Chromosome Lesions Induced in a Human Hematopoietic Cell Line by Infection with Epstein-Barr Virus[1] (35540)

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(Introduced by J. L. Ambrus)

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A small portion of cells in many continuous cultures derived from Burkitt lymphomas, leukocytes of patients with various disorders, and leukocytes of healthy individuals chronically harbor a herpes-type virus (1–6), generally referred to as Epstein-Barr virus (EBV). An etiological significance of this agent to Burkitt lymphoma or other disorders has been suggested but not established (1, 2,-6). Attempts have been made at demonstrating the biological activities of this virus in various *in vitro* systems. The evidence suggests that EBV is capable of transforming normal human leukocytes and can be transmitted to a limited number of human hematopoietic cell lines under suitable conditions (7–12). Chromosome lesions have been noted in several EBV carrier lines (13–17) and in cell lines transformed by the virus (7, 8, 10, 16). The present paper reports pronounced effects on the chromosomes of a human hematopoietic cell line after infection with cell-free EBV suspensions prepared from a cloned Burkitt lymphoma cell line.

Materials and Methods. Cell lines. RPMI 6410 was established from the peripheral blood of a male patient with myelogenous leukemia in February 1964 (18), and has been maintained in McCoy's medium 5A with 3% heat-inactivated fetal calf serum. Frequent electron microscopy and immunofluorescence tests have shown that this line is free of EBV (11).

P_3J-HR1K (HR1K) is a cloned subline (19) of the Burkitt lymphoma line P_3J (Jiyoye) (Pulvertaft, unpublished data). This subline contains high numbers of EBV-positive cells (19). The EBV used in the present study was prepared from this line.

Raji is a Burkitt lymphoma line that contains no detectable virus (2). EBV-free control material was prepared from this line. Both HR1K and Raji were maintained in PRMI medium 1640 with 10% heat-inactivated fetal calf serum.

Virus preparation and inoculation. EBV was concentrated from culture medium in which HR1K cells had been grown. A number of cultures were set up in 16-oz bottles containing 100 ml of medium/bottle, with a cell concentration of approximately 10^6 cells/ml. After incubation for 6 days at 37° and 4 to 5 days at 33° without changes of medium, the cells were removed by centrifugation at 2000 rpm for 30 min. The supernatant was filtered through first 0.8 and then 0.45 μ Millipore filters. The filtrate was centrifuged again in a Beckman L-4 no. 30 rotor at 20,000 rpm for 2 hr. The supernatant was decanted, and the resulting pellet was resuspended in the remaining fluid at about 1/300 of the original volume of fluid.

Three separate experiments were performed. In Expt. I, 1 ml of EBV suspension concentrated from about 300 ml of HR1K culture fluid, or 1 ml of medium (for control), was added to two tubes containing pellets of approximately 12.5×10^6 viable RPMI 6410 cells each. The mixtures were incubated for 90 min at 37° with frequent agitation. The cells were washed once with medium, and

[1] This study was supported in part by U.S. Public Health Service Research Grant FR-05648-03 from the division of Research Facilities and Resources, by Grant GR-10-RP-69 from the United Health Foundation of Western New York, by U.S. Public Health Service Grant CA-10465, and by NCI Contract PH43-63-593.

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were incubated at 37° with a cell density adjusted to 5–6 × 10^5 ml of medium.

Experiment II involved three tubes containing pellets of 12.5 × 10^6 viable RPMI 6410 cells each. In one tube, half of a 1-ml sample of EBV suspension, also concentrated from about 300 ml of HR1K culture fluid, was inoculated as described. To the other two tubes was added either the other half of the 1 ml of EBV suspension, which had been

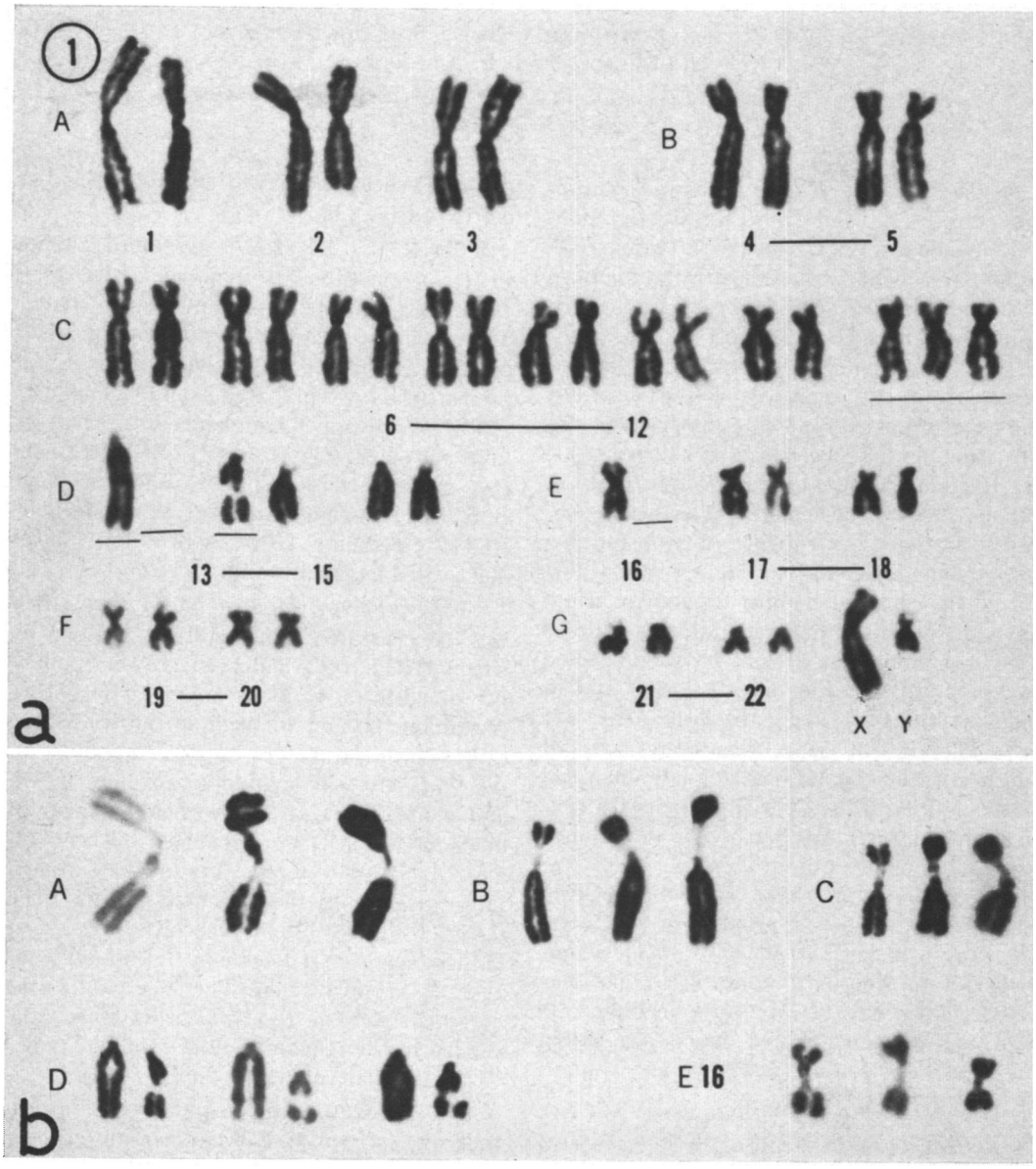

FIG. 1a. Hyperd'ploid karyotype (47 chromosomes) from cell line RPMI 6410. The deviations from the normal male karyotype include 3 extra chromosomes in group C, one large telocentric chromosome ass'gned to D, a secondary constriction in one D chromosome, the lack of one chromosome in group D, and monosomy of E16 (underlined). (b) Examples of centromeric attenuations in the chromosomes belonging to groups A, B, C, and E16 and the large telocentric chromosome as compared with one D chromosome possessing a secondary constriction, frequently observed in RPMI 6410 cells (cuttings from different cells).

heated for 30 min at 56°, or else an equal volume of medium.

Experiment III was the same as Expt. II, except that there was also a fourth tube containing the same number of RPMI 6410 cells. To this tube was added 0.5 ml of suspension concentrated from about 200 ml of culture fluid from the Burkitt lymphoma cell line Raji. The method for preparation of suspension from the Raji line was identical with that for preparation of EBV suspension from HR1K cells.

At various time intervals, portions of cells were removed for viability counts (trypan blue exclusion), immunofluorescence tests, and chromosome studies.

Immunofluorescence and chromosome analyses. Serum globulin containing high titers of EBV antibodies, obtained from a healthy donor, was labeled with fluorescein isothiocyanate, and acetone-fixed cell smears were overlaid with this labeled globulin. After 30 min of incubation at 37°, the cell smears were washed, and were mounted in 90% glycerol. The stained preparations were examined with a Leitz fluorescent microscope with an HBO-200 lamp (Osram).

Cells were treated with Colcemid (Ciba, Inc., Summit, New Jersey) for 1 hr prior to harvesting. Chromosome preparations were made as previously described (17).

Results. Chromosomes of RPMI 6410. The chromosomes of RPMI 6410 were studied in 1966 by Moore *et al.* (20), and again in 1967 by Zur Hausen (21). Cells with 46 chromosomes were predominantly observed in both studies. Chromosome analysis by Moore *et al.* (20) revealed a normal male karyotype.

Since numerical and structural changes of chromosomes in a cell line usually do occur after a long time *in vitro*, we studied the chromosomes of RPMI 1640 prior to its use for EBV infection. Our chromosome counts from a total of 200 metaphases showed that this line now has a modal number of 47 chromosomes (83%). Cells with 46 and 48 chromosomes were 13 and 4% of the total, respectively. Examination of 1000 randomly selected metaphases showed that the incidence of polyploid cells is about 2%. One telocentric chromosome that was appreciably longer than the chromosomes of the D group was seen in every cell studied. A secondary constriction in the middle of one D chromosome was observed in 61% of the cells studied, and a centromeric attenuation in one chromosome each of groups A, B, C, and E16 was observed in 16, 34, 44, and 38%, respectively (Fig. 1). Besides these abnormalities, nonspecific chromosome or chromatid breaks were observed in about 8% of the cells examined.

Karyotype analyses of 29 cells with 47 chromosomes were made. All of these cells had 2 to 4 extra chromosomes in group C and lacked 1 or 2 chromosomes in group E, one of the missing E chromosomes always being E16. The absence of one D chromosome was common (22 out of 29 cells). The lack or excess of one chromosome in groups of A, B, F, or G was observed, but not frequently. The most common karyotype (14 out of 29 cells) had 3 extra C chromosomes and lacked one D and one E16 (Fig. 1).

Effect of EBV on cell viability. In all three experiments, cell viability of the cultures inoculated with live EBV dropped, while the controls and the cultures treated either with heat-inactivated EBV or with Raji suspension maintained normal growth, except that cell concentrations declined during the later part of each experiment, but growth was far above that of the cultures treated with live EBV (Fig. 2). This decline may be due to depletion of nutrients in the medium. It should be noted that the enhancement of growth in the cultures exposed to heat-inactivated EBV or to Raji suspension relative to the control as shown in Fig. 3 was inconsistent.

When 12.5×10^6 cells were inoculated with 1 ml of EBV suspension concentrated from 300 ml of HR1K culture fluid (Expt. I), cell viability dropped sharply. On the third day, most of the cells in the EBV-treated culture were dead. When the same number of RPMI 6410 cells were inoculated with half of the same amount of EBV (Expt. II and III), cell viability declined but not as rapidly as in Expt. I. The EBV-inoculated cultures in Expts. II and III were terminated on 9 and 7 days after inoculation, respectively.

Immunofluorescence. Only two direct im-

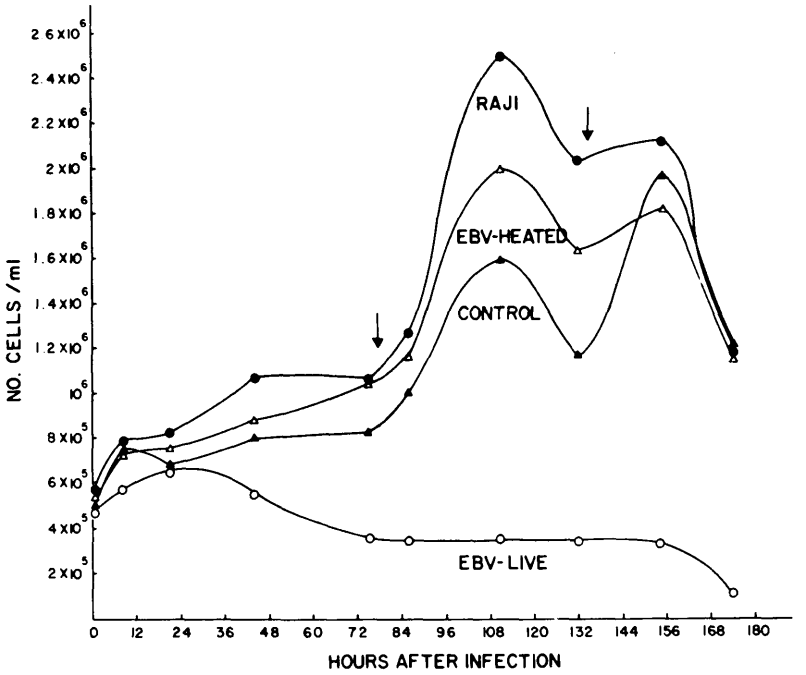

FIG. 2. Growth curves of RPMI cell line 6410. In the EBV-live culture, RPMI 6410 cells were infected with half of a 1-ml sample of EBV suspension concentrated from 300 ml of culture fluid of HR1K. In the EBV-heated culture, RPMI 6410 cells were treated with the other half of the 1 ml of EBV suspension, which had been heat inactivated. In the Raji culture, RPMI 6410 cells were treated with 0.5 ml of a suspension concentrated from 200 ml of culture fluid of the EBV-free line Raji. (arrows) indicate when fresh medium was added. Later cell concentrations are reported on the basis of the original culture volume.

munofluorescence tests were performed in each experiment. No immunospecific staining occurred in control cells or in cells treated either with heat-inactivated EBV or with Raji suspension in any of the experiments performed. On the other hand, the culture inoculated with 1 ml of EBV suspension had about 25% cells showing nuclear and cytoplasmic staining 24 and 48 hr after inoculation. The cultures inoculated with half of the same amount of EBV suspension were about 5 and 8% cells positive for immunofluorescence on days 3 and 5 after inoculation, respectively.

Chromosome aberrations. Cells with 47 chromosomes predominated in all cultures treated with EBV suspensions. Karyotypic analyses of 5 to 10 cells with only moderate breakage in each sample, except in samples where mitosis was extremely rare, all showed similar numerical deviations from normal male karyotype as described for the untreated RPMI 6410 cells.

Table I and Fig. 3 show chromosome aberrations induced by inoculation of RPMI 6410 cells with EBV. At all time intervals after inoculation with virus in all three experiments, a significantly high incidence of cells with chromosome aberrations was observed, except in the first sample of Expt. II, in which the incidence of cells with aberrations was at control level. Experiment I, in which 1 ml of EBV suspension concentrated from 300 ml of culture fluid of HR1K was added to 12.5×10^6 viable 6410 cells, had the highest incidences of cells with aberrations. All cells at metaphase had multiple aberrations two days after inoculation. Experiments II and III, in which half of the amount of EBV suspension used in Expt. I was added to the same number of RPMI 6410 cells, were characterized by comparatively mild effects.

The amount of aberrations generally correlated with the duration of infection: the longer the exposure, the greater the number of cells with multiple breaks and pulverized chromosomes.

In all three experiments, the subterminal and terminal regions of chromosomes of any group seemed to have more aberrations than did the other regions. Many chromosomes had minute chromosome material attached to the end of an arm; and when that minute piece was attached to the short arm, the chromosome involved resembled one with a clear satellite. No instance of dicentric or

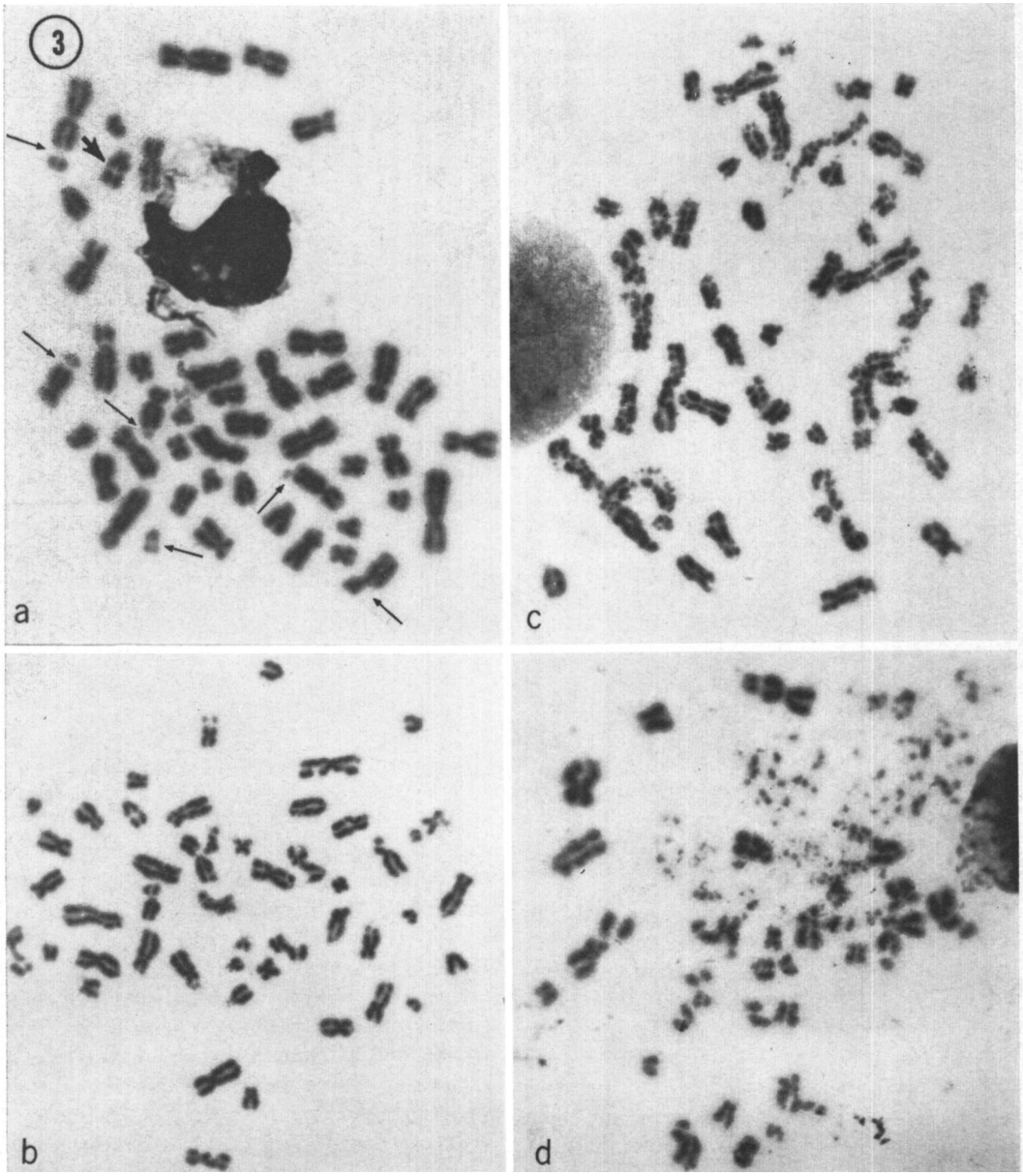

FIG. 3. Chromosome aberrations induced by infection with EBV. (a) There are 6 chromosomes with breaks. (short arrow with large point) indicates secondary constriction in one D chromosome, frequently observed in the controls. (b) More than 22 chromosomes have aberrations. (c) Aberrations in most of the chromosomes. (d) Chromosome pulverization.

TABLE I. Chromosome Aberrations Induced in RPMI Cell Line 6410 by Infection with EBV.[a]

Expt.[b]	After inoculation (days)	Total cells studied	Cells (%) with aberrations	Cells (%) with indicated no. of chromosome breaks[c] per cell										
				1	2	3	4	5	6	7–10	11–15	16–30	>30	Pulverization
I	0.5	100	40	20	9	5		2	2			1		1
	1	126	97	3	6	4	9	7	3	18	9	8	9	21
	1.5	62	96	3	8	8	6	8	6	27	8	3	2	17
	2	14	100		7			7		7		14		65
	3	(no metaphases)												
	Controls	50–100[d]	8[e]											
II	2.5	50	4	2	2									
	4.5	50	28	16	4	2	2			2				2
	5.5	100	27	11	9	2			1	3				1
	6.5	100	24	9	7	2	1	1		1		1		2
	7.5	100	38	11	7	4	2		1	3		5		5
	Controls	50–100[d]	0–12[e]											
	EBV-H[f]	50–100[d]	0–13[e]											
III	1	90	20	9	6	2	1	1				1		
	4	87	45	30	4	7	2		1			1		
	6	7	100	43	43	14								
	Controls	50–100[d]	8[e]											
	EBV-H[f]	50–100[d]	5–20[e,g]											
	Raji[h]	50–100[d]	6–14[e]											

[a] Secondary constriction in one D chromosome or centromeric attenuation in one A, B, C, or E16 chromosome was frequently seen in control cultures; such anomalies are not listed.

[b] Each culture in Expt. I was inoculated with 1 ml of EBV suspension concentrated from 300 ml of fluid from a culture of cell line HR1K; each culture in Expts. II or III was inoculated with half of that amount of EBV suspension.

[c] Chromosome and chromatid breaks and fragments are all counted as chromosome breaks.

[d] For each time interval.

[e] Cells with aberrations had 1 or 2 breaks/cell.

[f] Heat-inactivated EBV.

[g] One sample harvested on the fourth day was 20% cells with 1 or 2 breaks/cell.

[h] Instead of EBV suspension, 0.5 ml of suspension concentrated from about 200 ml of fluid from a culture of cell line Raji was used for inoculation.

exchange configuration was observed.

The approximate location of each break in each group of chromosomes was recorded for a total of 553 breaks in 91 moderately affected cells from samples harvested 12 and 24 hr after EBV infection in Expt. I. The distribution of the breaks is shown in Fig. 4. The terminal regions of each group of chromosomes, especially in the ends of the long arms, accumulated more aberrations than did the other regions.

Discussion. The results obtained in this study indicate that EBV concentrated from the virus-positive cell line HR1K is capable of inducing chromosome aberrations in the cells of RPMI line 6410. It is difficult to tell what stages of the cell cycle are affected by EBV. The fast decline in cell viability and the rapid increase in the number of cells with chromosome aberrations, especially in Expt. I, together with the absence of dicentric or exchange figures in EBV-infected cultures, suggest that the late S and G_2 periods are influenced by the virus. On the other hand, many open breaks of the chromosome type were observed in all experiments, and might have been induced during the G_1 period. The absence of dicentric or exchange figures also

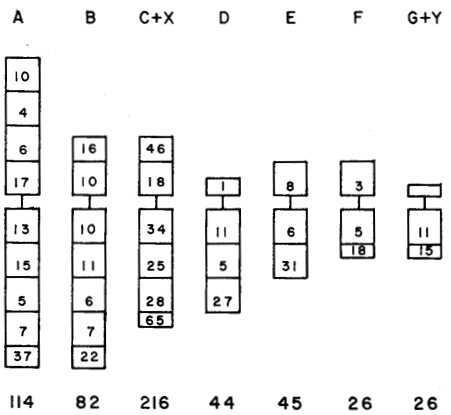

FIG. 4. Distribution of EBV-induced breaks in individual regions of the various groups of chromosomes in RPMI 6410 cells. The idiogram was constructed in accordance with the Denver Report (22). The length of the chromosomes in each group were averaged, and the resulting average chromosome was then divided into single units, starting from the centromere and proceeding to the ends of the arms. The region at the end of an arm was usually less than 1 unit, and the lengths of such regions were different in different groups of chromosomes. The number in each region of the idiogram indicates the number of breaks recorded for that region. A total of 553 breaks were recorded for 91 affected cells. It should be noted that the modal chromosome number of RPMI 6410 cells is 47, generally with 3 extra C chromosomes, and one chromosome lacking in each of group D and E.

indicates that the broken ends did not heal, and that most of the cells affected probably did not enter the next cycle.

The suspensions concentrated from HR1K culture fluid contained not only EBV, but also undefined subcellular components. The possibility that subcellular components had a major effect on the chromosomes of RPMI 6410 can be ruled out, at least partially, by the absence of such effect in the cultures treated with the suspension concentrated from culture fluid of the cell line Raji or in the cultures treated with heat-inactivated EBV suspension concentrated from HR1K culture fluid. Another factor to be considered is the possibility of contamination with mycoplasm. It has been reported that several strains of mycoplasma isolated from culture fluid of mammalian cell lines can induce chromosome changes (23, 24). Tests of the cell line HR1K at the time of our experiments showed that the line was free of mycoplasma. Furthermore, the reported effects of mycoplasma infections on chromosomes were significantly slower and less extensive than EBV effects described here.

Chromosome studies of several EBV-positive lines derived from Burkitt lymphomas and the peripheral blood of patients with infectious mononucleosis (IM) showed various incidences of cells with subterminal secondary constriction in chromosome pair no. 10 in the C group (13–16). This C chromosome marker was also observed in normal human leukocyte cultures after co-cultivation with lethally irradiated EBV-positive Burkitt cells, or after treatment with filtrate from an EBV-positive leukemic line (7, 8). Accordingly, a possible association of EBV with the C chromosome lesion has been suggested (7, 13, 14). In the present study, C chromosomes with subterminal secondary constrictions resembling the marker were observed in all EBV-treated cultures. Since secondary constrictions or chromosome breaks at various points in the subterminal or terminal regions of C chromosomes as well as chromosomes of other groups were very common in all EBV-treated cultures (Fig. 4), it was difficult to evaluate the incidence of cells with the particular marker in question. Furthermore, cells with this marker almost invariably had aberrations in other chromosomes.

In another study, Huang *et al.* (25) examined the chromosomes of 4 relatively new Burkitt lymphoma lines and 12 lines derived from the blood of patients with IM. Low percentages of EBV-containing cells were detected in all lines except one IM line. A search for the C chromosome marker revealed that only one EBV-negative and two EBV-positive IM lines had low incidences of cells with the marker, 1% in 2 lines and 4% in the other. The third line also had a high incidence of other aberrations, mostly in the subterminal or terminal regions of chromosomes belonging to various groups. Of the 4 Burkitt lines, 3 had high incidences of cells with distal secondary constrictions in one of the chromosomes of group A. Results of the

present study and others indicate that the terminal or subterminal regions of not only a C chromosome but also other chromosomes of various groups are vulnerable to EBV infection.

Summary. Epstein-Barr virus (EBV) was concentrated from culture medium in which the Burkitt lymphoma line P_3J-HR1K had grown. EBV infection of the virus-free line RPMI 6410, derived from the peripheral blood of a patient with myelogenous leukemia, resulted in a rapid decrease in cell viability, various incidences of cells with positive immunospecific staining for EBV, and high incidences of cells with chromosome aberrations. Control cultures and cultures treated either with heat-inactivated EBV suspension or with a suspension concentrated from the culture fluid of the EBV-free Burkitt lymphoma line Raji grew normally, lacking detectable EBV-containing cells and exhibiting relatively low incidences of cells with chromosome aberrations. Chromosome and chromatid breaks accumulated more in subterminal or terminal regions, especially in the long arms, of various groups of chromosomes. Metaphases with pulverized chromosomes but without dicentric or exchange figures were frequently observed in EBV-treated cultures.

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Received Sept. 8, 1970. P.S.E.B.M., 1971, Vol. 137.