

# Infection of Human Thymocytes by Epstein-Barr Virus

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## Summary

The Epstein-Barr Virus (EBV) causes infectious mononucleosis, and has been strongly associated with certain human cancers. The virus is thought to exclusively bind to B lymphocytes and epithelial cells via receptors (CR2/CD21) that also interact with fragments of the third component of complement (C3). Recent evidence, however, has challenged this belief. We have used two-color immunofluorescence analysis using biotin-conjugated EBV and streptavidin-phycoerythrin along with fluorescein-conjugated anti-T cell antibodies and demonstrated that CD1-positive, CD3-dull (immature) human thymocytes express functional EBV receptors. In four replicate experiments, the binding of EBV to thymocytes ranged between 8 and 18%. This interaction is specific as evidenced by inhibition with nonconjugated virus, anti-CR2 antibodies, aggregated C3, and an antibody to the gp350 viral glycoprotein that the virus uses to bind to CR2. EBV can infect the thymocytes as evaluated by the presence of episomal EBV-DNA in thymocytes that had been incubated with the virus as short as 12 days or as long as 6 weeks. Episomal DNA analysis was performed by Southern blotting with a EBV-DNA probe that hybridizes to the first internal reiteration of the viral DNA. The presence of the EBV genome is also supported by the detection of EBV nuclear antigen 1 in infected thymocytes, assessed by Western blotting with EBV-immune sera. The EBV infection is specific as determined by blocking experiments using anti-CR2 and anti-gp350 antibodies. Finally, virus infection of thymocytes can act synergistically along with interleukin 2 and induce a lymphokine-dependent cellular proliferation. In view of previously reported cases of EBV-positive human T cell lymphomas, the possibility is raised that EBV may be involved in cancers of T lymphocytes that have not been previously appreciated.

The EBV is the causative agent of infectious mononucleosis, and is associated with certain human neoplasias (1). EBV is thought to display exclusive tropism for B lymphocytes (2-7), follicular dendritic cells of tonsils and lymph nodes (8), and pharyngeal (9) and cervical (10) epithelia. The virus targets these cells via specific surface receptors which are also reactive with the C3d fragment of the third component of complement (EBV/C3d receptors/CR2/CD21) (11-13).

Reports from several laboratories, including ours, have provided evidence that challenges the exclusive B lymphocyte/epithelial cell tropism of EBV. In an early report, Menezes et al. (14) demonstrated the binding of EBV to the lymphoblastoid T cell line Molt 4. Fingeroth et al. (15) have purified

and have performed limited NH<sub>2</sub>-terminal sequencing of a CR2 protein found on the human leukemic T cell line HPB. In our own laboratory, we have demonstrated the reactivity of normal human thymocytes with anti-CR2 mAbs and have shown that these antibodies can immunoprecipitate a protein from thymocyte lysates that has the expected size (145 kD) for CR2 (16). Of particular interest are recent reports describing the cases of patients with EBV genome-positive T cell lymphomas (17-20), thus, suggesting that EBV could be involved in cancers of T cells not previously appreciated.

In this report we describe the specific binding of EBV to a subpopulation of phenotypically immature human thymocytes and provide evidence indicating that the virus can in-

fect the target cells and induce the expression of EBV nuclear antigen (EBNA)<sup>1</sup>. In addition, EBV can act synergistically along with IL-2 to induce thymocyte proliferation in a lymphokine dose-dependent manner.

## Materials and Methods

**Cells, Antibodies, and Other Reagents.** Human thymocytes were obtained from patients (up to 4 y of age) undergoing corrective cardiac surgery. Raji cells (CCL 86) were obtained from the American Type Culture Collection (Rockville, MD). The B95-8 marmoset leukocyte line (CRL 1612) was also obtained from the same source. Both cell lines were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>-air in RPMI 1640 (Mediatech, Washington, DC) that was supplemented with 10% FCS (Hyclone Laboratories, Logan, UT) and antibiotics (Gibco Laboratories, Grand Island, NY). The cell concentration was adjusted to 2 × 10<sup>5</sup> cells/ml every 3–4 d. The cultures were free of mycoplasma.

The hybridomas producing anti-CR2 antibody HB5 (HB 135) and anti-gp350 antibody 72A1 (HB 168) were obtained from the American Type Culture Collection. They were carried in culture as described above and used as culture supernatants. Purified antibodies OKB7 and OKT6 were purchased from Ortho Pharmaceuticals (Raritan, NJ). Purified antibodies Leu-12 and Leu-4 directly conjugated to fluorescein were purchased from Becton Dickinson & Co. (Mountain View, CA). Goat anti-mouse and goat anti-human IgG were obtained from Jackson ImmunoResearch Laboratories, Inc. (Avondale, PA); streptavidin-conjugated phycoerythrin (strep-PE) was purchased from Biomedica Corp. (Foster City, CA). <sup>125</sup>I-protein A (sp act 43 μCi/μg) was from ICN Biochemicals, Inc. (Irvine, CA). Moloney-murine leukemia virus reverse transcriptase was from Gibco Bethesda Research Laboratories (Gaithersburg, MD). Oligonucleotides were synthesized in our own facility using a synthesizer from Applied Biosystems (Foster City, CA).

**EBV Purification and Biotin Conjugation.** The EBV used in these experiments was purified from culture supernatants of the EBV-producing B95-8 cells. 2 liters of B95-8 cells, seeded at 2 × 10<sup>5</sup> cells/ml, were grown for 14 d in RPMI 1640 containing 10% FCS and 5 ng/ml PMA (Sigma Chemical Co., St. Louis, MO). Cells and cell debris were then removed by centrifugation (1,500 g, 30 min, 4°C), the supernatant was filtered through a 0.8-μm filter and centrifuged (15,000 g, 120 min, 4°C) to pellet the virus. The pellet was resuspended in TNB buffer (0.01 M Tris, 0.15 M NaCl, 0.1 mg/ml Bacitracin, pH 7.3) and layered onto a discontinuous dextran T-10 gradient containing equal volumes of 5, 10, 15, and 30% (wt/vol) dextran in TNB buffer. The gradient was then centrifuged (76,000 g, 60 min, 4°C) in an SW 41 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). The virus band was removed from the 15/30% interface and dialyzed against TNB buffer. Final volume of virus was 2 ml, resulting in a 1,000-fold concentration of the original culture volume. Conjugation of biotin to EBV was performed by addition of biotin-N-hydroxy-succinimide ester (Calbiochem-Behring, Corp., San Diego, CA) to 500 ml of purified EBV in 0.1 M NaPO<sub>4</sub>, 150 mM NaCl buffer (PBS), pH 7.54, to a final concentration of 2 mg/ml. This solution was allowed to incubate at room temperature for 2 h and was then layered onto a discontinuous dextran T-10 gradient as described above. Biotin-conjugated virus (bio-EBV) was isolated from the 15/30% inter-

face, dialyzed against TNB buffer, and titrated on Raji cells by immunofluorescence.

**EBV DNA Probe.** The EBV DNA probe pDK14 was the generous gift of Dr. E. Kieff (Harvard University, Boston, MA); it hybridizes to the long internal reiteration, IR1, of EBV DNA (21). This plasmid that contains BamHI fragments of EBV (B95-8) DNA cloned into the BamHI site of pBR322 was propagated in the JM109 strain of *Escherichia coli*. Plasmid isolation, purification, and nick translation were carried out by standard techniques (22).

**Two-color Immunofluorescence Analysis.** The analysis was performed as previously described (16). Determination of EBV binding was performed by incubating 10<sup>6</sup> cells with bio-EBV, in a final volume of 50 μl, in V-bottomed microtiter plate wells (Costar, Cambridge, MA) for 30 min. Cells were then washed with PBS, pH 7.3, and incubated with strep-PE in a 1:10 final dilution for an additional 30 min. Cells were washed twice in L15 medium (2% FCS), fixed in 1% paraformaldehyde, and analyzed in a flow cytometer (Ortho Cytofluorograph; Ortho Pharmaceuticals). Antibody staining was performed in a similar fashion with saturating amounts of fluorescein-conjugated antibodies either alone or in costaining experiments with EBV. In selected experiments, blocking of EBV binding was tested by preincubating thymocytes with nonconjugated anti-CR2 antibodies or with aggregated C3. The latter was purified and aggregated as previously described (23, 24).

**Polymerase Chain Reaction.** The PCR was performed by the method of Saiki et al. (25) as modified by Witsell and Schook (26) using a commercially available kit (Cetus Corp., Emeryville, CA). All reactions were performed in 1.5-ml microcentrifuge tubes. The RNA from thymocytes or a B cell line (JY) was isolated by the guanidinium thiocyanate method exactly as described by Witsell and Schook (26) and reversely transcribed by addition of random hexamers, deoxynucleotides, and Moloney-murine leukemia virus reverse transcriptase (26). The reaction proceeded for 1 h at 42°C and was terminated by heating at 90°C for 10 min. A pair of specific primers that amplify a 279-bp segment of the CD19 gene were constructed and used for amplification. The sequence of these primers is 5'-TCACCGTGGCAACCTGACCAT-3' (extends downstream) and 5'-GAGAGACAGCACGTT-3' (extends upstream). As a control, we also used a pair of primers that amplify 800 bp of an unrelated gene (GTP-binding protein stimulatory α subunit). The PCR was performed in a thermocycler (Ericomp Inc., San Diego, CA) exactly as described by Witsell and Schook (26) without, however, the addition of an internal primer. The detection of the PCR products was performed by agarose gel (2%) electrophoresis and by staining with ethidium bromide.

**EBV Infection and Episomal DNA Analysis.** Total thymocytes or thymocytes purified by SRBC rosetting, as previously described (27), were incubated at a concentration of 2 × 10<sup>6</sup> cells per ml in culture flasks (#3151; Costar) with B95-8 culture supernatants (1:10 dilution) for various days. Cells were harvested, episomal DNA was extracted, as previously described (28), and subjected to BamHI (Promega Biotec, Madison, WI) digestion. 10 μg of the digested DNA, corresponding to 2–3 × 10<sup>6</sup> Raji cells or 6–7 × 10<sup>6</sup> thymocytes, was analyzed by Southern blotting (29) with the probe pDK14 that specifically recognizes a 3-kb fragment corresponding to the first internal reiteration of the EBV genome (21). Episomal DNA isolated from the EBV-transformed Raji B cell line was treated in an identical fashion and used as a positive control. Episomal DNA from thymocytes that had not been exposed to EBV was also analyzed as a negative control.

**Detection of EBNA-1.** Detection was performed by Western blotting analysis as previously described (30). Briefly, EBV-infected thymocyte lysates or noninfected controls were incubated in RPMI

<sup>1</sup> Abbreviations used in this paper: bio-EBV, biotin-conjugated Epstein Barr Virus; EBNA, EBV nuclear antigen 1; SCR, short consensus repeat; strep-PE, streptavidin-conjugated phycoerythrin.

1640 with 10% FCS for 7 d. After harvesting and washing, cells were resuspended at  $10^8$  per ml of sample buffer (40 mM Tris-HCl, pH 7.4, 15% glycerol, 2% SDS, 5% 2-ME 1  $\mu$ g/ml pepstatin A, 2 mM PMSF, and 0.02% bromophenol blue), sonicated (60 s), and boiled. Debris was removed by centrifugation and an amount of lysate corresponding to  $5 \times 10^7$  cell equivalents was subjected to electrophoresis on preparative 10% polyacrylamide SDS slab gels and then electrophoretically transferred to NYTRAN filters (Schleicher and Schuell, Inc., Keene, NH). The filters were saturated with 1% powdered milk-0.1% gelatin solution in BBS (0.05 M sodium borate-0.14 M NaCl, pH 8.3) and cut into 3-mm strips. The strips were probed with a previously characterized EBNA-1-specific serum that was collected from a donor with a past EBV infection (30). The strips were also probed with two different EBV-negative sera, which were provided to us by Dr. R. Smith (Ortho Pharmaceutical, San Diego, CA). These latter sera are heterophile negative (Monospot; Ortho Pharmaceutical), negative in the Monolert assay (Ortho Pharmaceutical) for both IgG and IgM antibodies, and negative in Western blots using Wi-L2 and phorbol ester induced B95-8 cell extracts (31). They are also negative in immunofluorescence assays for viral capsid antigen IgG and IgM antibodies and early antigen and in anticomplement immunofluorescence assays for EBNA-1 (Granbio, Inc., Temecula, CA). The sera were diluted in 1% powdered milk, 0.1% gelatin-BBS, and incubation was carried out for 1 h at room temperature (RT). After washing in BBS, the protein bands were visualized by reacting with goat anti-human IgG for 1 h; RT, followed by incubation with  $^{125}$ I-protein A (1 h; RT) and autoradiography. Additional controls included neutralization of the anti-EBNA antiserum with a synthetic peptide (p62) that represents the glycine-alanine repeating major epitope of EBNA-1 (30). This was performed by incubating the sera overnight at 4°C with 10  $\mu$ g/ml of peptide in 1% powdered milk, 0.1% gelatin-BBS.

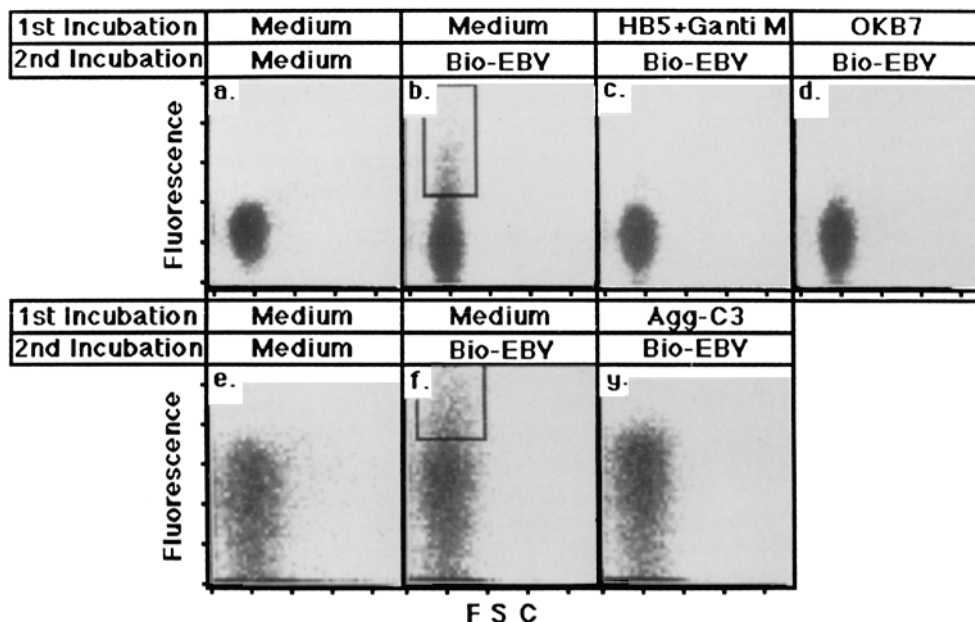
**Measurement of Proliferative Response.** Thymocytes, purified by SRBC rosetting, as previously described (27), were incubated (37°C, 5% CO<sub>2</sub>-air) at  $10^6$  cells per microtiter tray well (#3596; Costar) for 2 d in the presence of EBV or without any additions. Subse-

quently, various concentrations of rIL-2 were added, or no additions were made as a control, and incubation was continued for 5 d. Cellular proliferation was assessed as previously reported (32) by the incorporation of [ $^3$ H]-thymidine. The delay of rIL-2 addition was necessitated due to the fact that human thymocytes proliferate in response to IL-2, and this reaction is dampened if lymphokine addition is delayed by 2 d. In other experiments both virus and IL-2 additions were delayed for 2 d. The EBV used in the proliferation experiments was isolated from B95-8 tissue culture supernatants by centrifugation (15,000 g, 120 min, 4°C) and washing in order to remove PMA. The final virus concentration used in the cultures represented a 1/4 dilution of the original culture supernatant. Human rIL-2 produced in *E. coli* (33) was generously provided by the Cetus Corp. IL-2 Lot LP315 was shown by the supplier to be 98% pure by SDS-PAGE and contained <0.01 ng endotoxin per mg IL-2. 1 U of IL-2 activity is the amount producing half-maximal growth of an IL-2-dependent cell line.

## Results

**EBV Binds Specifically to Thymocytes.** Our previous observations on human thymocyte reactivity with anti-CR2 mAbs, along with the findings of others discussed above, prompted us to examine the binding and biological effects that EBV may have on thymocytes. To this end, thymocytes were incubated with purified bio-EBV and viral binding was visualized in a flow cytometer after staining with strep-PE. It can be seen from the data displayed in Fig. 1, *b* and *f* that a significant portion of thymocytes bound EBV (positive cells are enclosed in the rectangle).

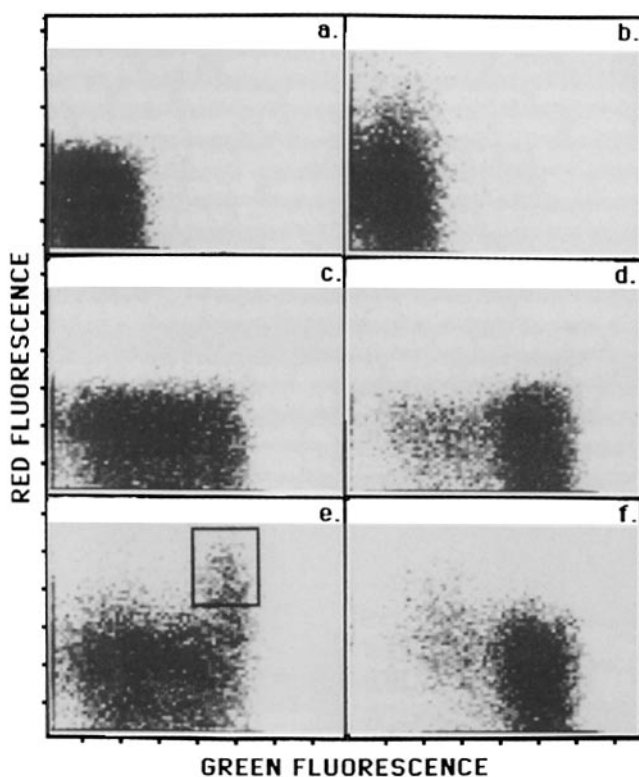
The specificity of EBV binding was tested by blocking with anti-CR2 mAbs (HB-5 and OKB7) and by the third complement component C3, since these modalities have been shown to block the binding of EBV to B cells (11-13, 34). Pretreatment of thymocytes with HB-5 plus goat anti-mouse



**Figure 1.** EBV binding to human thymocytes and blocking by anti-CR2 antibodies and by aggregated C3. Thymocytes were incubated with predetermined optimal amounts of bio-EBV without pretreatment (*b* and *f*), or after preincubation with 100  $\mu$ g/ml antibody HB-5 and goat anti-mouse immunoglobulin (G $\alpha$ M) (*c*), or 10  $\mu$ g/ml OKB7 (*d*), or 50  $\mu$ g/ml aggregated C3 (agg-C3) (*g*). Binding was visualized in a flow cytometer by the addition of strep-PE. The results are displayed as fluorescence (vertical axis) versus forward scatter (horizontal axis) in logarithmic scales. Nonspecific fluorescence was determined by treating cells only with strep-PE (*a* and *e*). The rectangles demarcate the positive cell area.

Ig or OKB7 alone practically eliminated EBV binding (Fig. 1, *c* and *d*). Pretreatment with aggregated C3 also caused a significant reduction in EBV binding (Fig. 1 *g*). Aggregated C3 was used in this experiment because of the low affinity of CR2 for monomeric ligand (35).

There are two experiments displayed in Fig. 1 (one in the upper panels, and one in the lower panels) each performed in duplicate with cells from different donors. The percentage EBV-reactive cells, in the four experiments, ranged between 8 and 18%. The percentage of EBV-reactive cells for the specific experiments displayed in Fig. 1 is 9% (population enclosed in rectangle, *b*) and 8% (population enclosed in rectangle, *f*), respectively. After HB-5 + G $\alpha$ M Ig treatment EBV binding was reduced to 0.9% (Fig. 1 *c*), after OKB7 treatment to 0.4% (Fig. 1 *d*), and after aggregated-C3 treatment to 4%



**Figure 2.** Characterization of the thymocyte subpopulation that binds EBV. Thymocytes were analyzed by two-color staining as follows: (a) antibody Leu-12 (green; anti-B cell specific), (b) bio-EBV (red) plus antibody Leu-12 (green), (c) antibody OKT6 (green; anti-immature thymocyte specific), (d) antibody Leu-4 (green; anti-T cell/mature thymocyte specific), (e) bio-EBV (red) plus antibody OKT6 (green), (f) bio-EBV (red) plus antibody leu4 (green). Binding of bio-EBV was visualized by the addition of strep-PE (red). The antibodies were directly fluorescein conjugated (green). Groups that did not receive bio-EBV were treated with strep-PE as control while the negative control for the antibodies was the isotype-matched (IgG1) antibody Leu-12 (B cell marker). All reagents were used at predetermined saturating concentrations. The results are representative of two replicate experiments with cells from different donors. Cells were analyzed in a flow cytometer (Ortho Diagnostic Systems) and results are displayed as dot plots of red fluorescence (PE) versus green fluorescence (fluorescein) in logarithmic scales. The rectangle demarcates the double-positive cell area.

(Fig. 1 *g*). Inhibition of viral binding with anti-CR2 antibodies in the other replicate experiments ranged between 90 and 96% and with aggregated C3 between 45 and 50%.

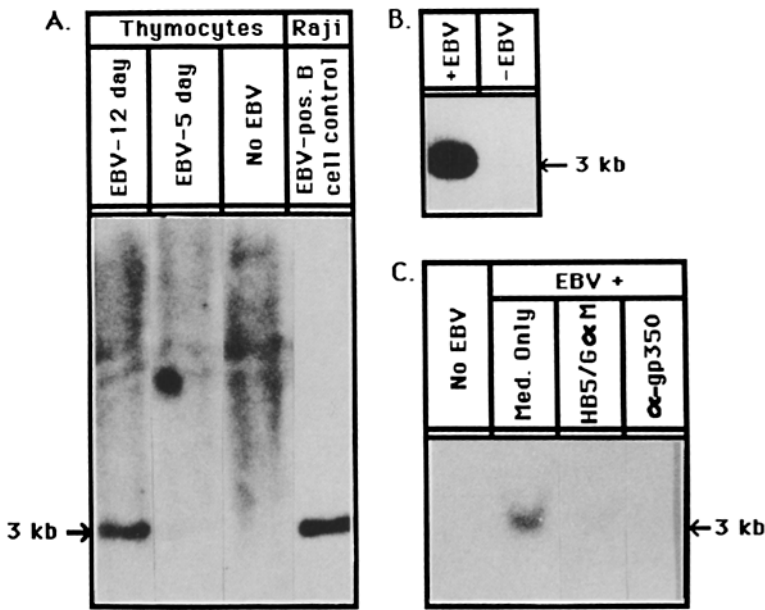
We further confirmed the specificity of EBV binding by preincubating thymocytes with nonconjugated virus or by pretreatment of bio-EBV with mAb 72A1 which reacts with gp350 (36), the EBV envelope glycoprotein that the virus uses to bind to its receptor (36, 37). In both cases binding of biotinylated virus was significantly inhibited (data not shown).

*The EBV Reactive Population Has an Immature Phenotype.* To identify the particular subpopulation of thymocytes that binds the virus, we performed two-color immunofluorescence analysis using bio-EBV/strep-PE (red fluorescence) and directly fluorescein-conjugated antibody OKT6 (anti-CD1; immature thymocytes; reference 38) or antibody Leu-4 (anti-CD3; mature thymocytes; reference 39) (green fluorescence). The results of such an experiment, shown in Fig. 2, indicate that practically all of the EBV-reactive thymocytes (11% in the displayed experiment) also reacted with the anti-CD1 marker (Fig. 2 *e*, cells enclosed in rectangle). Staining with both EBV and antibody Leu-4 revealed two distinct populations, one containing EBV-reactive cells that were dull for CD3 expression, while the other population was bright for CD3 and lacked EBV binding (Fig. 2 *f*). This indicates that the EBV-reactive thymocyte subpopulation has an immature phenotype.

*EBV Infects Thymocytes and Induces the Expression of EBNA-1.* We next examined the ability of EBV to infect human thymocytes. To this end, thymocytes were incubated with virus-containing culture supernatants (B95-8 marmoset cell line) for 5 and 12 d and isolated episomal DNA was tested for hybridization with an EBV DNA probe (pDK14) which hybridizes to the long internal reiteration, IR1, of EBV DNA (21). Autoradiography of the so-obtained Southern blots revealed a band of  $\sim$ 3 kb in size in the 12-d cultures, but not in the 5-d cultures (Fig. 3 *A*). The size of the signal is consistent with the reported size of the EBV DNA fragment to which the pDK14 probe hybridizes (21). The 3-kb signal was not seen in control thymocyte cultures that were not infected with virus (Fig. 3 *A*). Similar analysis of an EBV-positive B lymphoblastoid cell line, Raji, revealed a signal of the same size (Fig. 3 *A*).

The infection of thymocytes was inhibited by pretreatment of the cells with anti-CR2 receptor antibody HB-5 plus goat anti-mouse Ig or by preneutralization of the virus with the anti-gp350 antibody 72A1, thus, demonstrating the specificity of the infection (Fig. 3 *C*). In the results displayed in Fig. 3, the variability in the strength of the signals probably reflects differences in the specific activity of the radiolabeled DNA probe.

We further tested for the production of EBV-related proteins in virally infected thymocytes. Cells were incubated with EBV or medium for 7 d, cell lysates were prepared, subjected to electrophoresis, and blotted. The blots were then probed with a previously characterized EBV-positive antiserum containing reactivity against the glycine-alanine repeating major epitope of EBNA-1 or with EBV-negative sera (30). The data in Fig. 4 *A* (lane 2) indicate that a band of  $\sim$ 70 kD was

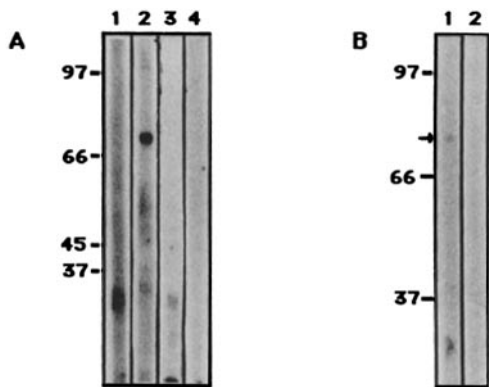


**Figure 3.** Infection of human thymocytes by EBV. Southern blotting analysis with the EBV DNA probe pDK14 of samples of episomal DNA (10  $\mu$ g per lane) isolated from (A) EBV-infected (5 and 12 d) or noninfected thymocytes and from Raji (EBV-genome positive) B cell lymphoblasts, (B) EBV-infected thymocytes that had been purified by SRBC rosetting before culturing with the virus, (C) thymocytes that had been pre-treated with antibody HB5 plus goat anti-mouse Ig (HB5/G $\alpha$ M) or not treated (medium only) and then infected with EBV which had been preincubated with anti-gp350 antibody ( $\alpha$ -gp350) or not treated (medium only). The position of the 3-kb marker was determined by analyzing BstEII-digested  $\lambda$  DNA. The data presented in each panel represent different experiments using specimens from separate donors.

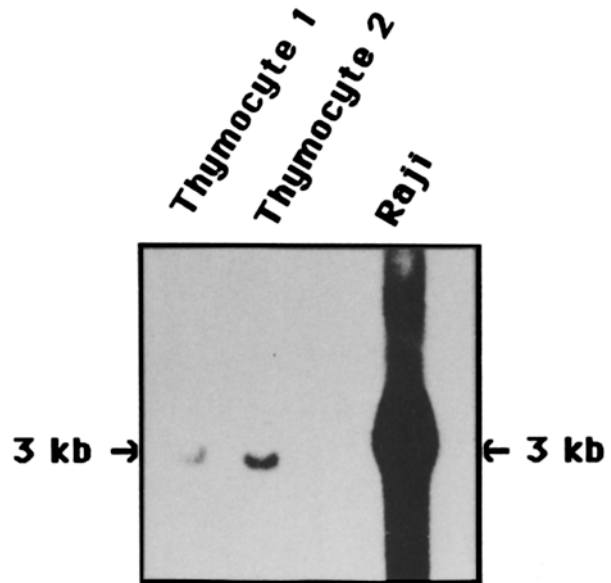
revealed by the EBV-positive serum in the virus-infected thymocytes, but not in the noninfected control (Fig. 4 A, lane 1). Probing with two different EBV-negative sera did not reveal a band (Fig. 4 A, lanes 3 and 4). The size of the detected band and the fact that the signal was revealed with EBV-positive, but not with EBV-negative, sera suggests that this protein may represent EBNA-1 (30).

Additional evidence that the detected band represents

EBNA-1 was obtained by blocking serum reactivity with a synthetic peptide, p62, representing the glycine-alanine repeating portion of the protein. This peptide represents the major epitope in humans and it inhibits the binding of >90% of the anti-EBNA-1 antibody in the serum used in these ex-



**Figure 4.** Detection of EBNA-1 expression. Purified (SRBC-rosetted) thymocytes,  $10^6$ /ml, were incubated (37°C, 5% CO<sub>2</sub>-air) for 7 d in flasks containing B95-8 culture supernatants (final dilution 1:10) or medium as control. Cells were washed and lysates prepared as described in Materials and Methods. (A) Lysates of noninfected (lane 1) or EBV-infected (lanes 2-4) thymocytes were probed with EBV-immune (lanes 1 and 2; 1:500 dilution) or two different nonimmune (lanes 3 and 4; 1:500 dilution) sera. (B) Lysates of EBV-infected thymocytes were probed with EBV-immune serum (1:6,000 dilution) that had been preincubated with 10  $\mu$ g/ml of p62 peptide (lane 2) or without it (lane 1). Molecular mass markers used were phosphorylase b (97.4 kD), bovine albumin (66 kD), egg albumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (37 kD); all were obtained from Sigma Chemical Co. Results represent those of two replicate experiments with thymocytes of different donors. Peptide neutralization represents a single experiment.

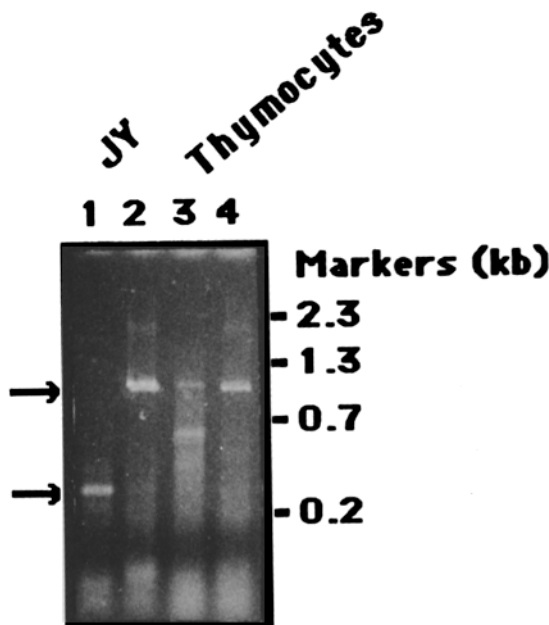


**Figure 5.** Presence of EBV genome in long-term thymocyte cultures. Purified (SRBC rosetted) thymocytes,  $2 \times 10^6$ /ml, were incubated (37°C, 5% CO<sub>2</sub> air) for 6 wk in flasks containing B95-8 culture supernatants (final dilution 1:10) and rIL2 (32 U/ml). Cells were maintained at  $1-2 \times 10^6$  cells/ml by weekly feedings with fresh medium containing 10 U of IL2 per ml. Cells were harvested, washed, and episomal DNA was isolated and analyzed by Southern blotting as described in Materials and Methods. Results display two cultures using thymocytes from different donors (thymocyte 1 and thymocyte 2). For comparison, episomal DNA isolated from the EBV-positive cell line Raji has been included. The position of the 3-kb marker was determined by analyzing Bst EII digested  $\lambda$  DNA.

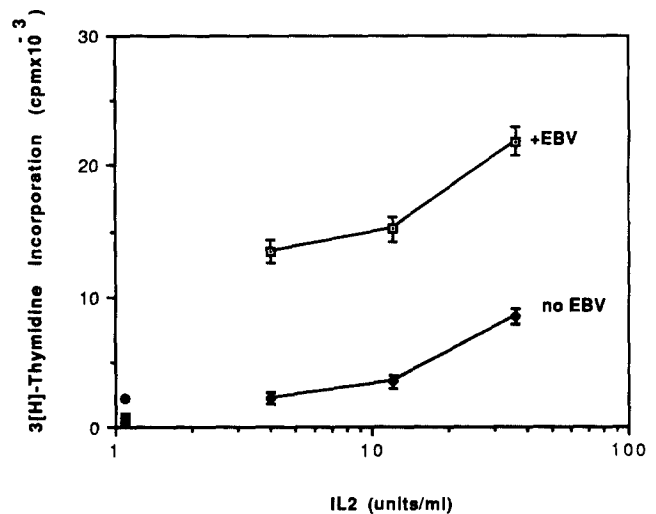
periments (30). Incubation of this serum with the peptide blocks binding to the 70-kD antigen found in the infected thymocytes (Fig. 4 B, compare lanes 1 and 2). This experiment was done at a higher serum dilution so the positive signal is weaker than the one seen in Fig. 4 A (lane 2).

In other studies, we assessed the stability of the EBV DNA in infected thymocytes. In long-term cultures, we incubated thymocytes with EBV in the presence of IL2 for a period of 6 wk and then assessed the presence of EBV in the cells by analyzing episomal DNA by Southern blotting as described above. The data in Fig. 5 indicate that in two different thymocyte cultures EBV DNA persisted during this period of time.

Evidence that the above observations represent infection of thymocytes and not of another contaminating cell type is supported by several observations. First, EBV binding is associated with CD1 expression (see Fig. 2 e), which is a specific marker of immature thymocytes (38). Second, immunofluorescence staining and cytofluorographic analysis revealed that the thymocyte populations consisted of 99–100% CD2-positive cells and did not contain any significant number of cells (<1%) reactive with an anti-CD19 antibody (B cell marker; reference 40) either before or after incubation with virus for 12 d. It is reasonable to expect that if B cells were contaminating our thymocyte population, we should have been able to detect higher anti-CD19 reactivity after EBV infection. Third, repeat of the experiment displayed in Fig. 3 A



**Figure 6.** Absence of B cell specific messages from the thymocyte population. Total RNA (5  $\mu$ g) from the JY B cell line (lanes 1 and 2) or SRBC-rosetted thymocytes (lanes 3 and 4) was reversely transcribed and amplified as described in Materials and Methods with specific primers that hybridize to either the B cell-specific CD19 DNA (lanes 1 and 3) or with primers that hybridize to the DNA coding for the  $\alpha$  subunit of the stimulatory GTP-binding protein (lanes 2 and 4) which is expressed in both B cells and thymocytes. The marker ladder was obtained by analyzing BstEII-digested  $\lambda$  DNA. The results are those obtained from a typical preparation of purified thymocytes.



**Figure 7.** Induction of thymocyte proliferation EBV. Thymocytes,  $10^6$  per microtiter tray well, that had been purified by SRBC rosetting were cultured in microtiter tray wells in the presence or absence of EBV for 2 d, then various concentrations of IL-2 were added and cultures incubated for an additional 5 d. Delay of IL-2 addition was necessitated to allow a spontaneously IL-2-responsive thymocyte population to become quiescent. Proliferation was measured by the incorporation of [ $^3$ H]thymidine. Controls receiving EBV only are denoted by the closed circle and those receiving medium only by the closed square. The results are displayed as the averages of triplicate determinations ( $\pm$  SD). The results are representative of three experiments using cells from different donors. In one of the experiments,  $0.5 \times 10^6$  cells per well were used with identical results.

with highly purified (SRBC-rosetted) thymocytes yielded identical results (Fig. 3 B). Fourth, analysis of the RNA from purified thymocyte population by PCR, using oligonucleotide primers that amplify the B cell-specific marker CD19 (40), revealed no specific product in the thymocytes (Fig. 6, lane 3), while the expected product of 279 bp was amplified in the B cell control RNA (Fig. 6, lane 1). Primers hybridizing to a gene ( $\alpha$  subunit of the stimulatory GTP-binding protein), which is unrelated to CD19, amplified the expected 800-bp segment in both B cells (Fig. 6, lane 2) and thymocytes (Fig. 6, lane 4). Collectively, the above observations indicate that the cells that interact with EBV are indeed thymocytes.

**EBV and IL-2 Have a Synergistic Effect on Thymocyte Proliferation.** We wished to ascertain the functional consequences, if any, EBV may have on thymocytes. Addition of the virus to purified thymocytes caused a small, but significant degree of proliferation (Fig. 7). Since we knew, from other studies of ours (41), that thymocytes can respond to IL-2, we tested the ability of this lymphokine to act synergistically with the virus. To minimize the spontaneous proliferative response that thymocytes have with IL-2, we delayed the addition of the lymphokine, since such a manipulation greatly reduces this spontaneous response. Thus, thymocytes were cultured with EBV for 2 d and then various concentrations of IL-2 were added. Controls included cultures that received no EBV during the preincubation period. Under these experimental conditions, it was observed that EBV and IL-2 act synergistically

**Table 1.** Proliferation of Thymocytes in Response to EBV and IL-2

Additions		[ <sup>3</sup> H]Thymidine incorporation
Day 0	Day 2	
Medium	Medium	226 ± 155
Medium	IL-2	11,254 ± 1,560
EBV	Medium	608 ± 220
Medium	EBV	141 ± 28
EBV	IL-2	18,918 ± 540
Medium	EBV + IL-2	9,260 ± 723

The experiment was performed in a fashion similar to the one displayed in Fig. 7 with the exception that EBV was added either 2 d before IL-2 or along with IL-2 after the cells had been incubated in medium for 2 d. The appropriate controls for each order of additions are also displayed. The data displayed are from a single experiment using thymocytes from a different donor than the ones used in Fig. 7.

in a lymphokine dose-dependent fashion to induce thymocyte proliferation (Fig. 7). To determine the need for EBV presence during the 2-d preincubation period, we cultured thymocytes without EBV for 2 d followed by simultaneous addition of virus and IL-2. In these cultures no synergistic proliferative effect was seen (Table 1).

## Discussion

In the present investigation, we provide evidence that indicates that a subpopulation of immature human thymocytes expresses EBV receptors. The virus binds specifically to these cells, infects them, induces EBNA-1 expression, and synergistically with IL-2 causes their proliferation.

The binding of EBV to thymocytes is specific. This is ascertained by the inhibition of viral binding with anti-CR2 mAbs and aggregated C3, manipulations known to specifically inhibit the binding of EBV to B lymphocytes (11–13, 34). Furthermore, EBV targets an immature thymocyte subpopulation. This is evidenced by the fact that the virus binds to cells that display the CD1 marker and are dull for CD3 expression, both of which are consistent with an immature phenotype (38).

The nature of this thymocyte receptor is not well understood. The CR2 expressed on B lymphocytes has been recently cloned and characterized (42–44). It consists of 15 or 16 60–70 amino acid repeats called short consensus repeats (SCRs) followed by a transmembrane and an intracytoplasmic domain. Investigators have used truncated CR2 mutants that bear serial deletions of their SCRs, or chimeric proteins containing SCRs from both CR2 and CR1 in order to map the epitopes that bind ligands such as C3 and EBV or monoclonal anti-CR2 antibodies (45, 46). It has been observed

that these ligands bind to epitopes structured by different SCRs (45, 46). For instance, antibodies OKB7 and HB5 react with epitopes that are formed by the first four SCRs, while antibody B2 reacts with an epitope shaped by SCRs 9–11 (45, 46). In view of the previously reported differential reactivity of anti-CR2 antibodies with thymocytes (16), we propose the possibility that the thymocyte EBV-binding molecule may represent an alternate form of CR2. This could be manifested by either a missing SCR or by the presence of a novel SCR. This possibility is supported by information on a limited amino acid sequence obtained from a CR2-like protein that was isolated from a leukemic T cell line (15). This sequence is significantly different from the sequence of CR2 on B lymphocytes (15).

It is well established that EBV receptors on B cells are identical to C3d receptors (11–13). Several earlier reports by other laboratories had described the presence of C3 binding proteins on cells of the T lineage (47–52). In these studies, the C3-reactive molecules were assessed by rosetting techniques and no structural or functional characterizations were performed. Thus, the nature and significance of these receptors could not be fully determined. Menezes et al. (14), using rosetting techniques, and later on Shapiro et al. (53), using radioactive EBV, reported the presence of EBV receptors on the T cell leukemia Molt 4. These reports claimed that although the virus could bind to the cells, it failed to internalize and thus, they proposed the possibility that this T cell EBV-binding protein might be different than the CR2 found on B lymphocytes. In view of the data presented here, it is possible that either the virus receptor on Molt 4 is different, due to the leukemic state of the cells, or that EBV may bind to different receptors that vary in their functional and/or structural properties. The latter possibility is supported by data from our laboratory (J. Hedrick, D. Watry, C. Speiser, P. O'Donnell, J. Lambiris, C. Tsoukas, manuscript submitted) where a T cell leukemic line although capable of binding and internalizing EBV, lacks expression of CR2 as assessed by anti-CR2 antibodies and Northern blotting analysis. Recent data from Sauvageau et al. (54) also support the contention that EBV receptor heterogeneity may exist by providing evidence of the existence of such virus receptors on peripheral CD8<sup>+</sup> T cells of healthy individuals. It was noted that these receptors do not react with the anti-CR2 antibody OKB7.

EBV can infect the thymocyte subpopulation it binds to as evidenced by the presence of episomal EBV-DNA in thymocytes after 12 d in culture with the virus. Furthermore, the fact that EBV-DNA could be detected in thymocytes even after 6 wk postinfection, indicates that the viral genome is stable in these cells. Additional evidence of the stability of EBV in thymocytes is the production of EBNA-1 which is known to be necessary for EBV episome maintenance (55).

EBV acts synergistically with IL-2 to induce a lymphokine-dependent thymocyte proliferation. It is interesting that delayed addition of EBV, after thymocytes have been incubated quiescent for 2 d, eliminates this synergistic effect suggesting that the thymocyte target population is short lived and infection by EBV is probably necessary early after thymocytes have been placed in culture. Although the exact biologic significance

of the thymocyte EBV receptor is not understood at the present time, the synergistic effect of EBV and IL-2, and the selective expression of the receptor on immature thymocytes, suggest the possibility that this molecule may be involved in T cell development. Several observations have indicated that CR2 on B lymphocytes may be involved in the differentiation and/or activation of these cells (6, 7, 56-59). Finally, there exists the potential that use of EBV and IL-2 may provide tools for the in vitro expansion and maintenance of thymocytes and the generation of clones for the study of human thymocyte differentiation.

The recent reports of EBV DNA-bearing T cell lymphomas is particularly intriguing. In these studies, Kikuta et al. (17) reported the case of a patient, who after clinical EBV infection, developed a Kawasaki-like disease with the interesting characteristic of circulating T cells that carried EBV genome. Jones et al. (18) described three different cases of patients with T cell lymphomas that carried EBV genome who, similar

to Kikuta's observations, had primary EBV infections before the development of the lymphoma. More recently, Harabuchi et al. (19) reported EBV-positive nasal T cell lymphomas in five patients with lethal midline granulomas. In all of the above cases the T cells were predominantly of the helper phenotype as determined by the expression of the CD4 protein on their surfaces and expressed EBNA-1.

In summary, our data challenge the current belief that EBV tropism is limited to B lymphocytes and epithelial cells. A subpopulation of human immature thymocytes is capable of binding EBV, becoming infected, and proliferating when IL-2 is also present. In view of the reports on EBV-positive T cell lymphomas, we propose that further investigations on the association of EBV and T cell malignancies, the characterization of the thymocyte EBV receptor, and the elucidation of its biologic significance should provide new insights on the association of this virus and T cells.

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