An Alloresponse in Humans is Dominated by Cytotoxic T Lymphocytes (CTL) Cross-reactive with a Single Epstein-Barr Virus CTL Epitope: Implications for Graft-Versus-Host Disease

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Summary

The phenomenon of T cell allorecognition is difficult to accommodate within the framework of a T cell repertoire positively selected in the thymus, unless allorecognition results from the cross-reactions of self-major histocompatibility complex restricted T cells. Herein, we demonstrate the dual specificity of cytotoxic T lymphocyte (CTL) clones for the immunodominant Epstein-Barr virus (EBV) epitope FLRGRAYGL, presented on HLA-B8, and the alloantigen HLA-B*4402. CTL which recognized peptide FLRGRAYGL in association with HLA-B8 could be reactivated in vitro from healthy individuals who had been exposed previously to EBV, using stimulator cells expressing the cross-reacting alloantigen HLA-B*4402. Limiting dilution analysis of the alloresponse to HLA-B*4402 in eight healthy individuals revealed that HLA-B8$^+$, EBV-sero$^+$ donors had higher CTL precursor frequencies for alloantigen HLA-B*4402 than EBV-sero$^-$ control donors. It is surprising that the majority (65–100%) of anti-HLA-B*4402 CTL, generated in limiting dilution mixed lymphocyte reactions between responder cells from HLA-B8$^+$, EBV-sero$^+$ individuals and HLA-B*4402$^+$ stimulators, also recognized the EBV CTL epitope FLRGRAYGL/HLA-B8. In contrast to previous studies showing extensive diversity in the T cell repertoire against individual alloantigens, these data demonstrate that the response to an alloantigen can be dominated by CTL cross-reactive with a single viral epitope, thus illustrating a possible mechanism for the frequent clinical association between herpesvirus exposure and graft-versus-host disease after bone marrow transplants.

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The response of T lymphocytes to intraspecies allelic variants of MHC molecules, namely the alloresponse, is displayed in vitro in the mixed lymphocyte reaction and in vivo during allograft rejection and GVHD. It is now clear that, in most cases, T cell recognition of alloantigens involves both the allo-MHC molecule and its associated peptide ligand (1). The vigor of the primary immune response to allogeneic MHC molecules is consistent with the high precursor frequency of T cells that respond to these molecules (2). Several models have been proposed to explain the high frequency of alloreactive T cells (1). The model first proposed by Matzinger and Bevan (3) is perhaps the easiest to incorporate within the framework of current knowledge of antigen presentation. It proposed that a single MHC product can interact with many different cellular proteins and that each individual "binary complex" can be recognized by a different T cell clone. Thus the variety of antigenic peptides processed from possibly hundreds of cellular and serum proteins and presented for allorecognition explains the magnitude of the response. This theory is supported by studies, mostly of murine alloresponses, showing extensive diversity in the TCR repertoire against individual alloantigens (4–7).

Responsiveness to foreign MHC molecules appears to violate the established dogma that only self-MHC–restricted T cells are positively selected in the thymus. It has therefore been suggested that the allospecific T cell repertoire overlaps with the repertoire which recognizes antigen in the context of self-MHC (1, 2). That is, allorecognition may result from molecular mimicry, whereby the allogeneic MHC molecule can resemble the self-MHC complexed with a nominal antigen. Several examples of CTL clones with dual specificity of this kind support this contention (8). Analyses of multiple cloned T cell populations have demonstrated that between 20 and 60% of antigen-specific, MHC-restricted T cell clones cross-react with alloantigens (9, 10). It has also been shown that approximately half of a "primary" alloresponse is contributed by previously primed T cells, which by definition, must be self-MHC restricted (11, 12).

Two previous studies have demonstrated CTL with dual specificity for autologous EBV-transformed cell lines and HLA

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Volume 179 April 1994 1155–1161

alloantigens (13, 14). EBV is a herpesvirus that latently infects more than 90% of all humans. In developed countries, primary infection with EBV is often delayed until adolescence, when it is usually clinically evident as infectious mononucleosis (15). The virus persists systemically as a latent infection in B cells and is apparently under the immune control of EBV-specific CTL that recognize epitopes derived from an array of latent viral proteins (15). Studies of in vitro–reactivated CTL have demonstrated very high frequencies of EBV-specific memory T cells in PBMC from normal asymptomatic EBV-sero$^+$ individuals of between 1/3,000 and 1/400 (16), precursor frequencies that are not dissimilar to those estimated for allospecific CTL in humans (17). This report describes the antigen mimicry between HLA-B*4402 as an alloantigen, and an immunodominant EBV-encoded CTL epitope that binds to HLA-B8 and that has been mapped to a nine–amino acid peptide from the EBV nuclear antigen EBNA 3 (18). We demonstrate that the response to an alloantigen can be dominated by CTL cross-reactive with a single EBV epitope which contrasts with previous studies which concluded that alloresponses are heterogenous. This illustration that memory T cells to a herpesvirus can significantly influence an alloresponse is discussed with reference to previous studies establishing herpesvirus exposure as an important risk factor for GVHD after bone marrow transplant.

Materials and Methods

Establishment and Maintenance of Cell Lines. CTL clones LC13 and SC17 were isolated from EBV-sero$^+$ donors LC (HLA-A1, -, B8,B18,DR3,DR11) and SC (HLA-A1,A31,B8,B51,DR3,DR4) after in vitro stimulation of fresh PBMC with the autologous lymphoblastoid cell lines (EBV-LCL)[1] transformed with EBV derived from the IARC-BL74 cell line (18). Both CD8$^+$ clones are HLA-B8 restricted and recognize the EBV CTL epitope FLRGRAYGL (19).

PHA blasts were generated by stimulating PBMC with PHA (Commonwealth Serum Laboratory, Melbourne, Australia) and after 3 d, growth medium (10% FCS/RPMI 1640) containing MLA-144 supernatant and rIL-2 (20, 21) was added. PHA blasts were propagated with biweekly replacement of rIL-2 and MLA-144 supernatant (PHA free) for up to 8 wk.

EBV-LCL were established by exogenous A-type EBV transformation of peripheral B cells and maintained in growth medium (22). All cell lines were regularly screened for mycoplasma contamination. Blood donors used in this study were healthy laboratory staff and were tested for previous EBV exposure using standard methods to detect IgG antibody to the EBV capsid antigen (viral capsid antigen) (23).

Cytotoxicity Assay. CTL clones were screened in the standard 5-h chromium release assay (E/T ratio 1:1 unless otherwise stated) (18). In some experiments, ^{51}Cr-labeled PHA blast targets were incubated for 45 min with or without peptide epitope FLRGRAYGL (50 μg/ml) before effectors were added. The peptide was synthesized using the simultaneous multiple peptide synthesis technique originally described by Houghten (24). In some experiments, target cells were incubated with mAb to HLA class I and II before CTL addition (W6/32, American Type Culture Collection (ATCC) HB-95 and antibody 2.06, ATCC HB-104; final dilution of 1/20 of ascites fluid). For cold target inhibition assays, varying numbers of unlabeled targets were added to labeled targets before the addition of effector cells.

Polyclonal CTL populations were also used as effectors. These were generated by culturing PBMC with γ-irradiated (2,000 rad) PBMC from an HLA-mismatched donor (responder/stimulator ratio 2:1) for 7 d. At this time, the stimulated T cells were harvested by E-rosetting and used in the standard 5-h chromium release assay (E/T ratio 20:1). In these experiments, peptide FLRGRAYGL was washed off target cells before plating out so that the large number of effector cells would not bind peptide and compete with lysis of ^{51}Cr-labeled targets.

Limiting Dilution Analysis. PBMC were distributed in graded numbers from 5×10^3 to 5×10^4 cells per well (EBV-sero$^-$ responders) or from 2.5×10^3 to 2×10^4 cells per well (EBV-sero$^+$ responders) in round-bottomed microtiter plates. Approximately 5×10^4 γ-irradiated (2,000 rad) PBMC from a HLA-B*4402$^+$ donor (SJ: HLA-A2, A3, B7, B*4402, DR1, -) were added to give a total volume of 100 μl. 24 or 36 replicates were used at each concentration in each experiment, and an equal number of control wells were set up in which no stimulator cells were added. Cultures were fed on days 4 and 7 with 50 μl of medium supplemented with 20 U of rIL-2 and 30% (vol/vol) supernatant from MLA-144 cultures. On day 10, each CTL microculture was split into three replicates and used as effectors in a standard 5-h ^{51}Cr-release assay against HLA-B8$^+$ PHA blasts (SC PHA blasts: HLA-A1,A31,B8,B51,DR3,DR4), precoated with peptide FLRGRAYGL or left uncoated, and HLA-B*4402$^+$ PHA blasts (PW PHA blasts: HLA-A1, A28, B8, B*4402, DR3, DR6). Wells were scored as positive when the percent specific chromium release exceeded the mean release from control wells by 3 SD. Limiting dilution analysis (LDA) was performed by the method of maximum likelihood estimation (25). Data from all experiments were compatible with the hypothesis of single-hit kinetics (p >0.4) and precursor estimates are given with 95% confidence limits.

Results

*CTL Clones Show Dual Specificity for an EBV Epitope Presented on HLA-B8 and the Alloantigen HLA-B*4402.* 12 CD8$^+$ CTL clones were isolated from four HLA-B8$^+$, EBV-sero$^+$ unrelated individuals after in vitro stimulation of PBMC with their autologous EBV-LCL. All clones that recognized the EBV epitope FLRGRAYGL, presented on HLA-B8, also lysed uninfected target cells (PHA blasts) expressing the alloantigen HLA-B*4402. Fig. 1 illustrates the specificity of one such clone from donor LC (CTL LC13). HLA-B8$^+$ cells were recognized by the CTL if infected with EBV (Fig. 1 *A*, TARGET EBV-LCL) or if peptide FLRGRAYGL was added exogenously to PHA blast targets (Fig. 1 *B*), whereas HLA-B*4402$^+$ cells were lysed with or without EBV infection. Cells expressing other HLA antigens, including the other major subtype of HLA-B44, B*4403, were not recognized. The addition of peptide FLRGRAYGL to HLA-B*4402$^+$ targets did not significantly increase levels of CTL lysis unless the targets were also HLA-B8$^+$.

Fig. 2 shows the cross-reactivity of a CTL clone (SC17), raised against the autologous EBV-LCL and selected for recognition of peptide FLRGRAYGL, isolated from another unrelated HLA-B8$^+$ donor. The target cells were again EBV-

[1] *Abbreviations used in this paper:* CTLp, CTL precursor; LCL, lymphoblastoid cell line; LDA, limiting dilution analysis.

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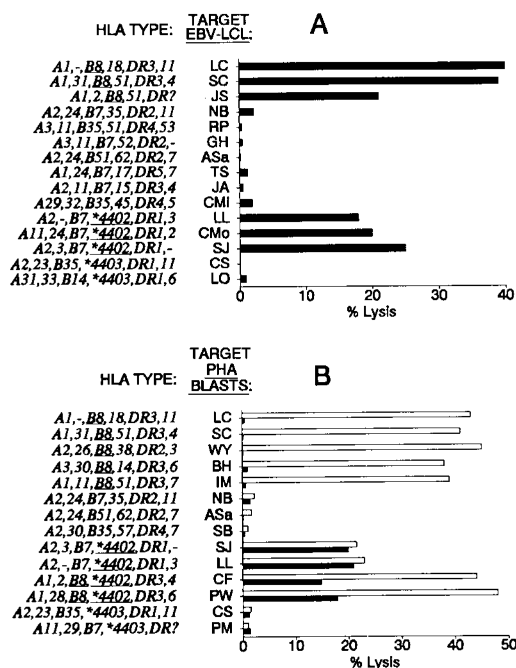

Figure 1. Lysis by CTL clone LC13 of EBV-LCL (*A*) and PHA blast targets (*B*) in the absence (■) or presence (□) of peptide FLRGRAYGL (E/T ratio = 1:1).

LCL (Fig. 2 *A*) and PHA blasts in the presence or absence of peptide FLRGRAYGL (Fig. 2 *B*), tested at a range of effector/target ratios. A similar pattern of lysis to that shown in Fig. 1 for clone LC13 was observed. The addition of

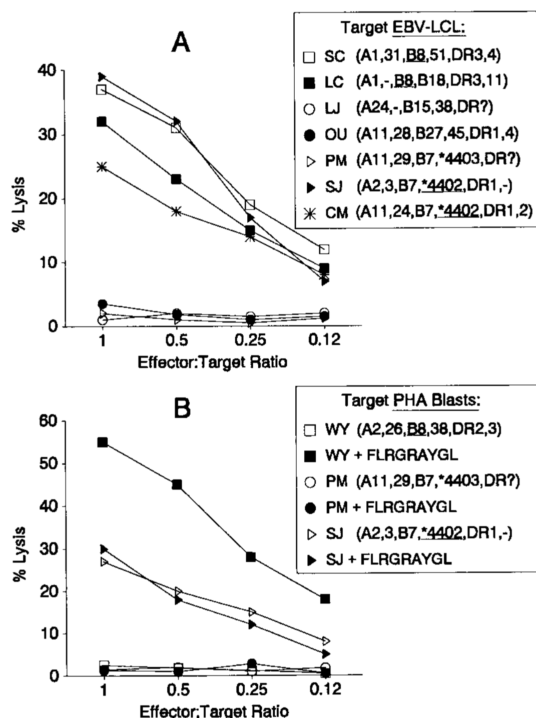

Figure 2. Lysis by CTL clone SC17 of EBV-LCL (*A*) and PHA blast targets (*B*) in the absence or presence of peptide FLRGRAYGL over a range of E/T ratios.

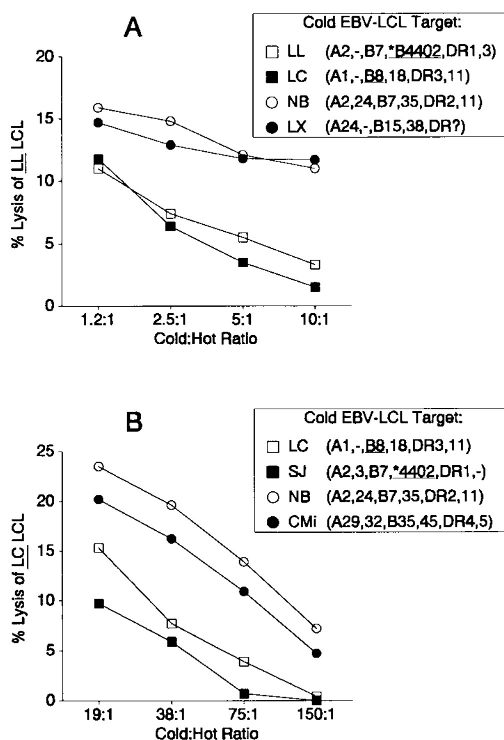

Figure 3. Cold target competition of CTL lysis by clone LC13 of a HLA-B*4402+ EBV-LCL (LL LCL - *A*) and a HLA-B8+ EBV-LCL (LC LCL - *B*) (effector/hot target ratio = 1:1). Specific cytotoxicity of the target cells in the absence of competing cells was 19.0% for LL LCL and 42.4% for LC LCL.

in Fig. 1 for clone LC13 was observed. The addition of
anti–class I antibody to HLA-B*4402+ and -B8+ targets significantly reduced lysis by these CTL clones, whereas anti–class II antibody had little effect (data not shown).

Cold target competition experiments were carried out on CTL LC13 to demonstrate that the dual reactivity pattern was not caused by a contaminating CTL population. Lysis by CTL LC13 of a HLA-B*4402+ cell line (Fig. 3 *A*) and the autologous EBV-LCL (Fig. 3 *B*) was more significantly inhibited by unlabeled HLA-B8+ and -B*4402+ EBV-LCL than by cell lines not expressing these antigens. The cross-reactivity is, therefore, clearly mediated by the same TCR on a single population of CTL. Higher cold/hot target ratios were necessary to compete with lysis of the autologous EBV-LCL than the HLA-B*4402+ cell line, suggesting that the self-HLA–restricted recognition by CTL LC13 is more efficient than the allorecognition.

EBV Memory T Cells Can Be Reactivated In Vitro Using Allostimulation. Studies in this laboratory have shown that, of those tested, the majority of HLA-B8+, EBV-sero+ individuals (but not EBV-sero− individuals) carry EBV memory CTL that recognize the epitope FLRGRAYGL (our unpublished observations). To determine if these memory cells could be reactivated using allostimulation, PBMC from two HLA-B8+, EBV-sero+ (ASu and LC) and two EBV-sero− (BHo and MW) individuals were stimulated in vitro with γ-irradiated PBMC from HLA-mismatched donor DM (HLA-A24, A29, *B*4402*, B47, DR1, DR7). The resulting poly-

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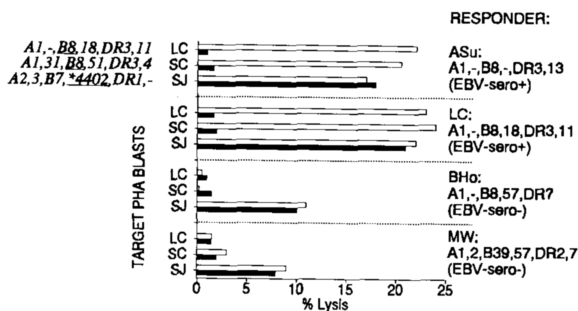

Figure 4. Lysis of PHA blast targets, with (□) or without (■) peptide FLRGRAYGL presensitization, by polyclonal CTL raised from two EBV-sero$^+$, HLA-B8$^+$ and two EBV-sero$^-$ individuals after stimulation with PBMC from HLA mismatched, HLA-B*4402$^+$ donor DM (E/T ratio = 20:1).

clonal CTL populations were tested for lysis of PHA blast targets in the presence or absence of EBV peptide FLRGRAYGL. Effectors generated from EBV-sero$^+$ responders not only gave significant lysis of targets sharing HLA-B*4402 with the stimulator PBMC (with or without exogenous peptide) but also lysed HLA-B8$^+$ PHA blasts in the presence of the EBV peptide (Fig. 4). In contrast, CTL from EBV-sero$^-$ donors lysed HLA-B*4402$^+$ targets less efficiently, and showed negligible recognition of FLRGRAYGL/HLA-B8 (Fig. 4). CTL clones that recognize EBV epitope FLRGRAYGL have also been raised from HLA-B8$^+$, EBV-sero$^+$ donor LC after in vitro stimulation with allogeneic PBMC expressing HLA-B*4402. The pattern of lysis for these clones parallels that of CTL clones LC13 and SC17 (Figs. 1 and 2) which were established after stimulation with the autologous EBV-LCL (data not shown). These data demonstrate that the alloantigen HLA-B*4402 can restimulate EBV memory CTL (which recognize peptide FLRGRAYGL) in HLA-B8$^+$, EBV-sero$^+$ individuals.

*CTL Precursor Frequencies for the Alloantigen HLA-B*4402 in EBV-sero$^+$ HLA-B8$^+$, and EBV-sero$^-$ Individuals.* Precursor frequency estimates of CTL against EBV epitope FLRGRAYGL/HLA-B8 previously have been shown to be high (1 in 7,530 to 2,200 PBMC) in most EBV-sero$^+$, HLA-B8$^+$ individuals (26, and our unpublished observations). To determine if these individuals also have elevated CTL precursor (CTLp) frequencies against alloantigen HLA-B*4402, PBMC from four EBV-sero$^+$, HLA-B8$^+$ donors were stimulated with γ-irradiated PBMC from a HLA-B*4402$^+$ donor in LDA. PBMC from four EBV-sero$^-$ individuals, which should not include memory CTLp for EBV epitope FLRGRAYGL/HLA-B8, were also analyzed for comparison. Effector wells were tested against target PHA blasts sharing only HLA-B*4402 with the stimulator cells. As shown in Fig. 5, the frequencies of CTLp which responded to HLA-B*4402 in four EBV-sero$^+$, HLA-B8$^+$ individuals were 1 per 10,100, 14,900, 17,600, and 13,800. These frequencies were significantly higher than those estimated for EBV-sero$^-$ donors (1 per 32,700, 196,000, 328,000, and 334,000). These data suggest that prior exposure to EBV might influence CTLp frequencies to an alloantigen.

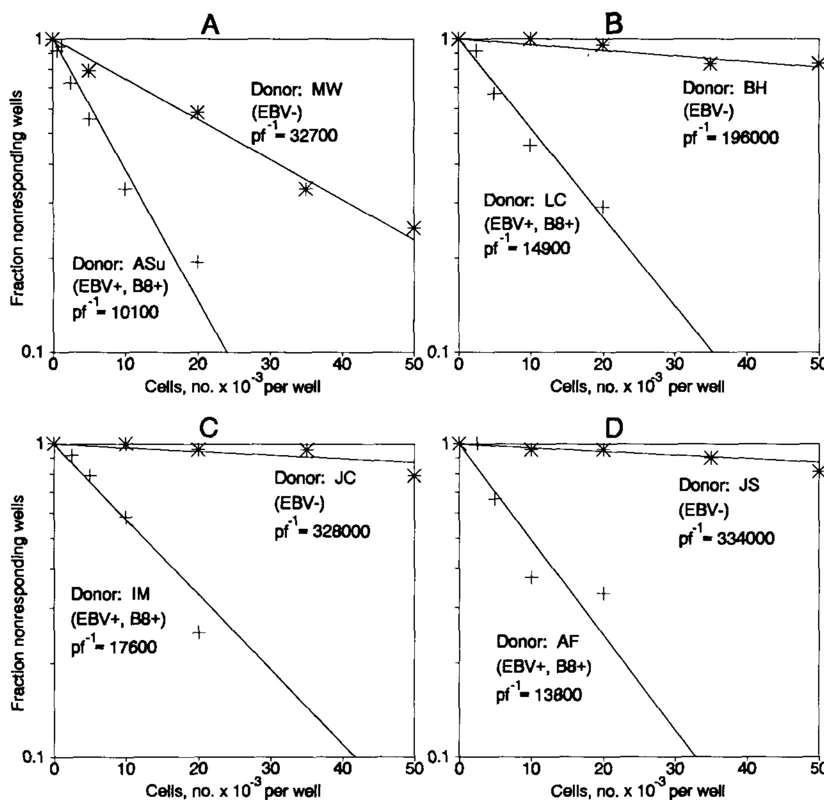

Figure 5. Comparative CTLp frequencies in four EBV-sero$^-$ and four EBV-sero$^+$, HLA-B8$^+$ individuals for alloantigen HLA-B*4402, using LDA. Reciprocal values of responder frequencies (pf^{-1}) are indicated. The 95% confidence limits for each pf^{-1} are as follows: (*ASu*) 7,500–13,500; (*MW*) 23,600–45,200; (*LC*) 10,600–20,900; (*BH*) 144,000–295,000; (*IM*) 12,600–24,700; (*JC*) 142,000–755,000; (*AF*) 9,900–19,200; and (*JS*) 173,000–656,000.

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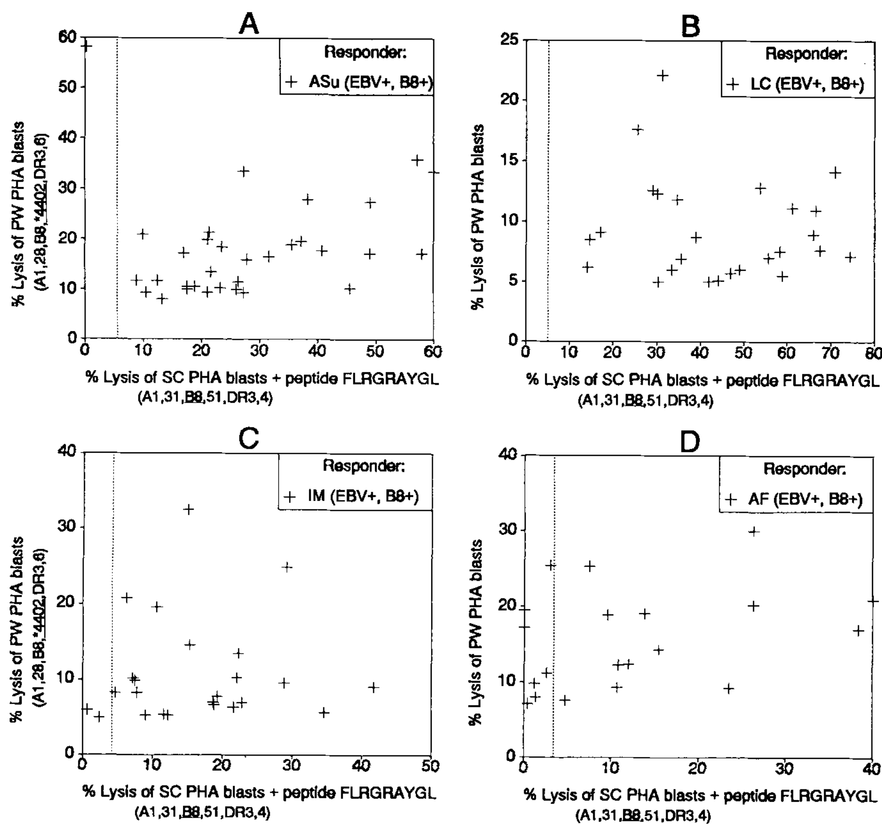

Figure 6. Split-well analysis of CTL microcultures generated in LDA from responder cell concentrations below the estimated CTLp frequencies for alloantigen HLA-B*4402 and which were positive for HLA-B*4402 recognition. Each point on the graph indicates the levels of lysis for each CTL microculture of two target populations: PW PHA blasts, which share only HLA-B*4402 with the stimulator cells (*y-axis*); and SC PHA blasts preincubated with peptide FLRGRAYGL, which are HLA-B8$^+$ but which share no HLA antigens with the stimulators (*x-axis*). SC PHA blasts were also used as targets without peptide presensitization (data not shown); these were lysed by <5% of CTL microcultures, and data from these CTL were omitted from the analysis. (*Dashed vertical lines*) Values calculated from control wells to define positive cytotoxic activity against SC PHA blasts plus peptide FLRGRAYGL.

*The Majority of CTLp for Alloantigen HLA-B*4402 in HLA-B8$^+$, EBV-sero$^+$ Individuals Are EBV Memory T Cells that Cross-react with the Alloantigen.* To determine if the higher frequencies of CTLp for alloantigen HLA-B*4402 in the EBV-sero$^+$, HLA-B8$^+$ individuals were a result of cross-reacting EBV memory T cells, "split-well" LDA assays were conducted. Each microculture in the above LDA was divided and assayed separately for CTL activity against HLA-B8$^+$ PHA blasts (SC PHA blasts), with and without peptide FLRGRAYGL, and HLA-B*4402$^+$ PHA blasts (PW PHA blasts). These target cells shared no HLA antigens, other than HLA-B*4402 in the case of the latter target, with the stimulator cells. Fig. 6 shows the reactivity pattern of CTL microcultures derived from responder cell concentrations below the estimated CTLp frequencies for alloantigen HLA-B*4402 (thus, most were likely to be derived from one anti-HLA-B*4402 precursor) and which were positive for HLA-B*4402 recognition. The majority of CTL from EBV-sero$^+$, HLA-B8$^+$ individuals which recognized HLA-B*4402 also lysed HLA-B8$^+$ targets presensitized with peptide FLRGRAYGL (97, 100, 92, and 65% of CTL microcultures for donors ASu, LC, IM, and AF, respectively). In contrast, only 1/55 CTL microcultures with anti-HLA-B*4402 activity, raised from EBV-sero$^-$ donors, cross-reacted with the HLA-B8–restricted EBV CTL epitope (data not shown). These data demonstrate that a response to an alloantigen can be dominated by CTL cross-reactive with a single viral epitope.

Discussion

The recognition of specific allo-MHC molecules by CTL clones generated in response to antigen presented by autologous cells, as described for CTL LC13 and SC17 (Figs. 1 and 2) and in several previous reports (8), suggests that allorecognition represents the cross-reactions of self-MHC–restricted T cells. This report extends evidence in support of this view with the demonstration that T cells which recognize an EBV epitope could be restimulated in vitro from EBV-exposed individuals using allostimulation (Fig. 4). This observation raised the possibility that the high frequency of memory T cells for the EBV epitope FLRGRAYGL, which appear to be present in most EBV-sero$^+$, HLA-B8$^+$ individuals (26, and our unpublished observations), could significantly influence the response to HLA-B*4402 as an alloantigen. Indeed, considerably more anti-HLA-B*4402 CTLp were detected in four EBV-sero$^+$, HLA-B8$^+$ donors used in this study compared with four EBV-sero$^-$ donors (Fig. 5). Although precursor frequencies of T cells reactive to particular MHC alloantigens in humans vary considerably between unrelated individuals (27), "split-well" analysis of CTL lines raised from single anti-HLA-B*4402 CTLp confirmed an important role for EBV memory T cells in the response to that alloantigen in EBV-sero$^+$, HLA-B8$^+$ donors. The majority of anti-HLA-B*4402 CTL from these individuals also recognized the EBV epitope presented by HLA-B8 (Fig. 6). These results suggest that FLRGRAYGL-specific memory T cells from different

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individuals frequently express a TCR with the common trait of cross-reacting with HLA-B*4402. This is consistent with the results of a parallel study in this laboratory which has demonstrated TCR sequence identity between FLRGRAYGL-specific CTL clones raised from four unrelated individuals after in vitro stimulation with their autologous EBV-LCL (Argaet, V., C. Schmidt, S. Burrows, A. Suhrbier, D. Moss, and I. Misko, manuscript in preparation).

The potency of the alloresponse is thought to reflect the profusion and diversity of potentially antigenic peptides naturally presented on MHC molecules, which stimulate a large variety of T cell clonotypes (1, 2). An early study supporting this model showed that a murine alloresponse to a single MHC antigen contained a minimum of 50 different T cell specificities (4). Repertoire diversity has also been demonstrated at the level of expression of different TCR genes (5–7). We have shown that, in some individuals, a highly restricted T cell repertoire is activated for a human alloantigen. The strength of the anti-HLA-B*4402 alloresponse in these individuals is therefore not induced by multiple alloantigenic determinants but by the high frequency of memory CTL to a single EBV epitope which fortuitously cross-reacts with the alloantigen.

Of the two major subtypes of HLA-B44, B*4402 and B*4403, only the former was recognized by the cross-reactive CTL. These two subtypes differ by a single amino acid at position 156, the most variable residue of the MHC $\alpha 2$ domain helix (28). It is interesting to note that HLA-B*4402 shares aspartic acid with HLA-B8 at this position whereas HLA-B*4403 has the consensus residue leucine (28), suggesting that the aspartic acid may be critical for recognition by these clones. Although the side chains of amino acids at position 156 point into the peptide-binding groove, they may remain partially accessible to direct contact with the TCR (29). Indeed, TCR contact with the MHC at position 156 appears to occur for several EBV-specific CTL clones recently isolated in our laboratory, which recognize an EBV epitope known to bind to both major subtypes of HLA-B44, but which lyse only target cells presenting the EBV epitope in association with HLA-B*4402 and not HLA-B*4403 (Burrows, S., R. Khanna, and D. Moss, manuscript in preparation).

The results presented herein support other evidence (11, 12) that memory T cells may play a major role in human alloresponses. The frequency of memory CTL to the common herpesvirus, EBV and human cytomegalovirus (HCMV), in healthy immune people are higher than those reported for any other virus (16, 30), with the possible exception of HIV (31). Virtually 100% of healthy EBV-sero$^+$ and HCMV-sero$^+$ individuals show a clear virus-specific CTL response in vitro (32, 33). The potency of the response to these viruses probably relates to their lifetime persistence and the repeated antigenic challenge with multiple viral epitopes. When this is considered in the context of reports that at least 60% of HLA-restricted CTL clones cross-react with alloantigens (10), it is not unlikely that memory T cells to these viruses significantly influence human alloresponses. It is interesting to note that pretransplant serological studies have established herpesvirus exposure as an important risk factor for GVHD after bone marrow transplant (34). Expanding on the present study, it will be important to establish what proportion of CTL clones reactive to other allo-HLA antigens cross-react with autologous cells infected with herpesviruses such as EBV and HCMV. In addition, a more extensive analysis comparing alloreactive CTLp frequencies between individuals sero$^-$ or sero$^+$ for the common herpesviruses, may confirm an important correlation between virus exposure and alloreactivity.

We thank Dr. S. Serjeantson, The John Curtin School of Medical Research, Canberra, Australia, and Mrs. N. Strachan, Tissue Typing Laboratory, Princess Alexandra Hospital, Brisbane, Australia for HLA typing our blood donors and cell lines.

This work was supported by a grant from the National Cancer Institute (USA), CA-57952-02.

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Received for publication 18 October 1993 and in revised form 22 December 1993.

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