CROSS-REACTIVITY OF SELF-HLA-RESTRICTED EPSTEIN-BARR VIRUS-SPECIFIC CYTOTOXIC T LYMPHOCYTES FOR ALLO-HLA DETERMINANTS*

By J. S. H. GASTON,‡ A. B. RICKINSON, AND M. A. EPSTEIN

From the Department of Pathology, University of Bristol, Bristol BS8 1TD, U.K.

The major histocompatibility complex (MHC)[1] was originally defined by means of alloreactive immune responses, (1–3), but more recent studies have revealed a physiological role for the MHC in the presentation of foreign (non-MHC) antigens to immune T cells (4, 5). However, any model of the MHC's physiological role in controlling T cell differentiation and function must be able to explain why the mature animal possesses such a high frequency of lymphocytes reactive to the MHC alloantigens of its own species; this appears to be at least an order of magnitude greater than that of self-MHC-restricted clones specific for any one individual antigen (6–8). One possible explanation for these findings is to postulate that MHC alloantigens, either alone (9) or in some form of association with other cell surface components (10, 11), display structures that mimic the immunogenic determinants that are presented to the T cell system by certain combinations of foreign antigen and self-MHC molecule. Viewed in this way, an alloreactive response represents the sum of responses of a variety of antigen-specific immune T cells all fortuitously induced through cross-reactive epitopes present on the allogeneic cell. A substantial amount of experimental evidence has been presented recently to support the existence of such cross-reactivity (12–17) but, to date, all of this stems from work with inbred strains of mice; the generality of this phenomenon remains an important and unresolved question.

MHC restriction of cytotoxic T cell function in man has been demonstrated in the response to haptens (18), to minor histocompatibility antigens (19), and to viruses (20, 21). Cytotoxic T cells specific for Epstein-Barr (EB) virus can be generated in vitro by appropriately challenging the blood mononuclear cells of previously infected (seropositive) individuals with autologous EB virus-transformed B cells and the effector cells thus obtained show clear evidence of class I MHC (HLA-A, B, C) antigen restriction (22). A much closer analysis of the restriction operative in this system has been made possible through the in vitro expansion of these effector cell populations as interleukin 2 (IL-2)-dependent T cell lines (23). The testing of such effector cells on large panels of allogeneic EB

---

virus-transformed B cell lines has now begun to reveal instances in which certain EB virus-specific cytotoxic T cell preparations, which display classically HLA-restricted recognition of the great majority of targets tested, also show an anomalous lysis of particular HLA-mismatched target cell lines. The present work shows that this anomalous lysis is directed against epitopes on class I HLA allo-antigens expressed not only on EB virus-transformed but also on mitogen-stimulated target cells, and is mediated by the same effector cells as mediate virus-specific self-HLA-restricted cytolysis. This, to our knowledge, is the first demonstration that such cross-reactivity exists within the T cell repertoire of an outbred species.

Materials and Methods

Blood Donors and HLA Typing. Blood samples were obtained from healthy adult donors whose immune status with respect to EB virus was assessed by measuring antibodies to the EB virus capsid antigen (24), and who were typed for HLA-A, -B, and -C, and HLA-DR antigens using peripheral blood mononuclear (PBM) cells and EB virus-transformed lymphoblastoid cell lines as described previously (25).

Cell Lines and Culture Medium. Cell lines were prepared and passaged as described previously (25). RPMI 1640 culture medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS) was used for maintenance of all cell lines and experimental cultures, unless otherwise stated.

Preparation of EB Virus-specific Effector Cells. This has been described in detail elsewhere (22–25). Briefly 2 × 10⁶ PBM cells were cultured with 5 × 10⁴ X-irradiated autologous EB virus-transformed lymphoblasts for 10 d; at this time the stimulated T cells were harvested by E-rosetting with sheep erythrocytes treated with 2-aminoethylisothiouronium bromide hydrobromide and cultured for 4–6 d with autologous X-irradiated stimulator cells at a responder/stimulator ratio of 5:2. The resulting effector population was expanded using IL-2–containing culture supernatants and repeated addition of X-irradiated stimulator cells.

Cytotoxicity Assays. The conduct of cytotoxicity assays, including cold target competition experiments, and assays testing the effect of monoclonal antibodies upon cytotoxicity, was exactly as described previously (24, 25). Mitogen-stimulated lymphoblasts were prepared from cultures of PBM cells either 3 d after exposure to phytohaemagglutinin (PHA) or 5 d after exposure to pokeweed mitogen (PWM). On some occasions mitogen-stimulated lymphoblasts were cultured in medium containing 15% human AB serum in place of 10% FCS.

Monoclonal Antibodies. The following monoclonal antibodies were used in cytotoxicity blocking studies:- W6/32 and BB7.7 (26), both specific for a framework determinant on all HLA-A, -B, and -C antigens, and TDR 31.1 (27) specific for an HLA-DR common framework determinant (all kindly provided by Dr. W. F. Bodmer and Dr. M. J. Crumpton, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London) and MA2.1 (28), specific for a polymorphic determinant on HLA-A2 and HLA-B17 (Bw57/w58) antigens (kindly provided by Professor A. McMichael, Nuffield Department of Medicine, John Radcliffe Hospital, Oxford).

Analysis of Results of Cytotoxicity Assays. In order to allow comparison of results of repeated testing of the same effector/target cell combination, the specific lysis was expressed on each occasion as a percentage of the autologous target cell lysis observed in the same experiment at the same effector/target ratio. The mean and standard deviation of the relative percentage lysis was then calculated for each effector/target combination tested across a range of different effector/target ratios (2.5:1 to 20:1).

Limiting Dilution Culture and Assay Procedure. Appropriate numbers of T cells from an IL-2–dependent cytotoxic T cell line from donor StG were cultured in U-shaped 0.2-ml volume wells in the presence of 2 × 10³ X-irradiated stimulator cells, either from the autologous cell line StG or from the HLA-Bw62–bearing cell line JU, and 25% IL-2–

containing culture supernatant. IL-2 and culture medium were replaced twice weekly. After 10 d, wells in which a proliferating colony was visible were subcultured to 2–6 further U-wells, with 2×10^3 stimulator cells per well. Subculturing and feeding with IL-2 and stimulator cells was continued until each colony was of sufficient size to allow assay for cytotoxicity.

In order to obtain further information on the cytotoxicity of a large number of colonies, an in situ cytotoxicity assay was used (29), instead of waiting for sufficient numbers of cells to be available for conventional cytotoxicity testing. This was necessary as colonies were often slow-growing. Growing colonies were harvested and split into equal aliquots, some of which were retained for further culture, while the others were transferred to fresh U-shaped well microtest plates for cytotoxicity assay. After feeding with fresh culture medium (without IL-2) the plates were centrifuged briefly, and the supernatant removed and replaced with further fresh medium. Cells were then cultured overnight. Before assay, replicate colony wells were checked visually to ensure that approximately equal numbers of effectors cells were present in each assay well. 150 μl of medium was removed from each well by careful suction; 50 μl of fresh medium and 10^4 chromium^{51}-labeled target cells in 100 μl culture medium were then added. By assaying colonies at least 3 d after feeding with IL-2 and replacing the medium three times, negligible amounts of PHA were present in the final assay well and there was never any evidence of lectin-mediated cytotoxicity. The assay was completed in the normal way.

Assessment of Results from In Situ Cytotoxicity Assay.   Although low levels of percentage specific lysis were recorded by this technique, lysed and nonlysed targets could be clearly distinguished; thus wells were scored positive for lysis if >4.5% specific lysis was observed (representing an isotope release $\geq$3 standard deviations higher than the spontaneous release obtained from 4–6 control wells containing target cells alone). <2% specific isotope release was observed in wells scored as negative for lysis. Most colonies were assayed on 2–4 separate occasions with excellent agreement between the results on each occasion.

Experimental Procedure.   EB virus-specific cytotoxic T cell lines were established from virus-immune donors and tested on a large panel of HLA-typed EB virus-transformed lymphoblastoid target cells. From this analysis, the identity of the dominant self HLA-determinants restricting each effector cell population could be determined, and instances of "anomalous" lysis of HLA mismatched target cells noted. The nature of the "anomalous" cytotoxicity was further investigated, (*a*) by extending the target cell panel to include EB virus-genome-negative mitogen-stimulated lymphoblasts from a range of HLA-typed donors, (*b*) by monoclonal antibody blocking studies using antibodies specific for mono-morphic (framework) or polymorphic determinants on class I HLA antigens, and (*c*) by cold target inhibition experiments to compare the "anomalous" and EB virus-specific components of cytotoxicity.

Finally, one particular effector T cell line displaying both "anomalous" and EB virus-specific cytotoxicities was seeded at limiting dilutions and the resulting colonies assayed for both cytotoxic functions.

Results

Demonstration of Alloreactivity in an EB Virus-specific Self-HLA-restricted Cytotoxic T Cell Population.   Fig. 1 presents an analysis of the cytotoxicity displayed by an effector T cell line obtained from the seropositive (i.e., virus-immune) donor JuG (HLA-A1, A2, B8, B14) by appropriate in vitro stimulation with autologous EB virus-transformed B cells. It is clear from *a* that lysis was preferentially directed towards the autologous stimulating cells and towards those allogeneic EB virus-transformed target cells sharing HLA-B8 in common with donor JuG. A lower level of killing was observed with HLA-B14-matched target cells, whereas there was no significant lysis of target cells sharing only HLA-A1 or -A2 with the effector cells. The HLA-restricted nature of the cytotoxicity is further supported by the results obtained with the 10 HLA-mismatched EB
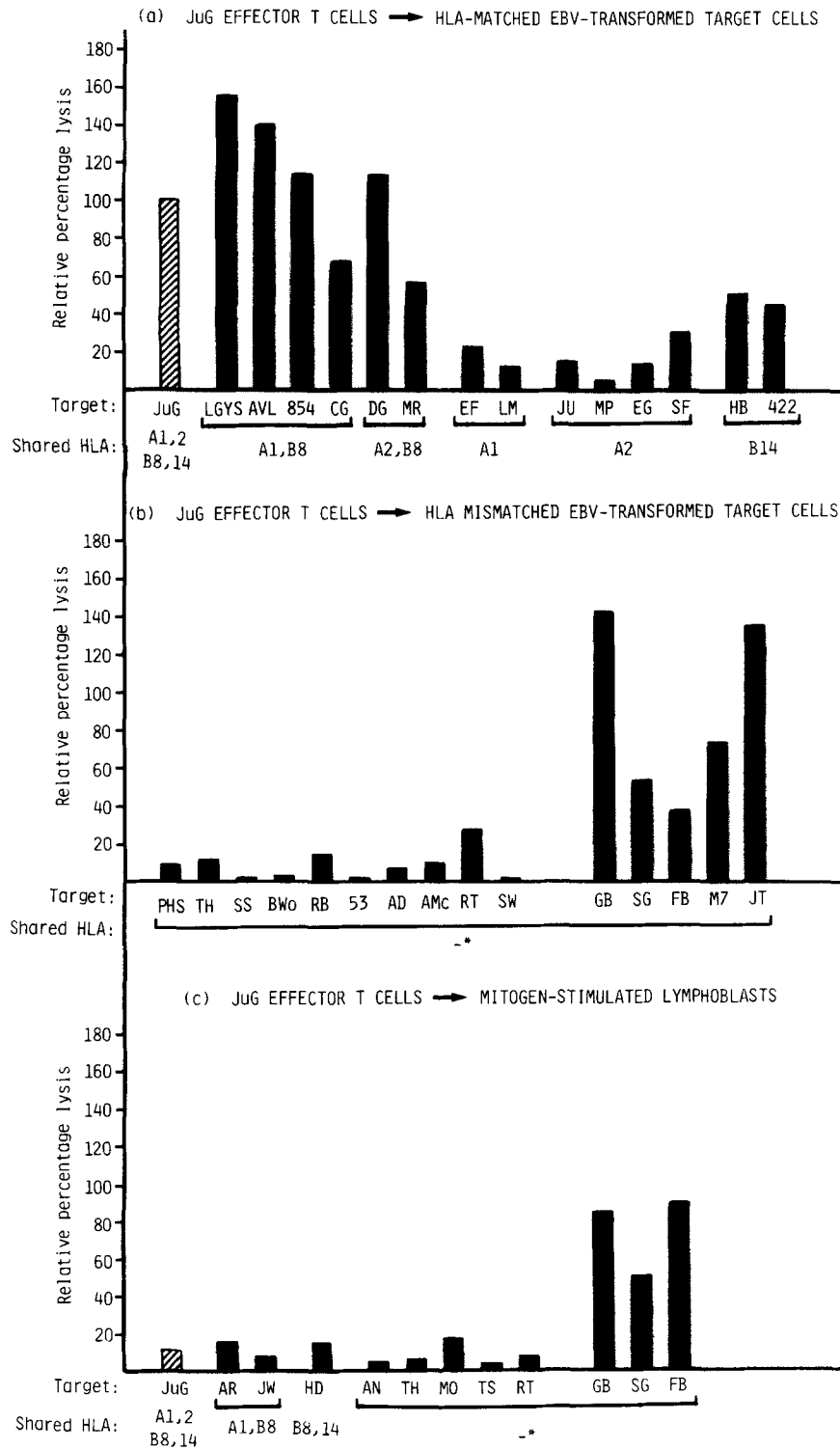
virus-transformed target cell lines shown on the left of *b*, none of which were lysed. Moreover, the EB virus specificity of these effector cells is apparent from their lack of reactivity when tested against autologous or HLA-B8/B14-bearing mitogen-stimulated lymphoblasts (*c*) or against the NK-sensitive target cell lines HSB2 and K562 (<10% relative lysis, data not shown).

However, as shown by the remaining results in *b*, five target cell lines completely mismatched for class I HLA antigens (or sharing only HLA-A2, an antigen that does not mediate virus-specific cytolysis by JuG effector T cells) were unexpectedly lysed at significant levels. This result did not reflect any unique sensitivity of these particular targets to cytolysis *per se* since in numerous other assays these same lines were not killed by EB virus-specific effector T cell preparations from other HLA-mismatched donors (data not shown). The alloreactive nature of this "anomalous" lysis was first suggested by the parallel results shown in *c*. Thus, where testing was possible, PHA-stimulated lymphoblasts prepared from these same individuals were also found to be sensitive to "anomalous" cytolysis by the JuG effector T cell line. This result was obtained on several occasions of testing, irrespective of whether the mitogenic stimulation was carried out using fetal calf serum or human AB serum (Fig. 1 legend).

Subsequent studies showed that high titer preparations of two different monoclonal antibodies (W6/32 and BB7.7), directed against framework determinants common to all class I HLA molecules, blocked this "anomalous" lysis of HLA-mismatched target lines very efficiently (74% and 82% inhibition for W6/32 and BB.7.7, respectively, cf. 81% and 52% inhibition of HLA-restricted virus-specific lysis in the same experiments). However, identification of the polymorphic class I HLA determinant against which "anomalous" lysis was directed in this particular case was difficult since no serologically defined HLA-antigen was common to all five sensitive targets (see Discussion).

Demonstration of Alloreactivity Against an Identifiable Serologically Defined HLA Alloantigen.. By contrast, Fig. 2 provides data obtained using another EB virus-specific cytotoxic T cell line with an alloreactive component, where it was indeed possible to identify the particular antigen against which the alloreactivity was directed. In this example, the effector T cell line from seropositive donor JU (HLA-A2, A2, Bw62, Bw62) displayed an EB virus-specific cytotoxic function predominantly restricted through HLA-Bw62, with little A2-restricted lysis. HLA-mismatched target cell lines were not lysed, with the striking exception of all four target cell lines tested that were positive for HLA-Bw57; these showed unusually high levels of lysis and again this "anomalous" reactivity of JU effector T cells extended to include HLA-Bw57–bearing mitogen-stimulated lymphoblasts (Fig. 2) in tests where all other EB virus-genome-negative target cells were not killed (data not shown).

The availability of the monoclonal antibody MA2.1, which is specific for an epitope shared only by HLA-A2 and -Bw57/Bw 58 antigens, allowed testing of the hypothesis that HLA-Bw57 was the target antigen for this "anomalous" lysis. As shown in Fig. 3, high titers of MA2.1 blocked "anomalous" lysis of the two Bw57-bearing target lines RT and TH just as efficiently as did the monoclonal antibody BB7.7 which binds to a common determinant on all class I HLA molecules; this result suggested that Bw57-directed alloreactivity was responsible

(a)   JuG EFFECTOR T CELLS ➡ HLA-MATCHED EBV-TRANSFORMED TARGET CELLS

Relative percentage lysis

Target: JuG   LGYS AVL 854 CG   DG MR   EF LM   JU MP EG SF   HB 422

Shared HLA: A1,2 B8,14   A1,B8   A2,B8   A1   A2   B14

(b)   JuG EFFECTOR T CELLS ➡ HLA MISMATCHED EBV-TRANSFORMED TARGET CELLS

Relative percentage lysis

Target: PHS TH SS BWo RB 53 AD AMc RT SW   GB SG FB M7 JT

Shared HLA: _*

(c)   JuG EFFECTOR T CELLS ➡ MITOGEN-STIMULATED LYMPHOBLASTS

Relative percentage lysis

Target: JuG   AR JW   HD   AN TH MO TS RT   GB SG FB

Shared HLA: A1,2 B8,14   A1,B8   B8,14   _*

for all of the observed lysis of RT and TH cell lines. In this same experiment, the EB virus-specific HLA-Bw62–restricted cytotoxicity of JU effector T cells directed against the autologous cell line was selectively blocked by the binding of BB7.7, but not of MA2.1, to these target cells.

Demonstration of Alloreactivity Against a "Public" Determinant Shared by Certain HLA Antigen Types/Subtypes. Table I summarizes the results obtained with repeated testing of an effector T cell line derived from another seropositive donor, StG (HLA-A1, A2, B8, B8), on a large panel of EB virus-transformed target cells. In this case, EB virus-specific cytolysis was restricted largely through HLA-B8 and to a lesser extent through HLA-A2, whilst there was no detectable HLA-A1–restricted component. Testing of HLA-mismatched target cell lines revealed a strikingly reproducible pattern of "anomalous" cytotoxicity affecting all HLA-Bw35–bearing, all HLA-Bw62–bearing and subset of HLA-B12–bearing (HLA-Bw44, w45) targets.

The alloreactive nature of these "anomalous" cytotoxicities of the StG effector T cell line was made clear by the results in Fig. 4. Thus, not only EB virus-transformed cells but also mitogen-stimulated lymphoblasts from relevant donors bearing either HLA-Bw62 or -Bw35 or -Bw44 were sensitive to "anomalous" lysis, whereas lysis of autologous or of HLA-matched targets was specific for EB virus-transformed cells only. Again the "anomalous" lysis of HLA-mismatched targets was strongly inhibited (<80%) by the class I HLA antigen-specific monoclonal antibody W6/32; in a parallel experiment the class II HLA antigen specific monoclonal antibody TDR31.1 inhibited lysis by <5% (data not shown).

Fig. 5 shows representative results of cold target inhibition experiments that were conducted in order to determine the relationship, if any, between the alloreactive responses directed against HLA-Bw62, -Bw35, and -Bw44 respectively. Unlabeled target cells expressing any one of these antigens were capable of significantly inhibiting the "anomalous" lysis of the Bw35-bearing cell line M7 (Fig. 5 *a*) or of HLA-Bw44- or HLA-Bw62-bearing cell lines (data not shown). Mitogen-stimulated lymphoblasts were just as effective cold target inhibitors as were the corresponding EB virus-transformed cells (see DW-blast results, Fig. 5 *a*).

An analogous series of cold target inhibition experiments was performed to

---

FIGURE 1. Cytotoxicity of an effector T cell line from donor JuG (A1, A2; B8, B14) tested on: (*a*) EB virus-transformed target cells whose degree of HLA-A and -B antigen matching with the effector cells is as shown; (*b*) EB virus-transformed target cells not sharing any HLA-A and -B antigens with the effector cells and (*c*) mitogen-stimulated EB virus-genome-negative lymphoblasts, both HLA-matched and -mismatched with the effector cells. Lysis of autologous target cells is shown by the hatched columns. The relative percentage lysis shown for each target represents the specific lysis of that target cell expressed as a percentage of the specific lysis of the EB virus-transformed autologous (JuG) target cell obtained in the same assay at the same effector/target ratio; the mean relative percentage lysis from between two and eight assays is shown for each target cell. (*Target cells AD, AMc, RT, and M7 were serologically typed as bearing HLA-A2 antigens but are as shown as HLA-mismatched targets since they express "variant" HLA-A2 antigens as defined by T cell-restricting determinants [reference 25].) In addition to the results shown in *c*, when PHA-stimulated lymphoblasts from donors GB and FB were cultured and assayed in medium containing either FCS or human AB serum, the results expressed in terms of relative percentage lysis (at effector/target ratio 5:1) were as follows: In FCS containing-medium, GB blasts 99%, FB blasts 89%; in AB serum containing-medium, GB blasts 85%, FB blasts 110%.
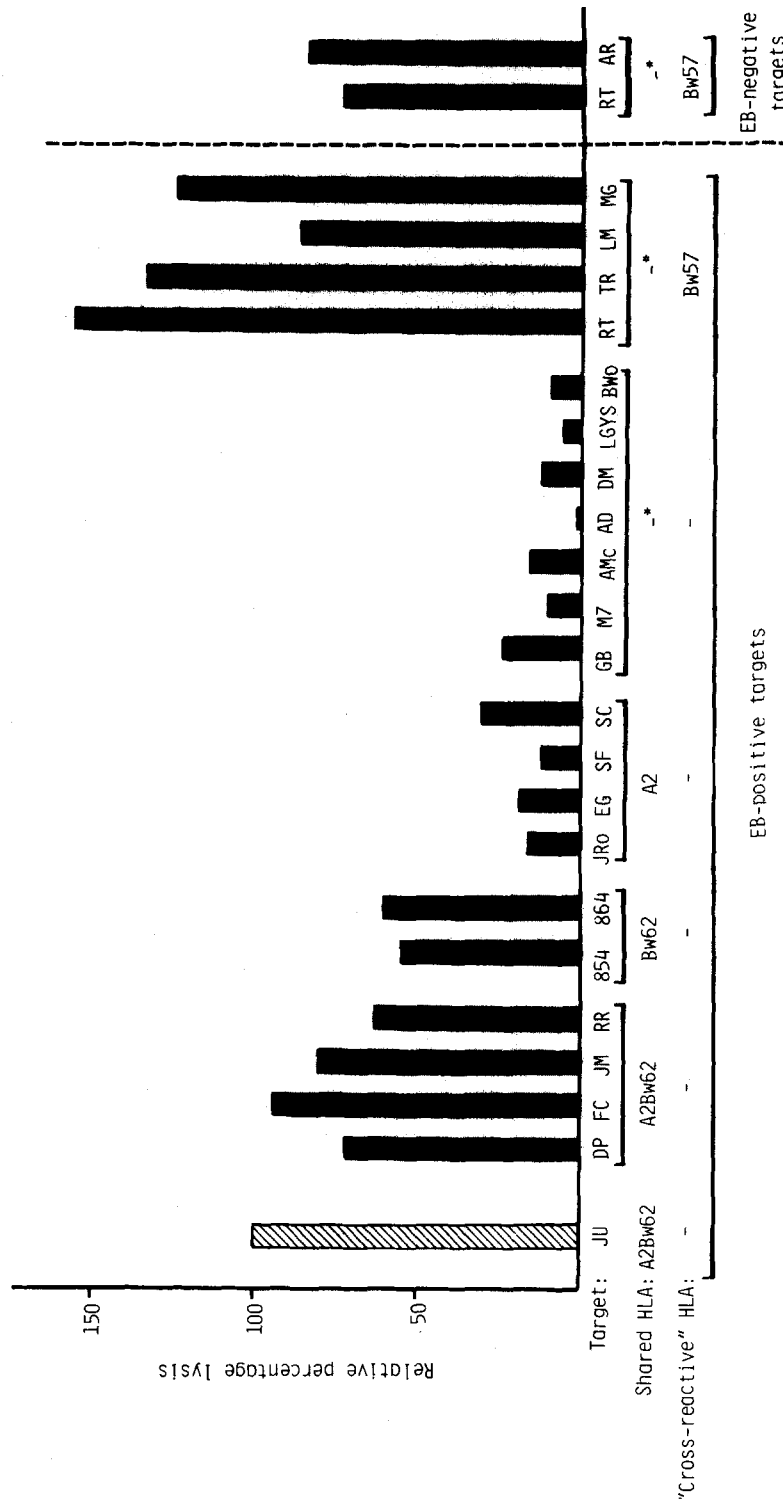
FIGURE 2.   Cytotoxicity of an effector T cell line from donor JU (A2, A2; Bw62, Bw62) tested on a range of EB virus genome-positive and -negative target cells whose degree of HLA-A and -B antigen matching with the effector cells is as shown. Target cells bearing the "cross-reactive" HLA-Bw57 antigen are also indicated. For each target cell, the results are expressed as relative percentage lysis as described in Fig. 1 legend. Lysis of the autologous target cell is shown by the hatched column. (*) As described in Fig. 1 legend, certain target cells (in this case, M7, AMc, AD, RT, and TR) express variant HLA-A2 antigens and are therefore shown as HLA-mismatched targets.
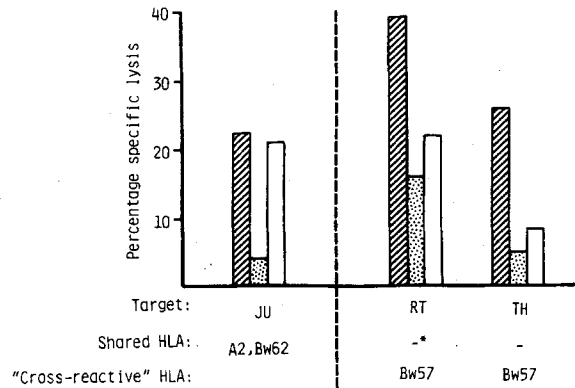
FIGURE 3. Effect of monoclonal antibodies on the level of EB virus-specific lysis of autologous target cells, and on the level of "anomalous" lysis of HLA-Bw57-bearing target cells mediated by effector T cells from donor JU. Results are expressed as percentage specific lysis in the absence of antibody (hatched columns) and in the presence of saturating concentrations of monoclonal antibody BB7.7 (stippled columns) or monoclonal antibody MA2.1 (open columns).

TABLE I

Summary of StG Effector T Cell Reactivities*

Type of reactivity	Targets grouped by HLA type	Targets per group	Mean relative lysis (m.r.l.)	Proportion of targets with >25% m.r.l.[‡]
EBV-specific	Auto (A1, 2, B8)	1	100	1/1
	A1 match	3	11 ± 7	0/3
	A2 match	4	44 ± 12	4/4
	A1B8/B8 match	6	71 ± 42	6/6
	A2B8 match	4	72 ± 16	4/4
	No match	15	9 ± 7	0/15
"Allospecific"	Bw35 cross-reaction	9	70 ± 37	9/9
	Bw62 cross-reaction	5	63 ± 20	5/5
	B12 cross-reaction			
	(a) Bw44/45 subgroup	9	56 ± 15	9/9
	(b) Bw44 subgroup	4	9 ± 2	0/4

* Effector T cells from donor StG were tested on a large number of target cells; for each target cell tested the relative percentage lysis was calculated as described in Fig. 1 legend from the results of several assays. Target cells are arranged in groups according to expression of HLA antigens shared with donor StG or expression of HLA antigens mediating alloreactivity; the mean relative percentage lysis (m.r.l.) values for all the target cells in each group is shown along with the standard deviation.
‡ The proportion of target cells within each group showing a relative percentage lysis of >25% when tested in several assays.

determine whether the alloreactivity of the polyclonal StG effector cell line reflected genuine cross-reactive lysis by virus-specific self-HLA–restricted effector T cells or was being mediated by a separate population of effectors. The results showed that unlabeled targets bearing any one of the relevant alloantigens, for instance either EB virus-transformed cells or mitogen-stimulated lymphoblasts from the HLA-Bw62-positive donor JU (Fig. 5 *b*), were capable of significantly inhibiting lysis of autologous EB virus-transformed targets by the StG effector
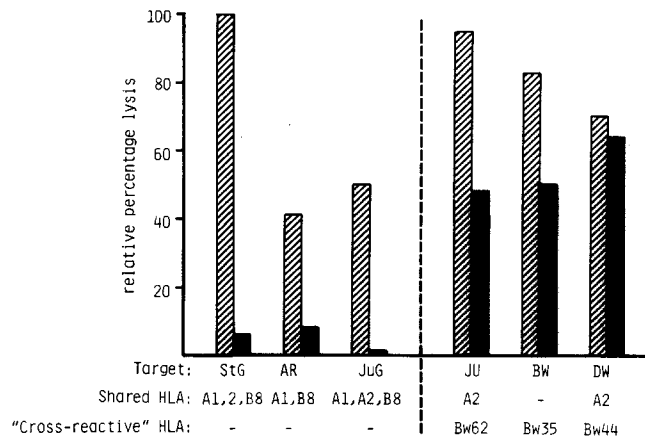
FIGURE 4. Cytotoxicity of an effector T cell line from donor StG (A1, A2; B8, B8) tested on either EB virus-transformed cells (hatched columns) or mitogen-stimulated lymphoblasts (filled columns) whose degree of HLA-A and -B antigen matching with the effector cells is as shown. Target cells bearing the "cross-reactive" antigens, HLA-Bw62 or -Bw35 or -Bw44, are also indicated. For each target cell, the results are expressed as relative percentage lysis as described in Fig. 1 legend.

cell line. The degree of inhibition, while less than that shown by unlabeled cells of the autologous cell line itself, was nevertheless equal to that shown by an HLA-matched cell line JuG (from the mother of donor StG) and clearly much greater than the background effects caused by irrelevant cold targets (Fig. 5 *b*). Conversely, alloreactive lysis by the StG effector T cell line could in each case be inhibited by unlabeled cells of the autologous virus-transformed cell line StG (Fig. 5 *a*) and of the HLA-matched cell line JuG (data not shown).

Analysis of Virus-specific and Alloreactive Cytotoxicities by Limiting Dilution of the Effector T Cell Line. Limiting dilution culture of the polyclonal StG effector T cell line provided an independent approach with which to assess the relationship between virus-specific and alloreactive cytotoxicities. In these experiments, the parent cell line was found to have a low plating efficiency and growing colonies were never obtained at seedings below 5 cells/well. The cell populations obtained could therefore not be designated as clones. Of the colonies derived from seedings of 5–40 cells/well, 24 of 72 colonies tested proved to be cytotoxic.

Fig. 6 presents the individual results from four such cytotoxic colonies chosen to illustrate the patterns of lysis most commonly obtained. In the upper panel, the two colonies (SB11, 20 cells/well; JD3, 5 cells/well) displayed autologous target lysis as well as lysis of all three cross-reacting targets (DW, JU, and M7 bearing HLA-Bw44, Bw62, and Bw35, respectively) while another HLA-mismatched target line, RC and the NK-sensitive target line HSB2 were not killed. In all, 18 colonies showed this pattern. In contrast the lower panel shows two colonies (SB9, 20 cells/well; SA11, 40 cells/well) whose cytotoxicity was preferentially directed towards the autologous cell line with no evidence of any crossreactivity against the relevant targets DW, JU, and M7; one further colony (40 cells/well) showed this pattern. A further three colonies (seeded at 20–40 cells/well) arising in the same experiment appeared to lyse the autologous target cells
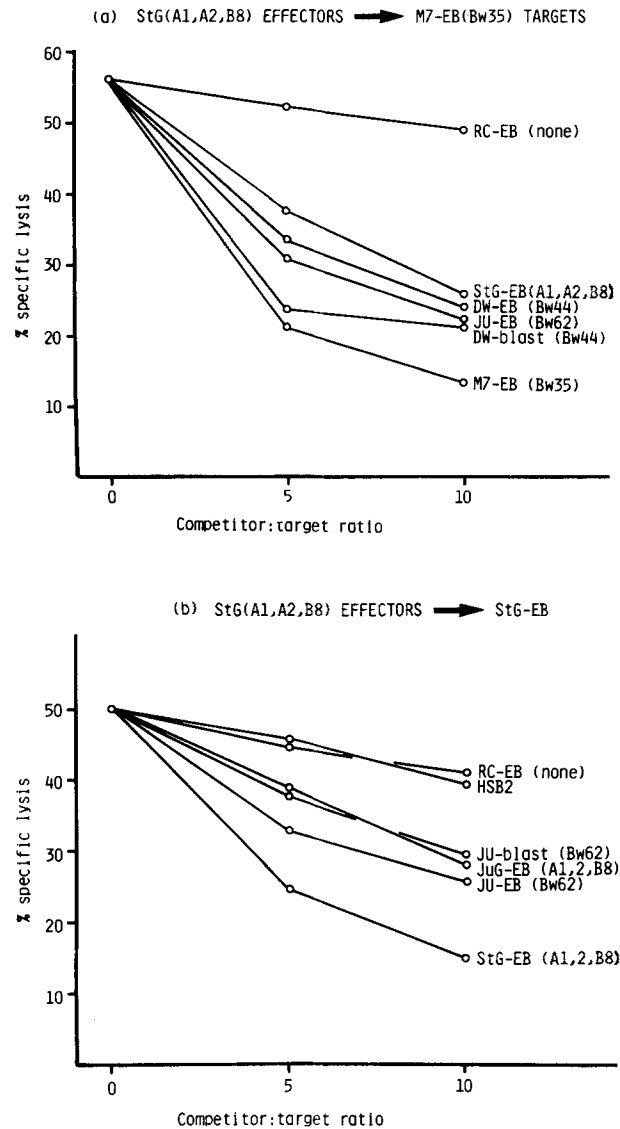
(a) StG(A1,A2,B8) EFFECTORS ➤ M7-EB(Bw35) TARGETS

(b) StG(A1,A2,B8) EFFECTORS ➤ StG-EB

FIGURE 5. (a) "Anomalous" lysis of the HLA-Bw35-bearing cell line M7-EB and (b) EB virus-specific lysis of the autologous cell line StG -EB by effector T cells from donor StG (effector/target ratio 5:1) in the presence of varying numbers of cold target competitor cells whose expression of HLA-A and -B antigens matched with the effector cells or of "cross-reactive" HLA antigens (Bw35, Bw44, Bw62) is shown. The notations -EB and -blast refer to EB virus-transformed and mitogen-stimulated cold target competitor cells respectively.

and only one of the cross-reacting targets, but in each of these three cases the pattern of reactivity could not be unequivocally identified because the levels of killing observed were borderline. Throughout this work, no colonies were found in which cross-reactive lysis occurred in the absence of cytotoxicity against the autologous virus-transformed line.
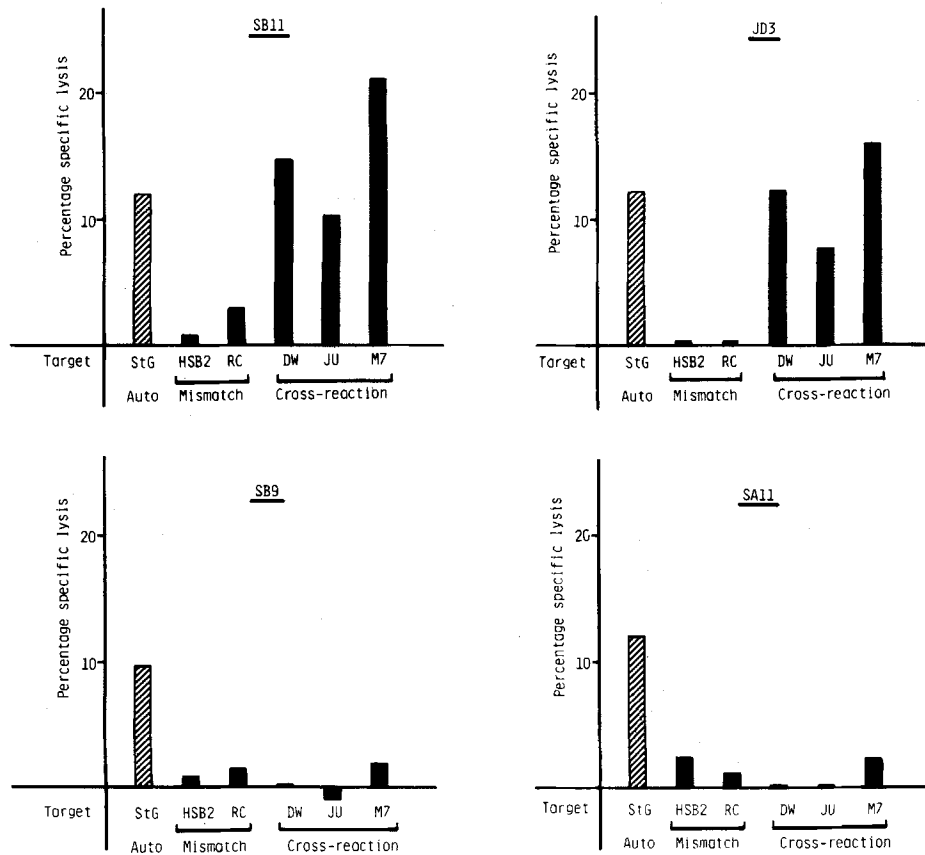
FIGURE 6. Cytotoxicity of four colonies derived from a limiting dilution analysis of effector T cells from donor StG, tested on the autologous EB virus-transformed cell line (hatched columns), on the EB virus genome-negative cell line HSB2 and on the HLA-mismatched EB virus-transformed cell line RC, and on target cells expressing the "cross-reactive" HLA-Bw44 (DW), -Bw62 (JU) or -Bw35 (M7) antigens. The results are expressed as percentage specific lysis. Colonies are designated according to initial number of cells seeded per well in limiting dilution as follows: A, 40 cells/well; B, 20 cells/well; C, 10 cells/well; D, 5 cells/well. Colonies are designated S or J depending on whether they were initially seeded on X-irradiated stimulator cells from the autologous (S) or from the HLA-Bw62-bearing JU (J) EB virus-transformed cell line. The plating efficiency was slightly higher with seeding on autologous stimulator cells but colonies showing autologous cell lysis and cross-reactive lysis were obtained by both methods.

Discussion

Investigations in this (21, 24, 30) and in several other (31–34) laboratories have shown that EB virus-specific cytotoxic T cells can be prepared by in vitro reactivation from memory cells in the blood of virus-immune donors, and that such effector cells are HLA-A and -B antigen-restricted in their function. However, a rigorous analysis of the restriction operative in this system has only recently been made possible through the development of IL-2–dependent effector T cell lines that retain the EB virus-specific HLA-restricted function of the original preparations from which they were derived (23). The present investigation was initiated once it became clear that certain effector T cell lines, which

in all other respects exhibited classical HLA-A and HLA-B antigen-restricted function, nevertheless showed "anomalous" lysis of particular HLA-mismatched targets. Detailed results from three effector T cell lines with such "anomalous" reactivity are described in the present paper. These are not isolated examples, however, for this same phenomenon has been observed in the functional analysis of several other EB virus-specific effector T cell lines derived from other donors and tested in this laboratory.

The results demonstrate that "anomalous" lysis of HLA-mismatched targets occurred irrespective of the EB virus genome status of the target cell, and appeared to be directed against class I allo-HLA determinants expressed on the target cell surface. Thus, in each case, both EB virus-transformed and mitogen-stimulated lymphoblasts from the particular allogeneic donors in question were sensitive to "anomalous" lysis (Figs. 1, 2, and 4), in contrast to the reactivity of these same effector cells against autologous or HLA-matched targets where lysis was confined to the EB virus-transformed cell line only. Moreover "anomalous" lysis could be specifically inhibited by monoclonal antibodies binding to framework determinants on all class I HLA antigens (Fig. 3), the degree of inhibition being at least as strong, if not stronger, than that observed when these same effector cells were simultaneously tested for EB virus-specific lysis of the autologous virus-transformed cell line.

The target structure for "anomalous" cytotoxicity therefore appears to be either a determinant expressed by a class I allo-HLA molecule *per se* or an "interaction antigen" (10, 11) formed by an allo-HLA molecule and some other (non-EB virus-associated) cell surface component. A precedent for the latter view comes from studies in the mouse system (35) in which cloned cytotoxic T cells specific for a minor transplantation antigen restricted by a self H-2K allele were found also to recognize a different minor transplantation antigen in the context of an allogeneic H-2D allele. The present experiments cannot determine whether a second antigen is being recognized in the context of allo-HLA, although they can eliminate fetal calf serum proteins in culture medium as possible contributors to such an "interaction antigen" since mitogen-stimulated lymphoblasts prepared in medium containing human AB serum were equally sensitive to "anomalous" lysis (Fig. 1 legend and unpublished results). It was necessary to check this particular point since there are circumstances in which fetal calf serum-directed cytotoxicity can contaminate otherwise EB virus-specific cytotoxic T cell preparations (36, 37).

Although the alloreactive nature of "anomalous" lysis has been clearly demonstrated, the identity of the target allo-antigen has not always been determined. Thus, in the first example shown by the JuG effector T cell line, no one serologically defined HLA-A, -B, or -C antigen was shared by all the five targets that were sensitive to "anomalous" lysis (Fig. 1). Yet in this case cold target inhibition studies clearly showed mutual cross-inhibition within this group of targets (data not shown), suggesting that each was being recognized through its expression of a common allodeterminant. Perhaps the most likely candidate for such a determinant would be an epitope shared by the serologically related HLA-A3 and -A11 antigens, since all five targets expressed one or other of these antigens.

In contrast, the target antigen responsible for allo-recognition by the EB virus-specific JU effector T cell line was positively identified as HLA-B17 (Bw57). Not only was "anomalous" lysis confined to Bw57-bearing target cells (Fig. 2), but also the killing was strongly inhibited by the monoclonal antibody MA2.1. In contrast, binding of MA2.1 to the HLA-A2-bearing autologous target cell line JU did not reduce its EB virus-specific lysis by the same effector population since most of this activity was restricted through another determinant (HLA-Bw62) on the target cell surface. These particular results suggest that in this case effector cells specific for the EB virus-induced lymphocyte-detected membrane antigen (LYDMA) presented in the context of HLA-Bw62 (self-HLA) were recognizing a cross-reacting epitope presented by the HLA-Bw57 molecule. It is interesting to note here that Bw62 and Bw57 do share some cross-reactive epitopes as revealed by HLA-typing sera (38) so that the interaction of LYDMA with Bw62 might present one or more such epitopes in an immunogenic form to the T cell system.

Analysis of the "anomalous" reactivity of the StG effector T cell line revealed a complex relationship between the target alloantigen recognized by T cells and the serologically defined polymorphism of HLA antigens. Thus, lysis involved all of the Bw35-bearing, all of the Bw62-bearing and a subset of the B12-bearing targets tested (Table I). This subdivision of B12, while clearly distinct from the serologically defined split into Bw44 and Bw45 sub-types, is strikingly similar to that recently reported by other workers using anti-B12 alloreactive T cells generated in a standard mixed lymphocyte culture (39, 40). The fact that all three alloreactivities, against Bw35, Bw62, and B12, respectively, showed considerable mutual cross-inhibition in cold target inhibition experiments (Fig. 5 and unpublished results) strongly suggested that they share a "public" specificity against which the "anomalous" cytotoxicity of StG effector T cells is directed. In this context, it is interesting to note that cross-reactions between Bw35 and Bw62 and between B12 and Bw35 have been identified by serological and cell-mediated lympholysis techniques (38, 41, 42). To our knowledge this is the first identification of a determinant common to all three antigens.

A central question posed by these findings is whether the alloreactivity observed is a genuine cross-reactivity displayed by the EB virus-specific effector T cells themselves or whether it represents a separate reactivity emerging pari passu with the expansion of virus-specific effector cells in IL-2. As a preliminary approach to this question, cryopreserved samples of the original effector cell preparations from which the IL-2–dependent StG and JuG effector T cell lines were derived were thawed and the cells tested on a representative series of targets; "anomalous" lysis of selected HLA-mismatched targets was again apparent even at this early stage (unpublished experiments). Subsequent cold target inhibition experiments supported the view that both virus-specific and alloreactive cytotoxicities were being mediated by the same effector cells. For example, using StG effector T cells, alloreactive lysis could be strongly inhibited by unlabeled target cells of the autologous EB virus-transformed cell line (Fig. 5 a) but not by autologous mitogen-stimulated lymphoblasts (data not shown), while EB virus-specific cytolysis was itself significantly inhibited either by EB virus-transformed cells or by mitogen-stimulated lymphoblasts bearing the relevant

alloantigen (Fig. 5 *b*). Several other examples of alloreactivity, including those displayed by the JuG and JU effector T cell lines, have been analyzed in a similar way and these have always shown significant cross-inhibition between the virus-specific and alloreactive components of the cytotoxicity, although the strength of mutual cross-inhibition did vary between individual examples.

The relationship between virus-specific and alloreactive cytotoxicities of the StG effector cell line was further pursued by limiting dilution culture. Although the relatively poor colony-forming ability of this line meant tht formal criteria for cloning could not be satisfied, colonies were isolated that maintained cytotoxicity against the autologous line but that, unlike the bulk culture, had no alloreactivity; on the other hand, all colonies that displayed alloreactivity also maintained virus-specific cytotoxicity (Fig. 6). This latter result could not be accounted for on the basis of a less stringent limiting dilution procedure used in these particular cases; in fact, those colonies showing only virus-specific lysis were all derived from wells at the higher cell seedings (20–40 cells/well), while those colonies derived from wells at the lowest cell seeding (5 cells/well) displayed both virus-specific and alloreactive cytotoxicities. The inference from these experiments, namely that the StG effector T cell line is composed of clones showing virus-specific lysis only and of clones showing virus-specific and alloreactive lysis, is entirely consistent with the earlier results from cold target inhibition experiments (Fig. 5).

The overall evidence therefore strongly supports the view that EB virus-specific, self HLA-restricted cytotoxic T cells can recognize cross-reactive epitopes presented by class I HLA alloantigens. To our knowledge this is the first demonstration in an outbred species (i.e., man) of a phenomenon that is becoming well recognized in studies with inbred mouse strains using either virus-specific (13, 16, 43) or minor transplantation antigen-specific (12, 14, 35, 44) cytotoxic T cells. Indeed it should be stressed that such cross-reactivities appear to be quite *common* in the EB virus system, having been detected in most if not all of the polyclonal effector T cell populations that have been closely analyzed in the course of this work. Such a result is not surprising in that the various target cells used in such an analysis are derived from many different individuals of an outbred species and thus provide a much greater variety of allodeterminants than are conventionally tested in analogous murine systems; the chances of recognizing fortuitous cross-reactions are therefore correspondingly increased.

Finally, the present studies give support to the thesis that alloreactive responses are derived from within the antigen-specific self-MHC-restricted T cell repertoire, a view that carries with it the rider that certain antigen-specific T cell clones restricted through one self-MHC determinant could show cross-reactivity against a different self-MHC molecule. In fact, such clones would be expected to be either suppressed or deleted in the self-tolerant animal (45, 46) and indeed this may explain why in certain mouse strains, for instance, particular combinations of class I MHC molecule and viral antigen do not appear to induce an effective T cell response (47, 48). Preferential restriction of effector T cells through some but not all of the available class I MHC antigens is also a well recognized feature of the cytotoxic T cell response to EB virus in man (24) and the present demonstration of the alloreactive potential of these virus-specific

cytotoxic T cells is particularly interesting in the light of the model outlined above.

Summary

Epstein-Barr (EB) virus-specific cytotoxic T cells, prepared from virus-immune donors by reactivation in vitro and maintained thereafter as IL-2–dependent T cell lines, have been tested against large panels of EB virus-transformed lymphoblastoid cell lines of known HLA type. Whilst the pattern of lysis of the majority of targets was always consistent with HLA-A and HLA-B antigen restriction of effector function, in several cases it was noticed that certain HLA-mismatched targets were also reproducibly lysed. When this "anomalous" lysis was investigated in detail, it was found to be directed against allodeterminants on class I HLA antigens; thus, mitogen-stimulated as well as EB virus-transformed lymphoblasts from the relevant target cell donors were sensitive to the killing, and in each case the lysis could be specifically blocked by monoclonal antibodies to class I HLA antigens. In one example the target for this alloreactive lysis could be identified as a single serologically defined antigen, HLA-Bw57, while in another example lysis was directed against a "public" epitope common to HLA-Bw35, -Bw62, and a subset of -B12 antigens. Both cold target inhibition experiments and limiting dilution analysis strongly suggested that this alloreactive lysis was being mediated by the same effector T cells that recognize EB viral antigens in the context of self-HLA. This is the first demonstration in man that alloreactive responses can be derived from within the antigen-specific, self MHC-restricted T cell repertoire.

References

1. Medawar, P. B. 1944. The behaviour and fate of skin autografts and skin homografts in rabbits. *J. Anat.* 78:176.
2. Mitchison, N. A. 1954. Passive transfer of transplantation immunity. *Proc. R. Soc. Lond. B. Biol. Sci.* 142:72.
3. Gorer, P. A. 1938. The antigenic basis of tumour transplantation. *J. Pathol. Bacteriol.* 147:231.
4. Zinkernagel, R. M., and P. C. Doherty. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature (Lond.).* 248:701.
5. Katz, D. H., T. Hamaoka, and B. Benecerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. *J. Exp. Med.* 137:1405.
6. Lindahl, K. F., and D. B. Wilson. 1977. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J. Exp. Med.* 145:508.
7. Teh, H-S., R. A. Phillips, and R. G. Miller. 1977. Quantitative studies on the

precursors of cytotoxic lymphocytes. II. Specificity of precursors responsive to alloantigens and to concanavalin A. *J. Immunol.* 118: 1057.

8. Ryser, J. E., and H. R. MacDonald. 1979. Limiting dilution analysis of alloantigen-reactive T lymphocytes. 1. Comparison of precursor frequencies for proliferative and cytolytic responses. *J. Immunol.* 122:1691.

9. Burakoff, S. J., R. Finberg, L. Glimcher, F. Lemonnier, B. Benacerraf, and H. Cantor. 1978. The biologic significance of alloreactivity. *J. Exp. Med.* 148:1414.

10. Matzinger, P., and M. J. Bevan. 1977. Why do so many lymphocytes respond to major histocompatibility antigens? *Cell Immunol.* 29:1.

11. Matzinger, P. 1981. A one-receptor view of T-cell behaviour. *Nature (Lond.).* 292:497.

12. Bevan, M. J. 1977. Killer cells reactive to altered-self antigens can also be alloreactive. *Proc. Natl. Acad. Sci. USA.* 74:2094.

13. Finberg, R., S. J. Burakoff, H. Cantor, and B. Benacerraf. 1978. Biological significance of alloreactivity: T cells stimulated by Sendai virus-coated syngeneic cells specifically lyse allogeneic target cells. *Proc. Natl. Acad. Sci. USA.* 75:5145.

14. von Boehmer, H., H. Hengartner, M. Nabholz, W. Lehnhardt, M. Schreier, and W. Haas. 1979. Fine specificity of a continuously growing killer cell clone specific for H-Y antigen. *Eur. J. Immunol.* 9:592.

15. Sredni, B., and R. H. Schwartz. 1980. Alloreactivity of an antigen-specific T-cell clone. *Nature (Lond.).* 287:855.

16. Braciale, T. J., M. E. Andrew, and V. L. Braciale. 1981. Simultaneous expression of H-2 restricted and allo-reactive recognition by a cloned line of influenza virus-specific cytotoxic T lymphocytes. *J. Exp. Med.* 153:1371.

17. Guimezanes, A., J-L. Davignon, and A. M. Schmitt-Verhulst. 1982. Multiple cytolytic T-cell clones with distinct cross-reactivity patterns co-exist in anti-self plus hapten cell lines. *Immunogenetics.* 16:37.

18. Dickmeiss, E., B. Soeberg, and A. Svejgaard. 1977. Human cell-mediated cytotoxicity against modified target cells is restricted by HLA. *Nature (Lond.).* 270:526.

19. Goulmy, E., A. Termijtelen, B. A. Bradley, and J. J. van Rood. 1977. Y-antigen killing by T cells of women is restricted by HLA. *Nature (Lond.).* 266:544.

20. McMichael, A. J., A. Ting, H. J. Zweerink, and B. A. Askonas. 1977. HLA restriction of cell-mediated lysis of influenza virus-infected human cells. *Nature (Lond.).* 270:524.

21. Rickinson, A. B., L. E. Wallace, and M. A. Epstein. 1980. HLA-restricted T cell recognition of Epstein-Barr virus-infected B cells. *Nature (Lond.)* 283:865.

22. Wallace, L. E., A. B. Rickinson, M. Rowe, D. J. Moss, D. J. Allen, and M. A. Epstein. 1982. Stimulation of human lymphocytes with irradiated cells of the autologous Epstein-Barr virus-transformed cell line. I. Virus-specific and non-specific components of the cytotoxic response. *Cell. Immunol.* 67:141.

23. Wallace, L. E., M. Rowe, J. S. H. Gaston, A. B. Rickinson, and M. A. Epstein. 1982. Cytotoxic T cell recognition of Epstein-Barr virus-infected B cells. III. Establishment of HLA-restricted cytotoxic T cell lines using IL-2. *Eur. J. Immunol.* 12:1012.

24. Moss, D. J., L. E. Wallace, A. B. Rickinson, and M. A. Epstein. 1981. Cytotoxic T cell recognition of Epstein-Barr virus-infected B cells. I. Specificity and HLA restriction of effector cells reactivated in vitro. *Eur. J. Immunol.* 11:686.

25. Gaston, J. S. H., A. B. Rickinson, and M. A. Epstein. 1983. Epstein-Barr virus-specific cytotoxic T lymphocytes as probes of HLA polymorphism: heterogeneity of T cell-restricting determinants associated with the serologically defined HLA-A2 antigen. *J. Exp. Med.* 158:280.

26. Brodsky, F. M., P. Parham, C. J. Barnstable, M. J. Crumpton, and W. F. Bodmer. 1979. Monoclonal antibodies for analysis of the HLA system. *Immunol. Rev.* 47:3.

27. De Kretser, T. A., M. J. Crumpton, J. G. Bodmer, and W. F. Bodmer. 1982.

Demonstration of two distinct light chains in HLA-DR-associated antigens by two-dimensional gel electrophoresis. *Eur. J. Immunol.* 12:214.

28. McMichael, A. J., P. Parham, N. Rust, and F. Brodsky. 1980. A monoclonal antibody that recognizes an antigenic determinant shared by HLA-A2 and -B17. *Hum. Immunol.* 1:121.

29. Wallace, L. E., A. B. Rickinson, M. Rowe, and M. A. Epstein. 1982. Epstein-Barr virus-specific cytotoxic T-cell clones restricted through a single HLA antigen. *Nature (Lond.).* 297:413.

30. Wallace, L. E., D. J. Moss, A. B. Rickinson, A. J. McMichael, and M. A. Epstein. 1981. Cytotoxic T cell recognition of Epstein-Barr virus-infected B cells. II. Blocking studies with monoclonal antibodies to HLA determinants. *Eur. J. Immunol.* 11:694.

31. Misko, I. S., D. J. Moss, and J. H. Pope. 1980. HLA antigen-related restriction of T lymphocyte cytotoxicity to Epstein-Barr virus. *Proc. Natl. Acad. Sci. USA.* 77:4247.

32. Tanaka, Y., K. Sugamura, Y. Hinuma, H. Sato, and K. Okochi. 1980. Memory of Epstein-Barr virus-specific cytotoxic T cells in normal seropositive adults is revealed by an in vitro restimulation method. *J. Immunol.* 125:1426.

33. Tsoukas, C. D., R. I. Fox, S. F. Slovin, D. A. Carson, M. Pellegrino, S. Fong, J-L. Pasquali, S. Ferrone, P. Kung, and J. H. Vaughan. 1981. T-lymphocyte mediated cytotoxicity against autologous EBV-genome-bearing B cells. *J. Immunol.* 126:1742.

34. Fukukawa, T., T. Hirano, N. Sakaguchi, T. Teranishi, I. Tsuyuguchi, N. Nagao, N. Naito, K. Yoshirmura, Y. Okuba, H. Tohda, and A. Oikawa. 1981. In vitro induction of HLA-restricted cytotoxic T lymphocytes against autologous Epstein-Barr virus-transformed B lymphoblastoid cell line. *J. Immunol.* 126:1697.

35. Hunig, T. R., and M. J. Bevan. 1982. Antigen recognition by cloned cytotoxic T lymphocytes follows rules predicted by the altered self hypothesis. *J. Exp. Med.* 155:111.

36. Misko, I. S., R. G. Kane, and J. H. Pope. 1982. Generation in vitro of HLA-restricted EB virus-specific cytotoxic human T cells by autologous lymphoblastoid cell lines: the role of previous EB virus infection and foetal calf serum. *Int. J. Cancer.* 29:41.

37. Misko, I. S., J. H. Pope, R. G. Kane, H. Bashir, and T. Doran. 1982. Evidence for the involvement of HLA-DR antigens in restricted cytotoxicity by foetal calf serum-specific human T cells. *Hum. Immunol.* 5:183.

38. Joysey, V. C., and E. Wolf. 1978. HLA-A, -B and -C antigens, their serology and cross-reactions. *Br. Med. Bull.* 34:217.

39. Kato, S., P. Ivanyi, E. Lacko, B. Breur, R. DuBois, and V. P. Eijsvoogel. 1982. Identification of human CML targets. HLA-B locus (B12) antigen variants defined by CTL generated between B locus identical (B12) responder-stimulator pairs. *J. Immunol.* 128:949.

40. Tekolf, W. A., W. E. Biddison, R. D. Aster, and S. Shaw. 1982. Two subgroups of HLA Bw44 defined by cell-mediated lympholysis that differ in Bw44 expression on platelets and in patterns of genetic linkage equilibrium. *J. Immunol.* 129:1474.

41. Schendel, D. J., R. Wank, and B. Dupont. 1978. Cell-mediated lympholysis: examination of HLA genetic fine structure and complementation using cytotoxic lymphocytes. *Eur. J. Immunol.* 8:634.

42. Schwartz, B. D., L. K. Luehrman, and G. E. Rodey. 1980. HLA public determinants are target antigens of cell-mediated cytotoxicity. *J. Exp. Med.* 152:341s.

43. Askonas, B. A., and Y. L. Lin. 1982. An influenza specific T-killer clone is restricted to H-2L^d and cross-reacts with D^k region. *Immunogenetics.* 16:83.

44. Wettstein, P. J., and R. W. Melvold. 1983. H-2 effects on cell-cell interactions in the response to single non-H-2 alloantigens. VI. H-2K^b mutants differentially regulate the immune response to the H-4.2 alloantigen. *Immunogenetics.* 17:109.

45. Mullbacher, A. 1981. Natural tolerance: a model for Ir Gene effects in the cytotoxic T cell response to H-Y. *Transplantation (Baltimore).* 32:58.

46. Mullbacher, A., R. V. Blanden, and M. Brenan. 1983. Neonatal tolerance of major histocompatibility complex antigens alters Ir gene control of the cytotoxic T cell response to vaccinia virus. *J. Exp. Med.* 157:1324.

47. Doherty, P. C., W. E. Biddison, J. R. Bennink, and B. B. Knowles. 1978. Cytotoxic T-cell responses in mice infected with influenza and vaccinia viruses may vary in magnitude with genotype. *J. Exp. Med.* 148:534.

48. Zinkernagel, R. M., A. Althage, S. Cooper, G. Kreeb, P. A. Klein, B. Sefton, L. Flaherty, J. Stimpfling, D. Shreffler, and J. Klein. 1978. Ir genes in H-2 regulate generation of anti-viral cytotoxic cells: mapping to K or D and dominance of unresponsiveness. *J. Exp. Med.* 148:592.