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 FLKGRAYGL, 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-FILA-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.
significant influence an alloresponse is discussed with reference to EBNA 3 (18). We demonstrate that the response to an alloantigen HLA-B*4402 can be dominated by CTL cross-reactive with a single EBV epitope which contrasts with previous studies which concluded that allospecific responses 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) 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/R.PMI 1640) containing MLA-144 cultures. On day 10, each CTL microculture was split into three replicates and used as effectors in a standard 5-h chromium 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 HB-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, isolated on 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 blasts 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-
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 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 allore cognition.

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 - (BH3 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-

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).
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.

**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).

**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.
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 re-
sponse 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 autolo-
gous cells, as described for CTL LC13 and SC17 (Figs. 1 and
2) and in several previous reports (8), suggests that allorecog-
nition 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 in-
dividuals 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, con-
siderably 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
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 FLGRAYGL-specific CTL clones raised from four unrelated individuals after in vitro stimulation with their autologous EBV-ICL (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 α2 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 these viruses 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.

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