SUPPRESSION OF IN VITRO EPSTEIN-BARR VIRUS INFECTION
A New Role for Adult Human T Lymphocytes

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Epstein-Barr Virus is associated with several neoplasms in man. It is presumed to be the causative agent of infectious mononucleosis (1), a self-limiting lymphoproliferative disease, and is consistently associated with nasopharyngeal carcinoma (NPC) (2) and African Burkitt's lymphoma (BL) (3). Viral isolates from the throat washings of patients with infectious mononucleosis can transform normal human lymphocytes in vitro giving rise to lymphoblastoid cell lines which express the Epstein-Barr (EB) nuclear antigen, carry multiple copies of the viral genome, and are of B-cell origin (4-6). Transforming Epstein-Barr virus (EBV) may also be used to induce lymphomas in marmosets from which virus-producing lymphoblastoid cell lines may be established (7). One such line, B95-8, has been used extensively as a source of transforming EBV (8).

Little is known about the mechanism by which EBV infects B lymphocytes, how the lymphoproliferation typical of infectious mononucleosis is controlled, and the nature of the breakdown in these controls during NPC and BL. A role of humoral immunity is protection against reinfection is suggested by the persistence of anti-EBV neutralizing titers after EBV infection (9). The possibility of cellular immunity during the acute phase of infectious mononucleosis (IM) has been suggested by the presence of cytotoxic cells specific for EBV-carrying cell lines (10); however, the levels of killing reported are variable. After recovery from IM the viral infection is not completely eliminated but is carried in some latent or suppressed form, as healthy seropositive individuals appear to carry infected cells at a very low number. These cells must exist because outgrowth of EBV-positive lymphoblastoid cell lines may be achieved from the lymphocytes of seropositive individuals without the addition of exogenous virus. In vitro culture of fetal cord lymphocytes or lymphocytes from seronegative donors does not result in such an outgrowth (11).

The studies presented here were prompted by the observation that adult lymphocytes transform and immortalize in vitro much less efficiently than lymphocytes from fetal cord blood (12). They indicate that there is little difference in the susceptibility of adult or fetal B lymphocytes to infection. However, the adult Ig-negative population has a strongly inhibitory effect when added to the infection mixture. This inhibition is not exhibited by the same fetal lympho-
cyte population. It is proposed that this in vitro effect is the result of the development in vivo of a mechanism during maturation for controlling the viral infection and maintaining it in a suppressed form.

**Materials and Methods**

**Virus.** Transforming virus was obtained from the supernate of the B95-8 marmoset lymphoblastoid line. The cell line (originally obtained from The John L. Smith Memorial for Cancer Research Inc., Maywood, N. J.) was carried in medium RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) containing, 10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 300 μg/ml glutamine. The cells were fed by diluting to 0.5 × 10^8 cells/ml every 3-4 days and were kept in a 5% CO2 humidified atmosphere at 37°C. To obtain maximal production of virus the cells were kept for 12 days from the last feeding at 37°C before harvesting. After this time the cells were removed by centrifuging at 2-3,000 g in the Sorvall RC-3. (Ivan Sorvall, Inc., Norwalk, Conn.) The clear supernate may be filtered through a 0.45-μm filter at this stage without loss of viral activity as judged by induction of DNA synthesis in B lymphocytes. The supernate was then stored in aliquots at -80°C. Viral activity was very sensitive to freezing and thawing so no preparation was refrozen after use.

**Cells.** Heparinized blood, usually diluted 1:1 with Hank’s balanced salt solution, was layered over Ficoll-Hypaque (13), and centrifuged at 1,000g for 30 min in the Sorvall RC-3. (Ivan Sorvall, Inc.) The lymphocytes at the serum Ficoll interface were removed and washed three times with a wash solution (medium containing only 5% fetal calf serum). Adult blood was obtained from donors working in this Institute whose age range was from 20 to 35 yr. Fetal cord blood was obtained by courtesy of Boston Lying In Hospital (Boston, Mass.).

**Cell Culture.** B and Ig-negative cells were cultured at appropriate concentrations (usually 1 × 10^8/ml unless otherwise stated) in a Linbro Mk II microtiter plate (Linbro Chemical Co., New Haven, Conn.) at a final vol of 0.2 ml of medium containing 20% fetal calf serum. Infection with virus was usually with a 1:10 or 1:20 final dilution of viral supernate. We have observed during the course of these experiments that a viral dilution of 1:10 or higher allows the suppression effect to be reproducibly observed. When necessary, cells were fed 5-6 days after infection and, thereafter, every 3-4 days by replacing 0.1 ml of culture medium with fresh medium containing 20% fetal calf serum.

**DNA Synthesis Assay.** This method was used to assay the transformation potency of B95-8 virus and is based on the original assay of Robinson and Miller (14). Triplicate cultures of cells (1 × 10^6 ml, 2 x 10^5/well) were pulsed for 4 h with [3H]thymidine (50 μl of 2 μCi/ml, sp act 2 Ci/mmol) and harvested onto glass fiber filters. Radioactivity was assayed by liquid scintillation counting by using a toluene/Liquifluor scintillation fluid (New England Nuclear, Boston, Mass.). All values for specific [3H]thymidine incorporation were calculated subtracting counts per minute (cpm) in mock infected culture from cpm in virus infected control.

**Outgrowth Assay.** Outgrowth of transformed lymphocytes was assayed visually at the time of appearance of transformed cells in the culture and assessed on a scale of one to four (Fig. 1). A score of one represents only dead cells, judged by phase contrast microscopy; two, living but not necessarily transformed cells and clumps; three, transformed cell clumps and large transformed single cells in the well (a score of two can sometimes represent a poor three which would be confirmed within 2 or 3 days); four, the well overrun by transformed and proliferating cells. Each experiment was performed in sextuplicate and the time of transformation in days was set as either the time when six wells obtain a score of three or alternatively four wells obtain a score of four. The plates were read microscopically every 2 days.

**Preparation of Rabbit Anti-Human Fab’ Immunoabsorbent Column.** The technique used was that described by Chess and Schlossman (15) except that 80 mg of anti-human Fab’ antibody was coupled lightly to 300 ml of Sephadex G-200 by allowing to stand at 4°C overnight with occasional stirring. Coupling efficiency was usually >70%, twice that obtained by the previously described procedure.

**Separation of B and Ig-Negative Lymphocyte Populations.** B and Ig-negative lymphocyte populations were prepared by passing whole lymphocyte populations over a rabbit anti-human Fab’ immunoabsorbent column as described by Chess and Schlossman (15). Recovery of cells was
Fig. 1. The appearance of PK1-transformed cells proliferating in culture 1, small cell-sized colonies 2, growing colonies 3, large untransformed proliferating colonies 4, the culture well overgrown with proliferating lymphoblasts.
greater than 90%. The B-cell population was 92% pure by the criterion of surface p23, 30 (16) antigens (a marker for B cells and a subpopulation of null cells) and 95% Ig positive. Both surface markers were assayed by the direct membrane immunofluorescence technique. The Ig-negative population contained 1% surface Ig-positive cells and approximately 2% p23, 30 positive cells due to the p23, 30 positive null cell population.

Preparation of Ig-Negative Lymphocyte Subpopulations. Adherent lymphocytes were removed by passage over nylon wool columns. T

and null lymphocytes were separated by means of the E-rosette technique. The methodology for these techniques has been described in detail elsewhere (15). Macrophages were removed from whole peripheral lymphocyte populations by treatment with iron carbonyl and magnetism (17).

Materials. Tissue culture medium and fetal calf serum were obtained from Microbiological Associates, Bethesda, Md.; [3H]thymidine from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. J.; cyanogen bromide from Eastman Kodak Co., Rochester, N. Y.; human gamma globulin from Sigma Chemical Co., St. Louis, Mo. All other chemicals were obtained from Fisher Scientific Co., Fair Lawn, N. J. and were all of high purity grade.

Results

DNA Synthesis in Separated B Cells from Various Donors. B cells purified by chromatography on a rabbit anti-human Fab' immunoabsorbent column provide sensitive targets for EBV-specific stimulation of DNA synthesis (D. A. Thorley-Lawson and J. L. Strominger, manuscript in preparation). No stimulation of DNA synthesis is observed with the Ig-negative lymphocytes from the column.

B cells from fetal cord lymphocytes and from the peripheral blood lymphocytes of EBV seropositive and seronegative adult donors were infected with EBV and the rate of DNA synthesis assayed at regular intervals up to 10 days after infection (Fig. 2). The rates of proliferation as measured by the slope and the absolute levels of [3H]thymidine incorporation at any time were similar for all three donors. This suggests that there was no significant difference in either the number of cells infected or the rate at which the infected cells proliferated.

Efficiency with which EBV Causes Outgrowth of Transformed Lymphocytes from Populations of Adult and Fetal Lymphocytes. Outgrowth experiments were performed on fetal and adult lymphocytes to determine whether the similarity in the rates and levels of DNA synthesis after infection correlated with the rate of appearance of transformed cells. It is known that lymphocytes from fetal cord blood transform more efficiently than those from the adult periphery (12) and this was confirmed by studies on several individuals (Table 1). The average time for outgrowth of adult peripheral blood lymphocytes was 25 days, compared to only 13 days for fetal cord lymphocytes. A more detailed study on two individuals (Fig. 3) indicated that by using unseparated adult lymphocytes only 8 of 33 infected wells grew out after 6 wk, while 24 of 24 wells containing fetal lymphocytes grew out after 2 wk.

Outgrowth studies were also carried out on separated Ig-negative and B cells. Adult B lymphocytes grew out as fast (13–14 days) and with the same efficiency (100% in 2 wk) as B cells from fetal cord blood (Table I, Fig. 3). In neither case did EBV have any effect on the Ig-negative cell population.

Transformation of Reconstituted Mixtures of T and B Lymphocytes from

\[3\text{T, thymus-derived lymphocyte, operationally defined as E-rosette positive, surface Ig negative.}\]
Fig. 2. Time-course of DNA synthesis in B Cells from different donors induced by infection with EBV. B lymphocytes from adult or fetal cord donors (2.10^5 cells in 0.2 ml) prepared by passage over a rabbit anti-human Fab column were infected with EBV (final dilution 1/10), or mock infected. Experiments were set up in triplicate and the cells pulsed at various times with [3H]thymidine to assess the level of DNA synthesis. The level of specific [3H]thymidine incorporation was estimated by subtracting the background counts obtained with the mock-infected cultures from the counts in the experimental cultures. Background counts were: 30-100 cpm EBV seropositive donor, 50-100 cpm EBV seronegative donor, 60-500 fetal cord lymphocytes.

**Table I**

**Time to Transformation by EBV of Unseparated and Isolated Fetal Cord and Adult B Lymphocytes**

| No. individuals tested | Cell origin     | Cell type | Time of transformation (Days) |
|------------------------|----------------|-----------|-------------------------------|
| 4                      | Fetal cord     | Whole     | 13 ± 2                        |
| 4                      | Fetal cord     | B         | 14 ± 2                        |
| 10                     | Adult PBL*     | Whole     | 25†                           |
| 6                      | Adult PBL      | B         | 13 ± 2                        |

* PBL, peripheral blood lymphocyte.
† Does not include four samples which had not grown out at time experiment stopped, usually 35 days.

**Adult Peripheral Blood and Fetal Cord Blood.** The experiments reported above strongly suggested that the susceptibility of human B lymphocytes to transformation does not depend on their fetal or adult origin. The question arises, therefore, as to the discrepancy in the efficiency with which unfractionated lymphocytes from the two sources transform (Table I, Fig. 3). One possible explanation for this result could be that there is a higher proportion of B...
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Fig. 3. Efficiency of outgrowth of different lymphocyte populations from adult peripheral blood and fetal cord blood. ○, whole lymphocytes; ●, B lymphocytes; X, uninfected B or infected Ig-negative lymphocytes.

TABLE II
Time to Transformation by EBV of Reconstituted Mixtures of Either Fetal Cord or Adult Lymphocytes

| Cell origin  | Cell mixture (B:T) | Days to transformation |
|--------------|--------------------|-----------------------|
| Fetal cord   | 100:0              | 13                    |
|              | 50:50              | 13                    |
|              | 20:80              | 13                    |
|              | Unfractionated     | 13                    |
| Adult PBL    | 100:0              | 13                    |
|              | 50:50              | 18                    |
|              | 20:80              | >21                   |
|              | Unfractionated     | >21                   |

Total cells = 2.10^5.

lymphocytes in fetal cord than in adult peripheral blood (18, 19). To test if this difference was the reason for the discrepancy, cells of both origins were separated, and then reconstituted into mixtures of 100, 50, and 20% B cells.

Fetal B lymphocytes transformed as efficiently whether containing 100 or 20% B cells: 80% autologous Ig-negative cells, or whether unseparated lymphocytes were used (Table II). By comparison adult B lymphocytes were strongly inhibited by the presence of 80% autologous Ig-negative lymphocytes (the ratio which is found in vivo) and were slightly inhibited by 50% Ig-negative lymphocytes (the ratio found in vivo with fetal cord blood).

Two explanations for this result may be suggested. Firstly, the number of B lymphocytes in a 20:80 B:Ig-negative cell mixture was less than in the 50:50 mixture, as the total cell number is constant. If adult B lymphocytes were more sensitive to dilution than cord B lymphocytes, a difference would have been
Fig. 4. Delay in outgrowth of adult B lymphocytes by addition of various proportions of Ig-negative lymphocytes after EBV infection. Mixtures were reconstituted keeping either the total cell number per well constant (2.10⁵ cells in 0.2 ml) •—• or keeping the number of B cells per well constant (1.10⁵ cells) and varying only the number of Ig-negative lymphocytes ○—○. Delay in outgrowth indicates number of days for infected B cells to grow out in the presence of Ig-negative lymphocytes minus the number of days for the same number of B cells to grow out alone. Viral dilution was 1:5.

observed in efficiency at the lower dilution. Secondly, the result could have been due to a suppression of the B-lymphocyte proliferation by the adult Ig-negative lymphocytes.

Reconstruction of Adult Peripheral Lymphocytes to Various Proportions Keeping Either Total Cell Number or B-Cell Number Constant. An experiment was performed with various mixtures of adult B and Ig-negative lymphocytes keeping either the total cell number constant or keeping the number of B lymphocytes constant and varying the number of Ig-negative lymphocytes (Fig. 4). Suppression of proliferation was measured as the days delay to outgrowth of infected cultures compared to controls with the same number of B cells but no added Ig-negative cells. Reconstitution of the lymphocytes to the in vivo ratio of 20:80, B:Ig-negative cells resulted in a marked suppression of the B-cell proliferation. This effect could not be explained by dilution sensitivity because: (a) the effect was compared to a control with the same number of B cells; (b) the effect was observed at both high and low total B-cell number. The observation that suppression occurred at the same ratio (>60% Ig-negative cells) in both the
Fig. 5. Delay in outgrowth of adult B lymphocytes by addition of various subpopulations of Ig-negative lymphocytes after EBV infection. For experimental details see legend to Fig. 6. Viral dilution used was 1:20. ●, ratios of B and Ig-negative lymphocytes varied keeping total cell number per well constant. ○, ratios of B and Ig-negative lymphocytes varied keeping number of B cells per well constant. X, these points represent cultures which still had not grown out when the experiment was terminated.

Identification of the Ig-Negative Population Responsible for In Vitro Suppression of EBV-Injected Adult B Lymphocytes. Ig-negative lymphocytes are predominantly T cells, however, they also contain small numbers of null cells and macrophages. A series of experiments were, therefore, carried out to investigate the role of these various populations in the in vitro suppression (Fig. 5). Removal of adherent cells by passage over a nylon wool column had no effect on the ability of Ig-negative lymphocytes to delay the outgrowth of infected B cells (50% Ig-negative lymphocytes delaying outgrowth by about 2 wk). T cells and null cells were prepared by taking E-rosette-positive and negative populations, respectively. The T cells were slightly more effective than the whole Ig-negative cells giving a 2-wk delay in outgrowth at a proportion of about 35–40%. By comparison only the highest proportion (80%) of null cells gave a significant suppression of the outgrowth and this may be readily explained by contamina-
tion of the null cells by a residue of T cells. These experiments showed that the T-cell population of Ig-negative lymphocytes is the one responsible for the suppression effect. The observation that 50% purified T cells provided very effective suppression whereas 50% null cells provided none is further evidence that the suppression is not the result of a cell culture artifact such as overcrowding.

As the B-cell population used in these studies contains a significant number of macrophages it was possible that they might play an indirect role in the suppression by interacting with T cells. Prior removal of macrophages by treatment with iron carbonyl and magnetism, however, had no effect on the ability of the T cells to suppress outgrowth (Fig. 6).

In agreement with other authors (20, 21) we have observed that depletion of macrophages somewhat retards the time at which proliferation and outgrowth of infected B cells occurs. However, the effect was not large (12-13 days compared to 8-10 days in the presence of macrophages) and there was not an absolute requirement for macrophages to obtain outgrowth.

Discussion

The results presented here indicate that T lymphocytes from adults but not fetal cord can suppress the outgrowth of in vitro EBV-infected B lymphocytes. Earlier work (22-24) and results presented here indicate that EBV infects only B cells. In the present study no significant difference was observed in the efficiency with which purified fetal or adult B lymphocytes were transformed by EBV as measured by either induction of DNA synthesis or time to outgrowth of proliferating cells. Addition of increasing numbers of autologous adult Ig-negative lymphocytes, however, resulted in the time to outgrowth of adult B cells becoming increasingly longer. This did not occur when fetal Ig-negative lymphocytes were added back to fetal B cells. Trivial explanations that this effect was due to culture artifacts such as cell crowding have been ruled out. The lymphocytes which were effective in this in vitro suppression of infection had the characteristics of being nonadherent to nylon wool and E-rosette positive indicating that they were T cells. It appears, therefore, that there is a function associated with human T lymphocytes which develops during maturation and allows them to limit EBV infection of B cells. This observation provides an explanation for the generally accepted view (12) that fetal cord lymphocytes transform more efficiently in vitro than those from adult peripheral blood.

The mechanism of in vitro suppression of proliferation may be explained by several known immune functions of lymphocytes. The most attractive comes from studies in mice where it has been shown that subpopulations of T lymphocytes are able to suppress the immune response of both B and T cells (25). Thus, murine B-cell proliferation and antibody production may be abrogated by the presence of a subpopulation of suppressor lymphocytes bearing the Ly 23 surface antigens (26).

It appears, therefore, that a mechanism for specifically suppressing B-cell proliferation already exists. Such a mechanism could play a role in recovery from EBV infection as it is known that individuals who have recovered from infectious mononucleosis carry EBV-infected cells which are suppressed in vivo
in the absence of cytotoxic lymphocytes. These cells will proliferate in culture to give rise to EBV-positive lymphoblastoid cell lines without the addition of exogenous virus (11). There must, therefore, be some mechanism by which these cells are kept under control in the healthy individual. There are two levels at which the control of the viral information could be exerted. First, it has been proposed that the virus is latent within the cell and only becomes activated on transfer to tissue culture (27). This would be a control working within the cell
itself. It has been demonstrated, however, that the large lymphoblasts from IM patients are Epstein-Barr virus nuclear antigen positive and this has been cited as evidence against the presence of a latent virus (28). The second method of control would be at the cellular level by means of suppressor cells and or release of soluble factors by T cells which prevent the B cells from proliferating further. Thus, EBV-carrying lymphocytes which persist at a low level after recovery from IM could be considered as EBV memory B cells.

An alternative explanation for the mechanism of suppression would be the induction of cytotoxic T cells sensitized by new antigens (29) on the surface of the transformed B cells. These cytotoxic lymphocytes would stop proliferation after infection by actually destroying the transformed cells. This mechanism is less likely because human fetal cord lymphocytes are themselves competent in T-cell-mediated cytotoxicity against blast transformed lymphocytes (D. A. Thorley-Lawson, unpublished observations) and yet are unable to suppress in vitro infection. Furthermore, it could not explain the in vivo situation as healthy adults do not carry sensitized T-killer cells after recovery from IM although the infected B cells persist in a suppressed state.

The ineffectiveness of purified null cells in the suppression of infection excludes any mechanism involving null cells such as the natural killer cell described in murine systems and recently a human system (30, 31) or the complement receptor-positive nonspecific killer cell described for human systems (10). A direct role of macrophages, for example, in macrophage-mediated cytotoxicity (32) has similarly been excluded although for technical reasons an indirect role of a small number of macrophages cannot be rigorously excluded.

The failure of fetal lymphocytes to suppress the in vitro infection suggests a wider potential significance for this phenomenon as both IM and African BL are diseases of childhood and adolescence (33, 34). Furthermore, failure of T-cell functions have been postulated as the cause of fatal X-linked EBV induced lymphoproliferation (Duncan's disease) (35). It is conceivable, therefore, that susceptibility to EBV-associated disease is also associated with a failure of the mechanisms involved in the in vitro suppression of EBV infection. It will be of critical importance in understanding this relationship to test the ability of genetically susceptible and immunosuppressed individuals to suppress in vitro EBV infection.

The results also lead to two important technical advances which are of general importance and, therefore, worthy of comment. First, the inefficient and unreliable transformation of whole adult lymphocytes makes the establishment of permanent cell lines difficult. However, when using separated B lymphocytes, it has, in our hands, been possible to obtain growing cell lines in less than 2 wk with 100% efficiency from all adult lymphocytes. Furthermore, the growing cells have not been subject to selection in the presence of, for example, potentially cytotoxic T cells. Second, purified adult B cells provide a more effective, reliable, and reproducible source of targets for transformation assays than the more commonly used fetal cord lymphocytes.

**Summary**

Studies have been performed on in vitro infection by Epstein-Barr virus
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(EBV) of subpopulations of human lymphocytes. B cells of adult peripheral or fetal cord blood transform with equal efficiency, whether assayed by DNA synthesis induction or by outgrowth of transformed lymphocytes. In contrast, unfractionated adult lymphocytes transform much less efficiently than those from fetal cord. Reconstitution experiments of different cell preparations indicated that this difference was due to a suppression of B-cell proliferation by adult Ig-negative lymphocytes which fetal Ig-negative lymphocytes were unable to perform. Separation of Ig-negative lymphocytes into various subpopulations revealed that the suppression was performed by T cells. Macrophages and null cells play little or no role in suppression. The relevance of this phenomenon to infection and recovery from EBV infection during and after infectious mononucleosis is discussed.

Received for publication 31 March 1977.

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