PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 193-196 (1976)

A Procedure for Quantifying Susceptibility of Human Lymphocytes to Transformation by Epstein-Barr Viruses[1] (39508)

R. SHIHMAN CHANG, RALPH A. FILLINGAME, TERESA PAGLIERONI, AND FRANK J. GLASSY

Department of Medical Microbiology, University of California, Davis, California 95616 and Department of Pathology, Sutter Memorial Hospital, Sacramento, California 95819

Epstein–Barr viruses (EBV) from diverse sources are capable of transforming human lymphoid cells with limited *in vitro* lifespan into established cell lines (1–11). Surface-marker studies have shown that all EBV-transformed human lymphoid cell lines possess immunoglobulins (Ig) (12–15), peripheral lymphoid cells depleted of the Ig-bearing population are less susceptible or nonsusceptible to EBV transformation (16, 17), and, only surface Ig-bearing cells have EBV receptors (18). These findings suggest that EBV may be specific for Ig-bearing or "B" lymphocytes (14–17). Little is known, however, as to whether all surface Ig-bearing lymphocytes are equally susceptible and all non-Ig-bearing lymphocytes are insusceptible to EBV transformation. To answer this question, one must devise a procedure to quantify cell susceptibility to EBV transformation. We have successfully devised such an assay which determines the minimal number of lymphoid cells that must be present in a culture in order for the culture to be transformed by the EBV; results obtained from this assay are reported here.

Materials and methods. Harvesting of lymphoid cells. In the early phase of this study, peripheral leukocytes were harvested by sedimentation in dextran (19), which yielded a population of leukocytes containing 40 to 60% lymphocytes. Later, leukocytes were collected by centrifugation of venous blood on a Ficoll cushion (20); leukocytes obtained by this method contained >85% lymphocytes.

Depletion of Ig-bearing cells. Immunoabsorbent columns were prepared by conjugating rabbit anti-human Ig or normal rabbit immunoglobulins to CNBr-activated Sepharose 4B beads. The procedures described by other investigators were used with minor modifications (21–24). Peripheral lymphoid cells harvested by the Ficoll method were passed through the anti-human Ig or the control column. Cells not retained were examined for surface markers and viability and then used in the study.

Surface markers. The proportion of cells that possess surface immunoglobulins was determined by staining with fluorescein-conjugated polyvalent antihuman Ig (25, 26). Under code, 100 to 300 cells were counted under the white light and the percentage of cells showing three or more specks of granular fluorescence under uv illumination was enumerated. If negative, a total of at least 1000 cells was examined for fluorescent specks. The percentage of cells which formed rosettes spontaneously with sheep erythrocytes was determined as previously described (27).

EBV preparations. One pool of a throat washing from a patient with infectious mononucleosis (7) and one pool of filtrate from the B95-8-transformed marmoset cell line (28) were used. The former contained $10^{1.5}$ and the latter contained $10^{4.5}$ transforming units per 0.2 ml when assayed on neonatal leukocytes (11).

Preparation of feeder cells. Human amnion cells were harvested and grown as described (29). A cell suspension containing 50,000 cells/ml of medium was prepared and distributed in 1-ml portions to 18 × 150-mm culture tubes which were kept in the upright position for 1–3 days. Prior to experimentation, the medium together with some nonadherent cells was removed.

Nutrient medium. Eagle's basal medium containing the nonessential amino acids (30), penicillin G (50 μg/ml), streptomycin

[1] Supported in part by a research grant (AI-12198) from the National Institute of Allergy and Infectious Diseases.

Copyright © 1976 by the Society for Experimental Biology and Medicine
All rights reserved.

(50 μg/ml), and 20% fetal calf serum was used.

Determination of the minimal number of lymphoid cells required for transformation (MNLRT). Quadruplicate cultures containing 10^6, 10^5, 10^4, 10^3, 10^2, and 10 viable leukocytes in 0.8 ml of medium were prepared in culture tubes (18 × 150 mm) filled with 5% CO_2 in air and closed with rubber stoppers. Two-tenths milliliter of EBV was added to each culture. Four cultures of 5 × 10^6 cells were kept as uninoculated controls to monitor for possible spontaneous transformation. The cultures were fed individually and observed for transformation as described (11). Transformation was signaled by the appearance of cell aggregates which increased in size and number together with increased metabolic activity 3 to 8 weeks after inoculation with the EBV. At the end of the eighth week, the MNLRT was calculated by the 50% endpoint method (31). No correction was made for the variation in the proportion of lymphoid cells in the leukocyte suspension. Assays in which uninfected cultures transformed spontaneously were considered not valid; of all the assays reported in this publication, there was only one assay whose control cultures transformed.

EBV antibody. EBV capsid antibody was tested by the indirect immunofluorescent test (33).

Results. Evaluation of the assay for the minimal number of lymphoid cells required for transformation (MNLRT). Results of 14 assays are summarized in Table I. Amnion feeder layers consistently reduced the MNLRT by about 1 log; this finding is consistent with the reports that feeder layers of macrophages or fibroblasts enhance transformation (16, 32). Increasing the dosage of EBV from $10^{1.5}$ to $10^{4.5}$ transforming units (TU) reduced the MNLRT by about 1 log. Using amnion feeder layers and $10^{1.5}$ TU, the MNLRT for the leukocyte of one donor tested on five occasions was 4.62 ± 0.19 log. With amnion feeder layers and $10^{4.5}$ TU, the MNLRT for the leukocytes of seven EBV-seronegative donors was 3.14 ± 0.27 log. These results indicated that the MNLRT assay yielded reasonably reproducible results and that the lowest MNLRT value was obtained by the use of amnion feeder layers and $10^{4.5}$ TU of EBV from the B95-8 marmoset cell line. These experimental conditions were used in all subsequent experiments.

Depletion of Ig-bearing cells on the MNLRT value. Results are summarized in

TABLE I. EFFECT OF FEEDER LAYERS, EBV DOSAGES, AND METHODS OF HARVESTING LEUKOCYTES ON THE MINIMAL NUMBER OF LYMPHOID CELLS REQUIRED FOR TRANSFORMATION (MNLRT).

Expt	Donors[a]	Harvesting of leukocytes	EBV[b] source and dose		MNLRT (in log)	
					With feeder	Without feeder
1	IS	Dextran	TW	$10^{1.5}$	4.3	6.0
2	IS	Dextran	TW	$10^{1.5}$	4.7	5.5
3	IS	Dextran	TW	$10^{1.5}$	4.7	6.0
4	IS	Dextran	TW	$10^{1.5}$	4.8	NT
5	IS	Dextran	TW	$10^{1.5}$	4.8	NT
6	HC	Dextran	TW	$10^{1.5}$	3.8	4.5
7	HC	Dextran	B95-8	$10^{1.5}$	4	NT
			B95-8	$10^{4.5}$	2.8	NT
8	NB	Dextran	B95-8	$10^{1.5}$	4	NT
			B95-8	$10^{4.5}$	3	NT
9	DV	Dextran	B95-8	$10^{4.5}$	3.5	4.5
		Ficoll	B95-8	$10^{4.5}$	3	4
10	DV	Ficoll	B95-8	$10^{4.5}$	3.5	NT
11	TC	Ficoll	B95-8	$10^{4.5}$	3.0	NT
12	BO	Ficoll	B95-8	$10^{4.5}$	3.0	NT
13	GC	Ficoll	B95-8	$10^{4.5}$	3.0	NT
14	IS	Dextran	B95-8	$10^{4.5}$	3.5	NT

[a] NB = newborn. All others were EBV-seronegative healthy persons (12–26 years old).
[b] TW = throat washing; B95-8 = filtered spent medium from the transformed marmoset line (28). Dosages are in transforming units (11).

TABLE II. EFFECT OF DEPLETION OF SURFACE Ig-BEARING CELLS ON THE MNLRT.

Expt[a]	Percentage of Ig-bearing and E-rosetting cells and MNLRT values		
	Predepletion[b]	Depleted[b]	Sham depletion[b]
1	(44) (56), 3.0[c]	(0.5) (77), 5.8	(5.5) (75), 2.8
2	(19) (53), 2.8	(<0.2) (72), 6.0	(6) (61), 2.8
3	(20) (53), 3.5	(<0.2) (69), >6.5	(10) (54), 4.0
4	(20) (45), 4.0	(1) (92), >6.5	(7) (75), 4.8

[a] The blood of EBV-seronegative donors was used in Expts. 1 to 3 and of an EBV-seropositive donor in Expt. 4.

[b] See Materials and Methods. Sham depletion = cells not adherent to normal rabbit Ig-sepharose column. All lymphoid cell suspensions contained >95% viable cells at the initiation of the assay.

[c] (44) (56), 3.0 = (44% surface Ig-bearing cells) (56% E-rosetting cells), and a MNLRT of 3.0 log.

Table II. The depletion of surface Ig-bearing cells from a lymphoid cell population increased the MNLRT value by at least 2.5 log. This finding substantiates quantitatively that depletion of surface Ig-bearing cells lowers susceptibility of lymphoid cells to EBV transformation (15–17).

The MNLRT values for lymphoid cells from patients with chronic lymphocytic leukemia (CLL). The MNLRT values for the peripheral lymphocytes (over 90% surface Ig-bearing cells) of three CLL patients were 6.5, >6.5, and >6.5 log. The assay for the fourth patient was not valid because of spontaneous transformation.

Discussion. A procedure for determining the minimal number of leukocytes required for transformation (MNLRT) by the EBV has been devised. Under the prescribed assay conditions, close MNLRT values were obtained. The average MNLRT value for the peripheral leukocytes of seven EBV-seronegative healthy persons was 3.14 ± 0.27 log. It seems reasonable to consider a difference of 1 log in the MNLRT value as a significant difference in susceptibility to EBV transformation; a higher value signifies a lower susceptibility to EBV transformation. Just why a minimum of about 3 log of peripheral leukocytes must be present for successful EBV transformation is not known. It is possible that only one of about 10^3 leukocytes is susceptible to EBV transformation; or, a minimum of about 10^3 EBV-transformed cells are necessary to create favorable culture conditions for some transformed cells to grow into an established line. Whatever the explanation may be, it appears reasonable to consider the MNLRT value as a measure of the *relative* susceptibility of a cell population to EBV transformation (defined as the ability of a cell population to grow persistently as a cell line). This statement is substantiated by our finding that depletion of the EBV-susceptible surface Ig-bearing cells from a lymphoid cell population increased the MNLRT values by at least 2.5 log.

Applying the assay to the peripheral lymphocyte of three patients with chronic lymphocyte leukemia (CLL), we found the CLL lymphocytes much less susceptible to EBV transformation. The MNLRT for the three CLL lymphoid cell populations were 6.5, >6.5, and >6.5 log in contrast to the value of 3.14 ± 0.27 log for the lymphoid cells from seven healthy persons. Since the CLL lymphocytes were predominately surface Ig-bearing cells, we conclude that there may be differences in susceptibility to EBV transformation among populations of surface Ig-bearing cells. The cellular basis for this low susceptibility of the CLL surface Ig-bearing cells to EBV transformation deserves further study.

Summary. An assay for determining the minimal number of leukocytes required for transformation (MNLRT) by the EB virus has been devised. Close MNLRT values of 3.14 ± 0.27 log were obtained for the peripheral leukocytes of seven EBV-seronegative persons. Depletion of surface Ig-bearing cells increased the MNLRT value by at least 2.5 log. Applying this assay to the leukocytes of three chronic lymphocytic leukemia (CLL) patients we obtained the values of 6.5, >6.5, and >6.5 log.

Addendum: Since the submission of this manuscript the following relevant article has appeared:

Mizuno, F., Aya, T. and Osato, T. Brief Communication: Growth in semi-solid agar medium of human cord leukocytes freshly transformed by EB virus. J. Nat. Cancer Inst. **56**, 171 (1976).

---

1. Henle, W., Diehl, V., Kohn, G., Zur Hausen, H., and Henle, G., Science **157**, 1064 (1967).
2. Pope, J. H., Horne, M. K., and Scott, W., Int. J. Cancer **3**, 857 (1968).
3. Gerber, P., Whang-Peng, J., and Monroe, J. H., Proc. Natl. Acad. Sci. (Wash.) **63**, 740 (1969).
4. Miller, G., Lisco, H., Kohn, H. I., and Stitt, D., Proc. Soc. Exp. Biol. Med. **137**, 1459 (1971).
5. Nilsson, K., Klein, G., Henle, W., and Henle, G., Int. J. Cancer **8**, 443 (1971).
6. Chang, R. S., Nature New Biology **233**, 124– 1971).
7. Chang, R. S., and Golden, H. D., Nature (London) **234**, 359 (1971).
8. Pereira, M. S., Field, A. M., Blake, J. M., Rodgers, F. G., and Bailey, L. A., Lancet **1**, 710 (1972).
9. Gerber, P., Nonoyama, M., Lucas, S., Perlin, E., and Goldstein, L. I., Lancet **2**, 988 (1972).
10. Miller, G., Neiderman, J. C., and Andrew, L-L., New Engl. J. Med. **288**, 229 (1973).
11. Chang, R. S., Lewis, J. P., and Abildgaard, C. F., New Engl. J. Med. **289**, 1325 (1973).
12. Shevach, E. M., Huberman, R., Frank, M. M., and Green, I., J. Clin. Invest. **51**, 1933 (1972).
13. Moore, G. E., and Minowada, J., New Engl. J. Med. **288**, 106 (1973).
14. Pattengale, P. K., Smith, R. W., and Gerber, P., Lancet **2**, 93 (1973).
15. Pattengale, P. K., Smith, R. W., and Gerber, P., J. Natl. Cancer Inst. **52**, 1081 (1974).
16. Schneider, U., and Zur Hausen, H., Int. J. Cancer **15**, 59 (1975).
17. Yata, J., Desgranges, C., Nakagawa, T., Favre, M. C., and De-The, G., Int. J. Cancer **15**, 377 (1975).
18. Jondal, M., and Klein, G., J. Exp. Med. **138**, 1365 (1973).
19. Chang, R. S., Hsieh, M. W., and Blankenship, W., J. Natl. Cancer Inst. **47**, 469 (1971).
20. Boyum, A., Scand. J. Clin. Lab. Invest. **21** (Suppl. 97), 31 (1968).
21. Schlossman, S., and Hudson, L., J. Immunol. **110**, 313 (1973).
22. Rocklin, R., MacDermott, R., Chess, L., Schlossman, S. F., and David, J. R., J. Exp. Med. **140**, 1303 (1974).
23. Chess, L., MacDermott, R., and Schlossman, S., J. Immunol. **113**, 1113 (1974).
24. Chess, L., MacDermott, R., and Schlossman, S., J. Immunol. **113**, 1122 (1974).
25. Rabellino, E., Colon, S., Grey, H. M., and Unanue, E. R., J. Exp. Med. **133**, 156 (1971).
26. Jondal, M., Holm, G., and Wigzell, H., J. Exp. Med. **136**, 207 (1972).
27. Mendes, N. F., Tolnai, M. E. A., Silveira, N. P. A., Gilbertsen, R. B., and Metzgar, R. S., J. Immunol. **111**, 860 (1973).
28. Miller, G., and Lipman, M., Proc. Natl. Acad. Sci. USA **70**, 190 (1973).
29. Chang, R. S., J. Natl. Cancer Inst. **40**, 491 (1968).
30. Eagle, H., Science **130**, 432 (1959).
31. Reed, L. J., and Meunch, H., Amer. J. Hyg. **27**, 493 (1938).
32. Pope, J. H., Scott, W., and Moss, D. J., Int. J. Cancer **14**, 122 (1974).
33. Henle, G., and Henle, W., J. Bacteriol. **91**, 1248 (1966).

Received April 7, 1976. P.S.E.B.M. 1976, Vol. 153.