PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 489–493 (1978)

Properties of Epstein-Barr Virus Transformed Woolly Monkey Lymphoblastoid Cell Lines (40083)

WARREN A. ANDIMAN AND GEORGE MILLER

Departments of Pediatrics and Epidemiology and Public Health, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

New world primates are useful experimental laboratory models for Epstein-Barr virus (EBV) infections (1). These species do not seem to harbor EBV or a related virus in nature, yet their cells are susceptible to transformation *in vitro* (2–4). In limited studies performed so far lymphoma has been induced in cotton-top marmosets (Saguinus) (3, 5), in owl monkeys (Aotus) (6) and in squirrel monkeys (Saimiri) (7). However, not all individuals of each species are susceptible to tumorigenesis or even to persistent infection. For example, squirrel monkeys with malaria developed fulminant lymphoproliferative disease after inoculation with autochthonous EBV-converted cells (7), but only transient antibody responses were found in normal squirrel monkeys (2). In some species, such as Callithrix marmosets, only mild lymphoproliferative disease has occurred after EBV inoculation (8).

These variable responses of non-human primates may be explained by differences in the amounts and types of virus or transformed cells inoculated and also by host differences, the nature of which remains to be elucidated.

In this report we described the derivation of continuous woolly monkey (WM) lymphoblastoid lines following *in vitro* transformation of leukocytes by an EBV strain (B95-8) which had previously been shown to be oncogenic in the cotton-top marmoset (CTM). Cultured WM cells differed from CTM cells in cell surface markers, virus production and oncogenicity. These differences serve as a basis for exploring factors which might account for the variations in pathogenic potential of EBV in different hosts.

Materials and methods. Cells. Mononuclear cell fractions were obtained from heparinized peripheral blood utilizing Ficoll–Hypaque gradients (9). Populations depleted of T lymphocytes were derived by a second Ficoll–Hypaque separation of cells which had formed rosettes with neuraminidase treated sheep erythrocytes (Vibrio cholera, Calbiochem). Lymphoblastoid lines were established following *in vitro* exposure of $2–8 \times 10^5$ cells to 10^4 transforming units of the B95-8 EBV strain (10).

Lymphocyte markers. Cells with receptors for C3 were identified by the "EAC" rosette test. The source of antibody was 19 S rabbit anti-sheep erythrocyte, and the source of complement was fresh mouse serum. Receptors for the Fc portion of IgG were sought with the "EA" rosette test. Two sets of reagents were employed: sheep rbc coated with a nonagglutinating dilution of 7 S rabbit anti-sheep rbc (Cordis Laboratories) and human type O Rh positive rbc coated with a 1:80 dilution of human anti-D γ globulin (Spectra Biologicals).

Cytogenetic and morphologic studies. Chromosome studies were performed with the Giemsa banding method (11) on metaphase cells derived from two immortalized woolly monkey cell lines as well as from freshly drawn peripheral blood leukocytes from an uninoculated monkey.

Tests for EBV in transformed cells. Lines were tested for EB nuclear antigen and viral capsid antigen using positive and negative human reference antisera (12, 13). Early antigen was sought following treatment with iododeoxyuridine (IUdR) and following superinfection with the P_3J-HR-1 EBV strain (14, 15). Cell profiles from several lines were examined for viral nucleocapsids by electron-microscopy.

Animal inoculations. Four monkeys (Lagothrix lagotricha) were inoculated with cell-free B95-8 virus with a titer of 10^5TD_{50}/ml. They received 1 ml intravenously, 1 ml intraperitoneally, and 0.5 ml applied to the posterior pharynx with a cotton swab. Two of the four monkeys received azathioprine (3

0037-9727/78/1573-0489$01.00/0

mg/kg) and prednisolone (0.15 mg/kg) for 20 days. Four monkeys received injections of 4×10^7–1×10^8 autologous cells first transformed *in vitro* by B95-8. Two of the monkeys which received autologous cells had received cell-free virus 9 months previously, but had not developed persistent antibodies. They received two sets of inoculations of autologous cells each: one was given intrathecally via the cisterna magna, and the second inoculation, 6 months later, was given intraperitoneally and into the buccal mucosa. The intraperitoneal inoculations were accompanied by immunosuppressive chemotherapy, as outlined above. Two other monkeys which had not previously received inoculations of virus were given $7–9 \times 10^7$ cells into the peritoneal cavity and 10^7 cells in each buccal mucosal surface; one was immunosuppressed.

EBV antibody. Antibodies to VCA and EBNA were measured in sera taken biweekly from inoculated animals (16).

Results. Establishment of lymphoblastoid cell lines (Table I). Woolly monkey peripheral blood lymphocytes were relatively resistant to *in vitro* transformation by EBV. Transformation in the EBV system is defined as continued proliferation of lymphocytes *in vitro* following virus exposure. Cell lines were established from four animals; considering only those animals in which transformation was successful (four of eight) only 10% (14/145) of virus-exposed leukocyte cultures resulted in cell lines. No lines were derived without *in vitro* exposure to virus.

We were unsuccessful in our attempts to increase transformation frequency by exposure of the leukocytes to mitogens (PHA, conA, PWM, LPS). Similarly we could not increase frequency of transformation by exposing the cells to virus after several days in culture when many of the cells had entered DNA synthesis. Depletion of T lymphocytes also did not affect transformation frequency.

Karyotypes were prepared from two cell lines (monkeys B and O) and from a fresh peripheral blood sample of monkey R. Primary cells from monkey R had a constant chromosome number of $2n = 62$. Most cells from line O contained 62 chromosomes; however, these appeared to be pseudodiploid. Cells from line B had a basic chromosome number of 61 with at least two probable translocation chromosomes and other abnormalities.

TABLE I. ATTEMPTS TO ESTABLISH LYMPHOBLASTOID CELL LINES FROM WOOLLY MONKEY LEUKOCYTES.

Monkey B[a]	3/15[c]	0/15
Monkey O[a]	3/14	0/15
Monkey G	0/7	0/7
Monkey D	0/7	0/7
Monkey P	2/96	0/53
Monkey M	0/4	0/4
Monkey W	0/18	0/19
Monkey R	6/20	0/12
Total[c]	14/181	0/132

[a] Animals immunosuppressed with azathioprine and prednisolone.

[b] B95-8 strain of EBV, with 10^4–10^5 transforming units per ml.

[c] No. cultures positive/no. attempted.

Expression of EBV in transformed cells. A low level of viral capsid antigen (0.1–1.0% of cells) was detected in four of five virus transformed lines. Supernatant fluids from these lines also contained transforming virus demonstrated by lymphoblastoid transformation of umbilical cord leukocytes with freeze-thawed and filtered materials. The transforming titer in all lines was 10^0–$10^{0.5}$ 50% transforming units per 0.1 ml. The amount of virus in the extracellular fluid was assayed by endpoint dilution transformation of fresh human umbilical cord lymphocytes. Titers were calculated by the Reed Muench formula. Of five lines tested for the presence of EBNA all were positive, but the reaction was less intense in the WM lines than in the Raji positive control and was characterized by a fine, flecked pattern rather than a coarsely granular one.

We attempted to detect early antigen (EA) in two WM cell lines and to influence its production by additon of IUdR (60 μg/ml) or by superinfection with HR-1 virus (5×10^4 EA inducing units/ml as measured in Raji cells (14, 15). Both cell lines spontaneously produced low levels (1–3%) of EA since more cells stained with the EA + VCA + serum than with a serum which only contained anti-VCA. Addition of HR-1 virus did not increase the number of fluorescent cells and the level of EA expression was not enhanced by IUdR.

We demonstrated herpesvirus particles by electron microscopy in a thin sectioned cell

pellet prepared from one cell line. These cells had been exposed to iododeoxyuridine (IUdR) for 72 hr 2 weeks earlier. One area of cell debris contained seven capsids; one capsid contained a core. No C-type particles were seen. Freeze-thawed supernatant fluids from these same cultures caused transformation of human umbilical cord leukocytes.

Lymphocyte markers on primary and EBV transformed WM leukocytes (Table II, III). About 50% of WM peripheral blood mononuclear cells isolated by the Ficoll Hypaque technique are T lymphocytes, i.e., make rosettes with sheep rbc (E). Nearly 50% of the cells in peripheral blood have a receptor for IgFc as identified by the EA rosette marker, and are presumably B lymphocytes and monocytes, or, possibly, activated T cells. Cells bearing the IgFc marker are about two to three times as prevalent in WM blood as in human or cotton-top marmoset blood (17). Approximately 20% of WM mononuclear cells have the C3 receptor as identified by the EAC rosette test.

Except for loss of T lymphocytes, the expression of lymphocyte markers on the transformed cells reflected the markers found on primary cells. The majority of cells in the lines expressed the IgFc receptor. Overnight incubation of cells in the WM lines with rabbit antibody-coated erythrocytes at 4° yielded 90% EA rosettes. EA rosettes were also formed when human anti-D coated Rh-positive type O human erythrocytes were used as the reagent (Table III). About 20% of cells in the WM lines exhibited the C3 receptor. Since cloning studies have not yet been performed with these lines, it is not known whether the EA and EAC receptors are present on the same cell and whether all cells have the capacity to express the EAC receptor.

Spontaneous expression of the EA receptor on WM cells is unusual for cells transformed *in vitro* by EBV and we have not encountered this receptor on comparable immortalized human or marmoset cells (17). We wished to determine the relationship of the EA receptor to that which is induced by herpes simplex virus, since we considered the possibility that WM cells might carry a related virus. After infection with HSV type 1, several Burkitt lymphoma cell lines and a WM line were tested for receptors for human and rabbit IgFc. From 2 to 4% of cells in the herpes infected Burkitt lymphoma lines developed receptors for rabbit IgFc but not human IgFc. EBV transformed WM cells expressed receptors for IgFc of both species, and these receptors were not altered following HSV infection. About 0.1% of cells in all four lines formed infected centers after HSV infection.

Response of animals to inoculation of virus and autologous EBV transformed cells. All monkeys remained well following inoculation of cell free virus and autologous transformed cells. No alterations in peripheral blood count, or blood leukocyte morphology were noted. No lymphadenopathy or masses were detected.

Autopsies were performed on three animals 6–22 months after inoculation of autologous transformed cells. Two monkeys had been inoculated with such cells in the cisterna magna. Both gross anatomy and histology were normal. Cell lines could not be estab-

TABLE II. EXPRESSION OF SURFACE RECEPTORS ON FRESH PERIPHERAL BLOOD LYMPHOCYTES OF WOOLLY MONKEYS AND ON WOOLLY MONKEY LYMPHOBLASTOID CELL LINES.

	Peripheral blood lymphocytes			Lymphoblastoid cell lines		
Animal	% EAC[a]	% EA(7S)[b]	% E[c]	% EAC	% EA(7S)	% E
WM P	18	45	55	18	67	0
WM P	18	52	56	29	75	0
WM R	18	43	47	27	45	0
WM O	ND[d]	ND	ND	21	48	0
WM B	ND	ND	ND	34	42	0
WM M	27	47	55	ND	ND	ND

[a] Percentage of cells which form rosettes with sheep erythrocytes coated with rabbit 19S antibody and mouse complement.

[b] Percentage of cells which form rosettes with sheep erythrocytes sensitized with rabbit 7S immunoglobulin.

[c] Percentage of cells which bind sheep erythrocytes at 4°.

[d] ND = Not done.

lished from various lymphoid tissues at autopsy, nor could EBNA be demonstrated in imprints of lymph nodes.

All animals lacked antibody to VCA initially. Low level anti-VCA, at a titer of 1:10, appeared in the sera of three of four animals inoculated with cell-free virus by weeks 4 and 5 following inoculation, but was no longer detected by week 9 after inoculation. Similarly, only low level transient anti-VCA responses were observed in animals given autologous transformed cells.

Many attempts were made to recover lymphoid cell lines from the peripheral blood of inoculated animals at various intervals after inoculation. This was unsuccessful, with one exception: cell lines were established from several cultures of blood leukocytes obtained one week after intracisternal inoculation of autologous cells in one monkey. These lines presumably represent transformed cells which found their way to the periphery and circulated transiently.

Discussion. We report these findings for two reasons. First, the surface properties of EBV-transformed WM lymphocytes are unusual. The lines express avid IgFc receptors as demonstrated by the EA rosette test and thus they may be useful in further work on this receptor. Second, although the number of inoculated animals is small, WM cell lines immortalized in vitro by EBV have not proved oncogenic when returned to the autologous host, even in an immunologically privileged site or when administered with immunosuppressive drugs.

The IgFc receptor found on transformed WM cells is not found on comparable human or marmoset cells transformed *in vitro* by EBV (17). The receptor appears to be qualitatively distinct from that induced by Herpes simplex, since it is capable of binding and forming rosettes with both human and rabbit immunoglobin coated erythrocytes whereas the HSV induced receptor forms rosettes with erythrocytes coated with rabbit but not human immunoglobin (Table III). The lines may be useful in detecting antigen-antibody complexes, or possibly in the purification and characterization of the chemical structure of this ligand receptor. We do not believe the IgFc receptor is induced by EB virus, since receptor activity is expressed on WM lymphocytes before transformation.

TABLE III. EXPRESSION OF THE IgFc RECEPTOR IN WOOLLY MONKEY LYMPHOBLASTOID LINES AND IN BURKITT LYMPHOMA LINES WITH AND WITHOUT HERPES SIMPLEX VIRUS INFECTION.

Cell Line	EBV Genome	HSV-1 Added	Fraction of Cells[b] making EA rosettes: Rabbit 7S anti-SRBC	Fraction of Cells[b] making EA rosettes: Human anti-D
WM	+	No	.88	.65
		Yes[a]	.90	.66
Raji	+	No	0	0
		Yes	.04	0
BJAB	−	No	0	0
		Yes	.02	
BJAB/HR-1	+	No	0	0
Clone B_1		Yes	.03	0

[a] Multiplicity of 0.75 PFU/cell. Rosette formation tested 24 hr after infection.

[b] In each rosette test, 100–200 cells were counted.

Several factors may correlate with the lack of oncogenicity of virus and transformed cells in this species of monkey. Firstly, WM lymphocytes are relatively refractory to transformation (Table I). For example preparations of EBV which contain 10^4 transforming units when assayed on human cells, will only transform a fraction of the cultures when added undiluted to WM cells. Since WM T lymphocytes spontaneously proliferate for several weeks *in vitro,* we entertained the idea that T cells might suppress transformation. However elimination of T lymphocytes and enrichment for cells bearing the Fc receptor did not increase the transformation rate (unpublished). The relative insusceptibility of lagothrix leukocytes to transformation cannot be explained by the absence of virus receptors since we were, in fact, able to induce transformation *in vitro,* albeit rarely. Ample numbers of lymphocytes with complement receptors, thought to be closely associated with the virus receptor, are found in WM blood (Table II) (17), but the subpopulation of EAC rosette-forming cells with virus receptors may be small or the virus receptors may be sparsely distributed on WM B lymphocytes. Susceptibility of cells to transformation and susceptibility of the host to infection may be related; higher transformation efficiencies of EBV are found in human cells, the natural host (18). Both the serologic studies and gen-

eral failure to recover cell lines from peripheral blood indicate that WM are not infected following inoculation of virus or cells.

Secondly, WM lymphoblastoid lines are relatively nonproductive of virus. They are similar to lines derived from adult humans and they contrast sharply with transformed marmoset cells which release relatively large amounts of cell free virus (19). While autochthonous EBV transformed marmoset cells have proved oncogenic in one instance (20) the tumor may have been induced by virus released from the transformed marmoset cell and not by the transformed cells *per se.* In fact, though there is ample evidence for oncogenicity of the virus in marmosets, there is as yet no evidence that lymphoblastoid cells of any species, derived following in vitro exposure to EBV, are inherently tumorigenic.

Thirdly, transformed cells were apparently readily eliminated, despite the absence of preexisting anti-viral immunity. In one instance transformed cells were retrieved from the peripheral blood seven days after inoculation, but not thereafter, this suggests an efficient mechanism for recognition and elimination of transformed cells.

Summary. Lymphoblastoid cell lines were derived from peripheral blood leukocytes of woolly monkeys by exposure to the B95-8 strain of EB virus. Primary cells were relatively resistant to transformation and the resulting lines produced little virus. Almost half the lymphocytes in fresh blood had a receptor for IgFc and 60–90% of continuously cultured cells expressed receptors for both rabbit and human IgFc as detected with the "EA" rosette test. The IgFc receptor on woolly monkey cell lines was qualitatively different from that induced by herpes simplex virus which could be detected with rabbit but not human Ig. Monkeys inoculated with cell-free virus and autologous transformed cells remained well and showed no evidence of infection. There was thus no evidence that woolly monkey cells transformed *in vitro* by EBV were oncogenic when replaced in the autologous host.

We would like to thank Elizabeth Grogan for expert help with the HSV infection and rosetting experiments, Dr. James Robinson for characterization of the surface markers on primary and transformed cell lines, Dr. Roy Breg and Mrs. Vicki Fritz for help with preparation and interpretation of the karyotypes, Mrs. Muriel Lipman for electron microscopic studies, Mr. Walter Webb for his expert animal care and Mrs. Katherine Papov for excellent technical assistance.

1. Frank, A., Andiman, W. A., and Miller, G. *in* "Advances in Cancer Research" (G. Klein and S. Weinhouse, eds.), Vol. 23, p. 171, Academic Press, New York (1976).
2. Shope, T., and Miller, G., J. Exp. Med. **137,** 140 (1973).
3. Falk, L. A., Wolfe, L., Deinhardt, F., Paciga, J., Dombos, L., Klein, G., Henle, W., and Henle, G., Int. J. Cancer **13,** 353 (1974).
4. Werner, J., Henle, G., Pinto, C. A., Haff, R. F., and Henle, W., Int. J. Cancer **10,** 557 (1972).
5. Shope, T., Dechairo, D., and Miller, G., Proc. Nat. Acad. Sci. U.S.A. **70,** 2487 (1973).
6. Epstein, M. A., Rabin, H., Ball, G., Rickinson, A. B., Jarvis, J., and Melendez, L. V. Int. J. Cancer **12,** 319 (1973).
7. Leibold, W., Huldt, G., Flanagan, T. D., Andersson, M., Dalens, M., Wright, D. H., Voller, A., and Klein, G., Int. J. Cancer **17,** 533 (1976).
8. Falk, L., Deinhardt, F., Wolfe, L., Johnson, D., Hilgers, J., and de The, G., Int. J. Cancer **17,** 785 (1976).
9. Boyum, A., Scand. J. Clin Lab. Invest. **29** (Suppl.), 1 (1968).
10. Henle, W., Diehl, V., Kohn, G., zur Hausen, H., and Henle, G., Science **157,** 1064 (1967).
11. Suu, N. C., Chu, E. H. Y., and Change, C. C., Caryologia **27,** 315 (1974).
12. Reedman, B. M., and Klein, G., Int. J. Cancer **11,** 499 (1973).
13. Henle, G., and Henle, W., J. Bacteriol. **91,** 1248 (1966).
14. Gerber, P., and Lucas, S., Proc. Soc. Exp. Biol. Med. **141,** 431 (1972).
15. Henle, W., Henle, G., Zajac, B., Pearson, G., Waubke, R., and Scriba, M., Science **169,** 188 (1970).
16. Andiman, W. A., and Miller, G. *in* "Manual of Clinical Immunology" (N. Rose and H. Friedman, eds), American Society for Microbiology, Washington, D. C. (1976).
17. Robinson, J. E., Andiman, W. A., Henderson, E., and Miller, G. Proc. Nat. Acad. Sci. U.S.A. **74,** 749 (1977).
18. Henderson, E., Miller, G., Robinson, J., and Heston, L. Virology **76,** 152 (1977).
19. Miller, G., and Lipman, M. Proc. Nat. Acad. Sci. **70,** 190 (1973).
20. Miller, G., Shope, T., and Coope, D. *in* "Mechanisms of Virus Disease" (W. S. Robinson and C. F. Fox, eds.), W. A. Benjamin, Inc. Menlo Park, CA. (1974).

Received June 6, 1977. P.S.E.B.M. 1978, Vol. 157.