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In vitro expansion of Epstein–Barr virus-specific HLA-restricted cytotoxic T cells direct from the blood of infectious mononucleosis patients

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SUMMARY

The cytotoxic T-cell response induced by primary Epstein–Barr virus (EBV) infection and detectable in the blood of infectious mononucleosis (IM) patients shows several unusual features when tested in *in vitro* assays. Lysis of EBV-transformed target lines occurs with no apparent HLA restriction, and the putative EBV specificity of the response has been seriously questioned. In the present work we show that the primary T-cell response in IM is polyclonal and indeed does contain a virus-specific HLA class I antigen-restricted component, which can be selectively expanded *in vitro* in the presence of appropriate stimulator cells and IL-2. This allows functional analysis of the virus-specific component of the response in the absence of co-resident reactivities. Studies on blood samples taken from individuals in the acute phase of IM and again post-convalescence suggest that functionally similar populations of HLA class I-restricted cytotoxic T cells are involved in the control of both the primary and persistent phase of EBV infection.

INTRODUCTION

Epstein–Barr virus (EBV), a B-lymphotropic agent, causes infectious mononucleosis (IM), a disease characterized by the appearance of large numbers of reactive CD8-positive T cells in the blood (Crawford *et al.*, 1981). This represents one of the few situations in man where the *in vivo* T-cell response to a defined agent is sufficiently strong and generalized to be easily accessible for analysis. The results obtained with IM-effector cells display several unusual features, however, and their interpretation has long been a source of controversy. Thus, in the initial studies (Svedmyr & Jondal, 1975; Royston *et al.*, 1975) and in some more recent work (Bakacs *et al.*, 1978; Seeley *et al.*, 1981) claims were made that IM mononuclear cell preparations, depleted of conventional natural killer (NK)-cell activity, gave a selective lysis of EBV-positive target lines; on the other hand, subsequent reports have questioned the putative virus specificity of the response (Klein *et al.*, 1980, 1981; Patel, Dorval & Menezes, 1982). The one consistent feature apparent from all these studies with IM effectors has been the lack of any obvious HLA restriction governing target-cell recognition. Indeed, this has led some to postulate (Klein *et al.*, 1981; Patel *et al.*, 1982) that IM-cell cytotoxicity is the *in vivo* correlate of the 'anomalous' T-cell killing which is often generated *in vitro* in situations where there is polyclonal T-cell activation (Seeley & Golub, 1978; Wallace *et al.*, 1982a; Dongworth *et al.*, 1985).

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We were led to re-examine these issues with the knowledge that the persistent, as opposed to the primary, phase of EBV infection is controlled by a memory T-cell response which, on *in vitro* re-activation, shows operational specificity for virus-infected cells and unequivocal restriction through HLA class I antigens (Moss *et al.*, 1981; Wallace *et al.*, 1981). Our recent work, testing cryopreserved IM effectors on a range of autologous and allogeneic targets, has suggested that the 'non-HLA-restricted' T-cell response in IM is actually composed of multiple HLA class I-dependent cytotoxicities (Strang & Rickinson, 1987). Lysis of the autologous EBV-transformed lymphoblastoid cell line (LCL) and of HLA-mismatched LCLs appeared to be mediated by separate populations of effector cells within IM blood. This, accordingly, raises the possibility that the primary response to EBV infection does contain a virus-specific HLA-restricted component functionally analogous to that which is subsequently established in the T-cell memory.

In the work presented here we provide evidence indicating that this is indeed the case. The autologous LCL-reactive component in IM blood has been selectively expanded in IL-2-conditioned medium, allowing it to be analysed free of other co-resident reactivities and to be compared directly with the memory T-cell response generated from the same individual post-convalescence.

MATERIALS AND METHODS

IM patients

All the patients studied showed the usual clinical symptoms of

acute IM, were heterophile antibody-positive in serological tests and had elevated numbers of circulating atypical lymphocytes. Blood samples for effector cell preparation were taken from such patients within 2 weeks of the onset of the disease. Certain patients also donated blood on a second occasion up to 6 months after resolution of the symptoms.

Preparation and storage of NK-depleted effector cells from IM patients

Full details are given elsewhere (Strang & Rickinson, 1987). Briefly, the unfractionated mononuclear (UM) cell population was separated from blood by isopycnic centrifugation on Ficoll–Hypaque (lymphocyte separation medium; Flow Laboratories, Rickmansworth, Herts). The CD16 (Fcγ receptor) -positive subpopulation, which included cells with NK activity, was removed either by EA rosetting or by treatment with anti-CD16 antibody plus complement. Following such depletion, replicate aliquots of CD16-negative cells were cryopreserved in liquid nitrogen. Subsequently individual aliquots were thawed and the effector cells used either directly in cytotoxicity assays or after *in vitro* cell culture.

Preparation and maintenance of target cells

All cells were cultured in RPMI-1640 supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS). EBV-carrying LCLs were prepared from the IM patients themselves either by spontaneous transformation or by experimental infection (using the B95-8 virus strain) of the CD16-positive EA-rosetted mononuclear cell fraction.

LCLs from all other donors were established by B95-8 virus infection of circulating B cells (Miller *et al.*, 1972). All LCLs, and the EBV-negative control cell lines HSB2 and K562, were maintained by twice weekly subculture. EBV-negative control populations of B lymphoblasts were prepared by 3-day mitogenic stimulation of purified circulating B cells using *Staphylococcus aureus* Cown strain I (SAC) (Walker *et al.*, 1986). SAC blasts prepared from IM patients were generated from circulating B cells purified from blood taken post-convalescence.

HLA typing

The IM patients used in this work and the various target cell lines were kindly typed for the antigens HLA-A, B, C, and DR by Ms L. Kennedy of Dr J. Bodmer's laboratory, ICRF Laboratories, London. Note that because serological typing at the HLA-C locus is not yet complete, it is possible that certain combinations of 'HLA-mismatched' effector and target cells could share an as yet undefined C locus allele; with other combinations, HLA mismatching is known to include the HLA-C locus.

Cytotoxicity testing

The ^{51}Cr-release assays were performed essentially as already described (Moss *et al.*, 1981), except that the incubation period varied between 5 hr and 8 hr, and effector : target (E : T) ratios of up to 100 : 1 were used in the case of effector cells freshly purified from the blood.

Monoclonal antibody (MAb) blocking studies

The MAbs used in this work, their antigenic specificities, immunoglobulin subclasses and final concentrations in the assay medium, are described fully elsewhere (Strang & Rickinson, 1987). Effector cells were preincubated with MAbs to CD3 (UCHT1), CD4 (Leu 3a), CD8 (Leu 2a, UCHT4), LFA-1 α chain (MHM24) and LFA-1 β chain (MHM23). Target cells were preincubated with MAbs to HLA class I antigens (W6/32, PA2.6), HLA class II antigens (NFK-1, Apo20) and the CD23 (MHM6) and p80 (AC2) antigens constitutively expressed on all EBV-transformed LCLs. Note that the p80 antigen was provisionally designated as CD39 at the Third International Workshop and Conference on Human Leucocyte Differentiation Antigens (Oxford, 1986). The effector or target cells were pre-exposed to these MAbs for 20 min at 37° in the assay plates before addition of the other cell population to the assay. In certain experiments, pre-exposed cells were washed free of the MAb-containing medium immediately before the assay.

Preparation of IL-2

The IL-2-producing cell line MLA144 (Rabin *et al.*, 1981) was maintained by twice weekly subculture. The residual cell suspension from the subculture was incubated at 37° for a further 7 days, then centrifuged at 400 ***g*** for 10 min to remove the cells, and the supernatant medium then filter sterilized and stored in aliquots at −20° as a source of IL-2.

In vitro *expansion of T lymphocytes from acute IM patients*

UM cells from acute IM patients, depleted of CD16-positive cells either by EA rosetting or by Leu 11b plus complement, were incubated at a density of 2×10^6 cells per 2-ml well or 3×10^5 cells per 0·2-ml well in flat-bottomed sterile culture plates. The IL-2-containing MLA144 supernatant was added to give a final concentration of 25% v/v, and γ-irradiated stimulator LCLs (4000 rads) were added to give a responder : stimulator ratio of 4 : 1. After 4 days, half the supernatant was removed and replaced with fresh medium containing 25% v/v IL-2-conditioned medium; subsequently cultures were refed in this way every 2–3 days. Freshly irradiated LCLs were added every 7 days, 2×10^5 being added to 2-ml wells and 2×10^4 to 0·3-ml wells. Wells containing expanding T-cell populations were subcultured when confluent, and aliquots of the cells were taken for cryopreservation or cytotoxicity testing.

In vitro *reactivation of EBV-specific memory T cells from post-convalescent patients*

EBV-specific cytotoxic T-cell preparations were generated by *in vitro* stimulation of UM cells from post-convalescent patients with autologous LCL stimulators at a responder : stimulator ratio of 40 : 1. The reactive cells were expanded in IL-2-conditioned medium, as already described (Wallace *et al.*, 1982b).

RESULTS

In vitro expansion of IM T cells

Several attempts were made to expand the *in vivo*-activated T cells in IM blood by resuscitating IM mononuclear cell preparations, depleted of Fcγ receptor-bearing cells before cryostorage, and exposing them to γ-irradiated autologous LCL cells and/or IL-2 in culture. Successful IM cell outgrowth, apparent from microscopic examination within 1–2 days of initiation of the cultures, was dependent upon two factors: (i)

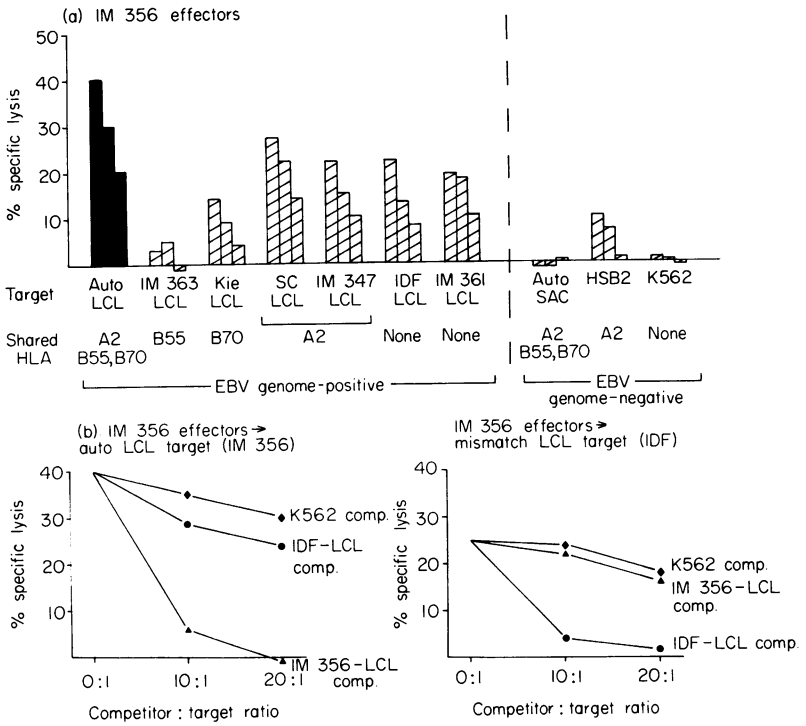

Figure 1. (a) Cytotoxicity of cryopreserved CD16-negative effector cells from acute IM 356 against a panel of target cell lines including autologous and allogeneic EBV-transformed LCLs, autologous SAC-stimulated B-cell blasts, and the NK-sensitive EBV genome-negative cell lines HSB2 and K562. Degrees of HLA-A, B antigen matching between effectors and targets are as shown. Results are expressed as the percentage-specific isotope release observed in an 8-hr cytotoxicity assay, the three columns for each target representing data obtained at E:T ratios of 50:1, 25:1 and 12·5:1. (b) Cold-target competition analysis of the IM 356 effector cells (see Fig. 1a) at a 50:1 E:T ratio against the autologous LCL target (HLA-A2, A2; B55, B70) and against an HLA-mismatched LCL target IDF (HLA-A26, Aw69; B18, B38), in the presence of unlabelled competitors (comp.) from the autologous LCL (▲), from the IDF-LCL (●), or from the K562 cell line (♦).

the presence of relatively high numbers of x-irradiated stimulator cells (initial responder:stimulator cell ratio 4:1); and (ii) immediate addition of IL-2. Unless both conditions were satisfied, the IM cell cultures rapidly degenerated. Rapid expansion could be maintained regularly for a period of at least 14 days, with IL-2 addition every 2–3 days and weekly restimulation with LCL cells, but thereafter the rate of growth slowed and usually cells could not be maintained beyond Days 30–35.

Functional analysis of *in vitro*-expanded cells

Once the above protocol was established, subsequent work concentrated on three particular patients (IM 356, IM 359, IM 363) from whom multiple aliquots of circulating CD16-negative cells had been cryopreserved during the acute phase of the disease. *In vitro*-expanded effector cell preparations were generated in all three cases and compared with the cytotoxic population initially present in the blood. Functional differences between the initial and the *in vitro*-expanded effector populations were observed in each case, the results following a consistent pattern, illustrated here with reference to IM 356.

Figure 1 presents the results of the functional assays involving CD16-negative effector cells cryopreserved direct from the blood of this patient. These effectors showed a complex pattern of cytotoxicity, with strong lysis of the autologous LCL plus a range of reactivities against allogeneic LCLs; allogeneic LCL lysis bore no obvious relationship to shared HLA antigens either of class I (Fig. 1a) or of class II type (data not shown). Killing of HLA-mismatched LCLs, in this case IDF and IM 361, is a characteristic feature of assays with IM effectors (Svedmyr & Jondal, 1975; Seeley *et al.*, 1981; Strang & Rickinson, 1987). However, cold-target competition experiments (Fig. 1b) clearly indicated that lysis of the autologous LCL and of the HLA-mismatched IDF-LCL was mediated by separate effectors within the IM 356 preparation. Thus isotope release from autologous LCL targets was inhibited by unlabelled competitor cells of the same line but not by IDF-LCL cells, and vice versa. Further cold-target competition experiments (data not shown) showed that residual lysis of the NK-sensitive target cell line HSB2 involved yet a third type of cytotoxic cell which, for some IM patients including IM 356, could not be completely removed by CD16-positive cell depletion (Strang & Rickinson, 1987).

Figure 2 shows the cytotoxic profile of *in vitro*-expanded effectors from IM 356 on a similar panel of targets. Whilst this effector population had retained strong reactivity against the autologous LCL, lysis of HLA-mismatched LCLs, including IDF, was now very low. There was also little reactivity against either the EBV-negative NK-sensitive targets or against autologous mitogen-stimulated B lymphoblasts. However there was significant lysis both of HLA-A2-matched and of HLA-B55-matched target LCLs, a finding at least consistent with an HLA class I antigen-restricted cytotoxicity. This possibility was

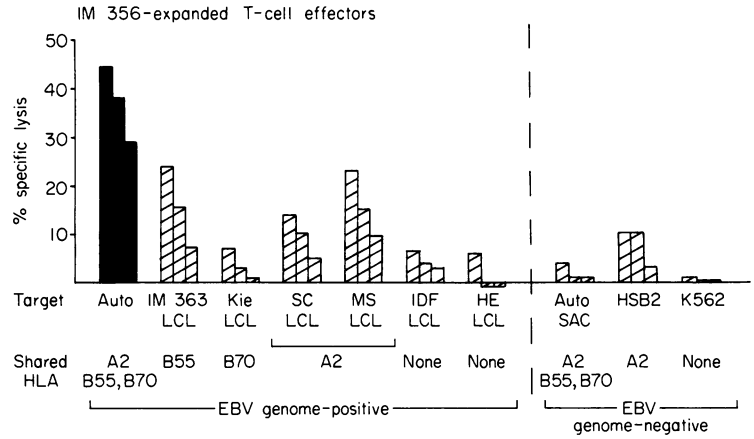

Figure 2. Cytotoxicity of IM 356 T-cell effectors, expanded *in vitro* in the presence of IL-2 and autologous LCL stimulators, against a panel of target cell lines including autologous and allogeneic EBV-transformed LCLs, autologous SAC-stimulated B-cell blasts, and the NK-sensitive cell lines HSB2 and K562. Degrees of HLA-A, B antigen matching between effectors and targets are as shown. E:T ratios are 18:1, 9:1 and 4·5:1.

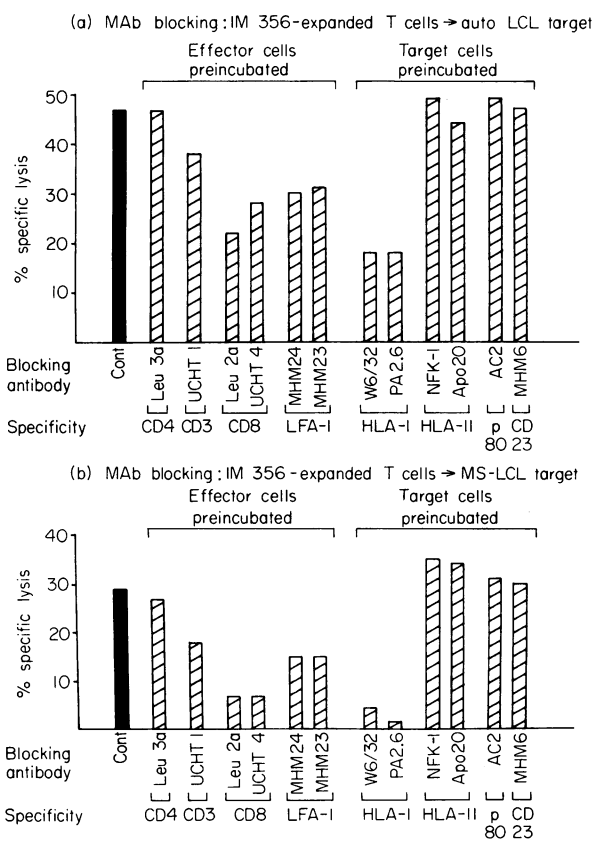

Figure 3. Monoclonal antibody (MAb) blocking of target cell lysis by IM 356 effector T cells expanded *in vitro:* (a) results with autologous LCL targets; (b) results with MS-LCL targets (HLA type A2, A2; B18, Bw44) matched through HLA-A2 with IM 356 (HLA type A2, A2; B55, B70). Filled columns represent levels of target cell lysis observed in the absence of MAb (cont), hatched columns represent lysis observed when either effector or target cells were pre-exposed to saturating concentrations of the relevant MAb before addition to the assay. The E:T ratio was 10:1.

further supported by the results of MAb blocking assays. Thus, as shown in Fig. 3, lysis of the autologous LCL and of the HLA-A2-matched MS-LCL by *in vitro*-expanded effectors was specifically inhibited by pre-treatment of the target cells with saturating concentrations of MAbs to framework determinants on HLA class I molecules; parallel exposure of the target cells to MAbs either against HLA class II molecules or against other cellular proteins strongly expressed on the surface of all LCLs (p80 and CD23) had no effect on their lysis. Pre-treatment of the effectors with MAbs to LFA-1, CD8 or CD3 also reduced the level of killing significantly, although the effect of the anti-CD3 MAb was less marked than usual in this particular experiment (Fig. 3). The levels of blocking observed in this work were like those already observed in MAb-blocking assays using *in vitro*-reactivated effector T cells from healthy virus-carrying individuals where target cell recognition is known to be immunologically specific and HLA class I antigen-restricted (Moss *et al.*, 1981; Wallace *et al.*, 1981).

Prospective study of primary and secondary T-cell responses to EBV

Prospective studies with two IM patients, IM 356 and IM 363, allowed the autologous LCL-reactive component of the primary T-cell response, expanded *in vitro* as above, to be compared with the EBV-specific memory T-cell response reactivated *in vitro* from the circulating T-cell pool of these same patients post-convalescence. The effector preparations thus produced were functionally very similar, a result illustrated here with reference to IM 363, where the similarity of the two responses extends to include an interesting additional reactivity.

Figure 4 presents the results of assays using *in vivo*-activated IM 363 effectors, cryopreserved after depletion of Fcγ receptor-bearing cells. Strong reactivity against the autologous LCL was accompanied by broad-ranging lysis affecting several HLA-mismatched LCLs, but particularly that from donor SB (Fig. 4a). In cold-target competition experiments, although unlabelled SB-LCL cells were unable to inhibit autologous (IM 363) LCL lysis, killing of SB-LCL targets was inhibited almost as

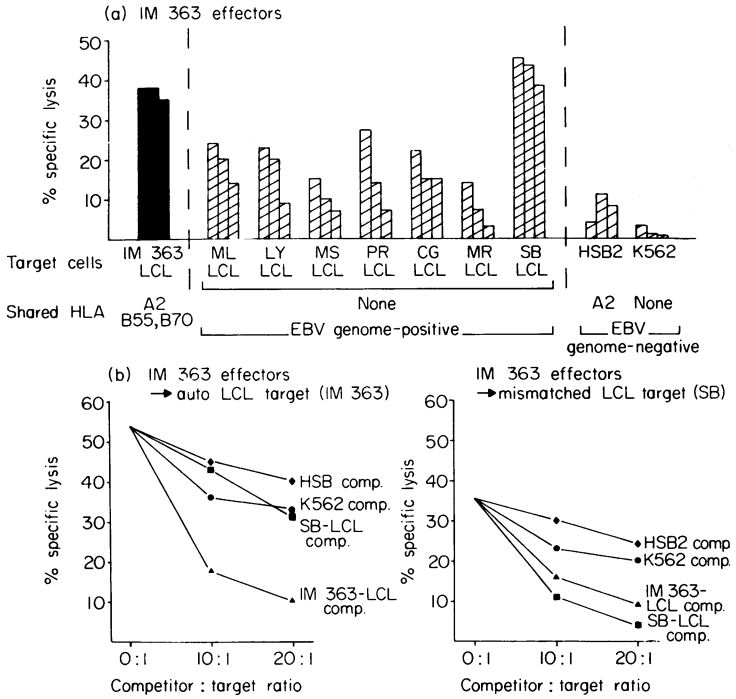

Figure 4. (a) Cytotoxicity of cryopreserved CD16 -negative effector cells from acute IM 363 against a panel of target cell lines including autologous and allogeneic EBV-transformed LCLs, and the HSB2 and K562 cell lines. Degrees of HLA-A, B antigen matching between effectors and targets are as shown. Results are expressed as the percentage specific isotope release observed in an 8-hr cytotoxicity assay, the three columns for each target representing data obtained at E:T ratios of 32:1, 16:1 and 8:1. (b) Cold-target competition analysis of IM 363 effector cells (see Fig. 4a) tested at a 60:1 E:T ratio against the autologous LCL target (HLA-A3, A3; B7, B55) and against a HLA-mismatched LCL target SB (HLA-A2, A11; B39, B44), in the presence of unlabelled competitors from the autologous LCL (▲), from the SB-LCL (■), or from the HSB2 (♦), or K562 (●) cell lines.

efficiently by unlabelled IM 363-LCL competitors as by SB-LCL competitors themselves (Fig. 4b). This was the *only* instance in our experience where a component of the autologous LCL-reactive response in acute IM appeared to be responsible also for the lysis of an HLA-mismatched target.

Interestingly, when this *in vivo*-activated IM 363 effector preparation was directly expanded *in vitro*, the proliferating cells displayed a pattern of reactivity against LCL targets that was essentially consistent with restriction through HLA class I antigens, except that strong lysis of the HLA-mismatched SB-LCL target had been retained (Fig. 5a, b). Moreover, when donor IM 363 was examined post-convalescence for evidence of EBV-specific T-cell memory, the *in vitro*-reactivated T-cell response also showed unexpected reactivity against SB-LCL cells (Fig. 5c). Extension of the target-cell panels used in these assays indicated that two further HLA-mismatched LCLs, from donors RB and CM, were also lysed both by *in vitro*-expanded IM 363 effectors and by memory T-cell preparations from the same patient (Fig. 6). The one common feature distinguishing these particular HLA-mismatched target lines from the rest was their expression of the HLA-A11 antigen. This recognition of SR-, RB- and CM-LCL cells appeared to be EBV-related since mitogen-stimulated B lymphoblasts prepared from these same donors were not killed (Fig. 6).

DISCUSSION

Functional studies on T-cell populations taken directly from the blood of IM patients have always been complicated by the presence of many functionally distinct components within the EBV-induced T-cell response. In particular, our recent work has indicated the existence of several distinct cytotoxic populations in IM blood; one is reactive against the autologous LCL whilst others, coincidentally activated during the disease process, appear to be largely responsible for the lysis of HLA-mismatched LCL targets (Strang & Rickinson, 1987). *In vitro* expansion, and ultimately cloning, of IM effector cells would clearly be useful in separating these various reactivities.

Whilst this work was in progress, Moss *et al.* (1985) reported that IM T cells survived very poorly *in vitro* in the absence of IL-2, appearing to be programmed towards cell death by apoptosis. Although Bishop *et al.* (1985) were able to achieve *in vitro* expansion of such cells by immediate provision of IL-2, in the absence of any stimulator cells, the expanded population showed broad-ranging cytotoxicity against both EBV-positive and EBV-negative target cell lines (Dr D. J. Moss, personal communication). In the present work, prior depletion of conventional NK-cell activity from the IM mononuclear cell pool, and subsequent stimulation of the depleted population with both autologous LCL cells and IL-2, has allowed the selective *in vitro* expansion of an autologous LCL-reactive component (Figs 1, 2, 4 and 5). This component specifically recognizes EBV-positive as opposed to EBV-negative autologous B lymphoblasts (Figs 2 and 6) and appears, both from its pattern of reactivity against allogeneic LCLs and from its sensitivity to MAb blocking, to be functionally restricted through HLA class I antigens on the target cell surface (Figs 2, 3 and 5). The involvement of CD3 molecules (associated with the

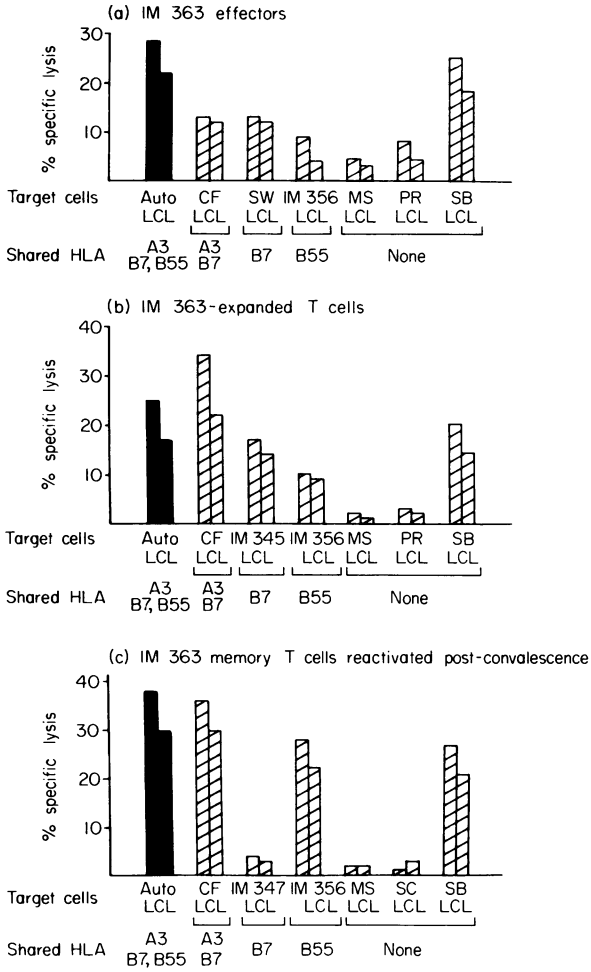

Figure 5. Cytotoxicity of three different populations of effector cells from donor IM 363 against a panel of target cell lines including autologous and allogeneic EBV-transformed LCLs. Degrees of HLA-A, B matching between effectors and targets are as shown. (a) Cryopreserved CD16 -negative effector cells from acute IM 363 tested in an 8-hr assay at E:T ratios of 32:1 and 16:1. (b) T cells expanded *in vitro* directly from the blood of acute IM 363 tested in a 5-hr assay at E:T ratios of 16:1 and 8:1. (c) Memory T cells reactivated *in vitro* from the blood of donor IM 363 6 months post-convalescence and tested in a-5 hr assay at E:T ratios of 2:1 and 1:1.

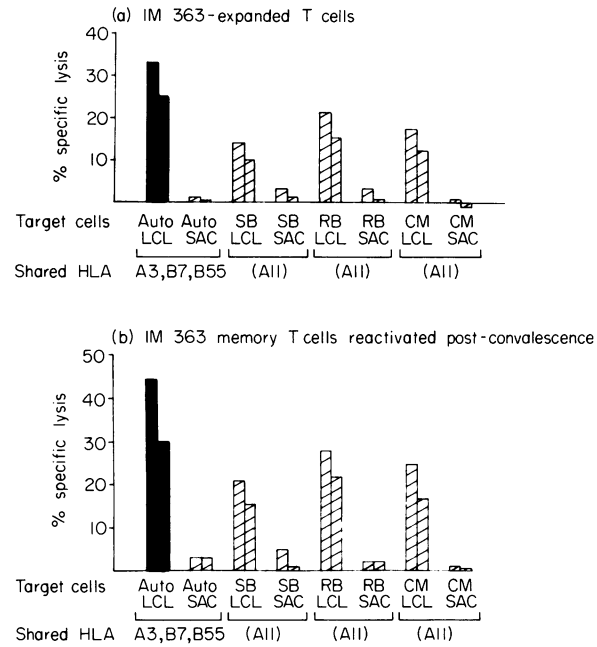

Figure 6. Cytotoxicity (a) of T cells expanded *in vitro* directly from the blood of acute IM 363, and (b) of memory T cells reactivated *in vitro* from the blood of donor IM 363 6 months post-convalescence. Target cells included EBV-transformed LCL cells and SAC-stimulated B-cell blasts of autologous type, and the corresponding pairs of targets from donors SB, RB and CM. These three donors share HLA-A11 in common but are HLA-A, B mismatched with IM 363. Results are from 5-hr cytotoxicity assays at E:T ratios (a) of 20:1 and 10:1, and (b) of 5:1 and 2·5:1.

T-cell receptor) and of CD8 molecules (thought to stabilize E:T association through binding to a non-polymorphic region of HLA class I molecules; Swain, 1983) again suggests immunologically specific recognition. Indeed, the MAb-blocking results with the *in vitro*-expanded IM effector population exactly mirror those seen with the autologous LCL-reactive component taken direct from the blood (Strang & Rickinson, 1987).

The protocol favouring *in vitro* expansion of IM effectors shows several important differences from that used to reactivate EBV-specific memory T cells from the blood of previously infected virus-immune donors (Wallace *et al.*, 1982a, b). In particular, the high numbers of autologous LCL stimulators necessary for IM cell expansion and the responder cells' immediate requirement for IL-2 would, if applied to the T-cell pool of virus-immune donors, generate an entirely non-specific 'anomalous' T-cell cytotoxicity (Wallace *et al.*, 1982a). Conversely, *in vitro* stimulation of IM cells using the memory cell reactivation protocol never achieved cell outgrowth; indeed, earlier studies have shown that the IM T-cell pool contains no EBV-specific T-cell memory of the kind revealed in the *in vitro* regression assay (Rickinson *et al.*, 1980). It should be noted that the autologous LCL-reactive T cells expanded from IM blood consistently showed only a limited *in vitro* proliferation, significantly less than that observed for reactivated memory T cells where cell proliferation can be maintained for several weeks, if not months (Wallace *et al.*, 1982b). This may reflect a real difference between the two types of cell. It may be that most effector T cells of the primary response have only a very limited potential for self-renewal, in line with their role as mediators of the acute response to infection. At the same time we cannot discount the possibility that cytotoxic T-cell growth *in vitro* is normally dependent upon accessory cell help, which is perhaps better provided in responder cell populations from healthy donors or which is antagonized by the suppressor cells known to be present in IM blood (Reinhertz *et al.*, 1980).

The present paper is the first to show the essential similarity between the autologous LCL-reactive component of the primary T-cell response in IM and the EBV-specific T-cell response which is subsequently established in T-cell memory. In the particular case of patient IM 363 (Figs 4, 5 and 6), this similarity even extended to an unexpected cross-recognition of three HLA-mismatched LCLs, all bearing the HLA-A11 alloantigen. Since this unexpected lysis was blocked by anti-HLA class I MAbs (data not shown), one possibility is that it represents

fortuitous cross-recognition of the alloantigen *per se*, just as has been described already in studies with murine (von Boehmer *et al.*, 1979) and with human (Gaston *et al.*, 1983a) T cells. In fact this was not the case, for only EBV-positive targets bearing the relevant alloantigen were lysed. The present results appear, therefore, to reflect a rare, but not unprecedented (Gaston *et al.*, 1983b), instance of EBV-specific effector T cells utilizing a restricting epitope on an HLA class I alloantigen.

It must be stressed that, in these initial experiments, our analysis of *in vitro*-expanded T-cell populations from IM blood has been confined to three patients; in particular we cannot yet say whether the *in vitro* protocol described here magnifies all or only some of the autologous LCL-reactive clones present *in vivo*. Nevertheless, the present results clearly demonstrate that the primary response to EBV infection includes a cytotoxic T-cell component which is both operationally EBV-specific and HLA class I-antigen restricted. The acute phase of IM is associated with EBV-induced B-cell proliferation *in vivo* (Klein *et al.*, 1976; Robinson, Smith & Niederman, 1980); we would suggest that it is the above EBV-specific component of the primary T-cell response which is principally responsible for bringing this B-cell proliferation under control.

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