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Specific lysis of mycobacterial antigen-bearing macrophages by class II MHC-restricted polyclonal T cell lines in healthy donors or patients with tuberculosis

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SUMMARY

The cytolytic capacity of mycobacterial antigen-stimulated peripheral blood mononuclear cells, from healthy Mantoux-positive volunteers and from patients with tuberculosis was investigated. Polyclonal T cell lines induced by 7 days of stimulation *in vitro* with PPD or a sonicate of *Mycobacterium tuberculosis* lysed both autologous macrophages and Epstein–Barr virus (EBV) transformed B cell lines which had been pulsed with mycobacterial antigens, to a greater extent than unpulsed target cells or target cells pulsed with an irrelevant antigen (streptokinase/streptodornase). The killing of mycobacterial antigen-pulsed macrophages and EBV-transformed B cell line targets was inhibited by monoclonal antibodies to MHC class II antigens but not by antibodies directed against MHC class I antigens. PPD-pulsed EBV-transformed lymphoblastoid cell lines (LCL) competitively inhibited the killing of mycobacterial antigen-pulsed macrophages, whereas natural killer (NK) sensitive K562 cells (with or without antigen pulsing) did not inhibit mycobacterial antigen-dependent cytolysis of macrophages. Patients with tuberculosis showed a spectrum of mycobacterial antigen-induced cytolytic capacity. Those with extensive tissue necrosis (e.g. cavitatory pulmonary tuberculosis or caseous, extrathoracic tuberculosis) had high levels while patients with disseminated (miliary) tuberculosis or disease refractory to treatment showed little evidence of mycobacterial antigen induced cytotoxicity. The ability of mycobacterial antigen-stimulated lymphoblasts to kill specific antigen-pulsed autologous macrophages was not significantly different between healthy donors and patients with tuberculosis. However, the 'mycobacterial antigen-specific' component of this cytolysis was significantly deficient ($P < 0.01$) in patients. We conclude that mycobacterial antigen-specific cytotoxic T cell responses may play a significant part in the immune response to mycobacterial infection.

Keywords tuberculosis class II MHC macrophages cytolysis T cells

INTRODUCTION

Mycobacterium tuberculosis is a facultative intracellular pathogen which replicates within human macrophages. Following the work of Lurie (1942) and Mackaness (1964), anti-tuberculous immunity has been thought to depend on T cell-mediated macrophage activation. This process results in increased bacteriostasis or killing of the intracellular organisms (Lowrie & Andrew, 1988). In mice it has been relatively easy to demonstrate inhibition of mycobacterial growth by T cell-derived lymphokines, especially interferon-gamma (IFN-γ) (Flesch & Kaufman, 1987). However, activation of human macrophages by lymphokines does not produce bacteriostasis or killing of intracellular mycobacteria to a degree sufficient to explain human anti-mycobacterial immunity (Rook, 1987).

Hence, alternative explanations of human anti-mycobacterial immunity need to be sought. In mice, recent evidence indicates that T cells can lyse macrophages bearing mycobacterial antigens (Chipulankar, De Libero & Kaufmann, 1986). Furthermore, adoptive transfer of cytotoxic T cell precursors (i.e. $CD8^+$, $CD4^-$ cells) can produce protective immunity against intracellular bacterial infections including tuberculosis (Orme & Collins, 1984). Conversely the selective depletion of helper ($CD4^+$) or cytolytic ($CD8^+$) T cells, respectively, can each render mice more susceptible to experimental infection with *M. Tuberculosis* (Muller *et al.*, 1987). Furthermore, recent

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evidence suggests that CD4 cells can be divided into 'helper' and 'inflammatory' subsets which can, respectively, provide 'help' to B cells or possess MHC class II-restricted cytolytic capacity and can mediate delayed-type hypersensitivity (DTH) reactions.

During studies aimed at activating BCG-infected human macrophages by mycobacterial antigen-reactive T cell lines, we observed that these phagocytic cells died within 48 h of adding the T lymphoblasts (Kumararatne *et al.*, 1989). Furthermore, recent work has shown that injection of PPD into skin lesions of lepromatous leprosy leads to the destruction of *M. lepraе*-infected macrophages in an environment containing recently immigrant CD4 and CD8 T cells (Kaplan *et al.*, 1988). Therefore we have investigated the cytotoxic capacity of mycobacterial antigen-stimulated peripheral blood mononuclear cells (PBMC) from healthy, Mantoux-positive volunteers, and from patients with tuberculosis. We found that polyclonal T cell lines, induced with PPD, or a sonicate of *M. tuberculosis*, can lyse both autologous macrophages and Epstein–Barr virus (EBV) transformed B cell lines which have been exposed to mycobacterial antigens. Compared with healthy donors, patients with tuberculosis (TB) showed a spectrum of antigen-specific cytolytic capacities ranging from high levels (in those with extensive tissue necrosis, e.g. cavitatory pulmonary TB or caseous extrathoracic TB) to insignificant levels in some patients with disseminated (miliary) TB or disease refractory to treatment. These results emphasize the dual role of T cells in mycobacterial diseases viz the capacity to cause tissue damage and to produce protective immunity.

MATERIALS AND METHODS

Subjects

Fifteen healthy adult volunteers studied were from the staff or students attached to the Department of Immunology at the Medical School, Birmingham. All except one were BCG-vaccinated and were Mantoux-positive. They included 10 Caucasians, two South-East Asians, one Ugandan Asian, one African and one South American (Caucasian) donor. Eleven patients with TB seen at the Dudley Road Hospital, or the East Birmingham Hospital were also studied. Ten had bacteriologically proven TB and one patient with lymph node TB had caseating granulomata, histologically compatible with TB.

Separating of PBMC

These were separated from defibrinated venous blood by Ficoll/Hypaque centrifugation (Böyum, 1968). These were washed three times in RPMI 1640 (Gibco Biocult) and resuspended in RPMI 1640 supplemented with glutamine (2 mM) penicillin (100 μg/ml) gentamycin (50 μg/ml) and 5% autologous serum (complete medium).

Generation of antigen-stimulated T cells

Isolated PBMC diluted to 10^6 cells/ml were incubated in 24-well tissue culture plates (Nunc) at 37°C, in 5% CO_2 in air for 7 days. Each well contained 2 ml of cells and replicates were stimulated for 7 days with optimum concentrations of the following antigens: PPD (Evans Medical) at 80 μg/ml, or PPD (State Serum Institut, Denmark) at 25 μg/ml; a sonicate of *M. Tuberculosis* (MTSE) kindly supplied by Dr Jackett, MRC Tuberculosis Research Unit, Hammersmith (Hewitt *et al.*, 1982) at a final concentration of 10 μg/ml; streptokinase/streptodornase (SK/SD) (Lederle) at 250 SK U/ml.

EBV-transformed B cells lines

These were prepared from PBMC as described in detail elsewhere (Rickinson *et al.*, 1984).

Preparation of macrophage targets

PBMC (150 μl at 1×10^6 cells/ml of complete medium) were plated in 96-well, round-bottomed microtitre tissue culture plates (Flow Lab). About 10% of the added PBMC would adhere as monocytes and this number was used to compute effector-to-target (E/T) cell ratios in cytotoxicity assays (see method for cytotoxicity assay). In some experiments, cells which adhered to gelatin-coated plastic were obtained from PBMC as described by Hassan, Campbell & Douglas (1986), resuspended in complete medium and cultured in 24-well tissue culture plates (Nunc) at a concentration of 10^5 cells/well. For the cytotoxicity assays, on day 6 of incubation the cells were pulsed with antigen (PPD; final concentration of 80 μg/ml for Evans PPD or 25 μg/ml for PPD from State Serum Institute, Copenhagen; mycobacterial sonicate antigen (MTSE) at 10 μg/ml final concentration; SK/SD at 250 SK U/ml final concentration) and radiolabelled with ^{51}Cr (2 μCi/well) (Amersham Radiochemicals). After 24 h of incubation with antigen and ^{51}Cr, the plates were washed three times in RPMI/10% normal human serum (NHS) and the plates replenished with 50 μl of RPMI/10% NHS per well.

Preparation of EBV-transformed lymphoblastoid cell lines (LCL) or K562 targets

These cells were used with or without antigen pulsing. For antigen pulsing 5×10^6 cells/2 ml of RPMI with 10% fetal calf serum (FCS) were added to each well of a 24-well tissue culture plate and incubated with antigen at the concentration indicated in the preceding section, for 24 h. Subsequently the cells were washed three times in RPMI/10% FCS, labelled in a pellet for 1 h with 200 μCi of ^{51}Cr at 37°C in 5% CO_2, washed three times with RPMI/10% FCS and adjusted to 10^5 cells/ml in this medium.

Cytotoxicity assay using macrophage targets

This was carried out using a ^{51}Cr release assay developed at the Armauer Hansen Research Institute, Addis Ababa, Ethiopia, which has been described in detail elsewhere (Ottenhoff *et al.*, 1988). Briefly, 7-day antigen-stimulated effector cells were added to target macrophages in a final volume of 150 μl of RPMI/10% NHS at E/T ratios ranging from 3/1 to 30/1 as indicated in Results. In a preliminary study, the numbers of adherent target cells remaining after washing on day 7 were determined by lysing the cells with Zapoglobin (Coulter Electronics, Luton, UK) and counting the nuclei using a coulter counter (Wickremesinghe, 1986). The average percentage of PBMC adhering per well, with or without antigen pulsing, was 10·9 (s.e.m. 0·8) and 10·8 (s.e.m. 0·6) respectively for 12 healthy controls; the corresponding figures were 11·0 (s.e.m. 0·7) and 11·2 (s.e.m. 0·5) for 12 patients with TB. Since the proportion of PBMC which remained adherent was, on average, 10% and this observation was in agreement with two published studies (Ottenhoff *et al.*, 1988; Djeu & Blanchard, 1988), this figure was used to calculate the E/T ratio throughout this study. As a further check that the E/T ratio of antigen-treated and

untreated replicate wells were uniform within any one experiment, the total number of ^{51}Cr counts incorporated per well were compared. These did not vary significantly for antigen-treated and untreated wells ($P < 0{\cdot}05$ Student's t-test) within any one experiment.

The percentage of isotope release for each well was calculated by the formula:

$$\frac{\text{ct/min supernatant}}{\text{ct/min supernatant} + \text{ct/min from Triton X pellet}} \times 100$$

The percentage of specific ^{51}Cr release was: % ^{51}Cr release from test − % spontaneous release.

The standard deviation between triplicate estimates of the % specific ^{51}Cr release value did not usually exceed 10% and spontaneous ^{51}Cr release did not usually exceed 15% of total radioactivity in cells.

Cytotoxicity assays using EBV-transformed LCL or K562 cells as targets

^{51}Cr-labelled target cells were adjusted to 5×10^5/ml in 10% FCS/RPMI. Target cells (10^4) in 100 μl of media were plated into 96-well microtitre tissue culture plates. Effector cells were added in 100 μl complete medium at E/T ratios ranging from 5/1 to 30/1 as indicated in Results. Each experimental combination was performed in triplicate. After incubation at 37°C, in 5% CO_2 for 4 h, the plates were spun at 500 ***g*** for 5 min, and 100 μl supernatant removed from each well for counting. Wells with 100 μl medium added instead of effector cells were used to determine spontaneous isotope release and wells with 100 μl 1% Triton X added instead of effector cells were used to determine total radioactivity in target cells:

$$\%\ \text{specific}\ ^{51}Cr\ \text{release} = \frac{\text{ct/min test} - \text{ct/min spontaneous}}{\text{ct/min total} - \text{ct/min spontaneous}} \times 100$$

The standard deviation of triplicates was usually less than 10% and spontaneous release was usually less than 20%.

Blocking of cytotoxicity with monoclonal antibodies

The mouse monoclonal antibodies W6/32 and BU26 were used for blocking MHC class I and MHC class II antigens, respectively. W6/32 (Sera Lab) is directed against non-polymorphic framework determinants of MHC class I antigens (Barnstable *et al.*, 1978) and BU26 against framework determinants of MHC class II antigens. This latter antibody in the form of ascitic fluid was kindly provided by Dr N. R. Ling, Department of Immunology, the Medical School, Birmingham. Before adding effector cells, the target cells were pre-incubated for 30 min with W6/32 at final dilutions of 1 μg/ml and 0·02 μg/ml and BU26 at final dilutions of 1/500 and 1/5000, respectively.

Competitive inhibition of macrophage lysis

Adherent cells (macrophages) were ^{51}Cr-labelled and prepared for use as targets as described earlier. Increasing numbers of unlabelled competing EBV-transformed LCL or cells of the natural killer (NK) sensitive, erythroleukaemic cell line K562 were added to triplicate wells containing ^{51}Cr-labelled macrophage targets. The total incubation volume at this stage was 100 μl of RPMI/10% FCS. Effector cells (3×10^6) were added to each well in 100 μl complete medium to give effector-to-adherent cell ratios of 20/1. The microtitre plate was incubated at 37°C in 5% CO_2 for 18 h and supernatant and radioactivity in pellet counted as described above. For each antigen-primed/unprimed target combination the corresponding spontaneous release was determined from triplicate wells without effector cells and subtracted from % isotope release in the presence of effector cells to give the % specific ^{51}Cr release.

Lymphocyte proliferation

Responses to each antigen used were determined as described in detail elsewhere (Oppenheim & Schecter, 1980), using a ^3H-thymidine incorporation assay. Briefly, 10^5 PBMC/well were incubated with antigens in microtitre plates in complete medium supplemented with 20% autologous serum. The wells were pulsed on day 6 with 1 μCi ^3H-thymidine (Amersham) and harvested 24 h later, and the radioactivity incorporated (as d/min), was measured by standard scintillation counting procedures. Each test was done in triplicate. Lymphocyte proliferation index was calculated as

$$\frac{\text{mean d/min in cultures with antigen} - \text{mean d/min in control wells}}{\text{mean d/min in control wells}}$$

Statistical analysis

This was by the Wilcoxon signed rank test for paired values except where indicated otherwise.

RESULTS

Cytotoxic activity of mycobacterial antigen-stimulated effector cells against autologous macrophages

Figure 1 shows results of cytotoxicity experiments using PPD- and MTSE-stimulated T cells from three healthy donors. Donors 1 and 2 were BCG-vaccinated and Mantoux-positive,

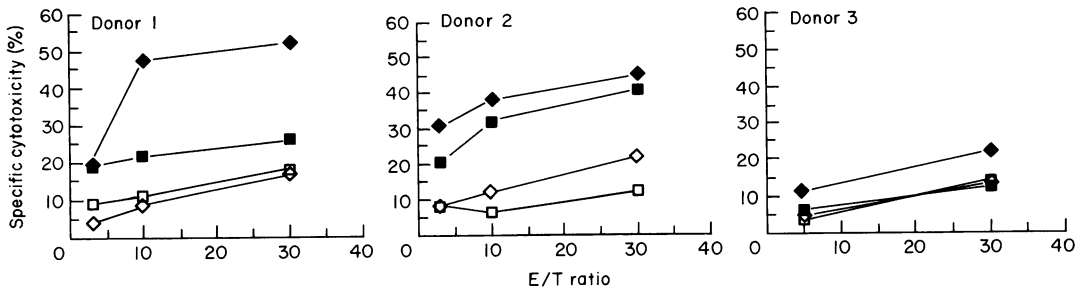

Fig. 1. Percentage of specific cytotoxicity of *M. tuberculosis* antigen-stimulated PBMC of healthy donors against autologous macrophages. □, No antigen; ◆, PPD; ■, MTSE; ◇, SK/SD. Donors 1 and 2 were Mantoux positive; donor 3 was Mantoux negative.

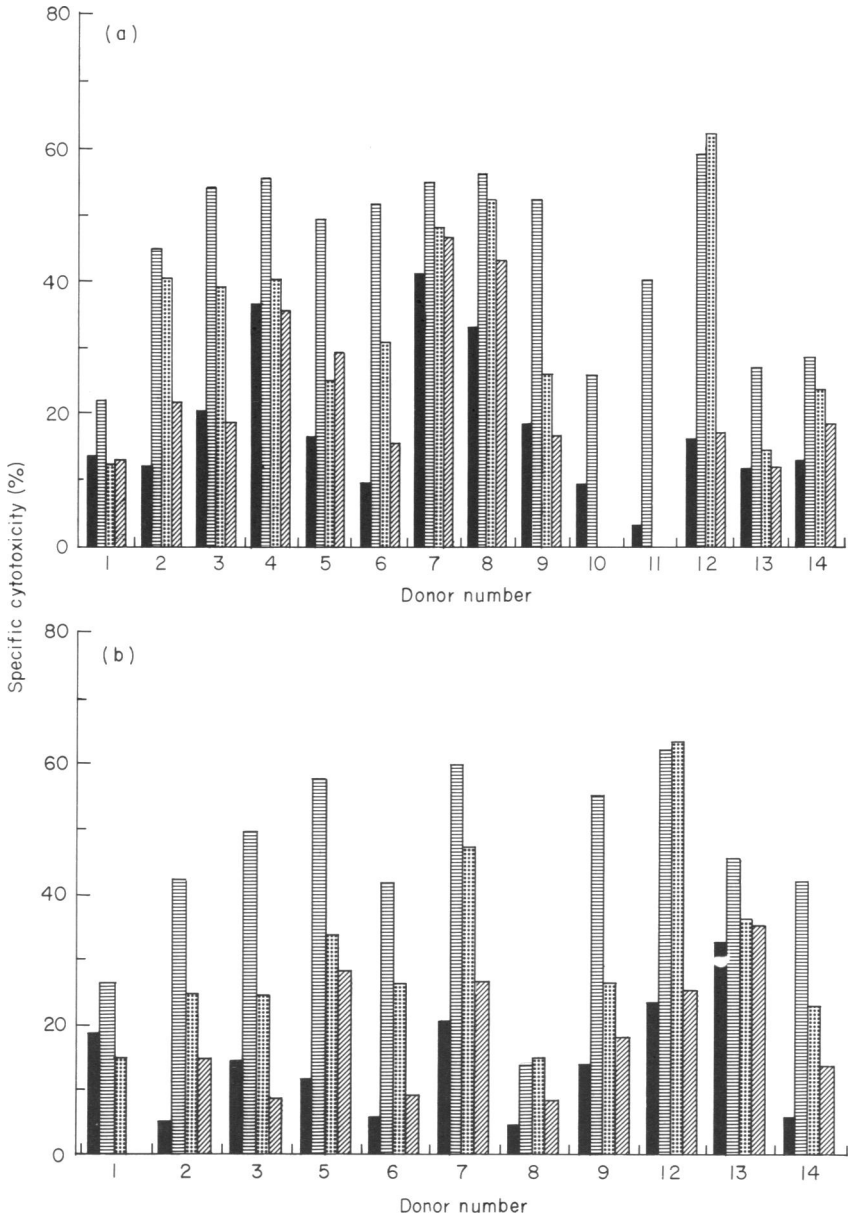

Fig. 2. Percentage specific cytotoxicity by PPD-stimulated (a) and MTSE-stimulated (b) effector cells from healthy donors against autologous macrophages pulsed with no antigen ■; PPD ■; a sonicate of *M. Tuberculosis* (MTSE) ▨; or SK/SD ■; as described in Materials and Methods. The effector-to-target ratio was 20/2 for donors 7–8 and 10–14, and 30/1 for donors 1–6 and 9. Pulsing of macrophages did not affect viability of the macrophages in the absence of effector cells. Thus the spontaneous release of isotope from antigen-unpulsed macrophages was 17% (s.e.m. 3·89; $n = 5$) after 18 h of incubation, while that of PPD-treated macrophages was 14·8 (s.e.m. 1·2; $n = 5$). MTSE and SK/SD pulsing not done for donors 10 and 11 (a). SK/SD pulsing of macrophages not done for donor 1 (b).

and donor 3 was a Mantoux-negative healthy Caucasian who had not received BCG. For donors 1 and 2, PPD- or MTSE-stimulated effector cells caused significantly more lysis of PPD- or MTSE-pulsed macrophages, compared with macrophages not pulsed with antigen or pulsed with an unrelated antigen (SK/SD). Effector cells from donor 3 produced substantial (4–14%) killing of antigen-unpulsed macrophages but there was no significant increase in the killing of MTSE-pulsed target cells over this level and only a modest (8–11%) increase in killing of PPD-treated macrophages.

The above observations were confirmed in a larger series of experiments (Fig. 2). It may be seen that peripheral blood lymphocytes from 14 healthy adult donors, when stimulated with PPD for 7 days, were capable of killing autologous macrophages (antigen-unpulsed) to a variable degree as assessed by an 18-h ^{51}Cr release assay. This antigen non-specific cytotoxicity varied from 3–41% depending on the individual donor. However, overnight pulsing of target macrophages with mycobacterial antigen (PPD or MTSE; Fig. 2a) caused significantly increased lysis when compared with antigen unpulsed

targets; the median increase was 28% and 10% for PPD- and MTSE-pulsed targets respectively ($P < 0.0001$ for both comparisons). The killing of macrophages pulsed with the unrelated antigen SK/SD was equivalent to that seen with antigen-untreated target cells or showed only a modest increase. The lysis of PPD- or MTSE-pulsed macrophages was significantly higher than that of SK/SD-pulsed targets ($P < 0.003$ and 0.025, respectively). Antigen pulsing of macrophages did not affect their viability in the absence of effector cells (see legend of Fig. 2). It can also be seen that the capacity of MTSE-induced effectors to cause antigen-specific and non-specific lysis was similar to that of PPD effectors (Fig. 2b). While PPD and MTSE were equally effective at inducing cytolytic effector cells from normal donors, PPD-pulsed targets were more efficiently lysed than MTSE-pulsed macrophages by both types of effector cells ($P < 0.001$ for PPD effectors and $P < 0.0045$ for MTSE effectors).

Table 1. Specific cytotoxicity of PPD-stimulated effector T cells using EBV-transformed target cells

Exp. no	Effector cell donor	Target cell donor	Target pulsed with	% Specific cytotoxicity at Effector/target cell ratios 5/1	10/1	12/1
1	A	A	No antigen	1·1	2·5	5·3
			PPD	9·0	14·5	21·2
2	B	B	No antigen	3·5	4·7	10·4
			PPD	18·2	24·1	30·5
3	B	B	No antigen	6·0	7·2	14·1
			PPD	13·1	18·9	25·4
		A	No antigen	3·2	5·4	8·2
			PPD	2·8	7·7	8·3
		C	No antigen	2·9	3·4	4·3
			PPD	4·1	3·0	5·1
4	A	A	No antigen	4·2	3·7	6·4
			PPD	13·4	14·0	19·0
		B	No antigen	5·8	10·2	13·5
			PPD	6·0	8·4	10·4
		C	No antigen	1·9	3·2	6·1
			PPD	4·0	7·6	9·9

Lysis of EBV-transformed LCL by mycobacterial antigen-induced effector cells from healthy Mantoux-positive, BCG-vaccinated donors

PPD- or MTSE-induced effector cells were also tested for their ability to lyse ^{51}Cr-labelled EBV-transformed LCL which were pulsed with mycobacterial antigens. Results of four representative experiments are summarized in Table 1. In experiments 1 and 2 it can be seen that the % specific ^{51}Cr release at each E/T ratio for target cells pulsed for 24 h with PPD, was greater than that of antigen-unpulsed targets. In experiments 3 and 4 the antigen-dependent increase in cytolysis was not seen with allogeneic E/T combinations, suggesting that the antigen-specific component of the cytolysis was MHC-restricted. Similar observations were made using autologous and heterologous macrophage targets (data not shown). It is noteworthy that both donors A and B were high responders to PPD with lymphocyte proliferative indices of 57 and 56, respectively.

Evidence that mycobacterial antigen-specific cytotoxicity against macrophage or EBV-transformed B cells is restricted by class II MHC antigens

Table 2 summarizes the results of representative experiments to test whether cytotoxicity by PPD- or MTSE-stimulated effector cells against autologous antigen-presenting cells could be inhibited by monoclonal antibodies directed against the non-polymorphic framework determinants of class I or class II MHC antigens. The results show that pretreatment with a monoclonal

Table 2. Anti-MHC class II antibodies inhibit PPD- or MTSE-induced cytotoxicity

EBV-transformed lymphoblastoid targets						Macrophage target cells					
						Specific lysis (%) at effector to target ratio of 20/1					
						Type of antibody added to target cells					
Effectors induced with	Type of monoclonal antibody added	Target cell pulsed with	Specific lysis (%) at effector/target ratios of 5/1	10/1	20/1	Exp. no.*	Effectors induced with	No antibody added†	Anti-MHC class I at a concentration of 1 μg/ml	0·2 μg/ml	Anti-MHC class II at a concentration of 1/500	1/5000
PPD	Nil	No antigen	1·1	2·5	5·3	1	PPD	49·3 (20)	45·3	41·4	22·9	25·5
PPD	Nil	PPD	13·6	14·0	19·0		MTSE	57·6 (12)	56·9	50·9	19·5	30·9
PPD	Anti-MHC class I (1 μg/ml)	PPD	13·7	22·5	32·5	2	PPD	51·4 (10)	46·8	43·5	14·2	27·1
PPD	Anti-MHC class II (1/500 final dilution)	PPD	3·6	3·8	6·3		MTSE	41·6 (5·9)	48·4	33·8	6·4	16·4
						3	PPD	42·2 (28)	49·7	50·8	30·2	37·6

* Experiments 1 and 2 were done with PBMC from healthy BCG-vaccinated donors. PBMC from a patient with tuberculosis was used for experiment 3.

† Figures in parentheses indicate % lysis of antigen-unpulsed macrophages.

Table 3. Inhibition of cytolysis of ^{51}Cr-labelled macrophage targets by 'cold' competing target cells

Exp. 1: Inhibition of cytotoxicity of PPD (or MTSE) effectors against antigen (PPD) pulsed macrophage targets

Effectors stimulated with	Cold targets* added	Specific lysis (%) of PPD pulsed macrophages† Cold/labelled target ratio 0	5/1	20/1
PPD	None	46	—	—
PPD	EBV LCL		54	41
PPD	EBV LCL+PPD		40	25
PPD	K562 cells		45	37
PPD	K562 cells+PPD		50	44
MTSE	None	45	—	—
MTSE	EBV LCL		49	53
MTSE	EBV LCL+PPD		36	21
MTSE	K562 cells		40	34
MTSE	K562 cells+PPD		40	37

Exp. 2: Inhibition of cytotoxicity of PPD (or MTSE) effectors against antigen unpulsed macrophage targets

Effectors stimulated with	Cold targets added	Specific lysis (%) of macrophages Cold/labelled target ratio 0	5/1
PPD	None	16	—
PPD	EBV LCL		18
PPD	K562 cells		3
MTSE	None	18	—
MTSE	EBV LCL		16
MTSE	K562 cells		12

* Cold targets were pulsed overnight with PPD or medium alone, and washed before use.

† Lysis of antigen-unpulsed macrophages was 10 and 11% for PPD and MTSE effectors, respectively.

EBV LCL, autologous Epstein–Barr virus-transformed lymphoblastoid line.

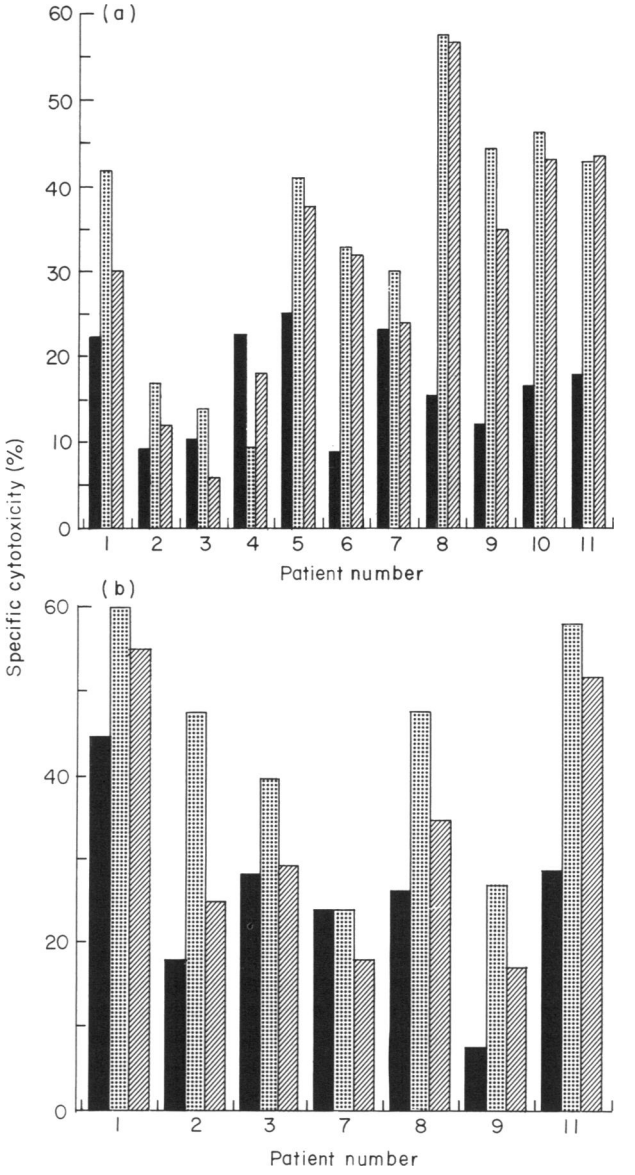

Fig. 3. Percentage specific cytotoxicity by PPD-stimulated (a) and MTSE-stimulated (b) effector cells from patients with TB, against autologous macrophages pulsed with no antigen ■;PPD ▨; or MTSE ■; as described in Materials and Methods. The effector-to-target cell ratio was 20/1 for patients 5–11 and 30/1 for patients 1–4.

antibody with broad specificity against class II MHC antigens inhibited lysis of antigen-pulsed EBV-transformed B cell lines almost to the level of killing of antigen-unpulsed targets (Table 2a). Similarly, antibodies to class II MHC antigens strongly inhibited the lysis of antigen-pulsed autologous macrophages (Table 2b); but the degree of inhibition of the mycobacterial antigen specific component of macrophage lysis was less complete. Treatment of targets with a class I MHC-specific monoclonal antibody did not cause inhibition of cytolysis but sometimes resulted in increased levels of cytotoxicity. Similar results were obtained in these blocking experiments, whether PPD or MTSE was used to stimulate effector cells or as a target antigen. In other experiments an HLA-DR-specific monoclonal antibody showed inhibition of antigen-specific cytolysis whereas anti-DP or -DQ antibodies were not inhibitory (data not shown).

Competitive inhibition of cytolysis of monocyte targets by EBV-transformed lymphoblastoid cells or K562 cells

The experiments detailed in the preceding sections suggested that activation of human peripheral blood lymphocytes with PPD or MTSE results in the generation class II MHC-restricted effector cells with similar cytolytic potential against antigen-pulsed autologous macrophages and EBV-transformed LCL. Cold target inhibition experiments were performed to demonstrate that the same effector lysed both kinds of target cells. The unlabelled target cells used to inhibit the lysis of macrophage targets were EBV-transformed LCL or K562 erythroleukaemic cells, and in each case the competing cells were used either

without antigen pulsing or after 24 h of exposure to PPD (80 μg/ml). The antigen-pulsed cells were washed three times with RPMI to eliminate unbound antigen before use in competitive assays. The results of a representative experiment are summarized in Table 3. Experiment 1 shows that antigen-unpulsed EBV-transformed LCL did not reduce the killing of PPD-pulsed monocytes by mycobacterial antigen-induced effector cells. The addition of a 20-fold excess of PPD-pulsed EBV-transformed cells to antigen-pulsed monocytes reduced their killing from 46% to 25% in the case of PPD effectors and from 45 to 21% in the case of MTSE effectors. In contrast, the presence of a 20-fold excess of competing K562 cells only reduced killing of PPD-pulsed macrophage targets from 46% to 37% and from 45% to 34%, respectively, when using PPD- or MTSE-induced effector cells; this minor inhibition did not increase if the competing K562 cells were pulsed with antigen. Thus, unlike EBV-transformed B cell lines, K562 cells did not inhibit the mycobacterial antigen-specific component of the target cell lysis. Table 3, experiment 2 shows that the addition of EBV cells (without antigen pulsing) did not reduce the non-specific lysis of antigen-untreated monocytes by PPD- or MTSE-induced effectors. The addition of K562 cells substantially reduced the killing of antigen-unpulsed macrophages.

The ability of patients with TB to generate mycobacterial antigen-specific cytolytic effector cells against autologous macrophages

Twelve patients with bacteriologically proven TB were tested for their capacity to generate mycobacterial antigen-specific cytolytic T cell responses directed against autologous macrophages. It can be seen from Fig. 3 that some patients (nos. 1, 5, 6 and 8–11) generated a high degree of cytolysis of mycobacterial antigen-pulsed macrophage targets, while others (nos. 2, 3, 4 and 7) generated a much lower degree of antigen-specific cytolysis. These differences appeared to correlate with the clinical picture in that the high responders were patients with cavitatory pulmonary TB (nos. 1, 5, 6, 8, 10), extrapulmonary TB with caseation (no. 9) and a patient tested 3 years after recovery from TB (no. 11). The patients with a poor cytolytic response had miliary TB (nos. 2 and 3), rapidly progressive pulmonary TB (no. 4), or pulmonary tuberculosis refractory to treatment (no. 7). This latter patient showed 23% lysis of antigen-unpulsed macrophages with PPD-stimulated effector cells at an E/T ratio of 20/1, while lysis of PPD-, MTSE- or SK/SD-pulsed targets by these effector cells was 30%, 24% and 30%, respectively. Results with MTSE effectors were similar; hence this patient appeared to be incapable of generating a significant level of mycobacterial antigen-specific cytolysis. Her lymphocyte proliferative response to PPD was high (lymphocyte transformation index of 50) and she had a strong Mantoux response (15 mm of induration at 48–72 h with 1 tuberculin unit). Thus she responded to PPD and MTSE with lymphocyte proliferation and the induction of DTH, but was incapable of generating antigen-specific cytotoxic T-cells during short term *in vitro* culture with mycobacterial antigens.

It may also be seen from Fig. 3 that PPD or MTSE stimulation of PBMC of patients with TB induced the generation of effector cells capable of antigen non-specific (NK type) killing of monocyte targets. The level of antigen non-specific cytotoxicity generated varied from individual to individual, in the same way as in healthy donors. Table 4 compares the ability of patients with TB and healthy controls to generate mycobacterial antigen-specific cytolytic T cells. The data summarized from Figs 2 and 3 show that patients and control mycobacterial antigen-stimulated effectors are not significantly different in their ability to kill antigen-unpulsed (or SK/SD-pulsed) autologous macrophages or PPD- or MTSE-pulsed macrophages. However, if for each individual donor the % killing of antigen-unpulsed (or SK/SD-pulsed) macrophages is subtracted from the degree of killing of mycobacterial antigen-pulsed macrophages, the patients with TB show a strikingly lower index of 'antigen-specific' cytoxicity compared with healthy controls ($P < 0.01$ by Wilcoxon test for unpaired samples).

Table 4. Patients with tuberculosis have reduced Mycobacterial antigen-specific cytolytic capacity

	Specific cytotoxicity (%) of mycobacterial antigen-stimulated effector cells against autologous macrophages pulsed with *		
	No antigen or SK/SD (1)	PPD or MTSE (2)	'Antigen-specific' cytotoxicity† (1)−(2)
Healthy donors ($n=15$)			
Mean (s.e.m.)	18·5 (2)	38·7 (3·4)	20·2 (3·2)
Median (Range)	16·3 (10–33·7)	38 (18·9–42·6)	20·1 (2·9–41·9)
Patients ($n=11$)			
Mean (s.e.m.)	20·1 (2·2)	34·7 (3·4)	7·3 (1·4)
Median (Range)	19·8 (4·4–44·1)	36 (9·8–57·8)	7·6 (0–20)
t-test	$P>0.6$	$P>0.4$	$P<0.001$
Wilcoxon (unpaired test)	$P>0.1$	$P>0.1$	$P<0.01$

* For each individual donor mean % specific cytotoxicity was calculated by averaging the killing of autologous macrophages by PPD and MTSE stimulated effectors.

† This was obtained by subtracting the mean % lysis of antigen unpulsed and SK/SD pulsed macrophages from the mean % killing of PPD and MTSE pulsed macrophages for each individual donor.

DISCUSSION

This study demonstrates that stimulation of peripheral blood lymphocytes of BCG-vaccinated, Mantoux-positive individuals with mycobacterial antigens results in the generation of cytotoxic effector cells, capable of lysing antigen-pulsed autologous macrophages. While the degree of cytolysis varied between individuals, mycobacterial antigen-pulsed targets were significantly more susceptible to lysis by these effector cells than unpulsed macrophages or those pulsed with an unrelated antigen (SK/SD).

We have also shown that antigen-pulsed autologous EBV-transformed LCL can be lysed in a similar manner by mycobacterial antigen-induced effector cells and that PPD-pulsed EBV-transformed B cell lines could competitively inhibit the killing of mycobacterial antigen-pulsed macrophages, suggesting that similar effector cells were responsible for killing both target cells. These data are not surprising, as EBV-transformed B cells can process and present antigen to T cells (Lanzavecchia & Bove, 1985).

Mycobacterial antigen-stimulated effector cells are also capable of lysing NK-cell sensitive targets like K562 cells (Eugui & Allison, 1987). We have shown that K562 cells can inhibit lysis of antigen-unpulsed macrophage targets, suggesting a similarity in the target structures recognized by the cytotoxic effector cells in each case. Thus it appears that stimulation of human PBMC with mycobacterial antigens results in the generation of a spectrum of cytolytic cells, including MHC-restricted antigen-specific killer cells and other killer cells, with a broad target specificity, which includes antigen-unpulsed macrophages and K562 cells. Similar data have been obtained in experimental infections with intracellular pathogens including *Listeria monocytogenes* (Chen-Woan, McGregor & Forsum, 1981; Kaufmann *et al.*, 1987), *M. tuberculosis* (De Libero, Flesch & Kaufmann, 1988), *L. pneumophila* (Blanchard *et al.*, 1987), and *Salmonella typhimurium* (Nencioni *et al.*, 1983).

Mycobacterial antigen-specific cytolysis was shown to be MHC-restricted, whereas non-specific lysis was not substantially diminished by using allogeneic E/T combinations. Furthermore, treatment with anti-MHC class II antibodies inhibited antigen-specific cytolysis of both macrophage and EBV-transformed B cell targets. Such inhibition was not produced by an anti-MHC class I antibody. The latter antibody used in our study has been shown to inhibit EBV and influenza-specific T-cell mediated cytotoxicity which is MHC class I-restricted (Wallace *et al.*, 1981). Thus in our experimental system, mycobacterial antigen-specific cytolysis was mainly produced by MHC class II-restricted effector cells. This is in agreement with the studies of Hansen & Kristensen (1986) who showed that PPD-induced cytolysis of autologous macrophages was MHC class II restricted. Data of Mustafa & Godal (1987) also suggested a dominant role for CD4-bearing effector cells in the cytolysis of BCG-pulsed antigen-bearing cells. It is well established that CD4-expressing T cells are MHC class II-restricted whereas CD8-bearing cells recognize antigen in the context of MHC class I molecules (Morrison *et al.*, 1986). Hence we may infer that CD4-bearing T cells and not CD8-bearing cells are mainly responsible for the mycobacterial antigen-specific cytotoxicity seen in the experiments documented above. Other investigators, however, have provided evidence for the generation of CD8-bearing cytotoxic T cells capable of lysing mycobacterial antigen-pulsed macrophages (De Libero *et al.*, 1988; Kaufmann, 1988).

What is the significance of cytolytic T cells to mycobacterial immunity? Following the original observations of Kaufmann (1988) that mycobacterial antigens induce the generation of $CD4^+$ or CD8-bearing cytolytic T cells in mice, it has been widely assumed that these cells are an important component of protective immunity against intracellular bacteria. The ability of virulent mycobacteria to resist killing by mononuclear phagocytes even when these cells are activated by lymphokines (Douvas *et al.*, 1985; Rook, 1987) made this hypothesis even more attractive. Our data indicate that most patients with tuberculosis (7/11) can generate T effector cells capable of pronounced levels of cytolysis of mycobacterial antigen-pulsed macrophages, equivalent to that seen in healthy control subjects. Hence the ability to generate such cytolytic capacity is a consequence of exposure to mycobacterial infection (analogous to a positive Mantoux response) which cannot be equated with protective immunity. Nevertheless, taken as a group patients with TB appeared to generate relatively less 'mycobacterial antigen-specific' cytolytic effectors than healthy controls (Table 4). A corollary is that antigen non-specific, MHC-unrestricted killer cells (NK type or lymphokine-activated killer cells) comprise the major cytolytic component found in mycobacterial antigen-stimulated lymphocyte cultures from patients with TB. Our data are therefore consistent with the view that antigen non-specific killer cells contribute to tissue damage within tuberculous lesions, while mycobacterial antigen-specific cytolytic cells may contribute to protective immunity. This is comparable to the tissue damaging but non-protective 'Koch type' hypersensitivity and 'listeria type' hypersensitivity which is protective (Rook 1987). The balance between these two processes may determine the outcome of natural exposure to the tubercle bacilli.

Our own preliminary data (Kale Ab *et al.*, 1990) suggests that lysis of BCG-infected macrophages by mycobacterial antigen-stimulated effector cells leads to a reduction of BCG colony-forming units.

Patient no. 7 described in this paper had recurrent TB in spite of chemotherapy for over 10 years. Although she developed strong delayed hypersensitivity to PPD and her PBMC showed a strong proliferative response to PPD and MTSE, she was unable to generate cytolytic T cells against macrophages primed with these antigens. Could the inability to generate mycobacterial antigen-reactive cytolytic T cells be relevant to this patient's poor response to chemotherapy? Anti-mycobacterial drugs are unable to eliminate bacilli which become physiologically dormant within mycobacterial lesions, and such persistent bacilli are thought to be responsible for relapses which may follow cessation of chemotherapy (Grosset, 1980; Toman, 1981). Crowle & Sbarbaro (1988) have demonstrated that isoniazid (INAH), which is a mycobactericidal drug, kills tubercle bacilli within human macrophages when the former are actively multiplying, but is ineffective against non-multiplying intracellular bacilli. Macrophage activation by lymphokines which induces bacteriostasis of intracellular organisms may help these bacilli to persist during chemotherapy. While *in vitro* (Khor, Lowrie & Mitchison, 1986a) and *in vivo* (Khor *et al.*, 1986b) studies in the murine model shows little effect of recombinant IFN-γ on the susceptibility of intracellular *M. microti* to INAH or Rifampicin, Mitchison & Selkon (1956),

showed that more pronounced immunological stimulation of guinea pigs with BCG vaccination was synergistic with anti-tuberculous chemotherapy. Thus ability to generate cytolytic T cells which can liberate bacteria contained within macrophages may help drugs to sterilize infected lesions. In contrast, inability to generate cytolytic T cells may contribute to the failure of drug therapy to achieve a bacteriological cure in mycobacterial diseases; the best examples being lepromatous leprosy (and cf. tuberculoid leprosy) (Waters, 1987) or *M. avium intracellulare* infections in patients with the AIDS (Pitchenik *et al.*, 1984). While most cases of treatment failure in tuberculosis are due to poor patient compliance, a few patients (like patient no. 7 documented above) have persistent or recurrent disease in spite of adequate chemotherapy. It would be particularly relevant to test whether such patients were unable to generate T cells cytolytic to *M. tuberculosis*-infected macrophages.

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