Demonstration of a Herpes Group Virus in Cultures of Peripheral Leukocytes from Patients with Infectious Mononucleosis

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Cultures were initiated with peripheral leukocytes from individuals: (i) in the acute stage of infectious mononucleosis (IM); (ii) with past histories of IM; and (iii) without histories of IM and without antibodies to EB virus (EBV). In confirmation of other reports, the first group of cultures developed readily and rapidly into lines of blastoid cells. Cellular replication commenced in 24 of 25 attempts within 17 to 28 days, regardless of the technique employed; i.e., initial seeding of the leukocytes with or without addition of phytohemagglutinin onto monolayers of human diploid cells (WI-38) or direct establishment of suspension cultures. EBV was detected in all cultures by immunofluorescence and also by electron microscopy when tested. Furthermore, the nine cultures which were examined cytogenetically revealed the C-group (#10) chromosomal marker previously found in cultured Burkitt tumor cells. These findings supported the earlier conclusion that EBV is related to, if not identical with, the causative agent of IM. Cultures of the second group commenced growth only within 30 to 60 days in five of seven attempts, depending apparently upon the early presence of WI-38 cells. These cultures also revealed the presence of EBV and, in the three examined, the C-group chromosomal marker. Leukocytes of the third group, seeded onto WI-38 monolayers, failed to become established in four attempts. The possible implications of these findings are discussed.

The herpes group virus, which has been found to be associated with more than 60 continuous cultures of blastoid cells, was recently reported (7, 17) to be related to, or identical with, the causative agent of infectious mononucleosis (IM). This virus, originally discovered in cultures of Burkitt's lymphomas established by Epstein and Barr (3), is referred to as EB virus (EBV). Patients in the acute stage of IM regularly showed high levels of antibodies to EBV, as determined by indirect immunofluorescence tests on smears of virus-positive Burkitt tumor cells (7). Sera collected in a prospective study of IM at Yale University were of particular value, since baseline sera, taken at entry into college, were available for students who subsequently developed the disease. All of these sera, some of which were collected within 3 months before onset, failed to reveal antibodies to EBV (7, 17). Limited efforts to isolate the virus from the blood, throat swabs or washings, or urine of

patients in various types of cultures thus far have failed. This is not surprising since EBV from continuous Burkitt tumor cell cultures has been found to be transmissible only to virus-free blastoid cell lines (10) or to peripheral leukocytes upon co-cultivation with lethally X-irradiated Burkitt tumor cells (9). It was shown earlier that cultures of bone marrows or peripheral leukocytes from IM patients readily grow and develop into continuous lines of blastoid cells (1, 4, 18). This was confirmed, as previously reported (7), when cultures of leukocytes from one IM patient (E. H.) were initiated 6 days and 2 months after onset. Both cultures, when 4 to 5 weeks old, harbored EBV in from 1 to 4% of the cells when examined by immunofluorescence and electron microscopy. To confirm and extend these observations, cultures were initiated with leukocytes from 25 patients in the acute stage of IM, from individuals who had histories of IM up to 20 years previously, and from young adults who had no past record of the disease and no antibodies to EBV. The results are reported below.

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MATERIALS AND METHODS

Peripheral leukocyte cultures. A modification of the method described by Mellman (15) was used. Briefly, 15 ml of venous blood was drawn into a 20-ml syringe which contained 5 ml of dextran solution (6%) and 0.1 ml of heparin (Liquemin, 1,000 USP units/ml). The syringe was kept in an upright position for 2 to 3 hr at room temperature to permit the red cells to settle. The leukocyte-rich plasma was then expelled through a bent needle into a centrifuge tube and spun at $900 \times g$ for 10 min. The cell-free plasma was saved for serological tests, and the sedimented cells were washed twice and finally suspended in 10 ml each of medium RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 4 mM glutamine, 20% fetal calf serum, 100 units of penicillin per ml, and 100 μ g of streptomycin per ml. Samples were removed and diluted in equal volumes of 0.1 N HCl for leukocyte counts. The suspension was then diluted in medium to attain the desired cell concentration. Phytohemagglutinin P (PHA) was added to some of the preparations to a concentration of 0.1%.

Three methods were used to set up the cultures. In the first method, 2×10^6 to 4×10^6 leukocytes were seeded onto test tube cultures of human diploid cells (WI-38) purchased from Flow Laboratories, Rockville, Md. or from Microbiological Associates, Bethesda, Md. The cultures were fed twice weekly, and floating cells in the spent media were separated and used to initiate or augment already existing suspension cultures in Erlenmeyer flasks. These suspension cultures were observed under an inverted microscope for evidence of cellular growth and ultimate establishment of lines.

In the second method, 10 ml of suspension containing 2×10^6 to 4×10^6 leukocytes/ml was transferred to petri plates (60×15 mm) containing cover slips. In some instances, the plates had been preseeded with trypsinized WI-38 cells. The cultures were placed in a CO_2 incubator and were fed twice weekly by partial medium renewal (30 to 50%); cells from the spent media were either returned to the petri plates or were used to set up suspension cultures as described above. Cover slips were removed at various intervals for immunofluorescence tests and cytological studies.

In the third method, 5 or 10 ml of leukocyte suspensions (2×10^6 to 6×10^6 /ml) was transferred to 25- or 50-ml Erlenmeyer flasks. Before feeding (twice a week), the cultures were kept at an angle of about 45° for 2 to 3 hr to permit the cells to settle in the curvature of the flask. About half of the medium was withdrawn without disturbing the sedimented cells and was replaced by fresh medium. When rapid growth of cells had commenced under any of the above conditions, cultures were fed on a weekly schedule by partial medium renewal or by sedimentation of the cells at $900 \times g$ for 10 min and resuspension in fresh medium to yield 3×10^5 to 5×10^6 viable cells/ml.

Detection of EB virus by immunofluorescence. Smears of fresh leukocytes and of cultured cells were prepared and tested for immunofluorescence by techniques which have been described (6). We primarily relied upon direct staining with fluorescein-isothiocyanate (FITC)-conjugated human γ -globulin, since

this reagent does not stain globulin-producing cells which are frequently present in this type of culture. For the indirect technique, several sera were used for the primary stage of the reaction: (i) a negative serum obtained prior to exposure to IM (7, 17); (ii) a positive serum obtained during the acute stage of the disease from the same individual; and (iii) a positive serum from a patient with Burkitt's lymphoma. Goat anti-human IgG globulin conjugate (Hyland Laboratories, Los Angeles, Calif.) was employed for the second stage of the reaction and also for direct staining as a control for detection of globulin-producing cells.

Detection of EB virus by electron microscopy. The technique employed has been described (11). These efforts were restricted to a few lines in which immunofluorescence yielded from 2 to 5% positive cells. We are indebted to K. Hummeler for performance of these tests.

RESULTS

Establishment of continuous cultures. Regardless of the method employed, continuous cultures of blastoid cells were rapidly established from peripheral leukocytes of patients with IM. This is evident from selected examples shown in Table 1. PHA was not a requirement. Similarly, implantation of the leukocytes onto monolayers of human diploid cells (WI-38) was not essential for initiation of growth, but the attachment of the cells to the fibroblasts permitted microscopic observation of some of the stages in the development of continuous cultures. This advantage was also provided to some extent by the most convenient method, i.e., direct cultivation of the leukocyte suspensions in Erlenmeyer flasks or petri plates with cover slips, in that macrophages, a few fibroblast-like cells, and debris from degenerating cells adhered to the glass surfaces to which, in turn, leukocytic elements attached.

It is evident from the examples, as well as from the results of an additional 19 cultures (Table 2), that blastoid cell lines were established in 24 of 25 attempts (one culture was lost due to bacterial contamination). The peripheral leukocytes were obtained as early as 4 and as late as 70 days after onset of IM. Differential leukocyte counts revealed from 3 to 58% atypical lymphocytes. All patients, with the exception of one, possessed antibodies to EBV at the time the cultures were initiated in titers ranging from 1:40 to 1:640. Most of them also showed high levels of heterophile antibodies. The first evidence of cellular growth was obtained in every instance within 17 to 28 days, and all cultures continued to grow at gradually increasing rates.

Cultures were also initiated from seven individuals who had well-documented histories of IM from 7 months to 20 years earlier (Table 2).

TABLE 1. Examples of blastoid cell cultures derived from peripheral leukocytes of a patient in the acute stage of IM

Patient					Cultural conditions		Established blastoid cell cultures		
Name	Time after onset	Atypical lymphocytes	Antibody titers		PHA ^a	WI-38 monolayers ^b	Start of growth	EBV + cells maximal	C-group marker
			Heterophile	EBV					
	days	%					days	%	
SCHE	10	13	3,584	640	+	+	26	2-3	+
MIL	40	3	1,792	240	+	+	26	1-3	+
MAZ	11	58	448	80	0	+	28	4-6	nt ^c
NON	19	13	14	160	0	+	24	0.5-1.0	+
WIR	11	29	1,280	160	+	0	20	0.1	+
COW	4	10	1,792	40	0	0	21	0.1	+

^a Phytohemagglutinin.
^b Seeded initially on WI-38 cultures.
^c Not tested.

TABLE 2. Cultures of peripheral leukocytes of IM patients and controls

Source of leukocytes	Time after onset	Anti-EBV titers	Results of cultures			
			Growth	Start of growth	EBV + cultures	C-group marker
				(days)	%	
Acute stage of IM	3-70 days	10-640	24/25 ^a	17-28	100	6/6 ^a
Past IM	7 months-20 years	10-80	5/7	30-60	100	3/3
No history of IM	—	<10	0/4 ^b	—	—	—

^a No. positive/no. tested.
^b Two individuals.

The titers of antibodies to EBV in these donors ranged from 1:10 to 1:80. Five of these cultures commenced to grow in 30 to 60 days as blastoid cells. The two unsuccessful cultures were set up in the absence of WI-38 cells (method 3), whereas the successful ones were initially seeded on monolayers of diploid cells (method 1). This indicated that the monolayers of WI-38 cells were advantageous for initiation of peripheral leukocyte cultures from individuals other than patients in the acute stage of IM.

One culture was initiated by chance during the incubation period of IM. The donor of the leukocytes, a technician in this laboratory (Y. B.), had no history of IM and no antibodies to EBV up to the middle of December 1967 (Table 3). She had donated leukocytes on 13 December 1967, i.e., 11 days before the onset of clinical signs of IM. This culture initiated on WI-38 cells commenced growth in 57 days. The second culture, started on the ninth day of illness in the absence of WI-38 cells, grew within 18 days.

Four cultures were initiated with leukocytes from donors without a history of IM and with-

out antibodies to EBV. Three of these were derived from one donor at different dates. All were started in the presence of WI-38 monolayers, but none proved to be successful (Table 2).

Cytological observations did not reveal any differences in the course of establishment of continuous cultures, whether the peripheral leukocytes were obtained during the acute stage of IM or a long time thereafter, and generally confirmed previous observations (4, 18, 19). The polymorphonuclear leukocytes degenerated rapidly and were no longer evident within 1 week. Three main cell types were noted in the early stages. (i) Macrophages with an eccentric nucleus, prominent nucleoli, and vacuolated cytoplasm often revealing ingested red or white cells or cellular debris. These cells attached immediately to the glass surfaces and served for attachment of lymphoid cells. They disappeared in time as the cultures started to grow at increasing rates. (ii) Small lymphocytes which in the absence of PHA appeared to remain unchanged before gradually degenerating within 10 to 14 days. (iii) Medium and large

TABLE 3. *Cultures of peripheral leukocytes of patient Y.B. during incubation and after onset of IM*

Date	Clinical status	Antibody titers		Peripheral leukocyte culture		
		Heterophile	EBV	Com- menced growth	EBV antigen	
					Day	Positive cells
				days		%
12/3/67	Pre-IM	nt ^a	<10	nt		
12/13/67	Incubation	<10	<10	57	0	0
					9	0
					27	0.3
12/24/67	Onset					
1/2/68	Acute stage (9th day)	480	40	18	0	0
					7	0.1
					18	1-2

^a Not tested.

lymphocytes and lymphoblastoid cells which gradually formed large aggregates. These cells often adhered to the macrophages and fibroblast-like cells but in time detached and floated in the medium. It appears that these cells, after latent periods of 2 to 4 weeks in which little or no growth was microscopically detectable, started to divide rapidly to form the elements of the continuous cultures (Fig. 1).

Tests for the presence of EBV in established cultures. Immunofluorescence tests on smears of leukocytes prepared prior to cultivation or within the first week of culture generally failed to reveal EBV antigen in any of the cells. In one instance (Table 3), a few fluorescent cells were noted on the seventh day and in a few more cases by the second week. All cultures gave positive tests by the fourth week and for some time thereafter (Tables 1 and 2). The incidence of brilliantly fluorescent cells obtained by the direct staining method with FITC-conjugated human γ -globulins ranged from <0.1 to about 6% in different cultures, and varied also in given cultures over a wide range depending upon the time of assay and cultural conditions. The percentages were generally highest when growth of the cultures was still slow, and they decreased with the onset of rapid cellular replication. Indeed, stainable cells were found to disappear in rapidly growing cultures within 2 to 3 months. Cultures established from the five individuals with histories of IM as long as 20 years ago also revealed the presence of EBV antigen in a small fraction of the cells.

The specificity of the staining was ascertained in indirect immunofluorescence tests. Sera collected from students prior to the onset of IM (7, 17) gave negative results as did sera with

antibodies to herpes simplex virus, varicella virus, or cytomegalovirus but devoid of antibodies to EBV. In contrast, sera from the above students taken during the acute stage of IM and from patients with Burkitt's lymphomas elicited brilliant immunofluorescence in the appropriate percentages of cells as established by the direct staining method with FITC-conjugated human γ -globulins. The results of the indirect method of staining were complicated to some extent, however, by the fact that all of the cultures contained a variable number of cells which produced globulin and were stainable directly by the goat anti-human IgG conjugate. In most instances, these reactions were largely or completely blocked by prior exposure of the cell smears to the control sera, and thus it is likely that they were equally reduced by sera which contained antibodies to EBV. Furthermore, the fluorescence elicited in globulin-producing cells can generally be differentiated from that seen in EBV antigen-containing cells. In the globulin-producing cells, fluorescence is restricted to the cytoplasm, appears granular, and usually assumes the form of crescents surrounding the dark nucleus. The EBV antigen-containing cells, in contrast, are stained throughout the cell, often show inclusion-like concentrations of fluorescence, and frequently exhibit varying degrees of degeneration. Because of the blocking effect of the sera used for the primary stage of the reaction, the globulin-producing cells in indirect tests are, as a rule, only weakly stained, if at all.

Several cultures which revealed from 2 to 5% immunofluorescent cells were also examined electron microscopically by K. Hummeler, using the methods previously described (11). Virus particles showing all of the characteristics of a member of the herpes group were readily demonstrable in all instances.

Cytogenetic studies. Three of the cultures derived from IM patients during the acute stage of illness were examined cytogenetically in detail, and the C-group ($\times 10$) chromosomal marker, detected earlier in Burkitt tumor cell lines (12, 16), was found in 13 to 21% of the metaphases (13). Another six cultures of this group were searched specifically for the C-group marker and it was readily found. The marker was also present in the three cultures derived from individuals with past histories of IM which were examined. Other chromosomal abnormalities (breaks and gaps) were of low frequency.

DISCUSSION

In confirmation of earlier reports (1, 4, 18), cultures of peripheral leukocytes from patients in the acute stage of IM readily developed into

FIG. 1. Stages in the development of blastoid cell lines from peripheral leukocytes of IM patients. The cells were seeded onto petri plates with cover slips. (a-c) Cells 8, 14, and 30 days after seeding. Macrophage-like cells, and small and large lymphocytes in increasingly larger aggregates are seen. $\times 1,600$. (d) Film of blastoid cells of an established line. $\times 4,000$.

continuous lines of blastoid cells. Cellular growth became evident within 17 to 28 days, whether the leukocytes had been stimulated with PHA, or had been seeded onto monolayers of human diploid fibroblasts, or were set up directly in stationary suspension cultures. Thus, as previously noted (4, 18), no special manipulations were required for the establishment of cultures. For initiation of cultures from leukocytes of individuals with long past histories of IM, however, implantation onto WI-38 monolayers appeared to aid the establishment of lines.

All of the cultures from IM patients, including

those previously reported (7), eventually revealed EBV in various percentages of the cells, as determined by immunofluorescence and confirmed, in the few instances tested, by electron microscopy. EBV antigen was not detectable in smears of fresh leukocytes made prior to cultivation, nor, with few exceptions, in cells cultured for 1 to 2 weeks. It is likely that antibodies to EBV were carried over from the donor's blood into the culture, and that these antibodies had to be diluted out during maintenance before the presence of virus could be ascertained. Stainable cells were regularly present at 4 weeks and for

various times thereafter. No more than a maximum of 6% and usually fewer cells produced viral antigen. The number of cells tended to decrease rapidly as cellular growth improved, and, indeed, the virus was nearly lost in several cultures after a period of 2 to 3 months of rapid growth. It will be essential to balance cellular and viral replication by a restricted feeding schedule, as was recently developed for cultured Burkitt tumor cells (10).

Cytogenetic analysis of several cultures initiated with leukocytes from IM patients (13) revealed, in all cell lines examined, the C-group chromosomal marker previously found in cultures of Burkitt tumor cell lines (12, 16) and cultures of blastoid cells obtained on co-cultivation of peripheral leukocytes from apparently normal infants with lethally X-irradiated Burkitt tumor cells from EBV-positive, but not EBV-negative lines (9; *in preparation*). The possible relationship of EBV to the chromosomal marker has been discussed elsewhere (13).

The demonstration of EBV in every culture of leukocytes from IM patients and the finding of the C-group chromosomal marker in those tested supports the earlier conclusion (7, 8, 17), based largely on serological evidence, that this virus is the probable cause of the disease. Results obtained on co-cultivation of normal peripheral leukocytes with lethally X-irradiated Burkitt cells suggested that EBV exerts a growth-stimulating effect (9; *in preparation*). The rapid establishment of continuous cultures from leukocytes of IM patients may denote that many of the cells were already stimulated *in vivo* by EBV. In line with this suggestion is the fact that a few leukocytes from IM patients take up ^3H -thymidine immediately after being placed in culture, whereas cells from healthy donors fail to do so (2, 5, 14).

It is conceivable that, after the primary infection, whether recognized as IM or not, EBV persists in the lymphopoietic system. Such a persistence would not be unusual for a member of the herpes group of viruses and could account for (i) the endurance of antibodies to EBV for long periods of time, if not for life (7, 8, 17); and (ii) the frequent emergence of the virus in continuous blastoid cell lines, whether derived from patients with Burkitt's lymphoma, leukemia, or other diseases or from healthy donors, including those with known past histories of IM. The available data further suggested that EBV might be required for successful initiation of continuous leukocyte cultures and that its concentration in the donor may determine the frequency and speed of the establishment of lines. In agreement with this concept, the four cultures

of lymphocytes from young adult donors without a history of IM and without antibodies to EBV, failed to grow. The first culture of Y.B., set up by chance during the incubation period, when antibodies were still undetectable and the virus concentration might have been low, grew only after a delay of 57 days. The second culture, started on the ninth day of illness, when the virus presumably had stimulated larger numbers of the appropriate target cells and antibodies had developed, commenced growth within 18 days; i.e., within the time range generally seen (17 to 28 days) with leukocytes of patients in the acute stage of IM. Cultures from individuals with long past histories of the disease, who may harbor only small amounts of virus but possess antibodies to EBV, started to grow only after a delay of 30 to 70 days. This suggested relationship of EBV to the establishment of continuous lines requires substantiation by further, more numerous attempts at cultivation of peripheral leukocytes under optimal conditions and by correlation of the results with the histories and antibody status of the cell donors.

There are two possible explanations for the fact that not all cultures of blastoid cells derived from healthy donors or from patients with diseases other than IM regularly revealed the presence of EBV: (i) they were examined merely by electron microscopy, a relatively insensitive assay; or (ii) they were examined too late; i.e., the virus may have been present initially but was lost during maintenance of the cultures under conditions for optimal cellular growth. This has been observed with Burkitt tumor cell lines in this laboratory and elsewhere (20; M. A. Epstein and J. T. Grace, Jr., *personal communications*) and now, even more strikingly, with peripheral leukocyte cultures derived from IM patients.

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