In Vitro Evaluation of Cell-Mediated Immunity to Epstein Barr Virus

Mahmood Mahmoodian Shooshtari, Mehdi Banitalebi Dehkordi, and Zohreh Sharifi

Research Centre, Iranian Blood Transfusion Organisation, Tehran, Iran

ABSTRACT

This study was conducted to measure cell-mediated immune response in healthy Epstein Barr virus (EBV)–seropositive individuals using a tissue culture "growth inhibition" assay (regression assay) where peripheral blood lymphocytes (PBLs) were tested for their ability to inhibit the outgrowth of the autologous lymphoblastoid cell lines (LCLs). Inhibition of the outgrowth of the autologous LCLs was seen after 4 weeks by the addition of PBLs from healthy EBV seropositive donors. The regression phenomenon was never seen when the donors of peripheral blood lymphocytes were EBV- seronegative**.** Regression assay showed that EBV- specific memory T cells were stable in healthy EBV seropositive over many years, which indicates the persistent nature of EBV infection.

Keywords: Cell lines; Cell mediated immunity; Epstein Barr virus

INTRODUCTION

The Epstein-Barr virus (EBV) is a human viral pathogen with a 170kb double stranded DNA genome and has demonstrable oncogenic potential.[1] In the course of primary human infection with EBV the agent infects epithelial cells of the oropharynx[2,3] and circulating lymphocytes which have differentiated toward immunoglobulin production.[4] Epithelial cell infection persists for years. Although lymphocyte infection also persists for life, there is little or no virus replication in lymphocytes. Infection is therefore said to be latent in these cells.[4] Lymphocyte infection results in cell proliferation.[4] In normal people, however, the latently infected lymphocytes are suppressed or killed by immune T and NK lymphocytes.[5] Since latently infected lymphocytes can be specifically killed by immune T cells they must be expressing a new cell surface antigen. When EBV infects B lymphocytes the cell becomes immortalized to perpetual growth[6] to produce EB virus transformed lymphoblastoid cell lines (LCLs).

Corresponding Author: Mahmood Mahmoodian Shooshtari, PhD; Iranian Blood Transfusion Organisation, Research centre, Tehran, Iran. Tel: (+98 21) 8828 8583, Fax: (+98 21) 8827 0001, E-mail: shooshtari@ibto.ir

LCLs express a limited set of EBV proteins known as the latency-associated proteins.[7]

EBV is widespread in the human population and more than 95% of adults are EBV seropositive and possess memory T-cells in their peripheral blood.[4] A key factor in maintaining a virus-host balance appears to be the cell-mediated immune response.[8,9] T cell response can be evaluated using a tissue culture growth inhibition assay (regression assay) or by a chromium-51 release assay.[10-12] Compared to radioactive isotope techniques such as a ^{51}Cr release assay, the growth inhibition assay is safer and economical (no radioactive isotopes are used, no additional reagents like scintillation fluid is required). Also the results obtained strongly correlate to those obtained with other techniques. The regression assay is so called because *in vitro*, EBV infected lymphocytes transform in the 14 days post infection and subsequently these infected B cells die or regress due to the activation of a specific T-cell response in the same culture.[10,12] In this study, cell mediated immune response to EBV in man was measured *in vitro* using a tissue culture "regression assay" where peripheral blood lymphocytes were tested for their ability to inhibit the outgrowth of autologous EB virus transformed lymphoblastoid cell lines (LCLs).

MATERIALS AND METHODS

Preparation of Peripheral Blood Lymphocytes (PBLs)

Blood samples (30-50 mls) were collected from healthy EBV seropositive donors [4 males (A, B, D, F), aged 30-40 years] into heparinzed RPMI 1640 medium. Then peripheral blood lymphocytes (PBLs) were separated using lymphocyte separation medium (ICN, FLOW ICN Biomedical, Thane Oxfordshire UK) and tested on the same day against autologous human lymphoblastoid cell lines (LCLs).

Cell Culture and Establishment of EBV-Transformed LCLs

Human EBV-transformed LCL was established from peripheral blood lymphocytes as previously described.[10] Briefly, frozen virus stocks prepared from the B95-8 cell line and having a titre of 10^5 lymphocyte transforming units per ml were thawed quickly and diluted 1 in 5 in tissue culture medium and 0.5 ml was added to 2 x 10^6 human lymphocytes. Cells were incubated in a CO_2 incubator at 37º C for 1 hour with regular shaking.

Cells were then washed and resuspended at the required cell concentration in 10% FCS RPMI 1640 medium containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 1% phytohaemaglutinin (PHA) (1% solution of a stock). The cells were plated out in 2ml wells and incubated in 5% CO_2 in a humidified incubator at 37ºC.

After 2-3 days, the cells were stimulated with PHA, clumped together and blast cells appeared. EBV transformed clumps were grown and cell numbers increased. The cells were sub-cultured into fresh wells for 2-3 weeks and then into 25 cm² plastic tissue culture flasks. After establishment, LCLs were fed twice a week by doubling the volume or replacing half the culture with fresh medium.

Regression Assay

PBLs from seropositive human donors (A, B, D, F) and one seronegative human donor (C) were tested for their ability to inhibit the outgrowth of their autologous LCL.

LCLs from the relevant autologous donor were plated out in round bottomed wells in 96 well plates (Corning, UK) as target cells at serial dilution ranging from 2.5 x 10^4 to 1.7 x 10^2 cells per 0.2 ml well. 12 wells were plated out per dilution. PBLs were prepared from the autologous sample and added to 6 wells of each dilution (48 wells) at 2.5 x 10^4 cells / well (Table 1).

The cultures were incubated at 37ºC in 5% CO_2 and observed twice a week for up to 4 weeks to determine the incidence of successful target cell outgrowth. The plates were fed weekly. Wells were observed using an inverted microscope and scored positive when characteristic progressively growing foci of the target LCLs were present. The effect of the PBLs on the outgrowth of the LCLs was calculated by the method of Reed-Munch.[13]

The data for this experiment was collected from the minimum number of autologous LCL cells required for a 50% incidence of successful outgrowth in the presence of PBLs in a multiwell dish with serial dilutions of target cells and after an appropriate incubation period when the wells showed successful outgrowth, the dilution was then estimated by determining the proportionate distance between the highest and lowest dilutions that would correspond to 50% of successful outgrowth.

RESULTS

Table 1 shows the results of a regression assay. Inspection of the data in donors (A, B, D, F) revealed that a 6x 10^3 dilution of target cells resulted a successful outgrowth in more than 50% whereas a 3x 10^3 dilution of target cells resulted in a successful outgrowth in fewer than 50%. Thus, the dilution that would yield a 50% incidence of successful outgrowth in all donors exactly lied between 6x 10^3 and 3x 10^3.

Figure 1 shows an ability of PBLs prepared from human seropositive donors (A, B, D, F), to inhibit the outgrowth of autologous LCLs.

The minimum number of autologous LCL cells required for a 50% incidence of successful outgrowth in the presence of PBLs for A donor being 4 x 10^3, B donor 4.5 x 10^3, D donor 3.8 x 10^3, and F donor 4.1 x 10^3. No inhibition of the outgrowth of the autologous LCL was seen after 4 weeks by the addition of PBLs with EBV seronagative (C donor).

DISCUSSION

The capacity of EBV to infect and transform B lymphocytes in vitro has provided an accessible

Table 1. Incidence of successful outgrowth after 4 weeks of EBV-transformed target cells (LCLs).

	Seronagative human donor C		Seropositive human donor A		Seropositive human donor B		Seropositive human donor D		Seropositive human donor F	
Number of target cells seeded per U well	LCL alone	LCL + 2.5 x10^4 PBLs	LCL alone	LCL + 2.5 x 10^4 PBLs	LCL alone	LCL + 2.5 x 10^4 PBLs	LCL alone	LCL + 2.5 x 10^4 PBLs	LCL alone	LCL + 2.5 x 10^4 PBLs
2.5 × 10^4	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
1.25 × 10^4	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
6 × 10^3	6/6	6/6	6/6	4/6	6/6	5/6	6/6	3/6	6/6	4/6
3 × 10^3	6/6	6/6	6/6	3/6	6/6	0/6	6/6	1/6	6/6	2/6
1.5 × 10^3	6/6	6/6	6/6	0/6	6/6	0/6	6/6	0/6	6/6	0/6
7 × 10^2	6/6	6/6	6/6	0/6	6/6	0/6	6/6	0/6	6/6	0/6
3.5 × 10^2	6/6	6/6	6/6	0/6	6/6	0/6	6/6	0/6	6/6	0/6
1.7 × 10^2	6/6	6/6	6/6	0/6	6/6	0/6	6/6	0/6	6/6	0/6

Seeded in the presence and absence of autologous PBLs from four EBV seropositive donors (A, B, D, F) and one seronagative donor (C).
6/6 indicates that LCLs were grown in all 6 wells; 0/6 all wells showed out growth of LCL at 4 weeks
3/6 indicates that 3 of 6 wells were grown

system with which to search for evidence of specific cellular immunity. This was first obtained with the demonstration that EBV-induced B cell transformation regresses in crowded cultures of infected lymphocytes from EBV immune, but not from EBV seronegative individuals.[10,11]

The regression is mainly due to reactivation of MHC class I restricted memory cytotoxic T cell (CTL) responses[11,12] though EBV-specific CD4 class II restricted cells have also been described.[14,16] Cytotoxic T Lymphocytes (CTLs) are important in the immune response to viral infection and play a crucial role in the host defence against viruses by recognizing viral antigens and eliminating virus- infected cells.[14,15] In the present work, cell mediated immune response was observed in four EBV-seropositive donors using a tissue culture growth inhibition assay after 4 weeks by the addition of peripheral blood lymphocytes whereas no inhibition of the outgrowth of the autologous LCL was seen with lymphocytes from EBV-seronagative donor.

The number of cells required for 50% regression of EBV-induced autologous LCLs in donors was similar to that in the other healthy subjects. This indicates the persistent nature of EBV infection, adequate memory cytotoxic T cell control of

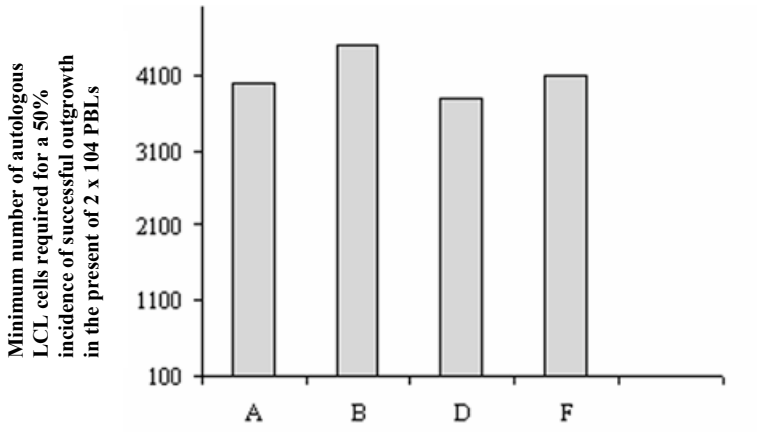

Figure 1. Ability of PBLs from human seropositive donors (A, B, D, F), to inhibit it the outgrowth of autologous LCLs.
Donor A: 50% end point =4×10^3; Donor B: 50% end point =4.5×10^3
Donor D: 50% end point =3.8×10^3; Donor F: 50% end point =4.1×10^3
Donor C: 50% end point = —

EBV-infected B lymphocytes. The regression phenomenon was never seen when the donors of PBLs were EBV-seronegative. Previous work has also shown that at least one in 10^3 to 10^4 circulating T lymphocytes are EBV-specific memory T-cells which are stable in seropositive donors.[10,11] This life-long virus carrier state is presumably due to chronic, low-grade replication of the virus at a permissive site in the oro-pharynx or naso-pharynx since EB virus can be detected in throat washings from the vast majority of seropositive human subjects.[2,3]

More works should aim to study T-cell responses to the EBV latent proteins which are constitutively expressed in LCLs and the cell-mediated immune response to EBV envelope glycoprotein's and other lytic cycle products.

ACKNOWLEDGMENT

The authors wish to thank the Iranian Blood Transfusion Research Centre for financing this research. We gratefully acknowledge Dr Suzan Finerty, Reader in virology, Department of pathology and microbiology, Bristol University, U.K for her advising and helpful comments.

REFERENCES

1. Thomas JA, Hotchin NA, Allday MJ, Amlot P, Rose M, Yacoub M, et al. Immunohistology of Epstein-Barr virus-associated antigens in B cell disorders from immunocompromised individuals. Transplantation 1990; 49(5):944-53.
2. Friborg JT. Aspects of the epidemiology of nasopharyngeal carcinoma and Epstein Barr virus infection in Greenland. Ugeskr Laeger 2005: 167(33):3065.
3. Friborg JT, Melbye M. Epstein Barr virus and nasopharyngeal carcinoma. Ugeskr Laeger 2002; 164(50):5908-11.
4. Rickinson AB, Elliott Kieff. Epstein-Barr virus. In: Fields BN, Knipe DM, editors. Field's Virology. New York: Lippincott-Raven Press, 2001.
5. Rickinson AB, Moss DJ. Human Cytotoxic T Lymphocyte responses to Epstein Barr virus infection. Annu Rev Immunol 1997; 15:405-31.
6. Henle G, Henle W. Seroepidemilogy of the virus. In: Epstein M, Achong B, editors. The Epstein Barr virus. Berlin: Springer-Verlay, 1999: 61-78.
7. Sample J, Young L, Martin B, Chatman T, Kieff E, Rickinson AB, et al. Epstein-Barr virus types 1 and 2 differ in their EBNA-3a, EBNA-3b, and EBNA-3c genes. J Virol 1990; 64(9):4084-92.
8. Khanna R, Burrows SR. Role of cytotoxic T Lymphocytes in Epstein Barr virus associated diseases. Annu Rev Microbiol 2000; 54:19-48.
9. Levitsky V, Masucci MG. Manipulation of immune responses by Epstein Barr virus. Virus Res 2002; 88(1-2):71-86.
10. Moss DJ, Rickinson A, Pope JH. Long-term T cell-mediated immunity to Epstein-Barr virus in man. I. Complete regression of virus-induced transformation in cultures of seropositive donor leukocytes. Int J Cancer 1978, 22(6):662-8.
11. Rickinson AB, Moss DJ, Wallace LE, Rowe M, Misko IS, Epstein MA, et al. Long-term T-cell-mediated immunity to Epstein-Barr virus. Cancer Res 1981; 41(11 Pt 1):4216-21.
12. Gudgeon NH, Taylor GS, Long HM, Haigh TA, Rickinson AB. Regression of Epstein-Barr virus-induced B-cell transformation in vitro involves virus- specific CD8+ T cells as the principal effectors and a novel CD4+ T-cell reactivity. J Virol 2005; 79(9):5477-88.
13. Reed LJ, Munch H. A simple method of estimating fifty percent end points. Am J Hygiene 1938; 27:493-497.
14. Misko IS, Sculley TB, Schmidt C, Moss DJ, Soszynski T, Burman K. Composite response of naive T cells to stimulation with the autologous lymphoblastoid cell line is mediated by CD4 cytotoxic T cell clones and includes an Epstein-Barr virus-specific component. Cell Immunol 1991; 132(2):295-307.
15. Khanna RS, Burrows R, Kurilla MG, Moss DJ. EBNA-1 as a target antigen for EBV-specific cytotoxic T lymphocytes. In: Cancer cells: Epstein Barr virus and associated diseases. New York: Cold Spring Harbor, 1994: 83.
16. Long HM, Haigh TA, Gudgeon NH, Leen AM, Tsang CW, Brooks J, et al. CD4+ T-cell responses to Epstein-Barr virus (EBV) latent-cycle antigens and the recognition of EBV-transformed lymphoblastoid cell lines. J Virol 2005; 79(8):4896-907.