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Virus-specific cytotoxic T cell responses are associated with immunity of the cottontop tamarin to Epstein–Barr virus (EBV)

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SUMMARY

The cytotoxic responses of peripheral blood lymphocytes from cottontop tamarins to *in vitro* re-stimulation with autologous lymphoblastoid cell lines (LCL) were assayed. Lymphocytes from immune tamarins that had recovered from EBV challenge developed potent cytotoxicity for natural killer (NK) cell targets and for autologous LCL. The cytotoxicity for LCL targets was EBV-specific, as B cell blasts uninfected with EBV were not killed. The cell lines could be maintained by repeated stimulation with LCL and the addition of IL-2. Flow cytometry showed that they were T cell lines expressing CD2, CD3, CD4, CD8 and CD25. Dual-colour flow cytometry revealed two subpopulations, one $CD4^+$ $CD8^+$ population and the other $CD4^-$ $CD8^+$. After separation by magnetic cell sorting both subpopulations were shown to be cytotoxic and the $CD4^+$ $CD8^+$ fraction was also shown to be MHC class II-restricted; the MHC restriction of the $CD8^+$ subpopulation could not be determined. The unseparated T cells and both the subpopulations were able to inhibit LCL outgrowth *in vitro*. In contrast, PBL from naive tamarins stimulated by autologous LCL developed less NK cell cytotoxicity and little cytotoxicity for LCL. The cytotoxic response was enhanced at higher levels of LCL stimulation, but the cells were unable to inhibit LCL outgrowth *in vitro*. We conclude that cytotoxic responses capable of inhibiting LCL growth *in vitro* correlate with *in vivo* immunity in the tamarin model and provide a basis for understanding the mechanism of vaccine-induced immune protection.

Keywords Epstein–Barr virus cytotoxic T cell natural killer cell tamarin

INTRODUCTION

The cottontop tamarin (*Saguinus oedipus oedipus*) has been proposed as an animal model of EBV infection in humans. Cottontop tamarins challenged with a large dose of EBV develop multiple B cell lymphomas within 14–21 days that contain the EBV genome [1]. In most cases the tumours are progressive and fatal, but a proportion of animals survive and in these individuals the tumours regress over a period of 8–12 weeks and these recovered tamarins are completely immune to a second virus challenge [1,2]. Although *in vivo* the B cell tumours of tamarins resemble those that develop in immunosuppressed humans and *in vitro* EBV-transformed lymphoblastoid cell lines (LCL) can be established from tamarin peripheral blood B cells [3], human and tamarin EBV infections differ in other respects. EBV does not establish a lifelong latent infection of tamarins at the same level that occurs in humans [2,4], and the dose of virus used to infect a tamarin is 50 000 times that estimated to transfer infection between humans [1]. Furthermore, attempts to infect tamarins via the mucosal route have not been successful (M. Finerty and A. J. Morgan, unpublished), nor has any disease resembled infectious mononucleosis been described in this species. This conflicting information raises the question of whether a vaccine capable of protecting tamarins would necessarily have any beneficial effect in humans.

Resistance to EBV-related disease in humans depends on an effective cellular immune response [5], and tamarins in which EBV-induced lymphoma regressed also possess a cellular immune response [2]. Prior studies have shown that not all potential vaccines protect tamarins from EBV challenge. Immunization with a recombinant vaccinia virus protected tamarins from EBV challenge in the absence of a neutralizing antibody response [6], but conversely, in one experiment vaccination with a gp340 envelope glycoprotein in liposomes induced high titres of *in vitro* neutralizing antibodies which were not protective *in vivo* [7]. These observations support the hypothesis that a cell-mediated immune response is essential to protect tamarins from EBV challenge, as is the case in humans.

Despite the difficulties described above, tamarins may still provide a useful model for the assessment of vaccines against EBV. However, in order that the data from such experiments can be interpreted it is essential that the nature of the protective

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immune response in tamarins can be measured and compared with immune responses in humans. In this study we describe the development of cytotoxic responses in tamarins that have recovered from EBV challenge, and characterize the cell types involved. We also describe the establishment of a cytotoxic T cell line from an immune tamarin and show that it can control outgrowth of EBV-infected LCL *in vitro*. Lymphocytes from naive tamarins do not share these properties. The data provide a basis for the objective assessment of protective immune responses induced by future candidate vaccines.

MATERIALS AND METHODS

EBV virus infection of tamarins

Adult tamarins were obtained from a breeding colony [8]. Two tamarins which had previously recovered from a tumourogenic dose of EBV prepared from B95-8 cells as previously described [1] were injected with a further dose of virus. A further group of six naive tamarins served as controls.

Cell culture

All cells were cultured at 37°C, 5% CO_2 in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO BRL, Paisley, UK). Tamarin EBV-transformed LCL were established from peripheral blood lymphocytes as previously described [2]. Cell lines K562 and HSB2 were obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK), cultured in RPMI as above and split twice weekly. The MLA cell line was acquired from the ECACC and used as a source of IL-2, where RPMI culture supernatant was collected weekly and filtered through a 0·22-μm filter, then stored at −20°C until use.

Cell phenotyping and separation

The phenotype of tamarin cell populations was determined by flow cytometry using cross-reacting mouse anti-human MoAbs as previously described [9]. Anti-CD2 (MT910), CD4 (MT301), CD14 (TUK4), CD20 (B-Lyl), CD23 (MHM6), and CD25 (ACT-1) were purchased from Dako (High Wycombe, UK). Monoclonal anti-human CD40 (G28.5), and monoclonal anti-MHC class I (BB7.7 and BB7.8) were acquired from the American Type Culture Collection (ATCC, Rockville, MD), Da6.231 anti-MHC class II came from the ECACC. Purified anti-CD3 (SP34) was a gift of Dr C. Terhorst (Dana Faber Cancer Institute, Boston, MA). Purified anti-CD8 (MT1014 and MT122) were provided by Dr P. Reiber (Institut für Immunologie, Universität Munchen, Germany). Anti-CR2 (CD21 (BU35)) was purchased from The Binding Site (Birmingham, UK). Briefly, cells were incubated with the appropriate MoAb diluted in PBS azide, washed in PBS azide and stained with FITC-conjugated affinity-purified Fab_2 fragment goat anti-mouse IgG absorbed with human IgG (Sigma, Poole, UK). For dual staining primary antibodies of different isotype were used followed by isotype-specific FITC or PE conjugates (Southern Biotechnology, Birmingham, AL).

Subpopulations of cells were separated by phenotype using magnetic activated cell separation. Cells were labelled in a two-stage procedure using the same MoAbs as used in flow cytometry and a secondary monoclonal rat anti-mouse IgG1 conjugated to paramagnetic microparticles (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The labelled cell population was then positively selected using a Minimacs column (Miltenyi Biotech), both labelled and unlabelled populations being retained for further use.

Cytotoxic assay

Target cells (10^6) were labelled by incubation for 1 h at 37°C with 50 μCi ^{51}chromium in a total volume of 50 μl and washed five times in PBS. Triplicate wells of V-bottomed microtitre plates (Costar, High Wycombe, UK) were prepared containing effector cells, labelled targets and, where indicated, blocking antibodies or excess unlabelled targets in a final volume of 200 μl. Control wells with targets alone or 1% Triton X100 were included to measure spontaneous label release and total label, respectively. The plates were incubated for 4 h at 37°C and centrifuged at 500 ***g*** for 1 min, 100 μl of supernatant were collected from each well and counted in a gamma counter (LKB Compugamma, Bromma, Sweden). The percentage specific chromium release was calculated as (specific counts − spontaneous counts)/(total counts − spontaneous counts) × 100/l.

In vitro *induction and maintenance of cytotoxic T cell lines*

Animals were sedated with 5 mg/kg ketamine hydrochloride (Park Davies, Morris Plains, NJ), and 2 ml of blood were collected from the medial femoral vein and placed in 2 ml Alsever's solution (Sigma). Peripheral blood lymphocytes (PBL) were isolated by centrifugation over lymphocyte separation media sp. gr. 1·0117 g/ml (Flow ICN Biochemicals, Thane, UK) at 500 ***g*** for 30 min. Cells at the interface were collected, washed twice in PBS and once in RPMI. Autologous LCL were treated for 1 h with 80 μg/ml mitomycin C (MMC) (Sigma) and washed five times in PBS. Cultures containing 2×10^6 PBL and autologous LCL at a ratio of 40 : 1 in a final volume of 2 ml were cultured for 10 days. The cultured cells were then washed, adjusted to 1×10^6 viable cells/well and re-stimulated with MMC-treated LCL at a ratio of two PBL to one LCL. On day 14 the viable cells were harvested and stimulated a third time with MMC LCL at a 2 : 1 ratio with the addition of 10% MLA supernatant and 10 U/ml recombinant IL-2 (Sigma). Thereafter the cultures were maintained by weekly re-stimulation with LCL at 2 : 1 ratio and bi-weekly addition of IL-2/MLA.

Growth inhibition assay

The ability of T cells to inhibit the outgrowth of autologous LCL *in vitro* as a measure of immune competence was carried out as previously described [2]. Briefly, six replicate serial dilutions of autologous LCL from $2{\cdot}5 \times 10^4$/well to $1{\cdot}7 \times 10^2$/well were placed in 96-well plates. A constant number of 2×10^4 effector T cells was added to each well and the cultures were incubated at 37°C for 4 weeks and observed for the outgrowth of LCL. Control wells without the addition of effector cells were set up at the same time. All control wells showed outgrowth of LCL at 4 weeks. The results of the inhibition were expressed as the highest ratio of T cells to LCL that inhibited LCL growth in 50% of wells.

In vitro *culture of non-EBV-transformed B cell lines*

B cells were purified from PBL using MACS. The cells were labelled with monoclonal anti-CD20 (Dako) and rat anti-mouse paramagnetic conjugate (Miltenyi Biotech) and separated on a Minimacs column. The purified B cells were eluted and cultured in RPMI 10% FCS supplemented with 10 ng/ml human recombinant

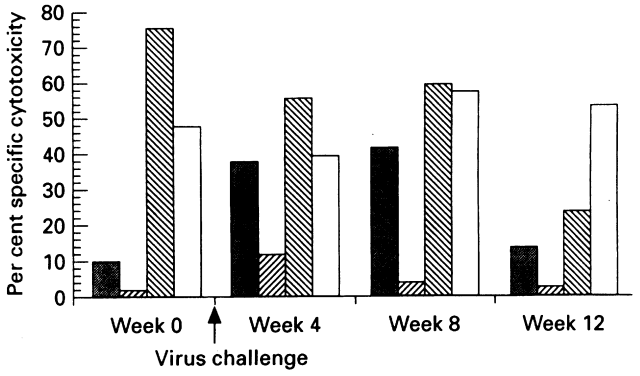

Fig. 1. The percentage specific cytotoxicity for various targets of peripheral blood lymphocytes (PBL) from a tamarin which had recovered from EBV infection. PBL were sampled prior to a second EBV challenge and at 4, 8, and 12 weeks after challenge. The PBL were cultured *in vitro* with autologous mitomycin-treated lymphoblastoid cell lines (LCL) at a ratio of 40 PBL:1 LCL and after 10 days were replated at a ratio of 2 PBL:1 LCL. The cells were used in the cytotoxicity assay on day 14 at an effector:target ratio of 10:1. The results show the percentage specific cytotoxicity for autologous LCL (■), heterologous LCL (▨), K652 natural killer (NK) targets (▨), and HSB2 (□).

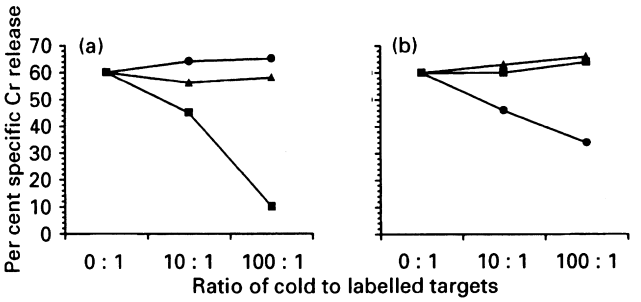

Fig. 2. Determination of specificity of cytotoxic responses by cold target inhibition. Peripheral blood lymphocytes (PBL) from an immune tamarin B152 were restimulated *in vitro* with autologous lymphoblastoid cell lines (LCL). Cytotoxicity assays were set up at an effector: target ratio of 10:1 using chromium-labelled autologous LCL (a) and K562 natural killer (NK) cell targets (b). Increasing numbers of unlabelled cells were added to compete with the labelled targets. (a) Killing of autologous LCL was inhibited by the addition of unlabelled autologous LCL (■) but not by unlabelled heterologous LCL (▲) (or K562 cells (●)). (b) Killing of NK cells was inhibited by the addition of unlabelled K562 cells (●) but not by autologous (■) or heterologous LCL (▲).

IL-4 (Sigma) and 0·2 μg/ml monoclonal anti-CD40 on a monolayer of mitomycin-treated CDw32 feeder cells [10].

RESULTS

The time course of the cytotoxic response of tamarin B158, which had recovered from primary EBV infection and was rechallenged with EBC at time 0, is shown in Fig. 1. Before challenge PBL restimulated *in vitro* with autologous LCL developed potent natural killer (NK) activity, as shown by their killing of K562 and HSB2 targets. The same cells had only slight ability to kill autologous LCL and showed minimal cytotoxicity for heterologous LCL. The high level of NK activity remained throughout the experiment. Killing of autologous LCL increased in the weeks post-challenge, peaking between weeks 4 and 5, thereafter tailing off toward resting levels. Killing of heterologous LCL increased slightly around week 4 but overall remained low throughout the experiment. Cells from the second immune tamarin B152 showed a similar time course of cytotoxicity (data not shown). The cultures of PBL could be maintained for several weeks by stimulation with LCL and addition of IL-2 to the medium. The cells continued to be cytotoxic for both NK and LCL targets.

The time course experiment suggests that the NK activity and the killing of LCL are separate functions. This was confirmed by cold target inhibition, showing that the addition of unlabelled LCL to the cytotoxicity assay inhibited killing of labelled LCL but not labelled K562 cells. Conversely, the killing of labelled K562 cells was inhibited by the addition of unlabelled K562 cells but not by autologous LCL. Addition of heterologous LCL from B158 did not inhibit either killing activity, implying that the killing of LCL is MHC-restricted (Fig. 2).

The EBV specificity of the cytotoxic response was confirmed by comparing the killing of autologous LCL and non-EBV-infected B cells by B152-derived T cells. The B cell line was demonstrated to be EBV^- by immunofluoresence for EBV antigens, and did not continue to grow in the absence of stimulation with IL-4 and anti-CD40. Flow cytometry showed that both the B cell line and LCL expressed a similar range of B cell surface antigens, i.e. surface immunoglobulin, CD20, 21, 23, 40, MHC class I and MHC class II. Two T cell lines derived 5 weeks and 6 weeks after virus challenge killed LCL but not B cells. Two further cell lines derived 6 months after virus challenge showed 19% and 11% specific killing of LCL, but only 6% and 0% killing of autologous B cells. The line with the high killing of autologous B cells also showed higher NK cell activity of 57% specific lysis of K562 cells (see Table 1).

Flow cytometry showed that the LCL-stimulated PBL cultures were predominantly T cells, i.e. >95% of the cells were $CD3^+$ and $CD2^+$. None of the cells expressed the B cell marker CD20 or the monocyte marker CD14. Dual staining with anti-CD4 and anti-CD8 showed two major subpopulations, one $CD4^-$ $CD8^+$ and the second $CD4^+$ $CD8^+$, and a minor population of $CD4^-$ $CD8^-$ cells (Fig. 3b). The distribution of cells stained for CD4 alone in FACS profiles was markedly closer to the abscissa, and this whole population shifted away to the right when anti-CD8 antibody was added. We conclude that most if not all $CD4^+$ cells expressed some CD8, although at a low level. These double-positives appear to be a feature of the immune responses to EBV in tamarins, since

Table 1. Cytotoxic activity against autologous EBV-infected lymphoblastoid cell lines (LCL), uninfected autologous B cells and K562 natural killer (NK) targets of two effector T cell lines taken from an immunized tamarin (B152) at several time points after challenge with EBV

Time course of effector T cell function after challenge	Target cell % cytotoxicity*		
	LCL	B cell	K562
5 weeks	10	0	9
6 weeks	11	0	13
6 months	19	6	57
6 months	11	0	43

* All assays conducted at the same time.

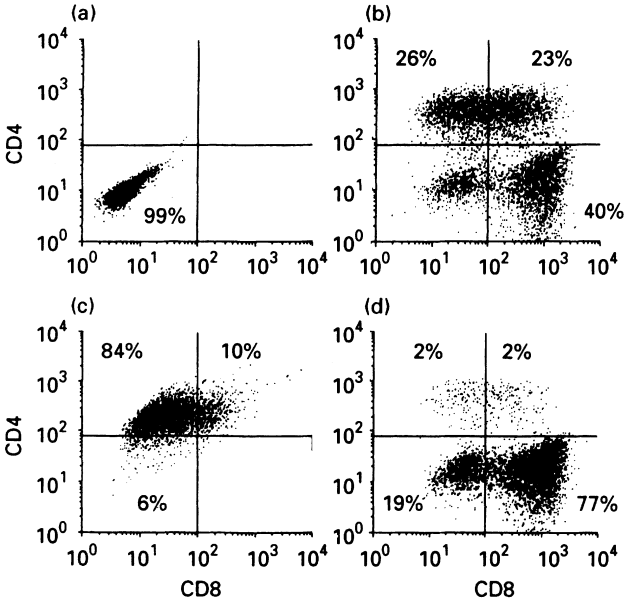

Fig. 3. B152 cells stained with CD4-PE and CD8-FITC. The cells were separated using a Minimacs magnetic sorting column with anti-CD4 and magnetic rat anti-mouse MoAbs. (a) Unstained controls. (b) Unseparated B152 cells. (c) Positively selected $CD4^+$ B152 cells. (d) $CD4^+$-depleted B152 cells.

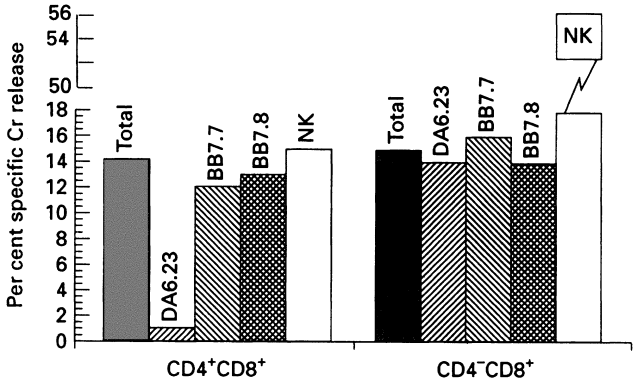

Fig. 4. After separation the $CD4^+$ $CD8^+$ subline was cytotoxic for lymphoblastoid cell lines (LCL) and natural killer (NK) targets. Killing of LCL was inhibited by MoAb DA6.231 against MHC class II, but was unaffected by MoAbs against MHC class I BB7.7 and BB7.8. The $CD4^-$ $CD8^+$ subline was cytotoxic for LCL and had a higher NK activity. The $CD4^-$ $CD8^+$ subline was not inhibited by any anti-MHC class I antibody.

cultures derived from naive tamarins have fewer dual-positive cells (Fig. 6e,f). Furthermore, the dual-positive cells are not seen in freshly isolated tamarin PBL nor in concanavalin A (Con A)-stimulated T cell blasts [9]. Prolonged culture led to a shift in the phenotype of the cells with a greater proportion becoming $CD4^+$ $CD8^+$ dual-positive. This change of pheno-type corresponded to a reduction of cytotoxic activity both for NK and LCL targets.

The subpopulations of the B152 T cell line were separated using magnetic cell sorting, with anti-CD4 antibody giving one positively selected population that was $CD4^+$ $CD8^+$ (Fig. 3c) dual-positive, and a second population of predominantly $CD4^-$ $CD8^+$ cells (Fig. 3d) which were further purified by positive selection of CD8 cells by MACS. The two subpopulations were re-stimulated in separate cultures with LCL and IL-2, where they retained their respective phenotypes. The $CD4^-$ $CD8^+$ cells grew more slowly than the $CD4^+$ $CD8^+$ cells, which may account for the shift in phenotype towards $CD4^+$ $CD8^+$ cells seen after several passages of unseparated cells. A third population of $CD4^-$ $CD8^-$ cells was also present which expressed CD3. If both CD4 and CD8 cells were removed by magnetic cell sorting the dual-negative cells responded to fresh stimulation with LCL and developed a phenotype identical to unseparated cells.

Both $CD4^+$ $CD8^+$ and $CD4^-$ $CD8^+$ subpopulations had reproducible (three experiments) cytotoxic activity against LCL and NK targets (Fig. 4), the $CD4^-$ $CD8^+$cells having greater activity against NK targets. Killing of autologous LCL by the $CD4^+$ $CD8^+$ subpopulation was markedly inhibited by a monoclonal anti-MHC class II (Da6.231), but was unaffected by antibodies BB7.7 and BB7.8 directed at MHC class I. None of the antibodies used had any effect on killing by the $CD4^-$ $CD8^+$ fraction (Fig. 4). Parallel experiments on human lymphocytes cultured in the same way showed that both anti-class II (Da6.231) and one of the anti-class I (BB7.8) antibodies were effective inhibitors of cytotoxic responses, demonstrating that the antibodies were active in this type of assay (data not shown). All three antibodies reacted with tamarin LCL as shown by flow cytometry, and were included in the inhibition assay at 10 times their optimal dilution for flow cytometry. The cytotoxicity of $CD4^+$ $CD8^+$ and $CD4^-$ $CD8^+$ subpopulations was measured against a range of heterologous tamarin LCL, and both sub-lines showed variable killing against different LCL targets, ranging from none to greater than that for autologous LCL (data not shown).

Unseparated B152 T cells were potent inhibitors of LCL growth *in vitro*, with 50% of wells showing no LCL growth at an effector:target ratio of 4:1. Either of the subpopulations of $CD4^+$ $CD8^+$ and $CD4^-$ $CD8^+$ cells were also capable of inhibiting LCL outgrowth *in vitro*, but with an effector:target ratio of 40:1 and 80:1, respectively.

In order to compare the above results with naive tamarins, PBL from six individuals were cultured with autologous LCL in the same way at a ratio of 40 PBL:1 LCL and also at the higher ratio of 4 PBL:1 LCL. The cytotoxic capacity of the cultured cells is shown in Fig. 5. PBL stimulated at 40:1 had a lower NK activity than PBL from immune tamarins cultured identically, also PBL from naive tamarins showed only marginal killing of autologous LCL in some individuals (Fig. 5a). In contrast, PBL from the same tamarins stimulated with a high ratio of LCL had NK activity comparable to that from an immune tamarin, and they were also capable of killing autologous LCL (Fig. 5b). Overall, the growth of cells was much less than that seen with immune tamarins. Three of the cultures from the high stimulator group and a further two from a repeat experiment had sufficient cells available after *in vitro* stimulation to be tested in the growth inhibition assay, and in no case was any inhibition of LCL outgrowth observed.

The phenotype of the cells cultured from naive tamarins was assessed by flow cytometry and compared with similar data from the immune tamarin B152. The results are shown in Fig. 6. Cells cultured from the immune tamarin did not contain any B cells, nor was the expression of the EBV glycoprotein gp340 detected (Fig. 6a). In contrast, all six cultures from naive tamarins contained B cells and cells expressing gp340. Two examples of the staining

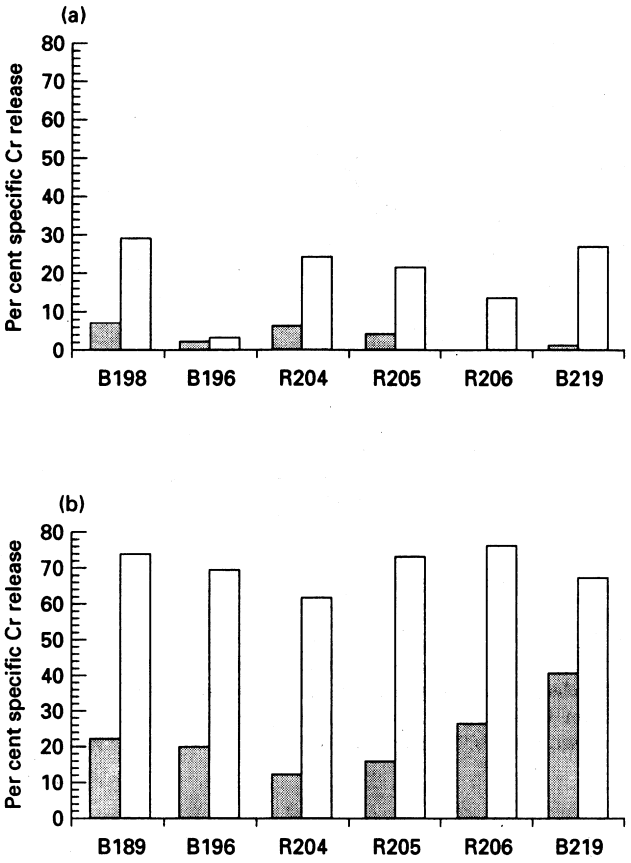

Fig. 5. Cytotoxic responses of peripheral blood lymphocytes (PBL) from tamarins not exposed to EBV. (a) PBL-stimulated with lympho-blastoid cell lines (LCL) at a low ratio of 1 LCL:40 PBL have little natural killer (NK) activity and a very low cytotoxicity for autologous LCL. (b) PBL stimulated at a higher ratio of 1 LCL:4 PBL give higher NK activity and marked cytotoxicity for autologous LCL.

pattern are shown in Fig. 6b,c. This provides further evidence that T cells from naive tamarins are incapable of inhibiting growth of EBV-infected cells *in vitro*. Figure 6d–f shows samples from the same cell populations stained for CD4 and CD8. The cells from the immune tamarin (Fig. 6d) have a higher proportion of $CD8^+$ cells and $CD4^+$ $CD8^+$ cells than those from naive tamarins (Fig. 6e,f).

DISCUSSION

Previous work has shown that resistance of vaccinated tamarins to EBV-induced lymphoma or tamarins in which EBV-induced lymphoma regresses is associated with a cell-mediated immune response [2,6,7]. In this respect EBV infection in tamarins parallels the findings in humans, where MHC-restricted cytotoxic T cells are considered to be a critical element of immunity to EBV [5]. The *in vitro* induction of strong cytotoxic responses by PBL from immune tamarins stimulated with LCL also parallels the responses of immune humans. In particular, tamarin LCL induced a potent NK cell activity in PBL. Human LCL are also potent inducers of NK cells, and the cytokine IL-12 was first identified as an NK cell-inducing factor produced by human LCL [11]. In addition to the presence of NK cells, immune tamarins also have a potent immune response that is specific for EBV antigens, as shown by the cold target inhibition experiment (Fig. 2) and the lack of killing of non-EBV-infected B cells.

Induction of NK cell activity and cytotoxicity for autologous LCL occurred in both naive and immune tamarins. The killing of LCL by cells from naive tamarins only occurred if large numbers of LCL were used to initiate the culture. PBL from naive tamarins stimulated with autologous LCL at a ratio of 40:1 did not develop cytotoxic responses against LCL despite having NK activity. The ability of LCL to stimulate cytotoxic T cells from naive tamarins at the higher ratio of 4:1 demonstrates the potent antigen-presenting capacity of LCL. However, the EBV specificity of the responses induced in this way is questionable, as despite the presence of LCL cytotoxicity and NK activity in both naive and immune tamarin cell cultures only cultures of PBL from immune tamarins were able to inhibit completely the growth of autologous LCL *in vitro*. Although the cells from unimmunized tamarins were more difficult to maintain in culture, this factor is unlikely to have influenced the results obtained, since comparable numbers of cells were always used in the assays. The non-specific cytotoxicity for LCL may represent the activity of T cells with specificities for non-EBV antigens present in the culture such as components of FCS. Induction of such non-specific responses has been described in human cell cultures [12]. Activation of non-EBV-specific T cells by LCL also has its *in vivo* parallel in human infectious mononucleosis, and has been blamed in part for the pathogenesis of the disease [13].

Further evidence for the lack of immune control of EBV in cells cultured from naive tamarins comes from the presence of B cells expressing EBV antigens in the cultures, since neither B cells nor cells expressing EBV antigens have so far been detected in cultures from immune tamarins. We have not seen any growth of LCL in control cultures containing mitomycin-treated LCL alone, and assume that the B cells are derived from *de novo* infection of peripheral blood B cells by virus released from the LCL stimulators when the cultures are initiated.

These observations also provide a basis for the use of the LCL growth inhibition assay as an *in vitro* measure of vaccine-induced resistance in tamarins. Together, these results show that NK cell activity alone does not provide protection from EBV *in vitro*, as presumable is the case *in vivo*. Rather, protection in tamarins is dependent on a potent EBV-specific T cell response, that is presumably MHC-restricted.

The MHC of tamarins has been extensively studied and has a number of unusual features. Both class I and class II MHC have very limited polymorphism and allelic diversity in this species [14]. The class I MHC has been shown to have three loci, but so far only 11 alleles have been discovered [15]. Furthermore, sequence data suggest that the tamarin has lost its conventional A, B and C loci and uses genes evolved from the so called atypical G and F loci in their place [16]. A further complication arises from the fact that most tamarins are born as dizygotic twins and in most cases the placental circulation is shared, resulting in bone marrow chimaerism, with equal numbers of mature lymphocytes from each sibling present in the circulation [17].

We have attempted to define the nature of MHC T cell interaction in the killing of tamarin LCL by a number of approaches. First, the cold target inhibition experiments show that killing of LCL is specific and not interfered with by the presence of excess NK cell targets, indicating that the two functions are not mediated by the same cellular mechanisms. Further evidence for MHC restriction comes from the lack of inhibition of

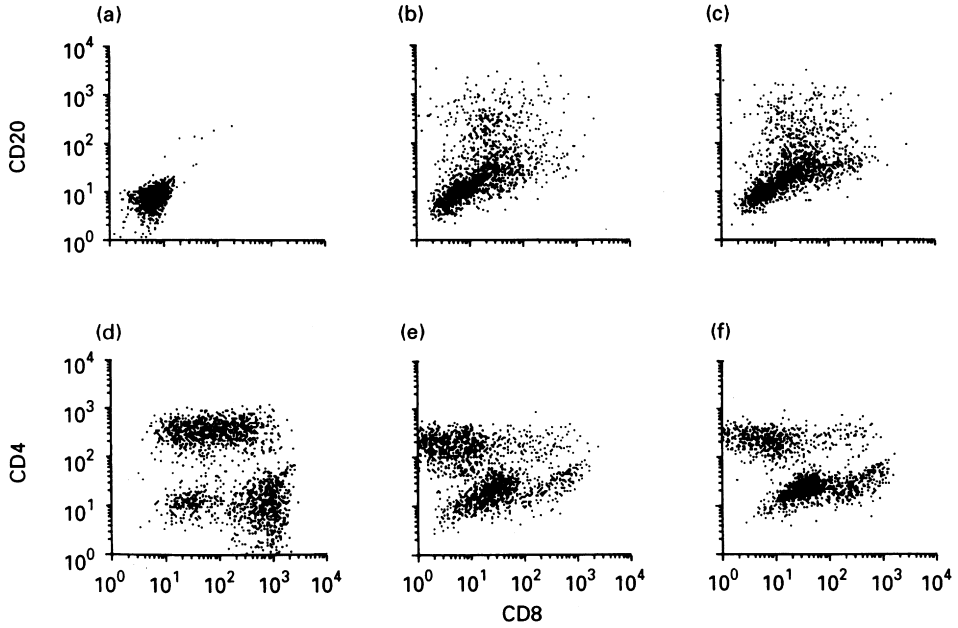

Fig. 6. Comparison of the phenotype of peripheral blood lymphocytes (PBL) from immune or naive tamarins after *in vitro* stimulation with autologous lymphoblastoid cell lines (LCL). The top row shows the cells stained with anti-CD20-PE and anti-gp340-FITC. The latter is an antibody reactive against the major EBV envelope glycoprotein. Cells cultured from an immune tamarin (B152) did not stain with either antibody (a), in contrast cells from naive tamarins expressed both CD20 and gp340 (b,c). (d–f) Cells from the same tamarins stained with anti-CD4-PE and anti-CD8-FITC.

B152 T cells killing autologous LCL by heterologous LCL from B158. The lack of MHC polymorphism and allelic diversity in tamarins make the demonstration of MHC restriction by failure to kill targets from unrelated individuals difficult. The results of such experiments were as predicted, i.e. T cells from one tamarin were frequently able to kill LCL from a variety of others with equal efficiency (data not shown). Fortuitously, B152 and B158 were two individuals who showed little cross-reactivity, either directly or in the cold target inhibition assay, and this is consistent with the view that some tamarins will not share MHC alleles and demonstrate genetic restriction.

We further examined the MHC restriction of the cytotoxic response using MoAbs to block killing by $CD4^+$ $CD8^+$ and $CD4^-$ $CD8^+$ subpopulations of cells. The $CD4^+$ $CD8^+$ dual-positive cells were completely inhibited by antibodies to MHC class II and unaffected by antibodies to MHC class I, and in this respect the dual-positive cells behave as ordinary $CD4^+$ cells. The significance of the $CD4^+$ $CD8^+$ dual-positive phenotype remains unclear, as this cell phenotype is rare in normal tamarin blood and does not appear on tamarin Con A blasts [9]. Dual-positive cells were detected at lower frequency in human T cell cultures stimulated with LCL (data not shown). Dual-positive cells are frequently found on immature populations of human thymic T cells, and occur at high frequency on resting lymphocytes in the circulation of other species such as pigs [18]. The co-expression of CD4 and CD8 has also been reported after prolonged activation of both rat and human T cells [19,20]. CD4 and CD8 normally determine the restriction of the T cell to MHC class II or class I antigen presentation [21]. The complete inhibition of the dual-positive tamarin cells by antibody directed against MHC class II suggests that the expression of CD8 on these cells may be redundant. Similar findings have been described for murine $CD4^+$ $CD8^+$ clones that expressed CD8 as a lyt2 homodimer and were MHC class II-restricted [22].

The evidence for class I restriction of $CD4^-$ $CD8^+$ tamarin lymphocytes is unclear. Antibody against class I had no effect on the killing by the $CD4^-$ $CD8^+$ fraction. This may be because the antibodies that cross-react with tamarin MHC class I recognize framework determinants and do not block MHC T cell receptor interaction. In support of this it has been shown by sequence analysis that tamarin MHC class I shows the most marked divergence from human sequences in the peptide binding region [13].

Overall the results from tamarin experiments are remarkably similar to those reported for humans [5,24]. However, the lack of clear evidence for MHC class I-restricted EBV-specific CD8 T cells remains a significant gap. Lack of evidence for MHC class I-restricted killing in primary EBV infection also proved to be an area of controversy in human studies, and this was not fully resolved until T cell responses were analysed at the clonal level [5,24]. We are currently working on characterizing cloned T cells from tamarins and developing tamarin-specific anti-MHC class I MoAbs in order to resolve this question.

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