheral blood leukocytes (WBC) were obtained from 10 to 20 ml of blood and were used after 3–4-days pretreatment with phytohemagglutinin-M, as outlined previously (4). The donors of WBC were 3 healthy adults, 2 women and 1 man. All donors possessed serum antibodies to EBV; none had a past history of infectious mononucleosis (IM).

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"Feeder layers." Human fibroblastoid cell strains were used as feeder layers in all experiments. Different passage levels of 4 strains of human placental cells (HPC) (5), were employed as well as a cell strain (A_2) from a whole 8 weeks human embryo. The A_2 cells were kindly made available by Dr. T. C. Shope.

Co-cultivation of HLCL and WBC. In one group of co-cultivation experiments, cells of a HLCL were lethally X-irradiated with approximately 4500 rads under the conditions in our previous report. From 0.5 to 2.0 $\times$ 10^6 X-irradiated cells were combined with the same number of WBC. In another group of experiments, crude extracts of HLCL were prepared by 3 cycles of freezing and thawing HLCL cells at a concentration of 0.5–2.0 $\times$ 10^7/ml. This material was lightly centrifuged (1000 rpm for 10 min); and, in some instances, the supernatant was passed through a 0.8-μ Millipore filter. An equal volume of extract and WBC at 8 $\times$ 10^5 cells/ml were combined. In all experiments inactivated HLCL alone and WBC alone were cultured as controls.

Cells were held at 5° for 15 min and at 35° for 2 hr on a shaker. The leukocytes suspended in RPMI1640 medium plus 20% FBS were then placed on a live feeder layer of HPC or A_2 cells. In earlier experiments feeder layers were grown on the sides of 15 $\times$ 150-mm test tubes, and tubes were held on a slant. In later experiments, feeder cells were started at the bottom of test tubes and cultures were incubated at 35° upright in 5% CO_2 and air. Part of the spent medium was removed and fresh medium was added weekly. Cultures were held from 3 to 6 months and were observed for appearance of clusters of enlarged, rapidly growing leukocytes, a sign that "transformation" had occurred. (In the context of this report "transformation" means this late phase of rapid leukocyte proliferation which continued indefinitely and is not to be confused with "blast transformation" which is an early short-lived phenomenon.) TNL which were to be tested for EBV antigen and chromosomes were subdivided, free of the feeder layer, until enough cells were available for the test.

Tests for EBV antigen and EBV antibody. Cell lines were examined for EBV antigen with the indirect immunofluorescence assay (6). The sources of EBV antibody were a serum from a healthy American adult and a serum from an African patient, "Mwangi," with nasopharyngeal tumor. The latter serum was a gift from Dr. W. Henle. Fluorescein isothiocyanate conjugated rabbit antihuman gamma globulin was purchased commercially (Antibodies, Inc., Davis, California). In measurements of serum antibody by the immunofluorescent method the EB_3 cell line was the source of antigen.

Karyotype. The chromosomes of 20 individual cultures of TNL were studied to determine the origin of the TNL. Chromosomes were examined only in situations in which HLCL and WBC of opposite sex were cocultivated. At least 20 metaphase spreads from each line were examined. Cultures were treated with 10^{-7} *M* colchicine for 2 hr prior to harvesting and preparation of cells. All results were evaluated as "unknowns."

Neutralization tests. When X-irradiated cells were the source of transforming factor, 2 unheated human sera with and without EBV fluorescent antibody were diluted 1:10 in medium, mixed with separate aliquots of the X-irradiated cells, and held at 37° for 1 hr, before the X-irradiated cells were added to WBC. The sera were also included in the medium of the culture system for 8 and 3 weeks in 2 experiments. In later neutralization tests, which employed clarified or filtered extracts of line 883L as transforming factors, a 1:2 dilution of unheated serum was mixed with an equal volume of the transforming factor and held in a water bath at 37° for 1 hr before the mixture was added to WBC. Human sera were not added thereafter. The transforming titer of the 883L extract was $10^{1.0}$ TD_{50}/0.25 ml.

Tests for mycoplasma. HLCL were tested for mycoplasmas by inoculation of agar plates according to the method of Hayflick (7). These tests were done by Miss Jean Emmons.

Results. Summary of co-cultivation experiments. Under the conditions of these experiments cell line formation did not occur in 72

TABLE I. Cell Lines Established by Co-cultivation of Killed Leukocyte Lines with Normal Peripheral Blood Leukocytes on Feeder Layers of Human Cells.

Method of inactivation	No. of expts.	Type of feeder layers	No. transformed/no. of cultures			Chromosome studies		
			Normal WBC	Inact. lines	Co-cult.[a]	Sex WBC donor	Sex TNL[b]	No. of TNL tested
X-Irradiation	6	HPC[c]	0/46	0/72	27/70	M	M	13
						F	F	6
Freeze–thaw	6	HPC; HEF[d]	0/26	0/42	79/100	M	NT[e]	0
						F	F	1

[a] The denominator includes all cultures of a group in which at least one replicate culture was transformed.

[b] TNL = transformed normal leukocytes.

[c] HPC = human placental cells; four different cell strains used.

[d] HEF = human embryonic fibroblasts; strain "A_2" was used (see text).

[e] NT = not tested.

control cultures of WBC alone or in 114 cultures inoculated with HLCL inactivated by X-irradiation or freezing and thawing (Table I). Cell line formation was evident only in cultures containing both an inactivated HLCL (or extract) and WBC. The frequency of transformation varied from experiment to experiment and was dependent on the source of transforming factor (Table II) and on other conditions such as the extent of initial destruction of the feeder layer by WBC.

Examination of the karyotype of the TNL invariably showed that they were derived from normal WBC (Table I). Twenty cultures were examined individually and in each instance the TNL proved to have the karyotype corresponding to the sex of the donor of the WBC.

Capacity of different HLCL to transform WBC. Of 11 HLCL tested, 6 were capable of inducing continuous proliferation of WBC. Three HLCL with the capacity to transform WBC were derived from leukemic children (one of whom had classical infectious mononucleosis when the leukocytes were placed in culture.) One line (883L) was obtained from the blood of an elderly patient who acquired a mononucleosis-like illness following blood transfusion. The other 2 lines capable of causing transformation originated from normal cells transformed *in vitro* by the LS-M line.

Correlation was found between the presence of EBV antigen and the capacity of an HLCL to transform. No transformation was induced by lines CCRF-CEM, NC37, or F265 which had no EBV antigen, or by line 516L, which had a very low level of antigen at the time it was tested. The highest frequency of transformation and the most rapid appearance of TNL occurred when line 883L was used as the source of transformation factor. This line also contained the most fluorescent cells at the time cell extracts were made. Intermediate frequency of transformation was observed with lines LS-M, 511L, and A87 which had intermediate levels of EBV antigen. Line EB_3, however, despite a fairly constant level of EBV antigen was not found to transform cells *in vitro*.

Evaluation of the role of mycoplasma in the transformation process. During the course of these studies the 511L line was found to contain an unidentified mycoplasma. Therefore, experiments were done to determine whether mycoplasmas could be directly responsible for the transformation. The evidence collected suggested that mycoplasmas were not essential for transformation. The 511L line "cured" by treatment with Kanamycin and Aureomycin (50 μg/ml of medium), was still capable of transforming leukocytes, and this group of TNL did not contain mycoplasma. Furthermore, several HLCL which were capable of supplying

TABLE II. Capacity of Different Human Lymphoblastoid Cell Lines to Induce Cell Line Formation from Normal Blood Leukocytes.[a]

Designation of HLCL	Origin of HLCL	Type of material	Cells with EBV antigen (%)	No. of expts.	No. transformed/no. of cultures[b]	Days to transformation [median (range)]	Tests for EBV in TNL[c]		
							No. of TNL		Cells with EBV antigen (%; range)
							Tested	With EBV antigen	
LS-M	Leukemia	X-Rayed cells	0.5–2.8	4	11/24	63 (32–126)	11	6	<0.1–1.6
A-81	Leukemia	Extract	<0.1	2	1/8	63	NT	NA	NA
CCRF-CEM	Leukemia	X-Rayed cells	Neg.	2	0/12	NA	NA	NA	NA
883L	IM	Extract; filtrate	4.2	4	21/22	23 (21–31)	5	5	2.0–4.7
A87	IM + leukemia	Extract	0.9	2	6/8	35 (21–42)	NT	NA	NA
516L	IM	Extract	<0.1	2	0/8	NA	NA	NA	NA
511L	TNL	X-Rayed cells; extract	<0.1–0.4	4	25/34	45 (30–139)	9	9	<0.1–0.8
733L	TNL	X-Rayed cells	0.4	2	3/10	92 (65–134)	1	0	NA
EB_3	BL	Extract	0.9	2	0/19	NA	NA	NA	NA
F-265	Normal	Extract	Neg.	1	0/4	NA	NA	NA	NA
NC37	Normal	Extract	Neg.	1	0/4	NA	NA	NA	NA

[a] HLCL = human lymphoblastoid cell line; IM = infectious mononucleosis; BL = Burkitt lymphoma; NA = not applicable; and NT = not tested.
[b] Does not include cultures in which human sera were added as part of a neutralization test (Table III).
[c] TNL = transformed normal leukocytes—transformed by exposure to LS-M.

TABLE III. Neutralization of Cell Line Formation from Normal Leukocytes by Human Sera Containing EBV Antibodies.[a]

Human serum	Source	Anti-EBV titer in FA Test[b]	Materials used to transform cells[c]	
			Extract 883L	Filtrate 883L
G. M.	Adult: well	1:80	NT	0/4
D. S.	Adult: well	1:40	NT	0/4
S. W.	Adult: IM, conv. 6 months	≧1:160	0/4	0/4
N. P.	Adult: IM, pre-illness	<1:10	NT	3/4
N. P.	Adult: IM, conv. day 49	1:40	NT	0/4
N. P.	Adult: IM, conv. day 189	≧1:160	NT	0/4
R. D.	Adult: IM, pre-illness	<1:10	NT	4/4
D. B.	Child: respiratory infection	<1:10	4/4	4/4
T. G.	Child: arthritis	<1:10	NT	4/4
C. G.	Child: diarrhea	<1:10	4/4	4/4
S. B.	Child: fever and rash	<1:10	NT	4/4
None		NA	4/4	7/8

[a] NT = not tested; NA = not applicable; and conv. = convalescent.
[b] Tested against antigen present in the EB_3 line.
[c] Numbers shown = no. of transformed cultures/no. of cultures attempted.

transformation factor (for example, the A87, A81, and 883L lines in Table II) were free of mycoplasma, and the TNL derived from cocultivation with mycoplasma-free HLCL were also negative for mycoplasmas.

Examination of TNL for EBV antigen. EBV antigen was detected in 20 of 26 individual cultures of TNL which were examined by immunofluorescence (Table II). Antigen was present in 13 TNL on first examination with a healthy adult serum. In 5 additional TNL, antigen was detected with the same serum on cells which had been held without subdivision for 3 weeks. In 2 other TNL, negative by the first two methods, antigen was demonstrated with the "Mwangi" serum.

Neutralization tests. In early experiments to demonstrate the effect of EBV antibody, when X-irradiated cells were the source of transforming factor, the results showed partial inhibition of transformation by antibody (data not shown). However, when it was found that extracts prepared from the 883L line were capable of inducing transformation rapidly and regularly, consistent neutralization results could be obtained. The results with 11 different sera showed that sera with EBV fluorescent antibody either from 2 healthy persons without a history of IM (but working with EBV), or from 2 persons convalescent from IM, inhibited transformation when tested at a dilution of 1:4 (Table III). Sera free of EBV antibody, either from children with various illnesses or from adults prior to IM (taken as part of a prospective study and generously supplied by Dr. J. Niederman) failed to neutralize transformation. Included in the results was one set of sera from the same patient (N.P.) before and after IM. Neutralization of transformation. was brought about only by the convalescent sera.

Discussion. The results of these experiments amplify and solidify evidence for the hypothesis first proposed by Henle *et al.* (8), that EBV stimulates continuous proliferation of human leukocytes *in vitro*. The evidence may be summarized as follows: First, lines which supply the transformation factor all contain EBV fluorescent antigen, and lines which are free of detectable antigen, such as the Burkitt lymphoma Raji line in experiments of Henle *et al.* (8) and Gerber *et al.* (9) or the leukemic CCRF-CEM line, or the normal NC37, or F265 lines in our experiments do not contain transforming factor measurable by available assays. A notable exception which will be discussed is the EB_3

line. Second, sera with EBV antibodies inhibit transformation, as first shown by Pope and co-workers (10–11) in an assay employing cell-free extracts from a leukemia line and fetal leukocytes. Gerber *et al.* (9) have transformed adult leukocytes with partially purified EBV and neutralized the effect with a serum with EBV antibody. Sera without EBV antibody have thus far not proved to be inhibitory. We have confirmed the inhibitory effect of EBV antibodies in another transformation system and demonstrated the appearance of neutralizing activity in the convalescent serum of a patient with infectious mononucleosis. Further study of neutralizing antibody in infectious mononucleosis is underway. Third, leukocytes from infants (8), fetuses (10), or adults without EBV antibodies (9) acquire EBV antigens following transformation *in vitro*.

Attention should be given to the reasons for the failure of the EB_3 line to transform WBC, a finding which is in agreement with observations by Diehl and co-workers (12). Some hypotheses which might be explored are that EB_3 does not produce fully infectious particles, that freezing and thawing does not release infectious virus from EB_3, that an accessory factor, perhaps helper virus, necessary for transformation is absent in EB_3, or that inhibitors of transformation are present in EB_3. We have no experimental data as yet to enable a choice among these alternatives.

Assays for leukocyte transformation by EBV have included infant and fetal leukocytes, large numbers of adult leukocytes and smaller numbers of adult leukocytes on a feeder layer. The latter method, which we employ, has the practical advantage of availability and economy of materials, but it has the disadvantages that most adults have had previous experience with EBV and that transformation of small numbers of adult leukocytes is dependent on the presence of the feeder layer. We have recently found umbilical cord leukocytes to form lines readily in response to EBV and feeder cells are not required (unpublished observations).

A major area to be investigated is how leukocyte transformation is effected by EBV. Does it proceed by mechanisms analogous to those of the smaller oncogenic DNA viruses, or is it a different phenomenon, somehow related to the immunologic properties of the lymphoid cells which are the only known hosts for the virus? Recent evidence (13–15) that viral genome is present in all cells of a line, even though only a small proportion or perhaps none reveal EBV antigen, would support the suggestion that transformation by EBV is the result of intracellular persistence of the viral genome.

It remains to be determined, by appropriate studies in laboratory animals, whether EBV has the capacity to induce leukocyte proliferation *in vivo* as well as *in vitro*.

Summary. Eleven human lymphoblastoid cell lines (HLCL), inactivated either by X-irradiation or by freezing and thawing, were tested for their capacity to induce continuous proliferation of normal adult human leukocytes (WBC). The six HLCL which provided the transformation factor all contained Epstein-Barr viral (EBV) antigen. Three HLCL without EBV antigen, and one with a barely detectable level of EBV antigen did not transform WBC. Only one line with a significant amount of EBV antigen, EB_3, was incapable of transforming WBC. EBV antigen appeared in most, but not all, lines of transformed normal leukocytes (TNL). Six human sera which contained antibody to EBV by the immunofluorescent method inhibited transformation and 5 sera free of such antibody permitted transformation to occur. The results provide additional evidence that EBV is capable of inducing continuous proliferation of WBC *in vitro*.

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