*RELATION OF BURKITT'S TUMOR-ASSOCIATED HERPES-YTPE VIRUS TO INFECTIOUS MONONUCLEOSIS**

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A herpes-type virus has been detected with remarkable frequency in cell lines derived from Burkitt's lymphomas, leukemic tissues, or buffy coats of a variety of patients and healthy donors.[1–8] This agent is being named EB virus (EBV), for convenience, after the cell lines in which it was first observed.[1] Most of the virus particles seen by electron microscopy in a small proportion of the cells are judged noninfectious because they are defective.[1, 9, 10] To date, EBV has been transmissible only to cultured human cells of the hematopoietic system.[11, 12] Virus-producing cells are readily detectable by indirect immunofluorescence tests with various human sera or by direct staining with fluorescein isothiocyanate-conjugated human γ-globulins.[13–17] Attempts to identify the agent by appropriate virus-specific immunofluorescence tests have failed. Thus EBV appears to be a new member of the herpes group.[13–15] From human serum surveys it is evident that infections by EBV, or a close relative of it, are frequent, and that the agent has a world-wide dissemination.[13, 17, 18] The age distribution of antibodies to EBV among American children parallels that of antibodies to other common viruses, such as measles, mumps, or poliomyelitis in the prevaccination era.[18] Except for the fact that all Burkitt's tumor patients studied thus far[13, 17] and a high percentage of patients with carcinomas of the postnasal spaces[19] were found to have high titers of antibodies to EBV antigens, no other suggestive relationship of the virus to known disease entities has been recorded. The present report indicates that EBV is related to, and probably the cause of, infectious mononucleosis.

Materials and Methods.—The techniques for growth and maintenance of cell lines derived from Burkitt's tumors have been described, as well as the procedures for preparation of cell smears, detection of EB virus antigens, and corresponding antibodies by immunofluorescence.[13, 16] Cells of the EB-3 line were routinely used, which had been kept for 4–7 days on arginine-deficient Eagle's basal medium with 25% fetal calf serum (BME-25) obtained by preincubation at 37°C for 7 days or merely by omission of the amino acid. The arginine deficiency inhibits cellular growth but increases the number of EBV antigen-producing cells by a factor of 5–10 (to be published).

Results.—The indirect immunofluorescence test for antibodies to EB virus was used in a search for illnesses that might be caused by this agent. In one series of tests, paired acute stage and convalescent sera from pediatric patients with unidentifiable viral infections were examined with negative results.[18] As another approach, serial sera were tested that had been collected from children in prospective studies of viral infections. Such sets of sera were kindly supplied by Dr. John P. Fox from the "New York Virus Watch," and by Dr. John H. Dingle from the Cleveland Family Study. Of the first group, 33 sets were examined that had been collected over periods of from one to four

years from children initially two to seven years of age. No conversions from negative to positive were noted and in six children having the antibody, the titers remained at constant levels from the first to last serum specimen. From the Cleveland study, 12 sets of sera became available from children ranging in age from 1 day to 8 years at the time of the first bleeding and observed for periods between 5 and 14 years. Nine of these children remained free of antibodies to EBV throughout the observation periods, at the end of which their ages ranged from 7 to 17 years. Two children, both 5 years old, possessed antibodies at the start, and the titers persisted at nearly constant levels (1:20–1:80) for 10 and 12 years, respectively. The 12th child (4-5, Table 1)

TABLE 1. *Results obtained with sera of child 4-5, Cleveland Family Study.*

Date	Anti-EBV titer	Date	Anti-EBV titer
5/19/48*	1:80	9/18/51	1:80
4/6/50	<1:10	9/2/53	1:80
11/10/50	<1:10	10/28/56	1:80
		12/4/57	1:80

* Date of birth.

revealed antibodies at birth, none in the next two sera. Between $2^1/_2$ and $3^1/_3$ years of age, the reaction became positive, and the titers remained stable at 1:80 for the subsequent 6 years. In the 10-month period in which the antibody appeared, the child sustained three common colds, a gastroenteritis, and on September 2, 1951 a "nonbacterial tonsillitis" of 6 days' duration. The frequency in children of antibodies to EBV, both in the New York and Cleveland studies, was small in comparison to the much higher incidence of positive reactions (about 50%) among sera from randomly selected patients, aged 4–15 years, at the Children's Hospital of Philadelphia.[18] This difference presumably reflects the fact that the hospital patients came largely from low socioeconomic levels, whereas the prospective studies involved well-to-do families.

An additional lead in the search for EBV-induced illnesses developed as a result of the following experience. E. H., a research technician in this laboratory, had contributed peripheral leukocytes in January 1967 for the type of experiment described in a preliminary report.[11] Planted on monolayers of human diploid cells (WI-38), her cells failed to grow at that time, and her plasma was devoid of antibodies to EBV (Table 2). On August 10, 1967, E. H. contracted what was subsequently diagnosed as infectious mononucleosis (IM), on the basis of leukocyte counts, abnormal lymphocytes, and a positive heterophile antibody test. She returned to the laboratory on August 16, 1967, but was noted on that day to have developed a morbilliform rash. Blood was taken and her leukocytes, on WI-38 cells, started to grow within four weeks. Smears of the original leukocytes and of cells cultured for eight days failed to reveal immunofluorescence which could be clearly ascribed to the presence of EBV but, at four weeks and later, from 1 to 3 per cent of the cultured cells harbored EBV antigen. Cytogenetic analysis of these cells at six weeks of cultivation revealed in 6 per cent of the metaphases the C-group chromosomal

TABLE 2. *Tests on a patient (E. H.) with infectious mononucleosis (IM).*

Date	Status	Peripheral Leukocyte Cultures: Cell growth	Peripheral Leukocyte Cultures: Cells with EBV antigen (%)	Antibody Titers: EBV	Antibody Titers: Heterophile
1/11/67	Pre-IM	None (4 wk)*	—	<1:10	<1:80
8/10/67	Onset of IM	—	—	—	—
8/16/67	Rash	Yes (4 wk)	Day 0, 0 " 8, 0 " 28+, 1-3	1:40	1:640
9/27/67	Convalescent	—	—	1:40	1:240
10/16/67	"	Yes (4 wk)	Day 0, 0 " 35, 2-4	1:80	1:160
11/16/67		—	—	1:80	≦1:80

* No viable cells left.

marker previously observed in Burkitt's tumor cell lines.[20] The plasma obtained on August 16 now contained antibodies to EBV as well as to heterophile antigens in titers of 1:40 and 1:640, respectively. Sera collected on September 27 and October 16 showed continuing anti-EBV levels but a decline in heterophile antibodies. Leukocyte cultures set up at the last date are still under observation. While these results clearly revealed the development of antibodies to EBV between January and August 1967, E. H. could conceivably have become infected with this agent prior to contracting IM.

As a result of this experience, further sera from patients with infectious mononucleosis were examined. These were kindly furnished by Drs. Robert W. McCollum and James C. Niederman, Yale University School of Medicine, H. T. Wright, Jr., Children's Hospital of Los Angeles, and P. F. Schrode and his associates, Student Health Service, University of Pennsylvania. The sera from Yale were of especial value since they were derived from a prospective study of IM in which baseline sera were obtained from students on entry into school, with the expectation that some of them would subsequently contract the disease. Table 3 shows the distribution of anti-EBV titers among acute stage sera of IM patients. All sera were strongly positive, with titers as high as 1:1280. Of 50 baseline sera of Yale students, 10 randomly selected from each of 5 consecutive years, only 24 per cent were positive, and their titers did not exceed 1:80. The low percentage of reactive sera may be a reflection of the socioeconomic level of their donors, as mentioned earlier. Results obtained with sera from patients with Burkitt's lymphomas and from African control children are included in the table for comparison. While only about half of the control sera gave reactions, mostly at low titers, all of the Burkitt's tumor patients possessed antibodies to EBV, and the distribution of titers among their sera was similar to that seen with IM cases. The IM patients generally also showed high heterophile antibody levels, whereas the sera of the Burkitt's patients tested were negative. The table also includes results obtained with sera from five American and nine African patients with carcinomas of the postnasal space. Twelve of 14 revealed titers in the ranges seen with IM and Burkitt's patients.

The fact that all acute stage sera from IM patients contained antibodies to

TABLE 3. *Distribution of anti-EBV titers among patients with infectious mononucleosis, Burkitt's lymphoma, and cancer of the postnasal space.*

Group	No. of individuals	Number with Anti-EBV Titers of								Positive (%)
		<1:10	1:10	1:20	1:40	1:80	1:160	1:320	>1:320	
Infectious mononucleosis	42	0	0	0	5	8	14	11	4	100
Control (Yale students)	50	38	1	1	8	2	0	0	0	24
Burkitt's lymphoma	34	0	0	0	3	5	8	9	9	100
Control (African children)	188	97	44	21	22	2	2	0	0	48
Carcinoma of p.n.s.	14	1	1	0	6	3	2	1	0	92

EBV, generally at high titer, suggested a definite link between this virus and the disease. This was firmly established in tests with coded serial sera from IM patients in the prospective study at Yale University. After breaking of the code the results turned out to be as follows (Table 4): all baseline sera were devoid of antibodies to EBV, whereas every patient showed such antibodies in the acute and later stages of the disease. The intervals between baseline bleedings and onset of IM ranged from 22 days to >5 years. The first acute stage sera were mostly obtained within 1–2 weeks after onset of illness, but in a few cases 1–$1^1/_2$ months later. It is evident that patients Ma, E, and G became infected shortly after, or even before, admission to the prospective study, since the incubation period in adults has been estimated to be 4–7 weeks. In seven of the patients, antibodies to EBV were already at their peak level in the first acute stage serum, but in four (Be, A, D, and F), significant increases in antibody titers were found in later sera, indicating a response to a current infection. The antibody levels persisted in the above cases, as well as in five additional patients for whom no baseline sera were available, at only slightly diminishing levels for periods as long as 3 years (L) and presumably longer, judging by the persistence of antibodies for at least 10–12 years in the two children in the Cleveland Family Study. In contrast, the heterophile antibody titers rose and fell rapidly. A correlation of clinical data with anti-EBV and heterophile reactions will be published separately (J. C. Niederman *et al.*).

A number of additional tests will be summarized briefly. (*a*) Baseline and early acute stage sera of patients E. H., Be, Ma, and Va were titrated on fixed cell smears of other cultures harboring EBV antigen-producing cells, i.e., the *Jijoye* line (Burkitt's) and the HR-1-K clone derived therefrom,[21] the Na line obtained by co-cultivation of peripheral leukocytes with lethally X-irradiated *Jijoye* cells,[11] and the culture initiated with leukocytes of E. H. during the acute stage of IM. The antibody titers were indistinguishable from those obtained with routinely employed EB-3 cells. (*b*) Baseline and acute stage sera of patients D and F were used for antibody coating and agglutination of

TABLE 4. *Results of antibody titrations of preinfection, acute stage, and later sera from patients with infectious mononucleosis.*

		Baseline Serum		Acute Stage and Later Sera									
				1		2		3		4		5	
Patient	Test*	Date	Titer	Date	Titer	Date	Titer	Date	Titer	Date	Titer	Date	Titer
BE	EBV	5/6/62	<10	6/16/67	40	6/19/67	320	6/30/67	320	7/12/67	160	10/19/67	160
	HET		<80				640		640				<80
MA	EBV	9/22/59	<10	1/5/60	320	1/11/60	80	5/10/60	160				
	HET		<80		2560		<80		<80				
VA	EBV	9/15/58	<10	1/15/60	160	1/27/60	160						
	HET		<80		<80		<80						
A	EBV	9/10/63	<10	5/4/64	20	5/8/64	80	5/22/64	240	5/26/64	160		
B	EBV	9/21/59	<10	5/1/61	80	5/15/61	80						
C	EBV	9/11/58	<10	1/6/60	80	1/22/60	120	2/8/60	80	2/18/60	20		
D	EBV	9/19/60	<10	12/8/61	<10	12/13/61	320	1/15/62	240				
E	EBV	9/18/61	<10	10/10/61	320	1/8/62	160						
F	EBV	9/12/58	<10	11/10/61	20	12/6/61	80						
G	EBV	9/18/61	<10	1/10/62	120								
H	EBV	9/11/58	<10	6/6/60	80								
Ri	EBV	—	—	11/4/64	160	11/12/64	160	11/19/64	80	4/12/64	40		
	HET				2560						≦80		
Le	EBV	—	—	1/7/65	160	1/8/65	160	1/28/65	160	2/15/65	80		
	HET				1280		1280		640		≦80		
J	EBV	—	—	11/9/64	40	11/12/64	40	11/17/64	40	11/30/64	40	12/14/64	40
	HET				480		240		240		160		160
K	EBV	—	—	1/6/65	320	1/11/65	320	1/21/65	160	1/27/65	160	4/12/65	160
	HET				>2560		>2560		2560		1280		≦80
L	EBV	—	—	12/17/64	<10	12/19/64	40	12/21/64	80	1/4/65	160	11/14/67	40
	HET				<80		80		320		240		<80

* EBV = immunofluorescence test for EB virus; HET = heterophile test.

partially purified EB virus particles.[15] Electron microscopic examination revealed that the baseline sera failed to coat the virions, whereas after exposure to acute stage sera the virus particles were covered with antibodies. (*c*) Several such sets of sera were also used in a preliminary test for detection of surface antigens of live HR-1-K and E. H. cells by the membrane fluorescence test described by the Kleins and their associates.[22] The results indicated that the baseline sera were devoid of significant activity, whereas with acute stage sera fluorescence was noted in the form of dots and rings around the circumference of both types of cells, comparable to results obtained with a known positive serum of a Burkitt's tumor patient (Mutua) kindly supplied by Dr. George Klein, Karolinska Institutet, Stockholm. (*d*) Several sets of sera were examined by immunofluorescence for fluctuations in antibodies to another member of the herpes group using cells of an EBV-free Burkitt's cell line (Raji) infected with herpes simplex virus. No changes in reactivity of the sera were noted in the course of IM. (*e*) Examination of serial sera from six patients with nonicteric serum hepatitis, collected in a prospective study of the disease at the time of blood transfusion(s) and at two- to four-week intervals thereafter for up to eight months,[23] revealed no significant changes in anti-EBV reactions. (*f*) Tests with preinoculation and early and later convalescent serum pools from children with infectious hepatitis, supplied by Dr. Saul Krugman, New York University Medical School, likewise failed to disclose anti-EBV responses.

Discussion.—The evidence strongly implies that EB virus is etiologically related to infectious mononucleosis. This conclusion is supported by the fact that all patients thus far studied showed high levels of anti-EBV in their acute stage sera, whereas only 24 per cent of sera from an appropriate control group reacted, and then, as a rule, to lower titers. Preinfection sera, available from 12 of the patients, were all negative and some of these had been collected only 1–3 months prior to onset of the disease. Furthermore, five of the patients revealed rises in antibodies from the first to subsequent acute stage sera, indicative of a current EBV infection. The titers tended to decline slightly as time progressed, but evidence provided by children of the Cleveland Family Study suggests that they may persist for many years. Sera from individuals with histories of IM as long as 20 years ago revealed anti-EBV levels of 1:40 to 1:80.

One might speculate that IM activates a latent EBV infection and that therefore the agent is unrelated to IM. This appears unlikely because of the apparent persistence of antibodies to EBV and none of the baseline sera revealed even traces of reactivity. Furthermore, such a situation would not be expected to exist in 100 per cent of the patients. The same arguments also hold against the suggestion of an anamnestic antibody response. In addition, such recalls are usually more rapid than the rate of anti-EBV formation as observed, for instance, in patients A and L. It should be noted, too, that antibodies to herpes simplex virus remained stationary in the course of IM and that anti-EBV levels failed to fluctuate in patients with infectious or serum hepatitis.

The available information on EBV and its seroepidemiology seems to fit well with what is known about infectious mononucleosis. The virus appears to depend upon cells of the hematopoietic system for its replication. IM has a

world-wide distribution. It is a disease of children and young adults and one attack confers lasting immunity, as a rule, thus suggesting that it is caused by a single agent. EBV, on the basis of serum surveys, is also found in many parts of the world. Antibodies have been found in a high proportion of children, but their acquisition is delayed in population segments of high socioeconomic status. The antibodies to this virus apparently persist for many years, if not for life, and it has not been possible thus far to distinguish, by the immunofluorescence technique, between antibodies on the one hand, and virus particles found in cell lines on the other, which were derived from African, American, European, or New Guinean donors. Thus, these agents belong at least to one group, if they are not identical. In children the disease might often not be recognized, especially in the absence of heterophile antibody responses. There is now little doubt that the "nonbacterial tonsillitis" of child 4-5 in the Cleveland Family Study was in fact IM, even though the single acute stage serum available failed to give a positive heterophile antibody test. IM is rare in persons over 35 years of age, i.e., in age ranges in which over 80 per cent of the individuals possess antibodies to EBV.[13] The reported failures to transmit IM experimentally to volunteers (cf. ref. 24) could have several explanations: (*a*) the recipients were immune to EBV; (*b*) acute stage blood or serum used for inoculation contained high concentrations of antibody; and (*c*) many of the virus particles in the inocula might have been defective, if the experience with EBV in various cell lines[9, 10] applies also to fresh infections. That EBV is found so frequently in cell lines derived from the hematopoietic system may denote the establishment of persisting latent EBV infections of these tissues after an initial exposure. Such a persistence is not unusual for members of the herpes group, although they may involve other tissue or organs.

An identification of EBV with the agent of IM does not necessarily denote that it could not also be etiologically related to Burkitt's lymphoma, either *per se* or in conjunction with other yet unknown factors. It certainly is remarkable that all sera of more than 60 Burkitt's tumor patients studied in this and another laboratory (J. T. Grace, Jr., personal communication) showed high anti-EBV levels with a distribution of titers similar to that seen in IM cases. This does not seem to be due to chance, since only about 50 per cent of 188 control sera from African children reacted at usually much lower titers. The high incidence of anti-EBV reactions among sera of patients with carcinomas of the postnasal space[19] is also of interest since it suggests that some of these tumors might originate from lymphoid tissues, conceivably infected with EBV.

Thus far no clear-cut evidence has been obtained for an oncogenic potential of EBV. It should be noted, however, that cultures of leukocytes from patients with IM grow more readily than those obtained from other donors.[25] This was also seen with leukocytes of patient E. H., which revealed the presence of EBV after several weeks of cultivation, presumably when antibodies present in the original cell suspension had been sufficiently diluted in the course of maintenance. Furthermore, co-cultivation of peripheral leukocytes of children with lethally X-irradiated, EBV-positive Burkitt's tumor cells resulted in readily growing cultures, whereas X-irradiated EBV-free Burkitt's tumor cells had no such

growth-stimulating effect.[11] The leukocyte cultures so obtained had acquired a persistent infection by EBV, and many of the cells revealed the chromosomal marker previously observed in Burkitt's tumor cell lines[20] and now also found in cultured E. H. cells. While these results do not indicate oncogenicity of EBV, they certainly do not preclude the possibility.

Summary.—Patients with infectious mononucleosis regularly develop antibodies to the herpes-type virus (EBV) found in cultures derived from Burkitt's tumors or other cells of the hematopoietic system. The antibodies persist for many years and are distinct from heterophile antibodies. The epidemiology of IM and the seroepidemiology of EBV share many features. Thus, it appears that EBV, or a close relative of it, is the cause of IM. This conclusion does not preclude the possibility that EBV might also be involved, either directly or indirectly, in the etiology of Burkitt's lymphoma.

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