Class I Major Histocompatibility Complex-restricted Cytotoxic T Lymphocytes Specific for Epstein-Barr Virus (EBV) Nuclear Antigens Fail to Lyse the EBV-transformed B Lymphoblastoid Cell Lines against Which They Were Raised

By Ann B. Hill,* Steven P. Lee,‡ John S. Haurum,* Nick Murray,* Qing-Yen Yao,‡ Martin Rowe,‡ Nathalie Signoret,* Alan B. Rickinson,‡ and Andrew J. McMichael*

*From the *Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX39DU; and the ‡Department of Cancer Studies, University of Birmingham Medical School, Birmingham BI52TJ, United Kingdom*

Summary

We have raised CD8$^+$ cytotoxic T lymphocytes (CTL) from three Epstein-Barr virus–seropositive donors by incubating peripheral blood lymphocytes with irradiated autologous B95.8-strain EBV-transformed B lymphoblastoid cells (LCL). However, to detect lysis in a standard ^{51}Cr release assay of the LCL against which these CTL were raised, superinfection with recombinant vaccinia expressing the appropriate EBV protein or incubation with the peptide epitope was necessary. The untreated LCL were not lysed, even though Western blotting demonstrated that they expressed the EBV antigens containing the CTL epitopes. We have found CTL of this phenotype that are restricted by human leukocyte antigen–A2, –A3, –B7, or –B39, and which recognize the EBV latent proteins, EBV nuclear antigen (EBNA)-3A, EBNA-3C, or terminal protein. During these experiments, we identified a new human leukocyte antigen–A3–restricted EBNA-3A epitope, residues 603-611, RLRAEAGVK. We raised a spontaneous LCL, transformed by endogenous EBV, from one donor, but this was also not lysed. For at least one of the epitopes, CTL from another donor lysed the LCL without superinfection or addition of peptides. We conclude that the CTL were unable to achieve a high enough avidity of interaction with untreated LCL to trigger effector function, although the LCL were able to stimulate them to grow in vitro for up to 4 mo. To assess whether a small percentage of the LCL might possess a higher antigen density, we used an assay of tumor necrosis factor release from a CTL clone, which was able to detect antigen-bearing cells representing only 1% of a stimulating LCL population. Nevertheless, the untreated autologous LCL line failed to stimulate tumor necrosis factor release.

EBV is a gamma herpes virus of humans that productively infects epithelial cells, principally in the oropharynx, and establishes latency in B cells (1). Infection usually occurs asymptomatically in childhood or adolescence, but in some individuals a vigorous proliferative cellular immune response results in the clinical syndrome of infectious mononucleosis (IM)[1] (2). Thereafter, the immune response controls but does not eradicate the virus; virus continues to be shed from the oropharynx, probably for life, and latently infected B lymphocytes persist throughout life. Two main types of latent infection of B lymphocytes are recognized (3). In the first, only the latent protein EBV nuclear antigen (EBNA)-1 is expressed and is responsible for maintenance of the EBV episomes in infected cells. In the second, there is expression of all the latent proteins (EBNA-1, -2, -3A, -3B, -3C, -LP, and -LMP1 and 2); these cells proliferate indefinitely in vitro as lymphoblastoid cell lines (LCL), and polyclonal proliferations of EBV-infected B cells with the same pattern of gene expression can occur in vivo in immunosuppressed individuals (2, 3). Most infectious virus production in vivo occurs in the oropharyngeal epithelium, and it is from this site that infection of new hosts is established (2, 3). The oropharyngeal epithelium may be periodically reseeded by circulating latently infected B cells.

The main immune cell responsible for control of EBV in

[1] *Abbreviations used in this paper:* EBNA, EBV nuclear antigen; IM, infectious mononucleosis; LCL, lymphoblastoid cell lines; LD, limiting dilution; LT, Lymphocult T; rVV, recombinant vaccinia virus; SA, soft agar; XLP, X-linked lymphoproliferative syndrome.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/06/2221/08 $2.00
Volume 181 June 1995 2221-2228

vivo is thought to be the CTL (4). EBV-specific CTL can be found in infected individuals in high frequency throughout life, implying a high level of continued virus activity. CTL recognizing each of the EBV latent proteins except EBNA-1 have been identified (5–8) and are thought to perform an immunosurveillance function, destroying immortalized EBV-infected B cells. Thus, in the infected individual, CTL appear to be directed against cells that are the in vivo equivalent of LCL (9).

These CTL can be reactivated in vitro by exposure to LCL. The protocol usually adopted involves culturing PBL from infected individuals in the presence of small numbers of irradiated autologous LCL (5–8). CTL are maintained in vitro for extended periods by restimulation with irradiated autologous LCL in the presence of lymphokines. CTL lines so generated are usually screened for EBV specificity by their ability to lyse LCL in an EBV-specific, HLA class I–restricted manner. The target antigen recognized can then be identified by lysis of EBV-negative HLA-matched cells infected with recombinant vaccinia virus (rVV) expressing the relevant EBV latent genes. However, this protocol often produces lines or clones that either fail to lyse autologous LCL or for which no clear HLA+ virus specificity can be assigned.

Here we report that we have used the standard protocol to induce CTL in vitro, but we extended the screening procedure by testing all clones for lysis of rVV-infected targets regardless of whether or not they lysed LCL. In three individuals, we found that most of the CTL clones we generated showed classic patterns of class I restriction and specificity for EBV latent proteins but failed to lyse the LCL that were used to activate them in vitro.

Materials and Methods

Establishment and Maintenance of EBV-transformed Cell Lines. B95.8-strain EBV–transformed B-LCL were established by culturing PBL with supernatant from the marmoset line B95.8 in the presence of 1 μg/ml cyclosporin A. The endogenous-strain EBV–transformed LCL JB-spont was obtained by spontaneous outgrowth from JB PBL cocultured with cord blood lymphocytes in the presence of cyclosporin A. The origin of the line JB-spont was confirmed as JB rather than the allogeneic cord blood by isoelectric focusing of class I HLA.

CTL Lines and Clones. EBV-specific CTL lines were established as previously described (8). Briefly, PBL (3×10^7) from the EBV-seropositive donor JB were cultured with irradiated (2,000 rad) autologous B95.8-transformed LCL (3×10^5). For polyclonal lines, the cultures were restimulated with autologous LCL at a stimulator to responder ratio of 1:4 on day 11, and 20% supernatant of the T cell line MLA (MLASN) was added as a source of IL-2 on day 14. Thereafter, the lines were restimulated with autologous LCL weekly and maintained in 20% MLASN. Soft agar (SA) oligoclonal lines were established from PBL 3 d after initial stimulation as described (6), and they were maintained long term by weekly restimulation with irradiated autologous B95.8-transformed LCL and cultured in RPMI 1640 supplemented with 20 U/ml rIL-2 (Cetus Corp., Emeryville, CA) and 10% Lymphocult T (LT; Biotest, UK), or 20% MLASN. Limiting dilution (LD) oligoclonal lines and subclones were obtained by culturing CTL from established lines in 96-well plates at 0.3/well in the presence of pooled irradiated (3,000 rad) PBL from three donors (10^6/ml), autologous LCL (10^5/ml), and 0.5 μg/ml PHA (Wellcome Reagent Ltd., Beckenham, UK) ("cloning mix") in a volume of 100 μl. 3 d later, 100 μl of 20% LT was added to each well. After 11 d, growing colonies were transferred to 24-well plates (Costar Corp., High Wycombe, UK) in the presence of 1 ml cloning mix, a further 1 ml of 20% LT was added 3 d later, and the clones/lines were maintained thereafter in 10% LT with weekly restimulation with autologous LCL. Sometimes clones were maintained in 20 U/ml rIL-2 in addition to LT.

Peptide. Peptides were synthesized using Fmoc chemistry and analyzed by HPLC. The EBNA-3A peptide RPPIFIRRL was >95% pure. The biochemical analysis of the EBNA 3C peptides QPRAPIRPI and QPRAPIRPIPT is complicated by cyclization of the amino-terminal glutamine to pyroglutamate, which recurred after HPLC purification. The concentrations quoted here represent the major HPLC peak, corresponding to the native species. QPRAPIRPI is less well recognized in CTL assays than QPRAPIRPIPT (10).

rVV. rVV expressing the EBV latent genes EBNA-3A and EBNA-3C, the kind gift of Dr. E. Kieff (Harvard Medical School, Boston, MA), have been previously described (6, 8).

CTL Assay. LCL were incubated with peptide (10^{-6} M) or rVV (10 PFU per cell) overnight, and then labeled for 1 h with 100 mCi ^{51}Cr, washed three times, and added at 5×10^3 cells per well to round-bottomed 96-well plates with or without CTL as indicated. Maximum ^{51}Cr release was determined by lysis with 2.5% Triton X-100. After 4 h, 20 μl supernatant was harvested and counted on a beta plate counter. Percent specific lysis was calculated as 100× (cpm experimental − cpm medium)/(cpm Triton X-100 − cpm medium).

Detection of EBNA-3A and EBNA-3C. Expression of EBNA-3A and EBNA-3C in LCL was assayed by Western blotting using affinity-purified human antibodies as described previously (11). Briefly, one million cells were solubilized in gel sample buffer and were separated by discontinuous SDS-PAGE using a resolving gel containing 7.5% acrylamide and 0.2% bisacrylamide (12). The resolved proteins were transferred to nitrocellulose by electroblotting, and the filters were then blocked by incubating first with 5% dried skim milk in PBS and then with PBS containing 0.1% Tween 80 detergent. The viral proteins were detected by incubating replicate filters with purified human antibodies to EBNA-3A or EBNA-3C followed by ^{125}I-labeled protein A. The filters were then subjected to autoradiography to visualize specifically bound antibodies.

TNF Release Assay. Where indicated, for use as stimulators, JB B95.8–transformed LCL were incubated overnight with rVV (multiplicity of infection of 10) or peptide. They were then washed three times before adding to CTL. CTL, 10^5/well, were incubated in 96-well round-bottomed plates with stimulators at the indicated ratios for 14 h, after which the plates were spun and supernatant was harvested and stored at −70°C for later assay of TNF.

TNF concentration was measured in 50 μl of supernatant using an ELISA. ELISA plates (Immulon-Dynatech Ltd., Billinghurst, UK) were coated with anti–human TNF-α monoclonal CB006 (Celltech, Slough, UK) in 0.2 M *N,N*-bis(2-hydroxyethyl)-2-amino sulphonic acid, pH 7 (BES) (Sigma Chemical Co., Poole, UK) overnight, and then washed three times with 1% BSA in 0.2 M BES. 50 μl samples or TNF controls were added per well for 1 h at room temperature (RT) and removed, and 50 μl rabbit anti-TNF (Celltech) preincubated for 10–60 min at RT in sample diluent (1% BSA, 2% normal mouse serum in PBS) was added for 1 h at RT. The plates were aspirated again, and 50 μl peroxidase-conjugated donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West

Grove, PA), 1 mg/ml in sample diluent, preincubated at RT for 10–60 min, was added to each well for 30 min at RT. The plates were washed four times with PBS. 100 μl substrate (0.01 mg/ml TMB, 0.01% hydrogen peroxide in 0.1 M Na acetate, pH 5.2; TMB was prepared from 1 mg/ml stock in DMSO, and the TMB/Na acetate was filtered before addition of hydrogen peroxide) was added per well for 30 min, and the reaction was stopped with 2 M H_2SO_4. Plates were read at 450 nm (reference: 63 nm) within 10 min.

Results

Polyclonal and Cloned CTL Specific for EBV Latent Antigens Fail to Lyse EBV-transformed LCL. A polyclonal CTL line from a healthy adult donor, P.G. (HLA-A2, -A3, -B7, -B51), raised by stimulating PBL with irradiated autologous LCL, showed no lysis of autologous LCL. However, when superinfected with rVV expressing EBNA-3A or EBNA-3C, the autologous LCL were lysed efficiently (Fig. 1 *A*). A clone established by LD cloning from this line also failed to lyse autologous LCL, but use of rVV demonstrated that it recognized EBNA-3A restricted through HLA-A3 (Fig. 1 *B*). Using a panel of synthetic peptides covering the entire EBNA-3A sequence followed by prediction of the minimal epitope based on the published motif for HLA-A3–binding peptides (13), the epitope was identified as residues 603-611-RLRAEQVK (Fig. 1 *C*). The clone lysed autologous LCL incubated with this peptide. Further clones from a second bleed of P.G. were established by cloning in SA. Screening of these revealed eight clones with little recognition of the autologous LCL that were specific for EBV terminal protein (TP or LMP2), restricted by HLA-A2. Three examples are shown in Fig. 1 *D*.

EBV-specific CTL from Two Other Donors Also Fail to Lyse Autologous LCL. Five EBV-specific CTL clones from a second donor, J.B. (HLA-A3, -A29, -B7, -B51), which were obtained by cloning in SA or by LD using standard techniques, were investigated extensively; the clones had been kept in continuous culture for up to 4 mo by weekly restimulation with irradiated autologous LCL and lymphokine supplements. All were CD8 positive (data not shown), were restricted by HLA-B7, and were specific for EBNA-3A 379-387 (RPPIFIRRL) or EBNA-3C 881-9 (QPRAPIRPI) (10). Each clone showed little or no lysis of the LCL with which it was stimulated, unless the LCL had been exposed to the appropriate peptide or rVV (Fig. 2, *A–E*). Finally, clones from a third donor, A.B. (HLA-A2, -A3, -B39, -B51), whose polyclonal CTL line showed no clear preference for autologous LCL, were also tested using rVV. These clones also failed to lyse autologous LCL but were specific for either EBNA-3A or EBNA-3C, restricted through HLA-B39 (Fig. 2 *F*).

Neither Poor Expression of EBNA-3A and EBNA-3C Nor Strain Variation between B95-8 and Endogenous EBV Account for the Failure to Lyse LCL. We addressed the possibility that EBNA-3A and EBNA-3C were poorly expressed in the LCL that failed to be lysed. A Western blot of autologous LCL from the three CTL donors demonstrated expression of EBNA-3A and EBNA-3C (Fig. 3). Clinical isolates of EBV usually have EBNA proteins that differ in sequence from that of the prototype laboratory B95.8 strain (14), and the differences can affect CTL recognition (15). To exclude the possibility that the JB CTL were specific for an epitope in J.B.'s endogenous EBV that cross-reacted only weakly with the B95.8 strain, a spontaneous LCL, transformed with the endogenous EBV strain, was established. As is usual for clinical isolates, this line (JB-spont) showed EBNA of different molecular weights than the laboratory B95.8 strain (Fig. 3). However, Fig. 4 shows that JB-spont was not lysed by either EBNA-3A or EBNA-3C–specific CTL, although it could be lysed in the presence of the appropriate peptide or recombinant vaccinia. The same result was seen with clones JBLDN (EBNA-3C specific) and JBLD1.4 and JBSA58.1 (EBNA-3A specific) (data not shown).

The Same Epitope Is Recognized by CTL That Lyse Autologous LCL and by CTL That Fail to Lyse Autologous LCL. We had previously identified a donor whose CTL recognized EBNA-3C, restricted by HLA-B7, which lysed autologous and other HLA-B7–positive LCL without addition of peptide or rVV (Fig. 5 *A*). A polyclonal CTL line that had been established from this donor was screened on overlapping 15-mer peptides covering the entire EBNA-3C sequence and it was found to recognize two overlapping 15 mer that contained the epitope 881-891 (data not shown). This specificity was confirmed by lysis of HLA-B7–positive PHA blasts incubated with peptide EBNA-3C 881-891 (Fig. 5 *B*). No further studies could be performed with these cells, but this assay demonstrated that the same epitope could be recognized by CTL from two donors, one with the ability to lyse LCL, the other unable to lyse LCL without additional antigen. Therefore, nonrecognition of LCL is not a property of the epitope involved.

A TNF Release Assay That Can Detect 1% of LCL Bearing the Appropriate Antigen Is Not Stimulated by Untreated LCL. We were puzzled by the ability of the LCL to stimulate CTL activation and proliferation without being susceptible to lysis by the same CTL clone. We hypothesized that a fraction of the cell line, too small to be detected in the killing assay, might express adequate amounts of antigen to stimulate CTL. To assess this possibility, we studied antigen-specific TNF release by the clone LD1.4. The results are shown in Fig. 6. Uninfected JB-B95.8 LCL, JB LCL infected with irrelevant vaccinia, and the mismatched LCL basilio caused no TNF release up to a stimulator (target) to responder (CTL) ratio of 10:1. JB LCL infected with VV-EBNA-3A stimulated TNF release that was detectable at a stimulator:responder (S:R) ratio of <0.1. Even when VV-EBNA-3A–infected JB cells were mixed with varying numbers of the nonstimulatory basilio LCL cells so that the total LCL/CTL ratio remained fixed at 10:1, a VV-EBNA-3A–infected JB/CTL ratio of 0.1 still caused TNF release. Thus, in this assay, the CTL could detect antigen expressed at the density given by VV-EBNA-3A infection when present in <1% of the stimulating LCL population. Comparison with TNF release stimulated by LCL incubated with peptide from 10^{-5} M to 10^{-10} M (Fig. 6 *B*) indicated that the density of antigen achieved by VV-EBNA-3A infection was equivalent to that achieved by overnight incubation with $\sim 10^{-5.5}$ M peptide. We conclude that <1% of the untreated LCL population can express the peptide epitope at this density.

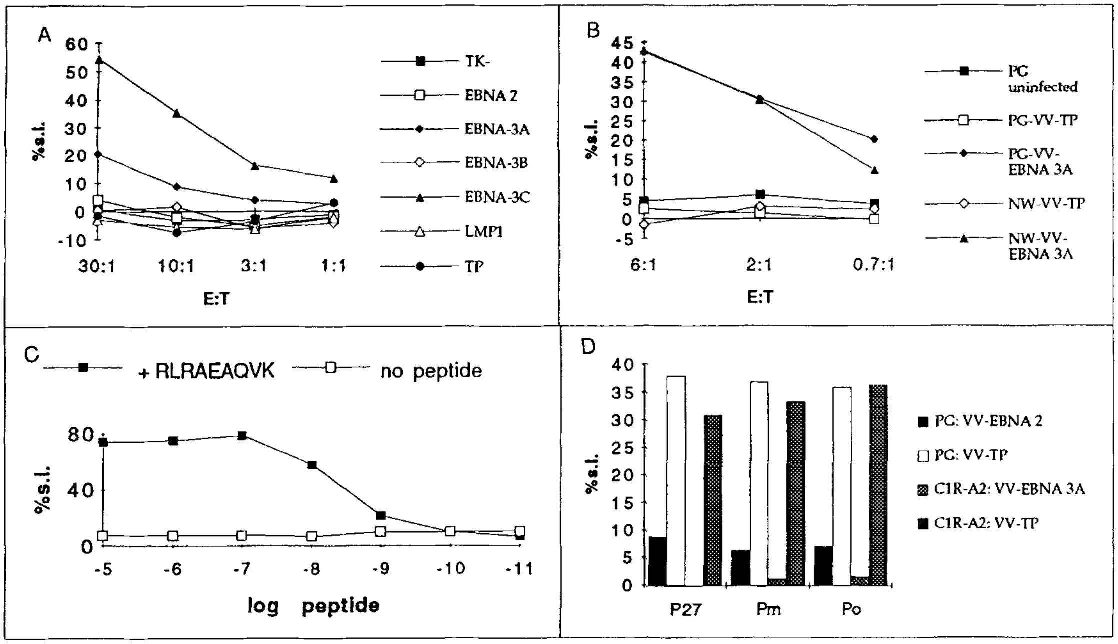

Figure 1. Polyclonal and cloned EBV-specific CTL from donor P.G. were able to lyse the autologous LCL only if they were infected with rVV expressing the appropriate EBV protein or incubated with peptide. (*A*) CTL assay of polyclonal CTL line from donor P.G. (HLA-A2, -A3, -B7, -B51) tested against autologous B95.8 LCL infected with irrelevant VV (thymidine kinase negative) or rVV expressing the EBV latent proteins indicated. (*B*) A CTL clone (PG1) failed to lyse uninfected autologous LCL but lysed autologous LCL or LCL matched only at HLA-A3 (NW:HLA-A3, -Aw33, -B8, -B2702) superinfected with VV-EBNA-3A. (*C*) Lysis by the clone PG1 of autologous LCL incubated with the peptide EBNA-3A 603-611 (RLRAEAQVK), E/T 5:1. (*D*) A second series of clones raised in SA from P.G. recognized autologous LCL only if superinfected with VV-TP; lysis of the transfectant C1R-A2 (HLA-A2, -Cw4) infected with VV-TP identified HLA-A2 as the restriction element.

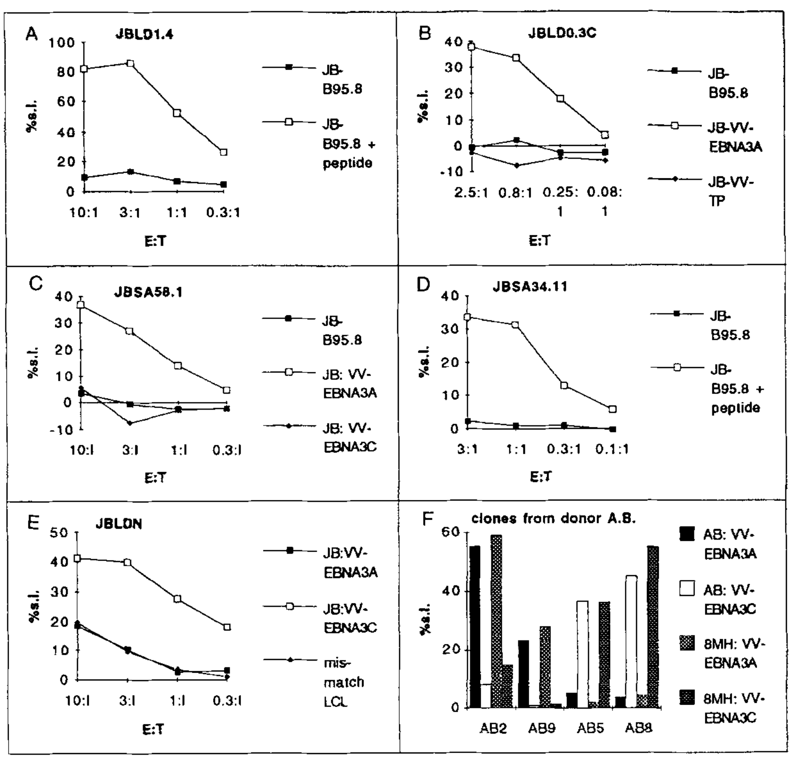

Figure 2. EBV-specific CTL from two other donors recognizing four other epitopes also failed to lyse autologous LCL. (*A–E*) Five clones raised from the donor J.B. by LD or by cloning in SA were specific for EBNA-3A 379-387 (RPPIFIRRL) (*A–C*) or EBNA-3C 881-9 (QPRAPIRPI) (*D* and *E*), restricted through HLA-B7. Each required either superinfection with rVV or incubation with the peptide epitope to lyse autologous LCL (JB-B95.8). (*F*) Clones derived by SA cloning from a third donor, A.B. (A2, A3, B39, B51), failed to lyse untreated autologous LCL but lysed autologous LCL or LCL matched only at HLA-B39 (8MH) infected with VV-EBNA-3A (clones AB2 and AB9) or VV-EBNA-3C (clones AB5 and AB8).

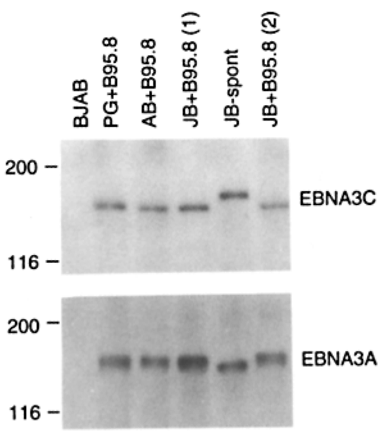

Figure 3. EBNA-3A and EBNA-3C expression in autologous B95.8-transformed and spontaneous LCL used in this study. Western blot of EBNA-3A and EBNA-3C in the autologous LCL used in this study that were not lysed by EBV-specific CTL shows normal levels of EBNA expression. The different molecular weight of EBNA-3A and -3C in the spontaneously arising LCL JB-spont confirm the endogenous origin of the virus responsible for transformation of this line.

JB EBNA-3A–specific Clones Show a Prominent Allo Cross-reactivity. Alloreactivity without apparent EBV specificity is a prominent feature of the CTL response to EBV and a striking feature of the CTL found in infectious mononucleosis (16, 17). In Fig. 7, an assay with JBLD1.4 shows lysis of three unrelated allo targets. The other EBNA-3A 379-387–specific clones from J.B. displayed the same pattern of alloreactivity (data not shown). Although a family study showed that the allospecificity was clearly encoded in the MHC, the antigen recognized has not been identified (data not shown). These results indicate that the common finding of alloreactivity without apparent EBV specificity in CLT-stimulated EBV may after all be due to classical class I–restricted EBV-specific CTL; however, the EBV specificity of the CTL may remain cryptic unless the antigen density is raised by superinfection with appropriate rVV or by exogenous peptide.

Discussion

We have found in three EBV-seropositive individuals CTL that fail to lyse the autologous CTL against which they were

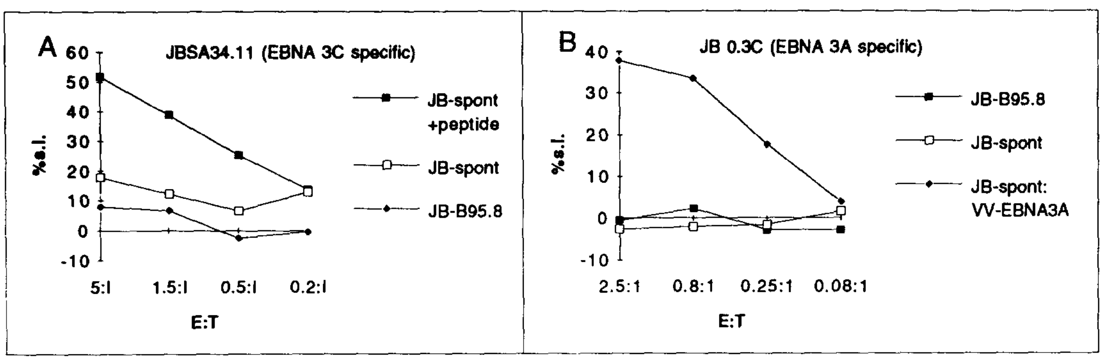

Figure 4. The spontaneously arising LCL from J.B. are also not lysed by EBNA-3A or EBNA-3C–specific CTL clones. The LCL JB-spont was derived by spontaneous outgrowth from PBL, indicating transformation by endogenous EBV. It was not lysed by the EBNA-3C–specific clone JBSA34.11 (*A*) unless incubated with the peptide QPRAPIRPIPT, or by the EBNA-3A–specific clone JB0.3C (*B*) unless infected with VV-EBNA-3A.

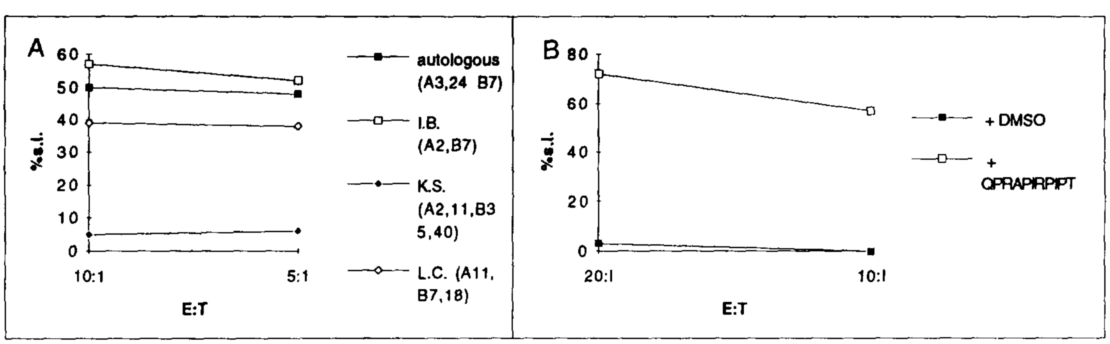

Figure 5. CTL from another donor that are able to lyse LCL also recognize EBNA-3C 881-891. A polyclonal line from the donor C.F. lysed autologous LCL, and all other LCL tested matched at HLA-B7. The assay shows lysis of PHA-blasts matched at HLA-B7 and demonstrates recognition of the epitope EBNA-3C 881-889.

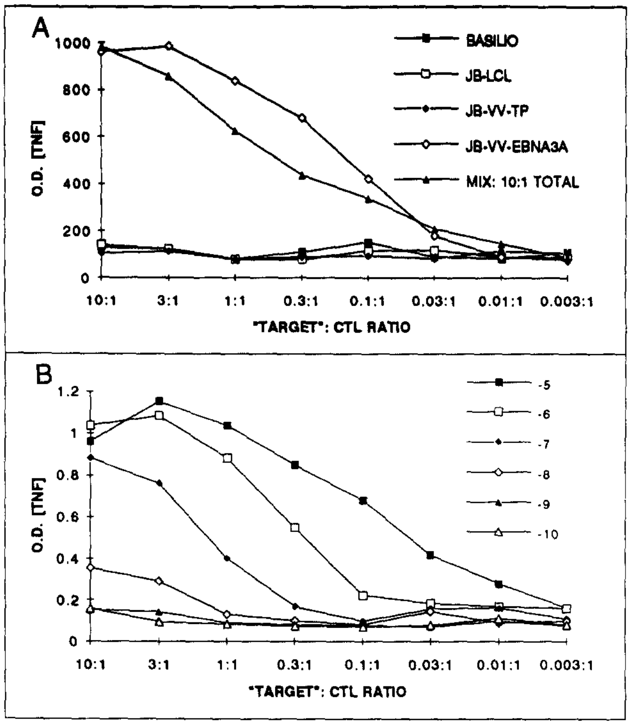

Figure 6. A TNF release assay can detect 1% of LCL bearing the appropriate antigen but is not stimulated by untreated LCL. TNF release by the clone JBLD1.4 (EBNA-3A specific) was used as an indicator of antigen-specific CTL-target (stimulator) interaction. (*A*) 10^5 JBLD1.4 CTL per well were incubated with mismatched LCL (basilio: HLA-A2, -B51), JB-B95.8 LCL, or JB-B95.8 LCL infected with irrelevant vaccinia (VV-TP), or VV-TP at stimulator (target)/CTL ratios indicated. The concentration of TNF release by the CTL was measured by ELISA and OD, proportional to TNF concentration, plotted against target/CTL ratio. Only LCL infected with VV-EBNA-3A caused TNF release at target/CTL ratios of up to 10:1. Basilio LCL were mixed with JB LCL infected with VV-EBNA-3A such that the total target (JB-VV-EBNA-3A + basilio)/CTL ratio was 10:1. The ratio plotted is that of JB-VV-EBNA-3A/CTL (*MIX 10:1*). This indicated that JB-VV-EBNA-3A caused specific TNF release even when it was present as only 1% of the target population. (*B*) JB LCL were incubated with peptide RPPIFIRRL overnight at concentrations from 10^{-5}–10^{-10}, washed three times, and used in the assay as above. Comparison of the curves in *A* and *B* indicates that infection with VV-EBNA-3A caused equivalent TNF release to that achieved by incubation overnight with $\sim 10^{-5.5}$ M peptide.

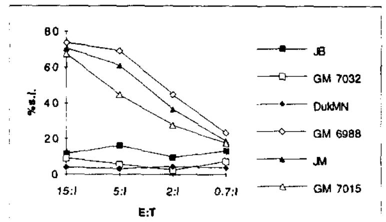

Figure 7. JBLD1.4 lyses mismatched LCL because of a cross-reactive allospecificity. JB LD1.4 was tested for lysis against a panel of LCL: autologous (JB), GM7032 (HLA-A1, -B8, -B52), DukMN (HLA-A2, -Aw33, -B51, -B53), GM6988 (HLA-A2, -B13, -B51), JM (HLA-A2, -B15, -B51), GM7015 (A1, A39, B52, B60). Lysis of the HLA-B7-negative LCL GM6988, JM, and GM7015 demonstrates that JBLD1.4 recognizes a cross-reactive allospecificity.

raised and repeatedly restimulated for long-term culture. These CTL display clear class I restriction and EBV specificity, and in one case we have shown that the specificity recognized is the same as that of "classic" EBV-specific CTL from another donor that are able to lyse the LCL. One previous example of CTL raised against an endogenous LCL that was unable to lyse the B95.8 strain–transformed LCL was shown to be caused by sequence difference in the epitope between B95.8 and the endogenous virus (15, 18). We have shown that this is not the explanation for our findings, as the LCL generated by transformation with J.B.'s endogenous virus were also not lysed by the JB clones specific for two different epitopes. This finding is also not due to intrinsic resistance to lysis by the LCL, as they were easily lysed when antigen concentration was increased by addition of peptide or recombinant vaccinia. For the same reason, the phenomenon cannot be attributed to poor lytic ability of the CTL, nor was there an absolute absence of antigen in the LCL, as expression of both EBNA-3A and -3C was detected by Western blot. Similarly, the level of the adhesion molecules ICAM-1, ICAM-3, and LFA-1 was typically high on the unlysed LCL and was not increased by infection with rVV (data not shown). We conclude that the avidity of interaction between native LCL and these CTL was inadequate to reach the threshold required to trigger effector function in the CTL. Alteration of either side of the avidity equation could lead to lysis. Increasing the antigen density on the LCL with peptide or rVV led to target lysis by the JB clones. Alternately, the untreated LCL could be lysed by CTL from another donor whose CTL displayed a higher avidity, presumably achieved either by a higher affinity TCR or by a higher level of TCR expression. High avidity CTL could not be generated from JB PBL even with an efficient peptide restimulation protocol; although this protocol efficiently restimulated CTL that were able to lyse targets treated with the appropriate peptide, they were nevertheless unable to lyse untreated LCL (data not shown).

The finding of CTL that have been raised against untreated LCL but are unable to lyse them is unexpected because it is in contrast to the general assumption made by immunologists that the antigen density required to trigger T cell activation should be higher than that required to trigger T cell effector functions, such as cytolysis and lymphokine release. We therefore postulated that a small percentage of the LCL may at any given time express a high enough density of antigen to trigger CTL activation and proliferation. Because a killing assay is insensitive if <10–20% of the cells express the antigen, we developed a cytokine release assay that was able to detect TNF release from the CTL clone when 1% of the stimulating LCL displayed the correct antigen (in the form of rVV). We found that the LCL did not cause TNF release in this assay unless they had been exposed to peptide or rVV. Increasing peptide concentration caused progressively more TNF release, evidenced by a shift to the right in the curve of the plot of TNF release vs S:R ratio (Fig. 6 *B*). Comparison of Fig. 6 *A* and *B* leads to the conclusion that the antigen density achieved by infection with rVV was equivalent to that achieved by incubating the cells overnight with $10^{-5.5}$ M peptide. Thus, we conclude that <1% of the LCL displayed antigen at the density equivalent to that achieved by exposure to $10^{-5.5}$ M peptide. We cannot, however, exclude the possibility that an even smaller percentage of the LCL displayed antigen at this concentration, or that a lower concentration expressed by more cells was sufficient to stimulate CTL.

Our findings have caused us to reexamine the assumption that a higher antigen density is required for T cell activation than is needed to trigger effector functions of cytolysis and lymphokine release. The assumption is based in part on teleology: It seems to make no sense that the immune system should allow activation of effector cells that are unable to exert their effector function. Thus, although T cell activation requires a second signal delivered prototypically through the B7/CD28 interaction in addition to the signal delivered though the antigen-specific TCR, we would still assume that the antigen-specific part of signaling for T cell activation would require a higher avidity of interaction with target than is required for effector functions. This is probably true for the initial activation of naive CTL precursors. To activate primary in vitro CTL with peptide-loaded antigen presentation–deficient T2 or RMAS cells, Melief and co-workers found that several logs higher concentration of peptide was required than was necessary to sensitize the T2 cells for lysis (19, 19a). The phenotype of the cell that activated the naive precursors of our CTL in vivo is not known and may differ in antigen density from the in vitro LCL; thus, we can draw no conclusions about the initial activation of our CTL. The evidence is less clear about the antigen density requirement for reactivation of memory CTL and for their continued propagation. The most relevant data comes from cross-reactive systems, where CTL have been activated in vivo and then restimulated in vitro with either a cross-reactive alloligand or a so-called "antagonist" peptide. Both types of ligands are assumed to present a lower affinity interaction to the TCR. The data are contradictory. CD4 clones were able to exert effector functions of lymphokine release and cytolysis but did not proliferate in response to an "antagonist" peptide (20). We have observed the opposite situation with $CD8^+$ CTL specific for an HIV peptide. Here an antagonist peptide could reactivate the CTL in vitro but was not able to sensitize targets for lysis (McAdam, S., P. Klenerman, L. Tussey, S. Rowland-Jones, D. Lalloo, R. Phillips, A. Edwards, P. Giangrande, A. L. Brown, F. Gotch, et al., manuscript submitted for publication; and Klenerman, P., U.-C. Meier, R. Phillips, and A. J. McMichael, manuscript submitted for publication). A similar situation was observed previously for vesicular stomatitis virus–specific CTL that could be cross-reactively reactivated by $H\text{-}2K^{bm8}$–expressing stimulators but did not lyse $H\text{-}2K^{bm8}$–expressing targets (21). Again, the inverse situation (ability of the cross-reactive ligand to trigger lysis but not reactivation) has also been reported (22). None of these experimental situations corresponds exactly to ours in that, in each case, the presumed lower avidity antigenic interaction is achieved by a qualitatively different ligand rather than by an alteration in ligand density. Furthermore, the involvement of high MHC density dendritic cells in stimulation but not in the lysis assay can often not be excluded. Nevertheless, we believe that the most likely interpretation of our data is that EBV-specific CTL clones were reactivated in vitro from memory CTL by an antigenic density on the LCL that was insufficient to trigger lysis.

We regard the major implication of this study as being for the investigation of the pathogenesis and control of infection by EBV. The methods usually used to detect EBV-specific CTL do not identify CTL of this phenotype. Acute symptomatic EBV infection, both in IM and in the usually fatal X-linked lymphoproliferative syndrome (XLP), is characterized by a marked expansion and activation of $CD8^+$ CTL, many of which lyse allogeneic cells in an EBV-independent, class I–dependent manner (16, 17, 23). Because autologous LCL do not competitively inhibit this lysis, it has been assumed that the alloreactive CTL in IM are the result of a nonspecific, antigen-independent "bystander" activation of $CD8^+$ T cells (17). Massive "nonspecific" activation of CD8 T cells is considered a hallmark of symptomatic EBV infection, and it has been thought that these may play a pathogenic role in XLP, with the concomitant suggestion that they be controlled by cytotoxic drug therapy (23). We report here that EBV-specific CTL frequently fail to lyse LCL in vitro and, furthermore, that some of these CTL clones exhibit alloreactivity highly reminiscent of that described in IM and XLP. We suggest that some or all of the alloreactive and other "nonspecifically activated" CTL seen in symptomatic EBV infection may after all be EBV specific but require additional antigen to demonstrate their specificity in vitro.

We do not know whether the in vivo T cells from which our CTL lines were derived are able to eliminate EBV-transformed B cells in vivo, although we note that our three donors are healthy and asymptomatic. The usual procedures used to screen for EBV-specific CTL would have precluded the identification of CTL displaying this phenotype. We predict that CTL of this phenotype could be identified in many EBV-seropositive donors. It is possible that screening for these CTL will demonstrate that the majority of the expanded activated $CD8^+$ T cell population in acute symptomatic EBV infection is in fact specific for EBV antigens.

We are very grateful to the three donors whose CTL are described in this paper, to S. Rowland-Jones for the gift of several CTL clones, and to M. Masucci and A. Bandeira for helpful discussions.

This work was supported by the Medical Research Council, United Kingdom, the Imperial Cancer Research Fund, and the Cancer Research Campaign.

Address correspondence to Dr. Ann B. Hill, Center for Cancer Research, Massachusetts Institute of Technology, 40 Ames Street, E17-322, Cambridge, MA 02139.

Received for publication 15 September 1994 and in revised form 26 January 1995.

References

1. Kieff, E., and D. Liebowitz. 1990. Epstein-Barr virus and its replication. *In* Virology. B.N. Fields, D.M. Knipe, R. Chanock, M. Hirsch, T. Melnick, T. Monath, and B. Roizman, editors. Raven Press, New York. 1889–1920.
2. Miller, G. 1990. Epstein-Barr virus biology, pathogenesis and medical aspects. *In* Virology. B.N. Fields, D.M. Knipe, R. Chanock, M. Hirsch, T. Melnick, T. Monath, and B. Roizman, editors. Raven Press, New York. 1921–1958.
3. Klein, G. 1994. Epstein-Barr virus strategy in normal and neoplastic B cells. *Cell.* 77:791–793.
4. Wallace, L.E., R.J. Murray, S.P. Lee, and A.B. Rickinson. 1993. Cell-mediated immunity to Epstein-Barr virus. *In* Viruses and the Cellular Immune Response. D.B. Thomas, editor. Marcel Dekker, Inc., New York. 447–472.
5. Burrows, S.R., T.B. Sculley, I.S. Misko, C. Schmidt, and D.J. Moss. 1990. An Epstein-Barr virus–specific cytotoxic T cell epitope in EBV nuclear antigen 3 (EBNA 3). *J. Exp. Med.* 171:345–349.
6. Khanna, R., S.R. Burrows, M.G. Kurilla, C.A. Jacob, I.S. Misko, T.B. Sculley, E. Kieff, and D.J. Moss. 1992. Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. *J. Exp. Med.* 176:169–176.
7. Gavioli, R., M.G. Kurilla, P.O. de Campos-Lima, L.E. Wallace, R. Dolcetti, R.J. Murray, A.B. Rickinson, and M.G. Masucci. 1993. Multiple HLA A11-restricted cytotoxic T-lymphocyte epitopes of different immunogenicities in the Epstein-Barr virus-encoded nuclear antigen 4. *J. Virol.* 67:1572–1578.
8. Murray, R.J., M.G. Kurilla, J.M. Brooks, W.A. Thomas, M. Rowe, E. Kieff, and A.B. Rickinson. 1992. Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus (EBV): implications for the immune control of EBV-positive malignancies. *J. Exp. Med.* 176:157–168.
9. Klein, G., E. Svedmyr, M. Jondal, and P.O. Persson. 1976. EBV-determined nuclear antigen (EBNA)-positive cells in the peripheral blood of infectious mononucleosis patients. *Int. J. Cancer.* 17:21–26.
10. Hill, A.B., A. Worth, T. Elliott, S. Rowland-Jones, J. Brooks, A. Rickinson, and A. McMichael. 1995. Characterisation of two Epstein-Barr virus epitopes restricted by HLA-B7. *Eur. J. Immunol.* 25:18–24.
11. Rowe, M., L.S. Young, K. Cadwallader, L. Petti, E. Kieff, and A.B. Rickinson. 1989. Distinction between Epstein-Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA 3 family of nuclear proteins. *J. Virol.* 63:1031–1039.
12. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
13. Di Brino, M., K.C. Parker, J. Shiloach, M. Knierman, J. Lukszo, R.V. Turner, W.E. Biddison, and J.E. Coligan. 1993. Endogenous peptides bound to HLA-A3 possess a specific combination of anchor residues that permit identification of potential antigenic peptides. *Proc. Natl. Acad. Sci. USA.* 90:1508–1512.
14. Sample, J., L. Young, B. Martin, T. Chatman, E. Kieff, A. Rickinson, and E. Kieff. 1990. Epstein-Barr Virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. *J. Virol.* 64:4084–4092.
15. Apolloni, A., D. Moss, R. Stumm, S. Burrows, A. Suhrbier, I. Misko, C. Schmidt, and T. Sculley. 1992. Sequence variation of cytotoxic T cell epitopes in different isolates of Epstein-Barr virus. *Eur. J. Immunol.* 22:183–189.
16. Strang, G., and A.B. Rickinson. 1987. Multiple HLA class I-dependent cytotoxicities constitute the "non-HLA-restricted" response in infectious mononucleosis. *Eur. J. Immunol.* 17: 1007–1013.
17. 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.
18. Misko, I.S., C. Schmidt, M. Honeyman, T.D. Soszynski, T.B. Sculley, S.R. Burrows, D.J. Moss, and K. Burman. 1992. Failure of Epstein-Barr virus-specific cytotoxic T lymphocytes to lyse B cells transformed with the B95-8 strain is mapped to an epitope that associates with the HLA-B8 antigen. *Clin. Exp. Immunol.* 87:65–70.
19. Houbiers, J.G.A., H.W. Nijman, S.J. van der Burg, J.W. Drijfhout, C.J.V. van der Velde, A. Brand, F. Momburg, W.K. Kast, and C.J.M. Melief. 1993. *In vitro* induction of human cytotoxic T lymphocyte responses against peptide of mutant and wild-type p53. *Eur. J. Immunol.* 23:2072–2077.

19a. De Bruijn, M.L., T.N. Schumacher, J.D. Nieland, H.L. Ploegh, W.M. Kast, and C.J. Melief. 1991. Peptide loading of empty major histocompatibility complex molecules on RMF-S cells allows the induction of primary cytotoxic T lymphocyte responses. *Eur. J. Immunol.* 21:2963–2970.

20. Evavold, B.D., and P.M. Allen. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science (Wash. DC).* 252:1308–1310.
21. Sheil, J.M., M.J. Bevan, and L. Lefrancois. 1987. Characterization of dual-reactive H-2K^b-restricted anti-vesicular stomatits virus and alloreactive cytotoxic T cells. *J. Immunol.* 138:3654–3660.
22. Müllbacher, A., and R.V. Blanden. 1979. Cross-reactivity patterns of murine cytotoxic T lymphocytes. *Cell. Immunol.* 43:70–81.
23. Sullivan, J.L., and B.A. Woda. 1989. X-linked lymphoproliferative syndrome. *Immunodefic. Rev.* 1:325–347.