An Epstein-Barr Virus–associated Superantigen

By Natalie Sutkowski,* Tessa Palkama,* Cristina Ciurli,‡
Rafick-Pierre Sekaly,‡ David A. Thorley-Lawson,* and Brigitte T. Huber*

*From the *Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts 02111; and the ‡Laboratory of Immunology, Institute for Clinical Research of Montreal, Montreal, PQ, Canada, H2W 1R7*

Summary

More than 90% of adults are latently infected with Epstein-Barr virus (EBV), the causative agent of infectious mononucleosis, a self-limiting lymphoproliferative disease characterized by extensive T cell activation. Reactivation of this herpesvirus during immunosuppression is often associated with oncogenesis. These considerations led us to analyze the early events that occur after exposure of the immune system to EBV. Strong major histocompatibility complex (MHC) class II–dependent, but not MHC-restricted, T cell proliferation was observed in vitro in response to autologous, lytically infected EBV-transformed B cells. By measuring the appearance of the early activation marker CD69 on individual T cell Vβ subsets, we could demonstrate selective activation of human Vβ13$^+$ T cells. This was confirmed with murine T cell hybridomas expressing various human BV genes. While EBV$^-$ Burkitt's lymphoma cells were nonstimulatory, they induced Vβ-restricted T cell activation after EBV infection. EBV specific activation was also demonstrated in cord blood cells, excluding a recall-antigen response. Thus, all of the characteristics of a superantigen-stimulated response are seen, indicating that induction of the EBV lytic cycle is associated with the expression of a superantigen in B cells. A model is presented proposing a role for the superantigen in infection, latency, and oncogenesis.

EBV has a strong tropism for B lymphocytes and has the capacity to activate them to proliferate continuously (reviewed in 1, 2). Exposure to EBV during childhood results in no symptoms or only limited symptoms. Infection during adolescence or early adulthood, however, can give rise to infectious mononucleosis (IM)[1] in ~50% of cases. IM is a self-limiting lymphoproliferative disease characterized by virus shedding into the saliva, growth of infected B cells, and a massive expansion of nonspecific T cells. Initially, both CD4$^+$ and CD8$^+$ T cells are activated; however, later on, the response comprises mainly atypical CD8$^+$ lymphoblastoid cells. Disease regression occurs gradually, manifested by a decrease in virus shedding as well as in the number of infected B cells. Presumably, this is mediated by virus-specific CTL (3).

EBV is a highly successful virus, which has evolved to inhabit and manipulate the immune system to its own advantage. The infection of antigen-presenting B cells, followed by a rapid activation of nonimmune T cells, led us to speculate that an EBV-associated superantigen might be influencing the immune response during the establishment of persistent infection.

Superantigens are a class of pathogen-derived proteins that elicit a powerful T cell response, activating whole families of T cells with identical or related TCR Vβ chains (reviewed in 4). Superantigens form a bridge between MHC class II molecules on APC and a region on the TCR Vβ chain outside of the unique antigen-binding domain. This bridging transduces a signal to the T cell as well as the APC, resulting in activation of both. The hallmarks of a superantigen-induced T cell response are (*a*) a vigorous activation of primary T cells; (*b*) an MHC class II–dependent, but not MHC-restricted, recognition; and (*c*) a TCR Vβ–restricted response. Exposure to superantigens in vivo results in skewing of the T cell repertoire, manifested either by amplification of particular T cell Vβ families, or deletion and/or anergy of specific subsets. A virally encoded superantigen could have irreversible, indirect effects on the human immune system by altering the response to pathogens, leading to disease or autoimmunity. An example is provided by the recent demonstration that the murine mammary tumor virus (MMTV) superantigen, Mls-1, predisposes mice to polyoma virus associated oncogenesis by deleting the cytotoxic T cells that control polyoma virus infection (5).

Superantigens are evolutionarily conserved in certain pathogens, and it is presumed that they confer a selective advantage for the microbe. It has been well documented

[1] *Abbreviations used in this paper:* CBMC, cord blood mononuclear cells; IM, infectious mononucleosis; LCL, lymphoblastoid cell line; MMTV, murine mammary tumor virus; SEB, *Staphylococcus* enterotoxin B.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/09/971/10 $2.00
Volume 184 September 1996 971–980

that stimulation of the host's immune system by the prototypic viral superantigen, Mls, is essential for transmission of infectious MMTV (6–8). Therefore, we set out to determine whether superantigen stimulation of the human immune system could likewise play a role in establishing persistent EBV infection and disease.

Materials and Methods

Proliferation Assays. Peripheral blood B cells from healthy adult volunteers were transformed to create lymphoblastoid cell lines (LCL), using viral supernatant from B95-8 cells, which produce a common laboratory strain of EBV (9, 10), or IM-1, an EBV strain isolated from an IM patient (a gift from M.A. Epstein, University of Oxford, England). LCL were stimulated with PMA (10 ng/ml) for 24 h to induce the lytic cycle, and were then treated with mitomycin C (100 μg/ml) for 1 h to arrest cellular division. After extensive washing with PBS, LCL were added to freshly isolated autologous PBMC or allogeneic umbilical cord blood mononuclear cells (CBMC). PBMC and CBMC were isolated by density gradient separation on Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden). All cells were cultured in complete RPMI media (GIBCO BRL, Gaithersburg, MD), supplemented with 10% FCS (Intergen, Purchase, NY), at 37°C with 5% CO_2. Each assay was carried out in quadruplicate wells of a 96-well plate with 10^5 responders/well, using stimulator to responder ratios of 1:3 and 1:10. Stimulation was compared to treatment with PHA (2 μg/ml). After an incubation period of 48 h, the cells were pulsed with [^{3}H]thymidine (1 μCi/well) and harvested 8–12 h later to assess DNA synthesis as a measure of cellular proliferation.

Similar assays were performed using as APC two EBV$^-$ Burkitt's lymphoma tumors, BL-2 and BL-41 (11), both before and subsequent to productive infection with B95-8 supernatant. APC were treated with PMA and mitomycin C and plated with allogeneic PBMC or CBMC at APC/responder ratios of 1:1, 1:3, or 1:10.

MHC Class II–blocking Experiments. Proliferation assays were performed as described above, with the following modifications: before the addition of responder cells, in some of the wells, LCL were preincubated for 1 h with D1-12 (80 μg/ml), an mAb that recognizes all HLA DR molecules (12), or the same concentration of an isotypic control mAb 116-13.1, anti–lyt-2. The mAb remained in the cultures for the entire 72 h. Each assay was set up using autologous PBMC from adult donors as responders, and with a stimulator/responder ratio of 1:10. As a control, D1-12 (80 μg/ml) was also added to PHA-treated cells.

CD69 Staining of Individual Vβ Subsets. Freshly isolated autologous PBMC were stimulated for 4 h with PMA-induced B95-8 LCL or IM-1 LCL in one well of a six-well plate using 10^7 PBMC and 1.25×10^5 stimulators (stimulator/responder = 1:80). As a control, PBMC were likewise stimulated either with PHA or *Staphylococcus* enterotoxin B (SEB; Sigma Immunochemicals, St. Louis, MO) (1 μg/ml) for 4 h. Stimulated cells were then immediately double stained with PE-conjugated anti-CD69 mAb, and a panel of anti-Vβ mAb that were either directly FITC conjugated, or if unconjugated, FITC-conjugated rabbit anti–mouse IgG (Zymed Laboratories, South San Francisco, CA) was used as a second step reagent. Cells were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA) to determine the percentage of each Vβ subset that became CD69$^+$. The results were calculated for each staining according to the following formula: number of double-positive cells (Vβ$^+$, CD69$^+$)/total number of Vβ$^+$ cells in each subfamily. This readout is independent of variations in size of individual Vβ compartments, since only the change in CD69 expression is recorded. Background levels of CD69 staining were ~3% on unstimulated cells from each donor.

Antibodies. D1-12, anti–HLA DR (12); PE-CD69 (Becton Dickinson); FITC-Vβ2, 14-23 (ImmunoTech Corp., Boston, MA); Vβ3a, 8F10; Vβ5b, W112; Vβ8a, 16G8 (T Cell Diagnostics, Woburn, MA); Vβ5a, 42/1C1 (13); Vβ5c, LC4 (14); Vβ6.7a, OT145 (15); Vβ8b, MX6 (16); Vβ12, S511 (17); Vβ13, H131 (18); 145-2C11, antimurine CD3 (19); OKT3, anti–human CD3, 116-13.1, antimurine CD8 (American Type Culture Collection, Bethesda, MD). The Vβ3-13 mAbs were supplied as a generous gift from Walter Tian of T Cell Diagnostics and Chuck Ritterhouse of T Cell Sciences (Cambridge, MA) to the TCR mAb Workshop participants (Human TCR Monoclonal Antibody Workshop, July 26, 1995, San Francisco, CA).

Stimulation of Murine T Cell Hybridomas Expressing Human Vβ13 Genes. The murine T cell hybridomas hVβ13.1, hVβ13.2, hVβ13.6, hVβ2, hVβ3, and hVβ9 were provided by Philippa Marrack (National Jewish Center for Immunology and Respiratory Medicine, Univ. of Colorado Health Sciences Center, Denver, CO). The derivation of the hVβ13 lines, hVβ13.1-1, hVβ13.2-1, and hVβ13.3-1, has been previously described (18, 20); the hVβ9 T cell hybrid DS3-A was constructed in a similar manner. They express the particular human Vβ gene in the context of murine Vα and CD3 chains. It should be noted that the hVβ13.3-1 hybridoma actually expresses Vβ13.6, according to a recent revision of the Vβ13 family nomenclature (21). The hVβ2 hybrid, TAL 8.1.9, has been previously described (22). The hVβ3 hybrid, 58α$^-$β$^-$/HAI-7α/β, expresses human Vα1.2 and Vβ3 fused to a murine constant region. The hVβ8 and hVβ17.1 hybrids, YLβ8#24 and YLβ17.1#15, were constructed by Victor Dumas in the Sekaly lab (unpublished data), by transfecting the murine T hybrid, YLβ$^-$ (a gift from Osami Kanagawa, Washington University School of Medicine, St. Louis, MO), with the RSV.5neo vector (23) containing the particular human genes. These express murine Vα and CD3 chains.

T cell hybridomas were cultured for 24–36 h with PMA-treated B95-8 or IM-1 LCL, or Burkitt's lymphoma cells, using 2×10^4 responders/well in quadruplicate wells of a 96-well plate, and stimulator/responder ratios of 1:1 or 1:3. As control, T cell hybridomas were stimulated by cross-linking the TCR on anti-CD3–coated wells (145-2C11 ascites, diluted 1:3,000). Cell lysates were prepared by freezing the plates for at least 12 h, and IL-2 production was monitored in a bioassay using HT-2 indicator cells (24).

Results

Peripheral Blood T Cells Proliferate in Response to Autologous, Lytically Infected EBV-transformed B Cells. To investigate whether a virally associated superantigen influences the establishment of persistent EBV infection, a classical T cell proliferation assay was performed. In this assay, lytically infected, EBV-transformed B LCL were used as APC, and autologous PBMC from healthy adult donors served as responders. This constituted an attempt to reproduce in vitro some of the initial events that occur during IM, leading to the massive T cell activation. It has not been possible to decipher these primary steps in vivo using IM patient blood samples, most likely because noticeable clinical symptoms

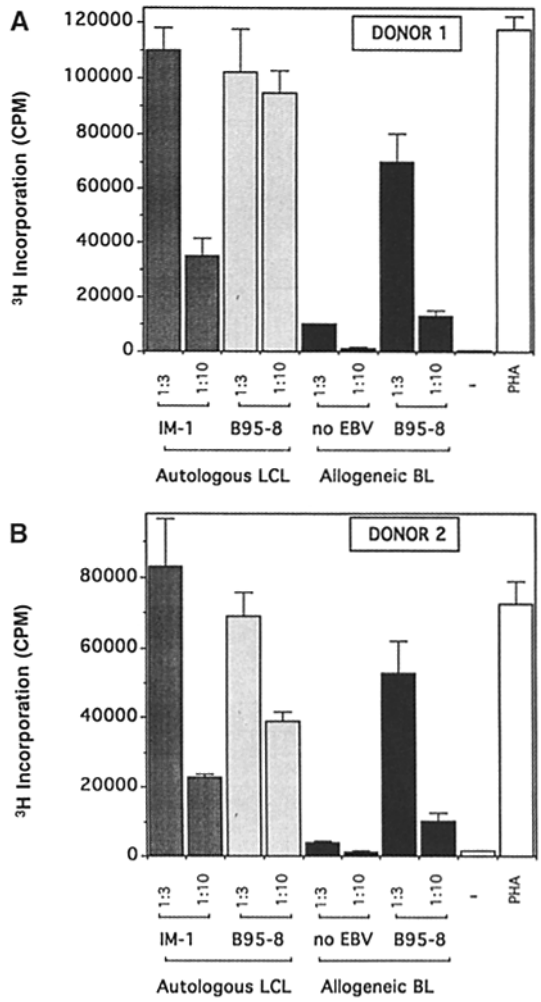

Figure 1. Proliferation of PBMC from healthy donors to autologous, lytically infected EBV-transformed B cells, and to an allogeneic EBV$^+$, but not EBV$^-$, Burkitt's lymphoma. B95-8 or IM-1 LCL, EBV$^-$ BL-2 cells, and B95-8–infected BL-2 cells were stimulated with PMA for 24 h to induce lytic viral replication and treated with mitomycin C for use as APC. Freshly isolated PBMC from donor 1 (*A*) and donor 2 (*B*) were then added to autologous LCL or allogeneic BL-2 cells. Each assay was carried out using stimulator to responder ratios of 1:3 and 1:10. Stimulation with PHA was used as a positive control. 48 h proliferation was assessed by [^{3}H]thymidine incorporation as a measure of DNA synthesis. Results were assessed in quadruplicate, and are expressed as the average cpm/well with the SD.

often do not appear until after the disease has progressed to the lymphoproliferative stage. At that point, there is significant bystander T cell activation, resulting from cytokine production and the generally activated immune status, which can obscure EBV-specific T cell activation.

LCL were established in vitro by infection of B cells with two different strains of EBV: B95-8, a common laboratory strain, and IM-1, a viral isolate derived from the peripheral blood of an IM patient. Since EBV is generally maintained in a latent form in LCL, we stimulated the cell lines for 24 h with the phorbol ester PMA, which induces the EBV lytic cycle and upregulates the production of viral proteins (25). Figs. 1, *A* and *B*, depict representative results obtained with PBMC from two healthy unrelated adult donors. In both cases, 48 h proliferation, as assessed by [^{3}H]thymidine incorporation, was demonstrated at levels comparable to the mitogen PHA. Maximum proliferation occurred at 72 h, although the absolute levels were not significantly higher (data not shown). To demonstrate that EBV is responsible for the strong proliferation, an EBV$^-$ Burkitt's lymphoma line, BL-2, was PMA treated for use as APC. Although allogeneic, these cells were found to be nonstimulatory or very weakly stimulatory at 48 h under identical conditions. Upon infection of the BL-2 cells with B95-8 EBV, strong proliferation was observed (Fig. 1, *A* and *B*).

The Early Activation Marker CD69 Is Preferentially Expressed on Vβ13 T Cells after a 4-h Stimulation with Autologous B95-8 LCL. The main characteristic of a superantigen is that it elicits a TCR Vβ–restricted response, resulting in a selective alteration of the T cell repertoire by either amplifying or deleting T cell clones that express the specific Vβ genes. If indeed the stimulation with autologous LCL is caused by the presence of a superantigen, we would expect to selectively activate T cells of a particular TCR Vβ phenotype. To test this, we developed a novel means of defining Vβ-specific T cell activation; namely, we analyzed the appearance of the early activation marker CD69 (26, 27) on individual T cell Vβ subsets 4 h after stimulation. Cytokines, which are potent activators of bystander T cells, are produced between 5–6 h after stimulation; thus, we deliberately chose an earlier time point to minimize the induction of nonspecific T cell proliferation. Moreover, the absence of proliferation at this early time point guarantees detection of a polyclonal response, excluding the readout of an oligoclonal expansion. This obviates the need for sequencing the VDJ region of numerous clones to establish polyclonality.

PBMC from the same two donors depicted in Fig. 1 were stimulated for 4 h with autologous B95-8 LCL or with PHA. The cells were then double stained with a PE-conjugated anti–CD69 mAb and a panel of FITC-labeled anti-Vβ mAbs. The stimulated cells were analyzed by flow cytometry to assess the percentage of each Vβ subset that expressed CD69. As can be seen in Fig. 2 *A*, stimulation with LCL led in both donors to a significant increase in CD69$^+$ cells in the Vβ13 T cell subset. As expected, the percentage of CD69$^+$ cells after stimulation with the mitogen PHA did not vary significantly between the Vβ subsets and was not significantly different from the overall percentage of CD69$^+$ cells in unfractionated PBMC. These results suggest that EBV induces in B cells the expression of a protein that is preferentially recognized by polyclonal Vβ13 T cells, implicating the presence of a superantigen. Similar results were obtained after stimulation with autologous IM-1 LCL, and with a third donor using autologous LCL as well (data not shown). After longer stimulation with PHA or LCL (>4 h), CD69 was expressed on a greater percentage of T cells (data not shown), probably because of activation of bystander T cells by the ensuing production of cytokines.

To ensure that the appearance of CD69 on particular Vβ

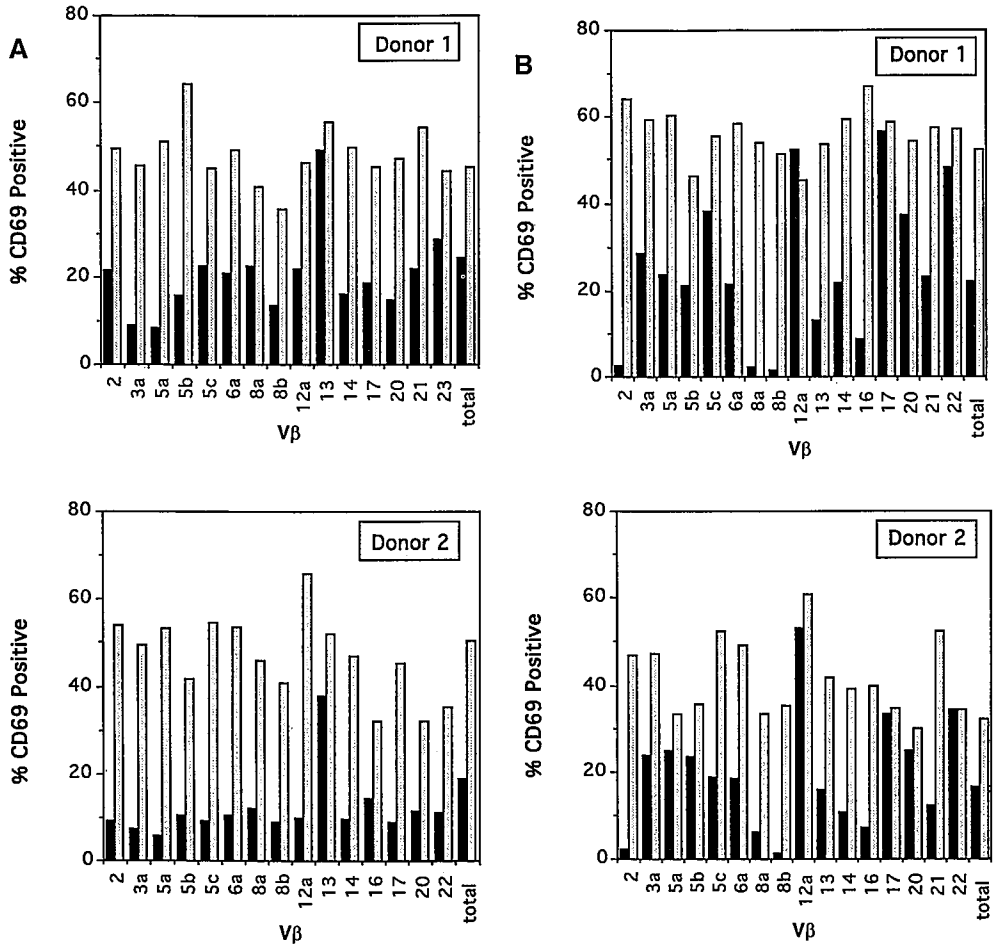

Figure 2. The early activation marker CD69 is preferentially expressed on Vβ13 T cells after a 4-h stimulation with autologous B95-8 LCL, and on Vβ12 T cells after SEB stimulation. Autologous PMA-induced B95-8 LCL were cultured for 4 h with freshly isolated PBMC from two donors, as described in Materials and Methods. As controls, PBMC from each donor were simultaneously stimulated with PHA (*A*). Stimulated cells were immediately double stained with PE-conjugated anti-CD69 mAb and a panel of FITC-conjugated anti-Vβ mAb. Analysis by flow cytometry determined the percentage of each Vβ subset that became CD69$^+$. ■, B95-8; □, PHA. (*B*) In a separate experiment, PBMC from the same two donors were stimulated with SEB for 4 h and stained as described in *A*; again, stimulation was compared to PHA. The graphs depict the percentage of cells from each Vβ family that were CD69$^+$ after stimulation with SEB (■) compared to PHA (▨).

subsets is truly indicative of superantigen activity, we analyzed the well-established superantigen response to SEB. The results obtained with PBMC of the same two donors are summarized in Fig. 2 *B*. The percentage of CD69$^+$ cells after SEB stimulation was significantly increased in the Vβ12, Vβ17, and Vβ20 subsets, consistent with published reports using conventional methods of Vβ analyses (28). The level of CD69 was highest on Vβ12$^+$ cells, the subset shown to be deleted in an in vivo study after immunization with SEB (29). On the other hand, the Vβ13 subset was not significantly elevated for CD69 staining after SEB treatment, indicating that the increased Vβ13 stimulation in Fig. 2 *A* was specific for EBV LCL. Thus, our assay system is sensitive for detection of Vβ-specific superantigen stimulation. It should be noted, however, that after 4 h incubation with SEB, CD69 expression was not significantly increased in the Vβ3a subset, despite the reported expansion of Vβ3 T cells after long-term SEB stimulation (28). Since our assay relies on the specificity of the Vβ mAb used for the detection of T cell subsets, it is our opinion that screening for CD69 expression is useful only as an initial test for detection of Vβ activation.

Murine T Cell Hybridomas Expressing Human Vβ13.1, Vβ13.2, and Vβ13.6 Produce IL-2 in Response to B95-8 and IM-1 LCL. The human Vβ13 family contains eight members, Vβ13.1–13.8, comprising a large portion of the overall T cell repertoire (GenBank/EMBL/DDBJ accession No. L36092). The anti-Vβ13 mAb we have used, H131, was originally developed against the human BV13.1 gene product, expressed in a murine T cell hybridoma as a chimeric TCR with the murine Vα and CD3 chains (18). The H131 mAb stains a relatively large percentage of peripheral blood cells from each donor tested (3–4.5%). Because the BV13 family members are highly homologous, it is difficult to rule out mAb cross-reactivity between the subfamilies.

To confirm the finding that LCL preferentially activate Vβ13 T cells, we tested murine T cell hybridomas expressing human Vβ13.1, Vβ13.2, and Vβ13.6 chains, respectively (18, 20). In addition, T cell hybrids expressing human Vβ2 (22), Vβ3, Vβ8, Vβ9, and Vβ17.1 were included in these experiments. These hybrids lack endogenous murine Vβ genes, although the constant region and CD3 complex are of murine origin. The hVβ2 and hVβ3 hybrids express human Vα as well, while all of the other hybrids express murine Vα chains. As can be seen in Fig. 3 *A*, the three Vβ13$^+$ T cell hybrids produced IL-2 after stimulation with either PMA-treated B95-8 or IM-1–derived LCL, but were only very weakly stimulated by latently infected LCL. On the other hand, the hVβ8 T cell hybrid was unreactive to the LCL, regardless of PMA treatment. Fig. 3 *B* shows that

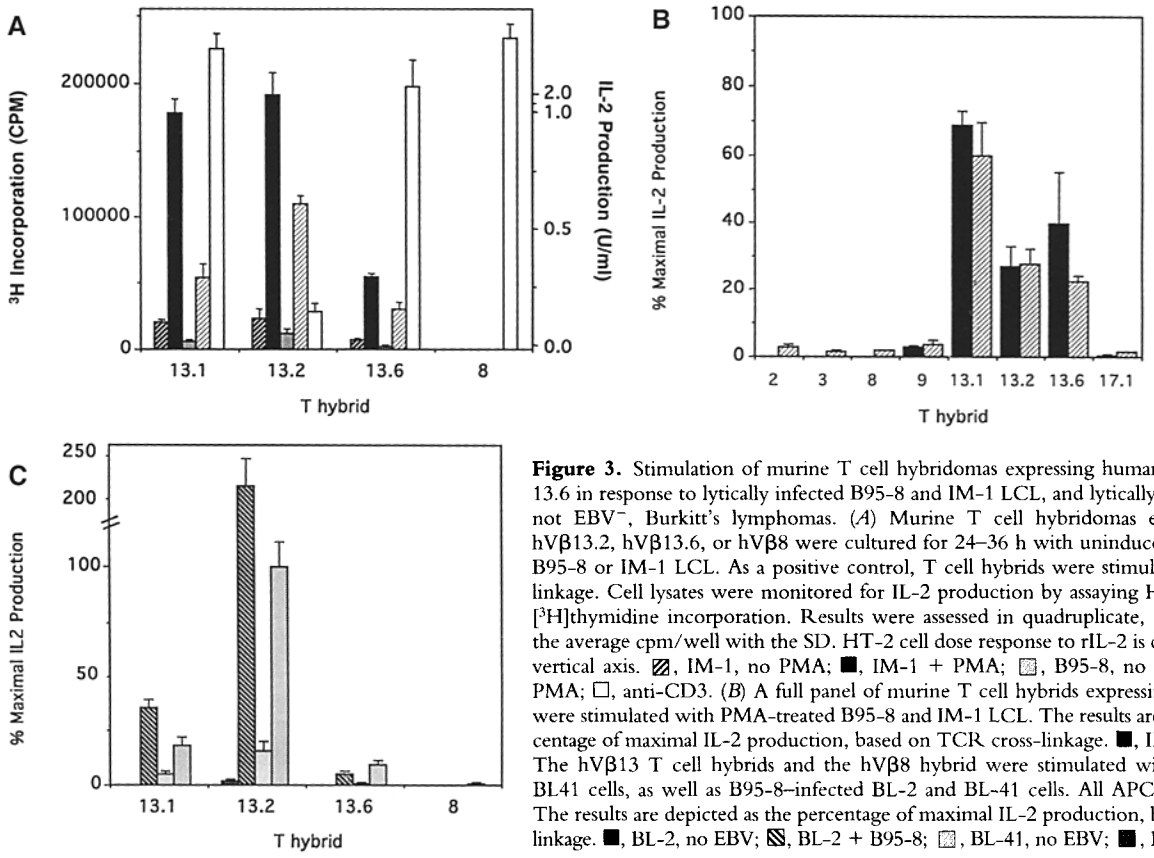

Figure 3. Stimulation of murine T cell hybridomas expressing human Vβ13.1, 13.2, and 13.6 in response to lytically infected B95-8 and IM-1 LCL, and lytically infected EBV$^+$, but not EBV$^-$, Burkitt's lymphomas. (*A*) Murine T cell hybridomas expressing hVβ13.1, hVβ13.2, hVβ13.6, or hVβ8 were cultured for 24–36 h with uninduced and PMA-treated B95-8 or IM-1 LCL. As a positive control, T cell hybrids were stimulated by TCR cross-linkage. Cell lysates were monitored for IL-2 production by assaying HT-2 cell growth by [^{3}H]thymidine incorporation. Results were assessed in quadruplicate, and are expressed as the average cpm/well with the SD. HT-2 cell dose response to rIL-2 is depicted on the right vertical axis. ▨, IM-1, no PMA; ■, IM-1 + PMA; ▩, B95-8, no PMA; ▨, B95-8 + PMA; □, anti-CD3. (*B*) A full panel of murine T cell hybrids expressing human BV genes were stimulated with PMA-treated B95-8 and IM-1 LCL. The results are depicted as the percentage of maximal IL-2 production, based on TCR cross-linkage. ■, IM-1; ▨, B95-8. (*C*) The hVβ13 T cell hybrids and the hVβ8 hybrid were stimulated with EBV$^-$ BL-2 and BL41 cells, as well as B95-8–infected BL-2 and BL-41 cells. All APC were PMA treated. The results are depicted as the percentage of maximal IL-2 production, based on TCR cross-linkage. ■, BL-2, no EBV; ▧, BL-2 + B95-8; ▩, BL-41, no EBV; ■, BL-41 + B95-8.

none of the other T cell hybrids expressing different human BV genes were stimulated by PMA treated IM-1 or B95-8 LCL. The results are presented as the percentage of maximal IL-2 production based on anti-CD3 cross-linking, since the overall level of response varied between the various T cell hybrids, reflecting fluctuations in the CD3 surface expression. Fig. 3 *C* demonstrates that EBV is responsible for stimulation of the Vβ13 T hybrids. In this assay, the Vβ13 hybrids produced IL-2 in response to two different PMA treated EBV$^-$ Burkitt's lymphoma lines, BL-2 and BL-41, only after infection of the cells with B95-8 supernatant. Again, the Vβ8 hybrid was unreactive. These experiments also indicate that the T cell response is not MHC restricted, since a similar pattern of stimulation was obtained with PMA-induced LCL and B95-8$^+$ BL lines, despite having different HLA haplotypes.

Proliferation to Autologous EBV LCL is MHC Class II Dependent, but Not Restricted. Presentation of all known superantigens to T cells is dependent on expression of MHC class II molecules. To determine whether the T cell response against EBV-transformed LCL requires MHC class II, we performed the proliferation assays in the presence of the mAb D1-12, which recognizes a nonpolymorphic region on all HLA DR molecules. As can be seen in Fig. 4, preincubation of PMA-stimulated B95-8 or IM-1 LCL with D1-12 mAb blocked the induction of autologous T

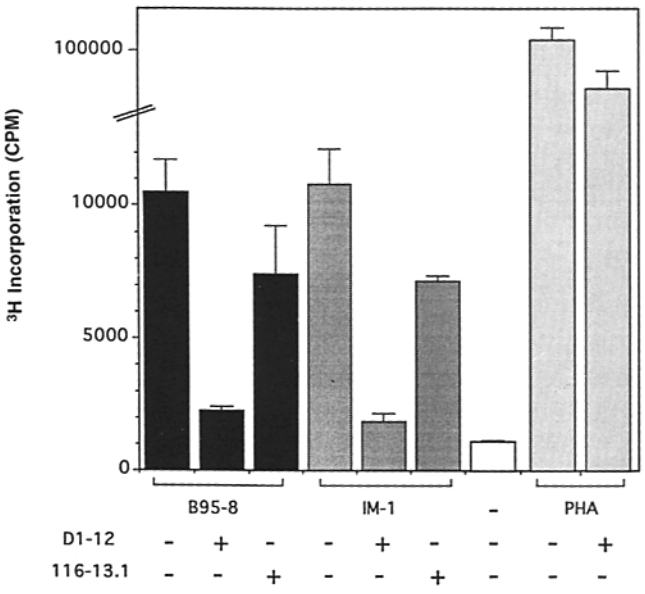

Figure 4. The anti-HLA DR mAb D1-12 blocks the 48-h proliferation of PBMC to autologous, lytically infected EBV LCL. Proliferation assay was performed as in Fig. 1; however, in some of the wells, LCL were preincubated with the mAb D1-12 or an isotypic control mAb 116-13.1, which remained in the cultures throughout the assay. Autologous B95-8 or IM-1 LCL were used as APC at a stimulator/responder ratio of 1:10. D1-12 added to PHA-treated cells did not affect stimulation.

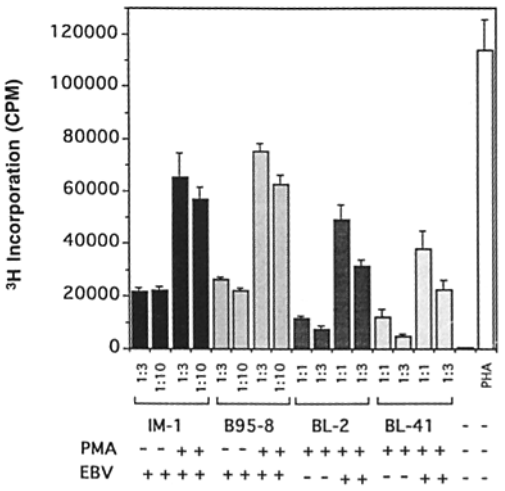

Figure 5. CBMC proliferate in response to lytically infected B95-8 and IM-1 LCL, and lytically infected EBV$^+$, but not EBV$^-$, Burkitt's lymphomas. Fresh CBMC were stimulated with allogeneic, uninduced, and PMA-treated B95-8 and IM-1 LCL at stimulator/responder ratios of 1:3 and 1:10. CBMC were also stimulated with allogeneic, PMA-treated EBV$^-$ and B95-8–infected BL-2 and BL-41 cells at stimulator/responder ratios of 1:1 and 1:3. The proliferation assay was performed as described in Fig. 1.

cell proliferation, while mitogen-induced stimulation was unaffected. Preincubation with an isotypic control antibody, 116-13.1, did not block 48-h T cell proliferation.

T Cell Proliferation to EBV-transformed B Cells Is Not Caused by a Memory Response. Since the vast majority of adults are EBV$^+$, the massive proliferation induced by EBV-transformed LCL could be caused by a recall antigen response. To test this possibility, we used CBMC as responders in this assay because newborns are EBV$^-$ and cannot have memory T cells. As can be seen in Fig. 5, CBMC vigorously proliferated at 48 h to allogeneic B95-8 and IM-1 LCL after PMA induction. Weak stimulation was observed in response to uninduced LCL. While the magnitude and the early kinetics of the response pointed to the action of a superantigen rather than an alloresponse, the Burkitt lymphoma lines actually confirmed that the stimulation was caused by EBV. Strong proliferation was detected only after PMA treatment of B95-8–infected BL-2 and BL-41 cells. PMA treated EBV$^-$ BL cells were weakly stimulatory, as would be expected from the alloresponse, which is not optimal at 48 h.

It should be mentioned that the LCL in all of our assays induce higher levels of T cell stimulation than do the B95-8–infected Burkitt's lymphoma lines. In Fig. 5, although the proliferation levels induced by all of the EBV$^+$ PMA–treated cells are comparable, the stimulator/responder ratios are 1:3 and 1:10 for the LCL, but 1:1 and 1:3 for the Burkitt's lymphomas. The difference in stimulation could be caused by several factors. LCL express higher levels than do most Burkitt's lymphoma lines of different cellular adhesion molecules, such as CD23, which possibly enhance T cell activation. In addition, since PMA treatment is necessary for optimal stimulation, we have surmised that the activation is most likely caused by an EBV lytic gene. LCL are easily induced into the lytic cycle with phorbol ester (2). Even in uninduced LCL, a small number of cells is present which spontaneously undergo lytic cycle viral replication. This number is significantly reduced in EBV$^+$ Burkitt's lymphoma lines (2). Spontaneous lytic cycle gene expression, in combination with the alloresponse, could account for the weak stimulation induced by non-PMA–treated LCL of the CBMC in Fig. 5, as well as of the T cell hybrids in Fig. 3 *A*.

Discussion

Taken together, the results presented here demonstrate the expression of a superantigen in EBV-transformed LCL and EBV$^+$ Burkitt's lymphomas that have been induced into the lytic cycle. The early activation of a large proportion of nonimmune T cells, combined with the fact that the stimulation is HLA-DR dependent, but not restricted, and the specific activation of Vβ13 T cells, all support this conclusion. Furthermore, the response of the CBMC to EBV$^+$, but not EBV$^-$ Burkitt's lymphoma cells, exclude the possibility of an anamnestic recall antigen response because CBMC are by definition EBV$^-$.

What are the possible advantages for EBV expressing a superantigen in infected B cells? We would predict that the superantigen is required for the establishment and/or the maintenance of persistent infection. The EBV latent genes have the capacity to directly induce B cell proliferation in vitro without T cell help, rendering superantigen stimulation superfluous. However, the site of viral persistence in vivo is a resting B cell that does not express the growth-promoting latent genes (30). We suggest, therefore, that the role of superantigen-activated T cells is to provide signals necessary for the growth and survival of these latently infected B cells in vivo, possibly through CD40–CD40L interaction (see model in Fig 6). This would replenish the pool of latently infected B cells, which does not decrease over time, but remains remarkably stable for years (30). T cell regulatory signals might also induce differentiation of latently infected B cells, driving some of them to terminally differentiate, reactivate the virus, produce more superantigen, and thereby complete the cycle (Fig. 6). This model proposes that viral replication is essential for viral persistence, not through reinfection of B cells, but through superantigen driven activation of T cells.

A role for T cells influencing EBV infection was suggested from earlier studies demonstrating that the spontaneous outgrowth of LCL in vitro from EBV-seropositive PBMC is more efficient in the presence of T cells (31). This requirement for T cells is usually obscured by the seemingly converse finding that EBV-specific CTL, which arise later in culture, must be depleted because they will eliminate the newly generated LCL (32, 33). Our model fits data obtained in vivo in SCID mice transplanted with EBV-seropositive human PBMC (34–36). These mice

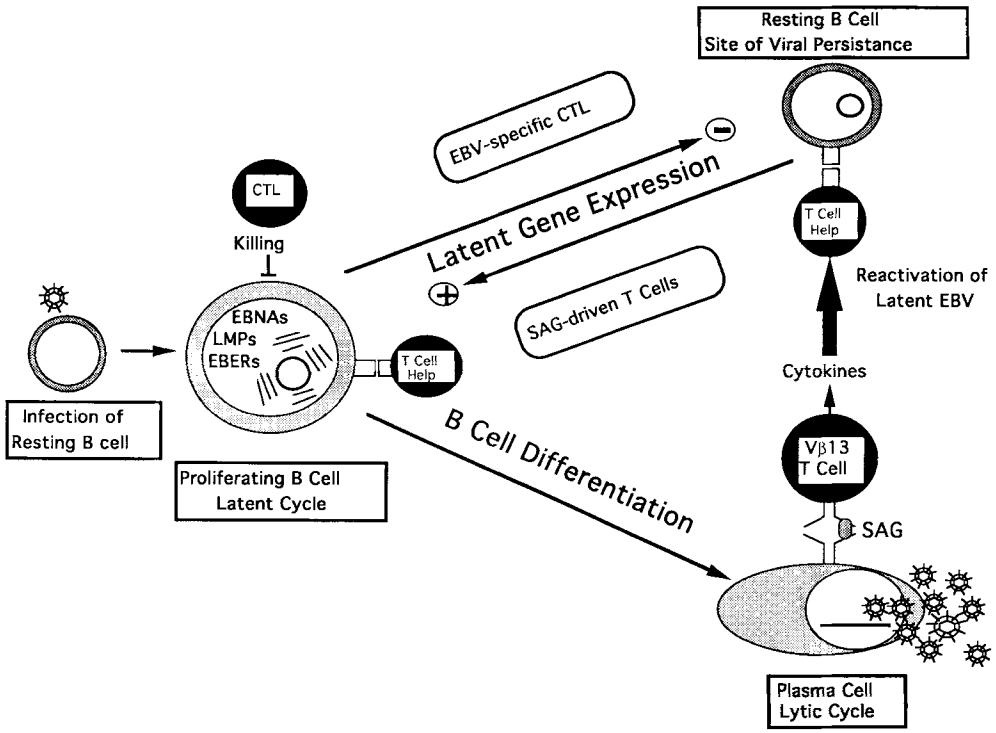

Figure 6. Role of the EBV-associated superantigen in infection, latency, and disease. After EBV infection of resting B cells, latent gene expression induces B cell proliferation. T cell help provides survival signals and signals for terminal differentiation. This enables lytic cycle viral replication and superantigen expression in the plasma cells. Activation of Vβ13 T cells initiates cytokine production and nonspecific activation of T cells, which can provide help to latently infected B cells. Primed EBV-specific CTL will kill B cells expressing the full latent gene repertoire, selecting for a population of cells that down-regulate latent gene expression and assume a resting phenotype. This becomes the site of viral persistence throughout the lifetime of the host. Periodically, these cells can be pushed into viral replication by T cell signaling, while memory CTL will counteract the process, maintaining a constant EBV burden.

spontaneously developed EBV-associated B cell lymphomas only when T cells were cotransferred, although the development of EBV-specific CTL limited tumor formation in some of the mice. Transplantation of highly purified B cells never yielded lymphomas, even in the presence of cytokines known to be secreted by activated T cells (35), suggesting that a direct T cell–B cell interaction is requisite. The tumors that develop are a mixture of latently infected proliferating B cells, and terminally differentiated, lytically infected B cells that do not divide (37). We would predict that the nondividing differentiated B cells provide an essential growth component for the growing tumor, namely superantigen-activated T cells. T cell signaling would promote B cell survival, allowing full latent gene expression, resulting in perpetual B cell proliferation. Simultaneously, T cells might push a fraction of cells to terminally differentiate, growth arrest, produce virus, and more superantigen (Fig. 6). Eventually, some of the latently infected, rapidly dividing cells would accumulate mutations in cellular oncogenes such as *myc*, allowing them to lose the T cell dependence and become transformed. This hypothesis is in fact supported by the SCID/hu mouse model, since the tumors that arise in SCID mice after long periods of time no longer contain the fully differentiated, lytically infected cells (37).

The other side of the equation, for healthy humans, is the cytotoxic T cell response (38). Persistent infection with EBV can thus be seen as a balance between the superantigen driven, T cell–dependent B cell growth, and the elimination of latently infected proliferating B cells by CTL (Fig. 6). The clinical symptoms of IM would arise as a result of the disruption of this balance. During IM, there is a rapid activation of both $CD4^+$ and $CD8^+$ T cells early after infection, presumably as a consequence of superantigen stimulation, mediated by the cytokine production of $V\beta13^+$ T cells. A characteristically massive expansion ensues of nonspecific, atypically activated, apoptotic $CD8^+$ T cells (39, 40). The nonspecific activation of CD8 cells possibly delays the development of virus-specific CTL. This could be a mechanism for EBV to evade a specific immune response in establishing latency.

Paradoxically, EBV infection during childhood leads to seroconversion with limited or no clinical symptoms. The following scenario provides an explanation for this apparent incongruity: In adolescents exposed for the first time to this pathogen, the mature T cell pool is poised to mount a rapid superantigen response, while the development of virus-specific CTL requires priming, and is therefore only effective after an initial lag period. CTL priming might be further delayed by the superantigen-induced activation of nonspecific T cells, resulting in the symptoms of IM. Children, on the other hand, would predictably mount a suboptimal T cell response to the EBV superantigen, as inferred from murine studies, indicating an impaired immune response to viral superantigens in immature animals (41). Thus, in children, the slower and weaker superantigen response would be balanced by the induction of EBV-specific CTL, preventing clinical manifestations. It is unclear whether IM represents an atypical immune response because the symptoms of IM are self-limiting and viral latency is established in both cases.

EBV reactivation in AIDS patients and transplant recipients often leads to the development of lymphomas and other lymphoproliferative diseases. Immunosuppression preferentially would affect the virus-specific memory CTL,

compared to the superantigen-reactive primary T cells, disturbing the balance in the favor of the latter. The loss of CTL, which control the spread of latently infected proliferating B cells, would lead to unregulated growth, favoring tumor development.

EBV has also been linked to the induction of autoimmunity, especially in Sjogren's syndrome, which is associated with elevated levels of the virus in patients (42). Of note are reports describing a significant increase in Vβ13 T cells in the lesions of patients with this autoimmune disease, indicative of the possible action of an EBV-associated superantigen (43–45).

Recently, it has been suggested that the presence of a superantigen in another herpesvirus, CMV, causes enhanced HIV-1 replication in Vβ12 T cells, which function as a reservoir for HIV-1 in infected patients (46). It is plausible that EBV might play a similar role as CMV in AIDS, particularly since EBV infection is ubiquitous, and it has been reported that perturbations in the Vβ13 compartment occur in patients with HIV infection (47). In addition, a recent report describes increased HIV-1 replication as a consequence of EBV lymphoma development in SCID mice transplanted with EBV-seropositive PBMC from HIV-1 infected patients (48).

Perhaps more importantly, we predict that some of the anergy and apoptosis of T cells occurring in HIV patients might actually be caused by activation-induced cell death, resulting as an indirect consequence of EBV superantigen stimulation, e.g., the increased cytokine environment. Furthermore, we speculate that the atypical, apoptotic $CD8^+$ T cells, which are massively expanded during IM, constitute the same T cell subset that is augmented in HIV infection (49, 50). In both diseases, elimination of $CD4^+$ T cells occurs, possibly caused in part by the nonspecific cytotoxic activity of these activated $CD8^+$ T cells. It is conceivable that these cells arise during HIV infection as a result of superantigen expression after EBV reactivation by HIV-1 Tat (51). In support of our postulate, a correlation was recently found between T cell apoptosis in HIV-1–infected children and an increased burden of EBV (52). Thus, interfering with the activation of Vβ13 T cells might have relevant clinical consequences.

We would like to thank Philippa Marrack for the kind gift of T cell hybridomas expressing hVβ chains, and we are indebted to David Posnett, Chuck Ritterhouse, and Walter Tian for the panel of anti-hVβ mAb. We gratefully acknowledge the technical assistance of Yuhong Gong and Raman Khiroya. Additional thanks go to Lisa Glickstein, Tracy Schmitz, and Bob Underwood for support and critical review of this manuscript.

The research work described in this review was supported by grants from the National Institutes of Health (RO1 AI14910 to B.T. Huber, AI 18757 and CA 31893 to D.A. Thorley-Lawson, 5T32 AR07570-04 to N. Sutkowski, the Human Frontiers (RG-544/95M to B.T. Huber and R.-Pierre-Sekaly, and by the Center for Gastroenterology Research on Absorptive and Secretory Processes (PHS NIDDK 1P30DK39428). T. Palkama was supported by a Research Training Fellowship from International Union Against Cancer, Lyon, France.

Address correspondence to Dr. Brigitte T. Huber, Department of Pathology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111.

Received for publication 27 December 1995 and in revised form 29 May 1996.

References

1. Miller, G. 1990. EBV biology, pathogenesis and medical aspects. *In* Virology. B.N. Fields and D.M. Knipe, R.M. Chanock, M.S. Hirsch, J.L. Melnick, T.P. Monack, and B. Roizman, editors. Raven Press, Ltd., New York. 1921–1958.
2. Kieff, E. 1996. Epstein-Barr virus and its replication. *In* Fundamental Virology. 3rd ed. B.N. Fields, D.M. Knipe, and P.M. Howley, editors. Lippincott–Raven Publishers, Philadelphia, PA. 1109–1162.
3. Tomkinson, B.E., R. Maziarz, and J.L. Sullivan. 1989. Characterization of the T cell-mediated cellular cytotoxicity during acute infectious mononucleosis. *J. Immunol.* 143:660–670.
4. Herman, A., J.W. Kappler, P. Marrack, and A.M. Pullen. 1991. Superantigens: mechanism of T-cell stimulation and role in immune responses. *Annu. Rev. Immunol.* 9:745–772.
5. Lukacher, A.E., Y. Ma, J.P. Carroll, S.R. Abromson-Leeman, J.C. Laning, M.E. Dorf, and T.L. Benjamin. 1995. Susceptibility to tumors induced by polyoma virus is conferred by an endogenous mouse mammary tumor virus superantigen. *J. Exp. Med.* 181:1683–1692.
6. Beutner, U., E. Kraus, D. Kitamura, K. Rajewsky, and B.T. Huber. 1994. B cells are essential for murine mammary tumor virus transmission, but not for presentation of endogenous superantigens. *J. Exp. Med.* 179:1457–1466.
7. Golovkina T.V., A. Chervonsky, J.P. Dudley, and S.R. Ross. 1992. Transgenic mouse mammary tumor virus superantigen expression prevents viral infection. *Cell.* 69:637–645.
8. Held, W., G.A. Wanders, A.N. Shakhow, L. Scarpellino, H. Acha-Orbea, and H.R. MacDonald. 1993. Superantigen-induced immune stimulation amplifies mouse mammary tumor virus infection and allows virus transmission. *Cell.* 74: 529–540.
9. Miller, G., and N. Lipman. 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proc. Natl. Acad. Sci. USA.* 70:190–194.
10. Baer, R., A.T. Bankier, M.D. Biggin, P.L. Deininger, P.J.

Farrell, T.J. Gibson, G. Hatfull, G.S. Hudson, S.C. Satchwell, C. Seguin, P.S. Tuffnell, and B.G. Barrell. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature (Lond.).* 310:207–211.

11. Calender A., M. Billaud, J.P. Aubry, J. Banchereau, M. Vuillaume, and G.M. Lenoir. 1987. Epstein-Barr virus (EBV) induces expression of B-cell activation markers on in vitro infection of EBV-negative B-lymphoma cells. *Proc. Natl. Acad. Sci. USA.* 84:8060–8064.
12. Accolla, R.S., R.P. Sekaly, A.P. McDonald, G. Corte, N. Gross, and S. Carrel. 1982. Demonstration at the single-cell level of the existence of distinct clusters of epitopes in two predefined human Ia molecular subsets. *Eur. J. Immunol.* 12: 166–169.
13. Brennan, F.M., S. Allard, M. Londei, C. Savill, A. Boylston, S. Carrel, R.N. Maini, and M. Feldmann. 1988. Heterogeneity of T cell receptor idiotypes in rheumatoid arthritis. *Clin. Exp. Immunol.* 73:417–423.
14. Takahashi, S., H.T. Maecker, and R. Levy. 1989. DNA fragmentation and cell death mediated by T cell antigen receptor/CD3 complex on a leukemia T cell line. *Eur. J. Immunol.* 19:1911–1919.
15. Posnett, D.N., A. Gottlieb, J.B. Bussel, S.M. Friedman, N. Chiorazzi, Y. Li, P. Szabo, N.R. Farid, and M.A. Robinson. 1988. T cell antigen receptors in autoimmunity. *J. Immunol.* 141:1963–1969.
16. Carrel, S., P. Isler, M. Schreyer, A. Vacca, S. Salvi, L. Giuffre, and J. Mach. 1986. Expression on human thymocytes of the idiotypic structures from two leukemia T cell lines Jurkat and HPB-ALL. *Eur. J. Immunol.* 16:649–652.
17. Posnett, D.N., R.D. Bigler, Y. Bushkin, D.E. Fisher, C.Y. Wang, L.F. Mayer, N. Chiorazzi, and H.G. Kunkel. 1984. T cell antiidiotypic antibodies reveal differences between two human leukemias. *J. Exp. Med.* 160:494–505.
18. Choi, Y.W., B. Kotzin, J. Lafferty, J. White, M. Pigeon, R. Kubo, J. Kappler, and P. Marrack. 1991. A method for production of antibodies to human T-cell receptor beta-chain variable regions. *Proc. Natl. Acad. Sci. USA.* 88:8357–8361.
19. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84: 1374–1378.
20. Choi, Y.W., A. Herman, D. DiGiusto, T. Wade, P. Marrack, and J. Kappler. 1990. Residues of the variable region of the T-cell-receptor beta-chain that interact with *S. aureus* toxin superantigens. *Nature (Lond.).* 346:471–473.
21. Wei, S., P. Charmley, M.A. Robinson, and P. Concannon. 1994. The extent of the human germline T-cell receptor V beta gene segment repertoire. *Immunogenetics.* 40:27–36.
22. Blank, U., B. Boitel, D. Mege, M. Ermonval, and O. Acuto. 1993. Analysis of tetanus toxin peptide/DR recognition by human T cell receptors reconstituted into a murine T cell hybridoma. *Eur. J. Immunol.* 23:3057–3065.
23. Long, E.O., S. Rosen-Bronson, D.R. Karp, M. Malnati, R.P. Sekaly, and D. Jaraquemada. 1991. Efficient cDNA expression vectors for stable and transient expression of HLA-DR in transfected fibroblast and lymphoid cells. *Hum. Immunol.* 31:229–235.
24. Kappler, J.W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2–restricted, interleukin, 2–producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J. Exp. Med.* 153:1198–1214.
25. zur Hausen, H., F. O'Neill, and U. Freese. 1978. Persisting oncogenic herpesvirus induced by the tumor promoter TPA. *Nature (Lond.).* 272:373–375.
26. Hara, T., L.K.L. Jung, J.M. Bjorndahl, and S.M. Fu. 1986. Human T cell activation. III. Rapid induction of a phosphorylated 28-kD/32-kD disulfide-linked early activation antigen (EA 1) by 12-*o*-tetradecanoyl phorbol-13-acetate, mitogens, and antigens. *J. Exp. Med.* 164:1988–2005.
27. Testi, R., J.H. Phillips, and L.L. Lanier. 1989. Leu23 induction as an early marker of functional CD3/T cell antigen receptor triggering: requirement for receptor crosslinking, prolonged elevation of intracellular [Ca^{++}] and activation of PKC. *J. Immunol.* 142:1854–1860.
28. Marrack, P., and J. Kappler. 1990. The Staphylococcal enterotoxins and their relatives. *Science (Wash. DC).* 248:705–711.
29. Waller, E.K., M.A. Sen, O.W. Kamel, G.A. Hansteen, M.R. Schick, and I.L. Weissman. 1992. Human T-cell development in SCID-hu mice: Staphylococcal enterotoxins induce specific clonal deletions, proliferation, and anergy. *Blood.* 80: 3144–3156.
30. Miyashita, E.M., B. Yang, K.M. Lam, D.H. Crawford, and D.A. Thorley-Lawson. 1995. A novel form of Epstein-Barr virus latency in normal B cells in vivo. *Cell.* 80:593–601.
31. v. Knebel Doeberitz, M., G.W. Bornkamm, and H. zur Hausen. 1983. Establishment of spontaneously outgrowing lymphoblastoid cell lines with cyclosporin A. *Med. Microbiol. Immunol.* 172:87–99.
32. Thorley-Lawson, D.A., L. Chess, and J.L. Strominger. 1977. Suppression of in vitro Epstein-Barr virus infection. A new role for adult human lymphocytes. *J. Exp. Med.* 146:495–508.
33. Moss, D.J., A.B. Rickinson, and J.H. Pope. 1978. Long-term T cell-mediated immunity in man. I. Complete regression of virus-induced transformation in cultures of seropositive donor leukocytes. *Int. J. Cancer.* 22:662–668.
34. Veronese, M.L., A. Veronesi, E. D'Andrea, A. Del Mistro, S. Indraccolo, M.R. Mazza, M. Mion, R. Zamarchi, C. Menin, M. Panozzo, A. Amadori, and L. Chieco-Bianchi. 1992. Lymphoproliferative disease in human peripheral blood mononuclear cell–injected SCID mice. I. T lymphocyte requirement for B cell tumor generation. *J. Exp. Med.* 176:1763–1767.
35. Veronese, M.L., A. Veronesi, L. Bruni, V. Coppola, E. D'Andrea, M.A. Del, S. Mezzalira, M. Montagna, G. Ruffatto, A. Amadori, et al. 1994. Properties of tumors arising in SCID mice injected with PBMC from EBV-positive donors. *Leukemia.* 8(Suppl)1:s214–217.
36. Veronesi, A., V. Coppola, M.L. Veronese, C. Menin, L. Bruni, E. D'Andrea, M. Mion, A. Amadori, and L. Chieco-Bianchi. 1994. Lymphoproliferative disease in human peripheral-blood-mononuclear-cell-injected SCID mice. II. Role of host and donor factors in tumor generation. *Int. J. Cancer.* 59:676–683.
37. Rochford, R., and D.E. Mosier. 1995. Differential Epstein-Barr virus gene expression in B-cell subsets recovered from lymphomas in SCID mice after transplantation of human peripheral blood lymphocytes. *J. Virol.* 69:150–155.
38. Khanna, R., S.R. Burrows, and D.J. Moss. 1995. Immune regulation in Epstein-Barr virus associated diseases. *Microbiol. Rev.* 59:387–405.
39. Moss, D.J., S.R. Burrows, J.D. Baxter, and M.F. Lavin. 1991. T cell-T cell killing is induced by specific epitopes: evidence for an apoptotic mechanism. *J. Exp. Med.* 173:681–686.
40. Uehara, T., T. Miyawaki, K. Ohta, Y. Tamaru, T. Yokoi, S.

Nakamura, and N. Taniguchi. 1993. Apoptotic cell death of primed CD45RO$^+$ T lymphocytes in Epstein-Barr virus-induced infectious mononucleosis. *Blood.* 80:452–458.

41. Le Bon, A., C. Desaymard, and M. Papiernik. 1995. Neonatal impaired response to viral superantigen encoded by MMTV (SW) and Mtv-7. *Int. Immunol.* 7:1897–1903.
42. Saito, I., B. Servenius, T. Compton, and R.I. Fox. 1989. Detection of Epstein-Barr virus DNA by polymerase chain reaction in blood and tissue biopsies from patients with Sjogren's syndrome. *J. Exp. Med.* 169:2191–2198.
43. Sumida, T., F. Yonaha, T. Maeda, E. Tanabe, T. Koike, H. Tomioka, and S. Yoshida. 1992. T cell receptor repertoire of infiltrating T cells in lips of Sjogren's syndrome patients. *J. Clin. Invest.* 89:681–685.
44. Yonaha, F., T. Sumida, T. Maeda, H. Tomioka, T. Koike, and S. Yoshida. 1992. Restricted junctional usage of T cell receptor V beta 2 and V beta 13 genes, which are overrepresented on infiltrating T cells in the lips of patients with Sjogren's syndrome. *Arthritis Rheum.* 35:1362–1367.
45. Sumida, T., T. Sakamaki, F. Yonaha, T. Maeda, T. Namekawa, Y. Nawata, K. Takabayashi, I. Iwamoto, and S. Yoshida. 1994. HLA-DR alleles in patients with Sjogren's syndrome over-representing V beta 2 and V beta 13 genes in the labial salivary glands. *Br. J. Rheumatol.* 33:420–424.
46. Dobrescu, D., B. Ursea, M. Pope, A.S. Asch, and D.N. Posnett. 1995. Enhanced HIV-1 replication in V β 12 T cells due to human cytomegalovirus in monocytes: evidence for a putative herpesvirus superantigen. *Cell.* 82:753–763.
47. Rebai, N., G. Pantaleo, J.F. Demarest, C. Ciurli, H. Soudeyns, J.W. Adelsberger, M. Vaccarezza, R.E. Walker, R.P. Sekaly, and A.S. Fauci. 1994. Analysis of the T-cell receptor beta-chain variable-region (V beta) repertoire in monozygotic twins discordant for human immunodeficiency virus: evidence for perturbations of specific V beta segments in CD4$^+$ T cells of the virus-positive twins. *Proc. Natl. Acad. Sci. USA.* 91:1529–1533.
48. Van Kuyk, R., and D.E. Mosier. 1995. Lack of pseudotype formation between human immunodeficiency virus type 1 and Epstein-Barr virus in productively coinfected B lymphoblastoid cell lines. *Virology.* 209:643–648.
49. Ho, H.N., L.E. Hultin, R.T. Mitsuyasu, J.L. Matud, M.A. Hausner, D. Bockstoce, C.C. Chou, S. O'Rourke, J.M. Taylor, and J.V. Giorgi. 1993. Circulating HIV-specific CD8$^+$ cytotoxic T cells express CD38 and HLA-DR antigens. *J. Immunol.* 150:3070–3079.
50. Lewis, D.E., D.S. Tang, O.A. Adu, W. Schober, and J.R. Rodgers. 1994. Anergy and apoptosis in CD8$^+$ T cells from HIV-infected persons. *J. Immunol.* 153:412–420.
51. Astrin, S.M., and J. Laurence. 1992. Human immunodeficiency virus activates *c*-myc and Epstein-Barr virus in human B lymphocytes. *Ann. NY Acad. Sci.* 651:422–432.
52. Lauener, R.P., S. Huttner, M. Buisson, J.P. Hossle, M. Albisetti, J.M. Seigneurin, R.A. Seger, and D. Nadal. 1995. T cell death by apoptosis in vertically human immunodeficiency virus-infected children coincides with expansion of CD8$^+$/interleukin-2 receptor$^-$/HLA-DR$^+$ T cells: sign of a possible role for herpes viruses as cofactors? *Blood.* 86:1400–1407.