

*TRANSFORMATION AND CHROMOSOME CHANGES  
INDUCED BY EPSTEIN-BARR VIRUS IN NORMAL  
HUMAN LEUKOCYTE CULTURES*

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*Abstract.*—Three separate attempts to establish long-term leukocyte cultures from the peripheral blood of a healthy adult who had no evidence of previous infection with Epstein-Barr virus were consistently unsuccessful. On the other hand, *in vitro* infection of buffy-coat cells of this donor with Epstein-Barr virus resulted in blastoid transformation of the lymphocytes and long-term growth potential. The transformation was accompanied by major chromosome changes. The significance of these findings in relation to lymphoproliferative diseases in man remains to be determined.

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In 1964 Epstein and Barr<sup>1</sup> first described the presence of a herpes-like virus in lymphoblast cultures of Burkitt's lymphoma. Subsequently, particles of similar morphology and antigenicity were detected in cultures of other Burkitt tumors,<sup>2-5</sup> in buffy-coat cell cultures of patients with leukemia<sup>6</sup> and infectious mononucleosis,<sup>7</sup> and of apparently normal individuals.<sup>8, 9</sup> This herpes-like virus, frequently designated Epstein-Barr virus (EBV), is apparently antigenically distinct from the other known human herpes viruses.<sup>10</sup> The frequent association of the Epstein-Barr virus with lymphoblastoid cells in long-term leukocyte cultures raises the question of its possible lymphoproliferative potential. Co-cultivation of leukocytes with irradiated Burkitt's lymphoma cells<sup>11</sup> gave suggestive evidence of a growth stimulatory effect, and more recently Pope *et al.*<sup>12</sup> reported transformation of fetal human leukocytes by extracts of a human leukocyte cell culture of leukemic origin.

The present study was undertaken to determine the *in vitro* effect of EBV on the growth and morphology of leukocytes from a healthy human donor who had no evidence of previous infections with Epstein-Barr or other known human herpes viruses.

*Materials and Methods.*—*Cell cultures:* Two lymphoblastic cell lines derived from Burkitt's lymphomas were grown in medium 1640<sup>9</sup> supplemented with 20% heat-inactivated fetal calf serum and 80 µg/ml of neomycin sulfate. One of these, the P3-HRIK line,<sup>13</sup> contains variable amounts of EB virus, while Raji, the other cell line,<sup>14</sup> contains no detectable EB virus. An established line of human leukocytes designated NC37, derived from a healthy donor<sup>15</sup> and free of detectable EBV, was grown in McCoy medium 5A<sup>16</sup> with 10% heat-inactivated fetal calf serum and 80 µg/ml of neomycin sulfate. Buffy-coat cells for culture were derived from 100 or 500 ml of fresh blood from a healthy male donor A by methods described previously.<sup>17</sup> Donor A had no detectable complement-fixing antibodies at 1:2 dilution of serum to four human viruses of the herpes group: EB virus, herpes simplex, cytomegalo virus, and varicella. Viable cell counts were performed at 4-day intervals; the medium was changed weekly, and the culture volume was adjusted

to maintain a cell density of  $4-5 \times 10^5$ /ml as long as possible. All cultures of donor A cells were maintained as long as viable leukocytes were detectable.

*Preparation of EB virus and virus-free control material:* After the Raji or the P3-HRIK cell cultures reached a cell density of about  $2 \times 10^6$ /ml, the spent medium was removed by centrifugation and the cells were seeded at a concentration of  $3 \times 10^6$ /ml in 1640 medium containing 10% heat-inactivated fetal calf serum. After a period of incubation of 10-12 days at 33°C without media changes, the cells were removed by centrifugation at 10,000 rpm for 20 min, the supernatant fluid was filtered through a 0.45- $\mu$  Millipore filter membrane and concentrated to approximately 160 ml by ultrafiltration through a Diaflo UM-10 membrane. The concentrated fluid was then centrifuged in a Spinco SW 25.2 rotor for 2 hr at 25,000 rpm. The pellets, consisting of subcellular components and virus in the case of P3-HRIK culture fluid, were resuspended in growth medium containing 10% dimethylsulfoxide in 1/100-1/400 of the original volume of culture fluid. These preparations were used either fresh or after storage at -90°C.

*Inoculation of leukocytes:* Pellets of fresh buffy-coat cells of donor A containing approximately  $40 \times 10^6$  cells were resuspended in 0.3-0.5 ml of EBV suspension. Controls received suspensions of pellets obtained from virus-free Raji culture fluid. Pellets of  $10^7$  NC 37 cells were treated similarly. In addition, cells suspended in medium were included as controls. The cell suspension mixtures were incubated at 37°C for 1 hr with frequent agitation. The cells were then washed in 20 vol of medium and planted in 8.0 ml of medium in 2-oz prescription bottles at 37°C and 5% CO<sub>2</sub>. For virus neutralization studies, equal volumes of 200 $\times$  concentrated virus suspension and 1:2 diluted human sera with or without EBV antibodies were incubated for 3 hr at 4°C and then used for cell infection.

*Immunofluorescence (IF) test:* Acetone-fixed films of cells of donor A or of NC 37 cells were treated with a fluorescein-isothiocyanate-labeled serum globulin of a healthy human donor containing high titers of EBV antibodies as determined by complement fixation. Virus-free NC 37 cultures served as controls. The stained preparations were examined under UV illumination (Osram HB 200-watt lamp).

*Complement-fixation (CF) test:* Cultured leukocytes of donor A were sedimented by centrifugation and washed in veronal-buffered saline; a 10% suspension in the saline was sonicated for 2-3 minutes. The sonicate was centrifuged at 500 rpm for 15 min, and the supernatant fluid was tested for CF activity with known positive and negative human sera. The microtiter technique<sup>18</sup> employing 1.8 units of complement was used. Virus-free Raji cells and NC 37 cells served as antigen controls.

*Demonstration of infectious virus in EBV-transformed cells:* Cell-free culture fluids obtained in the process of CF antigen preparation were filtered through a 0.45- $\mu$  Millipore membrane and centrifuged in a SW 25.2 rotor at 25,000 rpm for 2 hr. The pellets were resuspended in 1/100 of the original volume in growth medium and used to infect NC 37 cells, which were examined at 3-day intervals for the presence of viral antigens by IF tests.

*Interferon tests:* Cell-free supernatant culture fluids were inoculated into tube cultures of Wi38 human diploid cells. One ml of undiluted culture fluid was inoculated into each of six tubes. After 24-hr incubation at 36°C the cell sheets were thoroughly washed with Eagle's medium and inoculated with 0.2 ml of vesicular stomatitis virus containing 100 TCID<sub>50</sub>. After an adsorption period of 1 hr, 1.8 ml of Eagle's medium with 2% fetal calf serum was added; the cultures were incubated for 6 days and examined microscopically at daily intervals. The virus controls showed 3-4<sup>+</sup> cytopathogenicity about 2-3 days after inoculation. In each test, culture fluids of a human leukocyte cell line producing high levels of interferon were included as controls.

*Chromosome studies:* Chromosome studies were carried out on PHA-stimulated peripheral blood of the donor and on the established EBV-transformed leukocyte cultures by the method of Tjio and Whang.<sup>19</sup>

*Electron microscopy:* Cells for thin-section study were prepared and processed as described,<sup>17</sup> and examined with a Siemens-Elmiskop IA.

*Results.—Transformation experiments:* During a five-month period preceding the transformation experiments, three separate attempts were made to obtain long-term, established cultures of leukocytes from 500 ml of fresh citrated blood obtained from donor A. In each case the number of viable leukocytes decreased steadily after 10–14 days; most viable cells disappeared between 50 and 65 days, resulting in the ultimate loss of the cultures. When a sufficient quantity of infectious EBV was available, approximately  $400 \times 10^6$  fresh buffy-coat cells were divided into ten aliquots of  $40 \times 10^6$  cells, and the cell pellets were resuspended in 0.4 ml of the following preparations: growth medium (A/CO), Raji culture fluid (A/R), EBV neutralized with positive serum (A/V<sup>+</sup>), EBV treated with negative serum (A/V<sup>-</sup>), and  $400\times$ -concentrated EBV (A/V<sup>4</sup>). Each group (A/) was set up in duplicate. In addition, pellets of 10<sup>7</sup>NC37 leukocytes were treated with the same inocula. Virus infectivity and neutralization were evaluated by IF tests of inoculated NC 37 cells at 3 to 4-day intervals. At three days approximately 0.5 per cent of NC 37 cells inoculated with unneutralized virus showed nuclear and cytoplasmic staining with a fluorescein-labeled globulin of a positive human serum. The frequency and intensity of the staining reaction increased during the next ten days to a maximum of 5–7 per cent. On the other hand, NC 37 cells inoculated with neutralized EB virus, those treated with Raji culture fluid, and untreated controls gave consistently negative staining reactions. Neutralization of virus with a human serum containing a high titer of CF antibodies to EBV could be demonstrated repeatedly using NC 37 cells as indicator cells.

During the first 10–14 days all buffy-coat cell cultures showed a decrease in the number of viable cells. The cultures inoculated with a high concentration of EBV (A/V<sup>4</sup>) contained only 10 per cent surviving cells; about 15 per cent of these were medium- to large-sized lymphocytes. The cells of this culture group showed a rapid loss of viability and were completely degenerated on the 18th day of culture. All other cultures showed an approximately 70 per cent reduction in the number of viable cells at the end of the second week, and duplicate cultures of each group were combined. They consisted mainly of small lymphocytes and occasional macrophages. At 18 days the average viable cell count of the remaining cultures was between 300,000 and 400,000/ml; there was a striking change in morphology of cells in culture A/V<sup>-</sup> consisting of about 30–50 per cent of large cells seen frequently in small aggregates. The remaining cultures showed no significant morphologic changes. On day 21 there was a further reduction of viable cells to approximately 200,000/ml in all groups. Cultures A/CO, A/R, and A/V<sup>+</sup> showed a continued decline in viability and were finally lost around 40–45 days. By contrast, cells of the A/V<sup>-</sup> culture reached a concentration of  $10^6$ /ml on the 24th day with the appearance of macroscopic cell clumps. During the following three days the cell population doubled; the culture could be divided and has been subcultured two to three times weekly for the past seven months.

About eight weeks after the first transformation experiment, we obtained 100 ml of citrated blood from donor A for a repeat experiment which consisted of an uninfected control and cells treated with  $100\times$ -concentrated EBV. The con-

trol group lost viability at 42 days, while the infected cultures showed evidence of transformation at 26 days and could also be established in long-term culture. The two transformed leukocyte cell lines were designated AV-1 and AV-2, respectively.

*Properties of transformed leukocytes:* Cultures of AV-1 and AV-2 cells consist essentially of three cell types. Approximately 12 per cent appear to be small lymphocytes of about 7–10- $\mu$  diameter, with large rounded nucleus and sparse cytoplasm. Medium-sized (10–15  $\mu$ ) lymphocytes with similar morphology make up 70 per cent of the population. Approximately 14 per cent are large, immature lymphoblastoid cells up to 25  $\mu$  in diameter, with large pleomorphic nuclei containing prominent nucleoli. Between 3 and 5 per cent of the population consists of multinucleated giant cells. No inclusion bodies were observed in Giemsa- or Feulgen-stained preparations.

AV cells maintained in logarithmic growth phase have a generation time of 36–40 hr. In cultures maintained without regular refeeding, many cells begin to degenerate, particularly the multinucleated cell type. Such "aged" cultures also show an increase in the number of EBV-containing cells, suggesting that cell death is correlated with a lytic infectious cycle. Cultures of AV cells were tested at frequent intervals and were found to be free of mycoplasma and other organisms. Aliquots of AV cells frozen in the presence of 10 per cent dimethylsulfoxide and stored in liquid nitrogen could be readily revived.

*Serological studies:* After 26 days in culture, cells of groups A/CO, A/R, and A/V<sup>+</sup> failed to stain in direct IF tests. On the other hand, about 0.5 per cent of the cells of the A/V<sup>-</sup> group showed nuclear and cytoplasmic staining which increased in intensity and frequency during the following two weeks and reached a level of 10–15 per cent. Most of the multinucleated giant cells showed strong nuclear and cytoplasmic staining. The staining reaction could be blocked with unlabeled sera of patients with Burkitt's tumor or infectious mononucleosis. Acetone-fixed AV cells treated with fluorescein-labeled goat anti-human globulin serum failed to react, indicating absence of immunoglobulin-producing cells.

Sonicates of 10 per cent AV cell suspensions contained 16–32 units of CF antigens which reacted only with known positive human sera.

*Infectivity and interferon studies:* Pellets of NC 37 cells were inoculated with suspensions of 100 $\times$ -concentrated cell-free fluids of AV cultures. IF tests at three days postinfection revealed brilliant nuclear staining of 50 per cent of the cells. On the fifth day more than 90 per cent of the inoculated cells degenerated, presumably due to lytic infection. Infectivity could be transmitted serially to fresh NC 37 cells.

For the detection of interferon, cell-free fluids of A/CO and A/V<sup>-</sup> cultures were collected on the 26th day of culture, and additional AV culture fluids were obtained on days 55 and 85. None of these preparations, when tested undiluted, demonstrated detectable interferon activity.

*Electron microscopic studies:* The AV cells showed gross morphological characteristics similar to those seen in lymphoblastic cell lines derived from normal human subjects<sup>9, 17</sup> from Burkitt's tumor,<sup>20</sup> or from leukemic patients.<sup>21</sup> Most of the cells appeared enlarged, and several were multinucleate or had multilobulated nuclei.

Figure 1 shows a comparison between an infected cell (*a*) and a noninfected cell (*b*). The most obvious difference at this magnification is a considerable lessening in nuclear density in the infected cell. At a higher magnification (Fig. 2), a doubling of the nuclear envelope (*double arrows*) can be seen. This phenomenon has been previously observed in Burkitt's lymphoma cells,<sup>20</sup> in human leukemic lymphoblasts,<sup>21</sup> and in leukocyte cell lines derived from normal individuals.<sup>17</sup> Another characteristic of the AV cells is pairing of elements of the endoplasmic reticulum (Fig. 2, *arrows*). Herpes-like virus particles are visible in the cell nucleus (Fig. 2, *empty arrows*) and are shown at a higher magnification in Figure 3. The enlarged cells frequently contained nuclear virus particles, but the normalized (10- $\mu$ ) cells were also seen to be producing virus.

*Chromosome analyses:* A summary of the chromosome distribution and aberrations is shown in Table 1. A two-day PHA-stimulated culture of the donor's peripheral blood demonstrated that all cells had a normal male karyotype with 2 per cent breaks and 0.5 per cent polyploidy, well within normal limits.

The long-term transformed culture AV-1 derived from the same individual was harvested at different time intervals, and the results were as follows: at three

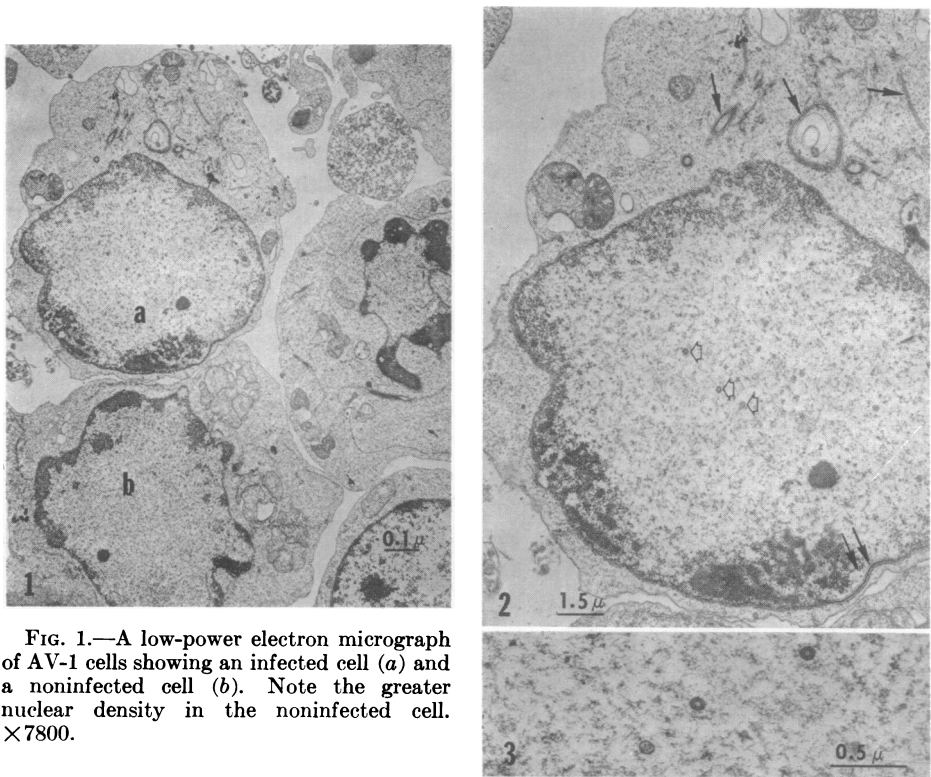


FIG. 1.—A low-power electron micrograph of AV-1 cells showing an infected cell (*a*) and a noninfected cell (*b*). Note the greater nuclear density in the noninfected cell.  $\times 7800$ .

FIG. 2.—A more highly magnified view of the infected cell seen in Fig. 1. Note the doubling of the nuclear envelope (*double arrow*), the paired and generally abnormal endoplasmic reticulum (*arrows*), and the nuclear virus particles (*empty arrows*).  $\times 15,000$ .

FIG. 3.—A highly magnified view of the herpes virus particles seen in Fig. 2.  $\times 38,400$ .

weeks, 18 per cent of the metaphases had a normal male karyotype, 65 per cent had 47 chromosomes with one extra chromosome in group C (Fig. 4), 6 per cent had 48 chromosomes (three of these cells were analyzable: two had an extra chromosome in both groups C and D; the third cell had an extra chromosome in both groups C and G), and 10% had other chromosomal aberrations.

At six weeks, there was an increase in the percentage of cells with 47 chromosomes from 65 per cent (at three weeks) to 90 per cent; 3 per cent of the cells had 48 chromosomes with one extra chromosome in both groups C and D; 5 per cent had 46 chromosomes (3 per cent were normal, 2 per cent had an extra chromosome in group C and were missing a chromosome in either group F or G); and 16 per cent had other chromosomal aberrations which included 3 per cent pulverization with or without syncytia (Fig. 5).

At 12 weeks 8 per cent of the AV-1 cells had 46 chromosomes (half of these were normal; the other half had an extra chromosome in group C and the missing chromosome varied from cell to cell), 83 per cent of the cells had 47 chromosomes, 6 per cent had 48 chromosomes with an extra one in both groups C and D, and 3 per cent had 49, 74, or 80 chromosomes. Other aberrations, including 9 per cent pulverization with or without syncytia, were seen in 33 per cent of the cells.

A repeat culture (AV-2) of the same donor was harvested at four weeks and contained 98 per cent of cells with 47 chromosomes, with one extra chromosome in group C; 2 per cent of the cells had 48 or 49 chromosomes, and 10 per cent had other chromosomal aberrations.

*Discussion.*—Blastoid transformation of human leukocytes has been demonstrated with a variety of agents: phytohemagglutinin,<sup>22</sup> pokeweed,<sup>23</sup> antiserum to leuko-

TABLE 1. *Chromosome analysis in control and infected donor cell cultures.*

Desig.	Days in culture	Chromosome Distribution										Chromosome Aberration				
		42	44	45	46	47	48	49	Total	Polyp. (%)	Total group (%)	Distal portion of C (%)	Breaks (%)	Pulveriz. or syncytia (%)	Ring or dicentric (%)	
Control with PHA	2	...	...	...	100	...	...	...	...	...	...	100	0.5	0	0	0
	21	1	3	3	18	65	6	...	51	1	52	63	1	3	0	0
AV <sub>1</sub>	42	...	...	1	5	90	3	1	...	...	...	100	5	2	1	3
	84	...	...	...	8	83	6	1	74	80	...	100	6	12	2	9
AV <sub>2</sub>	28	...	...	...	...	98	1	1	...	...	...	100	2	2	0	7

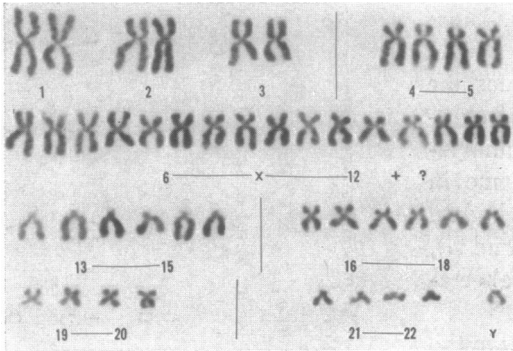


FIG. 4.—Karyotype of AV-1 cells containing 47 chromosomes with one extra C-group chromosome.

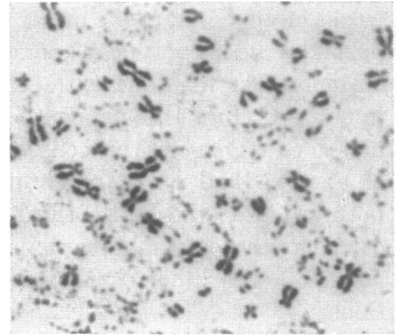


FIG. 5.—Mitosis and syncytia of AV-1 cells showing both normal-appearing and pulverized chromosomes.

cytes,<sup>24</sup> extracts of lymphocytes,<sup>25</sup> treatment of specifically sensitized cells with corresponding antigens,<sup>26</sup> and mixed culture with leukocytes of unrelated individuals.<sup>27</sup>

The following lines of evidence suggest a role of EBV in the induction of proliferative response, and morphologic and karyotypic changes of leukocytes from donor A: (a) consistent failure to observe spontaneous transformation of uninfected leukocytes of this donor and inability to establish the cells in long-term culture; (b) failure to induce transformation with concentrates of fluids from virus-free Raji cell cultures; (c) neutralization of infectivity of EBV, inhibited induction of transformation, while similar treatment with an antibody-free human serum had no effect; (d) ability to reproduce EBV-induced transformation and karyotypic changes.

Absence of detectable CF antibodies in donor A to EBV and other human herpes viruses five months before and eight weeks after initiation of the transformation experiments indicates that this individual had probably no previous infections with these viruses, since the corresponding antibodies usually persist for many months or years. The special immune status of this donor excludes the possibility of antigenic stimulation of sensitized lymphocytes by EBV.

It has been suggested by some investigators<sup>28</sup> that lymphoid cells endowed with a potential for long-term *in vitro* proliferation are present at low concentration in the circulating blood of apparently healthy individuals and are selected for long-term culture by the use of large quantities of blood in the initiation of buffy-coat cultures. The evidence presented does not support this argument, since we failed in three attempts to establish long-term leukocyte cultures from 500 ml of blood from a donor without previous EBV infection while the same culture techniques enabled us to establish leukocyte cell lines from six of eight healthy donors with serological evidence of previous EBV infections.<sup>8, 17</sup>

The successful neutralization of EBV infectivity with a human serum containing specific antibodies opens the way for further antigenic characterization of this virus.

The role of viruses in the induction of chromosome changes has been well established in recent years.<sup>29</sup> Kohn *et al.*<sup>30, 31</sup> noted a high frequency of cells with

distal secondary constriction of chromosome pair #10 of the C group in Burkitt lymphoma cultures and long-term leukocyte cultures of patients with infectious mononucleosis. We have not considered similar aberrations noted in AV cells to be significant. Additional investigations are in progress to determine whether chromosomal aberrations similar to those described in AV cells will be found in cells of other donors following EBV infection.

Further studies are required to determine the actual mechanism involved in EBV-induced blastoid transformation of leukocytes and its possible significance in relation to lymphoproliferative diseases of man.

*Note added in proof:* Since submission of this manuscript, Pope *et al.* (*Intern. J. Cancer*, **4**, 255 (1969)) reported transformation of fetal human leukocytes by EBV.

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