Clin Exp Immunol 1995; 99:82–89

Lysis of human macrophages by cytolytic CD4$^+$ T cells fails to affect survival of intracellular *Mycobacterium bovis*–bacille Calmette–Guérin (BCG)

N. FAZAL, D. A. LAMMAS, M. RAHELU, A. D. PITHIE*, J. S. H. GASTON† & D. S. KUMARARATNE
*Departments of Immunology, *Infection and †Rheumatology, University of Birmingham, Birmingham, UK*

(Accepted for publication 20 September 1994)

SUMMARY

Human CD4$^+$, mycobacteria-specific, cytolytic T cell clones were used to lyse BCG-infected macrophages, and the effect on the subsequent growth and viability of the organisms was examined. The survival of released bacteria following cell lysis was assessed by both ^{3}H-uridine labelling and colony-forming unit (CFU) estimation. The results indicate that even when effective antigen-specific or lectin-mediated cytolysis of the infected macrophages was achieved, there was no evidence for a direct mycobactericidal effect on the intracellular bacteria. This remained the case even if the period of co-culture of T cells and macrophages was extended up to 48 h. Pretreatment of the macrophages with interferon-gamma (IFN-γ) was not able to act together with T cell-mediated lysis to produce inhibition of mycobacterial growth.

Keywords mycobacteria cytolytic T cells CD4$^+$ human macrophages intracellular survival

INTRODUCTION

The recent global rise in reported tuberculosis associated with HIV infections has highlighted the importance of the cellular immune system in controlling this important disease. Acquired immunity to intracellular pathogens is thought to rely on antigen-specific CD4$^+$ T lymphocytes [1], which are typically represented as having helper functions, mediating the destruction of intracellular bacteria via cytokine activation of infected macrophages. It is known, however, that mycobacteria may persist within parasitized macrophages, and moreover that interferon-gamma (IFN-γ)-activated macrophages have not been convincingly shown to inhibit growth of *Mycobacterium tuberculosis* in humans [2,3]. Consequently other mechanisms of protection need to be investigated and their antimycobacterial effects examined. Class II-restricted T cells have been shown to have cytolytic activity *in vitro* against mycobacterial-infected cells [4,5]. The relevance of class II-restricted cytolytic T lymphocytes (CTL) in tuberculosis infections, however, remains controversial, with both protective and pathogenic roles being cited in the literature [6–8]. Evidence for the involvement of CTL in mycobacterial infections *in vivo* at the site of pathology has, however, been reported for both leprosy [9] and tuberculosis [10].

In this study, a panel of mycobacteria-specific human CD4$^+$ CTL clones was used to lyse BCG-infected macrophages. MHC-matched macrophages were lysed in an antigen-specific manner and mis-matched macrophages lysed by using the lectin concanavalin A (Con A). The effect which T cell-mediated lysis of the infected macrophages had on the subsequent growth and viability of the organisms was then examined.

Correspondence: Dr D. S. Kumararatne, Department of Immunology, The Medical School, University of Birmingham, Vincent Drive, Edgbaston, Birmingham B15 2TT, UK.

MATERIALS AND METHODS

BCG culture

BCG cultures were prepared from Statens Seruminstitut freeze-dried vaccine (Copenhagen, Denmark). Stock solutions were grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 10% Middlebrook ADC enrichment media (Difco) and 0·02% Tween 80 for 1 week at 37°C in a 5% CO_2 incubator. The bacterial suspensions were then centrifuged at 400 *g* for 20 min, resuspended in RPMI 1640 medium (GIBCO Biocult, Paisley, UK) supplemented with glutamine (2 mM) and 10% non-heat-inactivated A+ serum (Complete Medium (CM)), sonicated for 3 min and dispensed into 1-ml aliquots and stored at −70°C until required. The bacterial concentration in colony-forming units (CFU) of the BCG stock was then determined by the microcolony method (see below). For infection of target monolayers, BCG aliquots were thawed, sonicated for 3 min to obtain a uniformly dispersed BCG suspension of predetermined CFU concentration for appropriate dilution.

Preparation of mycobacterial antigen-specific T cell clones as effector cells

Two CD4$^+$ mycobacterial antigen-responsive T cell clones, clone MAW 10.15 (DR-3-restricted) and specific for amino

acids 4–13 of *Myco. leprae* hsp 60, and clone CF 10.23 (DR-4-restricted), which recognizes an uncharacterized component of *Myco. tuberculosis* purified protein derivative (PPD), and three PPD- or hsp 65-specific T cell clones, CF 92B2.8, CF 92B1.3, and CFP 1.2.7, were used throughout these experiments. These clones had previously been characterized and shown to have cytolytic activity against MHC-matched antigen-presenting cells [11,12]. They were maintained by twice weekly feeding with IL-2 100 U/ml in supplemented antibiotic-free medium, and bi-monthly restimulation using irradiated allogeneic peripheral blood mononuclear cells (PBMC) and phytohaemagglutinin (PHA).

Preparation of macrophage monolayers as targets for cytolytic effector cells

For BCG killing experiments, macrophage targets for CTL clones were prepared from five different donors as follows. PBMC were separated from defibrinated blood by centrifugation on Ficoll–Paque (Pharmacia LKB, Uppsala, Sweden) gradients, washed three times with RPMI and re-suspended in complete medium at a concentration of $2{\cdot}5 \times 10^6$/ml. Aliquots (200 μl) of PBMC (5×10^5 cells/well) were then placed in round-bottomed 96-well microtitre tissue culture plates (Corning, Corning, NY). We have previously shown that on average 10% of the added mononuclear cells adhere as monocytes [4], and this number was used to compute effector:target ratios in cytotoxicity assays.

After a 4-h or overnight (16 h) incubation period the cell cultures were gently washed three times to remove non-adherent cells, and the adherent monocytes incubated for a further 6 days to allow differentiation into macrophages. The cells were then infected overnight (16 h) with BCG at an infection ratio of five organisms per cell in a final volume of 200 μl complete medium. For cytotoxicity assays the cells were also simultaneously labelled with ^{51}chromium (2 μCi/well) (ICN Flow, Thame, UK), incubated overnight (16 h), washed three times in warm complete medium to remove both extracellular bacteria and excess chromium, and finally resuspended in 100 μl complete medium.

Cytotoxicity assays

This method has been described in detail elsewhere [4,8]. Briefly, T cell clones were harvested, washed once, and resuspended in complete medium. Effector cells were then dispensed in 100-μl aliquots to the target macrophages in triplicate wells at various effector:target ratios and the 96-well culture plates centrifuged at 500 *g* for 3 min to aid cell–cell contact. Triplicate wells with 100 μl of medium were also incorporated into the experimental design as medium controls. After 6, 16 or 48 h incubation, the plates were again centrifuged at 500 *g* for 3 min and the cells assessed for either: (i) cytotoxicity, by ^{51}Cr release; or (ii) BCG viability which was assayed by measuring both ^{3}H-uridine incorporation and CFU determination using the microcolony counting method (see below).

Measurement of cytotoxicity by ^{51}Cr release

For cytotoxicity assays, the supernatant was aspirated from individual wells into LP2 plastic counting tubes (Appleton Woods, Birmingham, UK). Aliquots of 100 μl 1% Triton X-100 (Sigma, Poole, UK) were then added to each cell pellet and incubated for 30 min at 37°C to lyse the remaining adherent cells. The entire volume was subsequently aspirated and transferred to another counting tube. The tubes were then sealed with wax and the activity of both the supernatant and pellet assessed by measurement of radioactive decay with a gamma counter (LKB, Selsdon, UK).

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

$$\text{Per cent isotope release} = \frac{\text{ct/min supernatant}}{\text{ct/min supernatant} + \text{ct/min pellet}} \times 100$$

The level of cytotoxicity (per cent specific release) in each well was determined as follows: cytotoxicity (per cent specific release) = % isotope release in test well − % isotope release in spontaneous release well.

Results are presented as the mean (s.d.) of triplicate estimates of the percentage ^{51}Cr release. The s.d. between triplicates was not normally greater than 5%. Spontaneous release in these assays was usually no greater than 20% of the total uptake of isotope.

Radiometric labelling assay for determining viability of BCG

To monitor differences in BCG growth following T cell lysis of infected targets, bacterial numbers (per well) were determined by radio-labelling the released bacteria with ^{3}H-uridine as previously described [13]. Briefly, following incubation of effector cells with non-^{51}Cr-labelled BCG-infected target macrophages, the cells were lysed by a combination of resuspension of adherent macrophages using a micro-cell scraper and the addition of Saponin (0·2% w/v, final concentration). The plates were then incubated for 30–60 min to allow complete lysis of the cells, which was monitored by frequent visual observation under an inverted microscope (Olympus CK2, Tokyo, Japan).

Following lysis, 100 μl of 7H9 Middlebrook broth supplemented with 10% ADC enrichment media were added to each well and the contents thoroughly mixed. Aliquots of 50 μl of cell suspension were then removed from each well and transferred to a fresh 96-well flat-bottomed microtitre plate for microcolony CFU determination (see below). 5,6-^{3}H-uridine (2 μCi/well; 10 mCi/ml specific activity; ICN Flow) was then added to the remaining 150 μl cell suspension in each well. The plates were then incubated at 37°C/5% CO_2 for 72 h to allow incorporation of the radiolabel into the proliferating bacteria. The bacterial cultures were harvested onto glassfibre filter papers (Printed Filtermat A, 1205-401; Pharmacia Wallac, UK), using an automated cell harvester (Skatron, Lier, Norway). The filtermats were air dried and radioactivity assessed using a beta counter (LKB Rackbetta 2). The results expressed are the sum values of the ct/min obtained for each sample.

Determination of BCG viability and growth by the microcolony counting method of CFU estimation

Viable counts of BCG were performed using a microcolony counting technique, the details of which have been published previously [13]. Briefly, 50-μl aliquots drawn from 200 μl of disrupted macrophage lysates were serially diluted (1:5) in triplicate in 200 μl liquid Middlebrook 7H9 broth (Difco) + 0·2% glycerol + 10% ADC (Difco) supplement in 96-well flat-bottomed microtitre plates. The plates were then incubated at 37°C in a 5% CO_2 incubator and individual discrete colonies were counted by inverted microscopy (Olympus CK2) after 10–14 days incubation. This time was found to

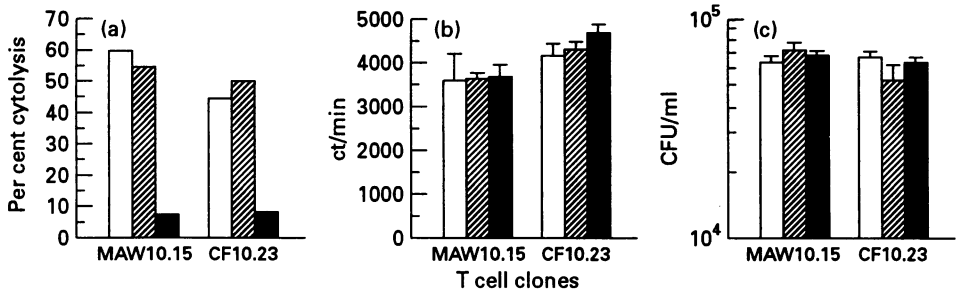

Fig. 1. Cytolysis of class II MHC-matched 6-day-old human macrophages by $CD4^+$ T cell clones (MAW 10.15 and CF 10.23) in the presence (□) or absence (▨) of specific antigen or peptide, compared with infected macrophage-alone control (■) (a). T cells were incubated with BCG-infected macrophage targets for 16 h at an effector to target ratio of 5:1. ^{51}Cr release was determined at 6 h post-incubation. The data represent means of triplicates of ^{51}Cr release with s.e.m. < 5% (data not shown). Bacterial survival/viability was also assessed by both radiolabelling with ^3H-uridine incorporation (b) and colony-forming unit (CFU) determination (c). The data represent the mean of triplicates ± s.d.

be optimal for culture of countable CFU (20–40 CFU/well), and the CFU concentration/ml of the individual wells was then calculated as follows: CFU/ml = mean CFU × Dil × 20, where mean CFU = average of counts in replicate wells, Dil = dilution factor of the well row, and ×20 = correction factor to express bacterial numbers as CFU/ml.

RESULTS

Effect of cytolysis of BCG-infected macrophages by antigen-specific $CD4^+$ T cells on intracellular bacterial survival

To examine the effect of T cell-mediated lysis of BCG-infected macrophages on bacterial viability, experiments were performed using antigen-specific T cell clones against target macrophages derived from DR-matched donors (Fig. 1). The survival of released bacteria was then assessed by both ^3H-uridine incorporation and CFU determination (Fig. 1b,c). The T cells were co-cultured with the 6-day-old BCG-infected, DR-matched macrophages for 16 h at an effector to target ratio of 5:1. In the presence of specific peptide or PPD for the respective clones, marked specific cytolysis (55–60%) of BCG-infected target cells was observed at 16 h post-incubation (Fig. 1a), in contrast to that achieved against uninfected control macrophages (10–15%). The levels of ^{51}Cr release in the absence of clone-specific antigens, however, was similar (55–52%), demonstrating that infection alone resulted in mycobacterial antigen presentation sufficient to stimulate effective T cell-mediated lysis (Fig. 1a). However, despite the successful

Table 1. T cell-mediated lysis[1] of BCG-infected macrophages bacterial survival assessed by ^3H-uridine incorporation[2] and colony-forming unit (CFU) determination[3] at 6 h post-incubation

Exp. no.	[1] Per cent specific cytolysis*				[2] ^3H-uridine incorporation (ct/min)				[3] CFU (BCG/ml×10^4)			
	T cell clones	Macrophage donors	+Con A	−Con A	+Con A†	−Con A	−T cells	%Δ†	+Con A†	−Con A	−T cells	%Δ†
1	CF 92B2.8	A	40	10	5248	3500	4606	+13·9	3·7	3·6	3·8	−2·6
2	CF 92B2.8	A	30	5	6157	6179	5160	+19·3	1·0	1·1	1·2	−16·6
3	CF 92B2.8	B	30	10	2171	2128	2690	−19·3	18·9	18·4	14·8	+27·7
4	CF 92B2.8	B	35	6	9106	10156	11154	−18·4	6·9	3·7	4·2	+64·3‡
5	CF 92B2.8	B	30	5	10058	9643	8741	+15	11·2	9·8	10·2	+9·8
6	CF 92B2.8	D	43	13	8745	7854	7653	+14·3	0·8	0·7	0·8	+0
7	CF 92B2.8	E	47	13	8745	8245	8653	+1·1	2·3	2·1	2·1	+9·5
8	CFP 1.2.7	C	40	12	9152	14297	12571	−27·2	5·5	4·8	5·1	+7·8
9	CFP 1.2.7	C	45	10	3470	3810	3850	−9·9	1·1	1·4	1·2	−8·3
10	CFP 1.2.7	C	42	9	6077	5825	5715	+6·3	3·4	3·1	3·4	0
11	CFP 1.2.7	D	50	14	5790	9860	4670	+24·0	11·1	11·7	11·7	−5·1
12	CFP 1.2.7	D	47	12	6981	5987	5999	+16·3	1·0	0·8	1·0	0
13	CFP 1.2.7	E	39	8	2896	2789	2985	3·0	1·5	1·6	1·4	+7·1
14	CF 92B1.3	A	30	6	8754	7895	8647	+1·2	3·3	2·5	3·3	0
15	MAW 10.15	A	35	5	7111	6134	7129	−0·3	5·8	6·4	4·2	+38·1‡

* Per cent cytolysis of macrophages alone controls was < 2.

† Per cent Δ, Percentage change in bacterial growth. Growth enhancement (+), inhibition (−).

‡ Statistically significant $P < 0{\cdot}5$. S.D. < 20% in all experiments. ND, Not determined.

Con A, Concanavalin A.

Table 2. T cell-mediated lysis[1] of BCG-infected macrophages bacterial survival assessed by ^{3}H-uridine incorporation[2] and colony-forming unit (CFU) determination[3] at times post-incubation

Exp. no.	[1] Per cent specific cytolysis* T cell clones	Macrophage donors	+Con A	−Con A	[2] ^{3}H-uridine incorporation (ct/min) +Con A†	−Con A	−T cells	%Δ†	[3] CFU (BCG/ml×10^4) +Con A†	−Con A	−T cells	%Δ†
a. *16 h post-incubation*												
1	CF 92B2.8	E	55	20	2872	2605	2616	+9·8	2·7	2·5	2·0	+35·0
2	CF 92B2.8	D	50	15	2657	2340	2612	+1·7	6·6	8·1	3·9	+69·2‡
3	CFP 1.2.7	A	40	10	1618	1509	1633	−0·9	2·6	2·7	2·6	0
4	CFP 1.2.7	E	48	8	3230	2313	2440	+32·4	5·3	4·3	3·7	+43·2‡
5	CF 10.23	D	40	20	1515	1639	1676	−9·6	3·3	2·2	2·1	+57·1‡
6	CF 10.23	A	60	15	1645	1341	1294	+27·1	3·6	3·9	3·2	+12·5
7	CF 10.23	A	55	12	1421	1232	1577	−9·9	0·9	0·7	0·9	0
8	CF 10.23	E	60	10	2231	2016	2696	−17·2	4·0	4·1	3·3	+21·2
9	MAW 10.15	B	38	8	2896	2754	2568	+12·8	3·8	3·7	3·5	+8·57
b. *48 h post-incubation*												
1	CF 92B2.8	A	35	7	ND	ND	ND	ND	5·9	6·2	4·7	+25·3
2	CF 92B2.8	E	35	5	ND	ND	ND	ND	12·0	10·7	11·7	+11·1
3	CFP 1.2.7	A	40	12	ND	ND	ND	ND	11·4	10·0	8·7	+31·0
4	CFP 1.2.7	D	40	14	ND	ND	ND	ND	8·2	10·2	7·4	+10·8
5	CFP 1.2.7	D	50	8	ND	ND	ND	ND	10·8	8·0	8·3	+30·1
6	CFP 1.2.7	E	47	11	ND	ND	ND	ND	7·5	7·0	7·2	+4·2

* Per cent cytolysis of macrophage alone controls was < 2.
† Per cent Δ, Percentage change in bacterial growth. Growth enhancement (+), inhibition (−).
‡ Statistically significant $P < 0{\cdot}5$. S.D. < 20% in all experiments. ND, Not determined.
Con A, Concanavalin A.

specific lysis of infected macrophages, there was no demonstrable inhibitory effect upon BCG growth or survival, compared with bacteria released non-specifically from detergent-treated macrophages, as assessed by either ^{3}H-uridine incorporation (Fig. 1b) or microcolony CFU determination (Fig. 1c). Infection *per se* did not, however, appear to affect macrophage viability, since spontaneous ^{51}Cr release was similar for uninfected (12 ± 5%) and infected macrophage target cells (10 ± 7%). In addition, there was no significant loss of adherent target cell numbers as a result of infection at this bacterial to target ratio, as determined by both control cell counts and by observed equivalent levels of ^{51}Cr incorporation by both infected and uninfected targets (data not shown).

Effect of Con A-stimulated cytolysis of BCG-infected human macrophages by CD4$^+$ T cells on intracellular bacterial survival
To extend these observations, and to circumvent the need for macrophages from class II MHC-matched donors, T cell clones were induced to lyse target macrophages using the lectin Con A. A panel of five CD4$^+$ MHC class II-restricted, PPD- or hsp 65-specific T cell clones was assessed for cytolytic activity against 6-day-old BCG-infected macrophage targets, derived

Table 3. T cell mediated lysis[1] of BCG-infected monocytes bacterial survival assessed by ^{3}H-uridine incorporation[2] and colony-forming unit (CFU) determination[3] at 16 h post-incubation

Exp. no.	[1] Per cent specific cytolysis* T cell clones	Macrophage donors	+Con A	−Con A	[2] ^{3}H-uridine incorporation (ct/min) +Con A†	−Con A	−T cell	%Δ†	[3] CFU (BCG/ml×10^4) +Con A†	−Con A	−T cells	%Δ†
c. *16 h post-incubation*												
1	CF 92B2.8	A	50	20	9248	10340	8606	+7·6	2·6	2·4	2·1	+23·8
2	CFP 1.2.7	C	55	15	9152	11573	10297	−11·1	5·8	5·1	6·0	−3·3
3	CFP 1.2.7	D	50	20	8790	4670	9860	−10·9	11·2	11·7	11·7	−4·3
4	CF 92B1.3	A	35	16	26906	33340	25945	−3·7	8·7	8·0	7·9	+10·1

* Per cent cytolysis of monocyte alone controls was < 2.
† Per cent Δ, Percentage change in bacterial growth. Growth enhancement (+), inhibition (−). S.D. < 20% in all experiments. ND, Not determined.

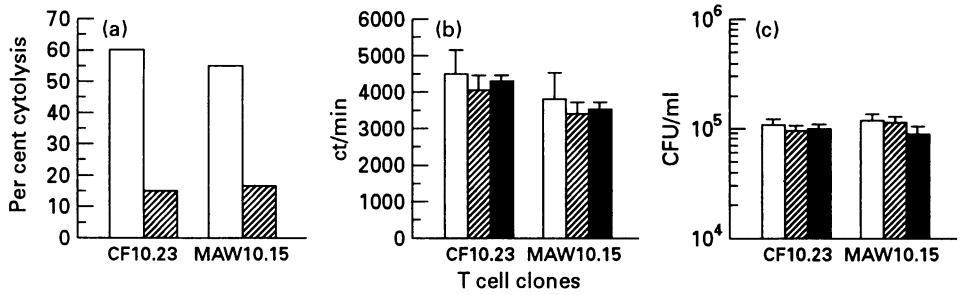

Fig. 2. Percentage cytolysis of IFN-γ-activated MHC-non-matched 6-day-old human macrophages by two mycobacterial-specific $CD4^+$ T cell clones (CF10.23 and MAW 10.15) in the presence (□) or absence (▨) of concanavalin A (Con A) (10 μg/ml) at a clone to target ratio of 5:1, compared with infected macrophage-alone control (■) (a). The macrophage targets were primed with IFN-γ 1000 U/ml for 16 h before infection with BCG, and ^{51}Cr release was determined at 16 h post-incubation. Bacterial survival/growth was then assessed by both ^3H-uridine incorporation (b) and colony-forming unit (CFU) determination (c). The data represent the means of triplicate values ± s.d.

from five different cell donors. Target lysis was determined at 6 or 16 h post-infection in the presence or absence of Con A. As illustrated in Table 1, the degree of Con A-induced cytolysis at 6 h was found to vary for different clone–macrophage combinations (30–59%), but in all cases was significantly greater than the lysis seen in the absence of lectin, which was < 13%. The presence of Con A in the absence of co-culture with T cells, however, had no discernible affect on isotope incorporation or spontaneous release by infected macrophages.

Having determined that Con A could be used satisfactorily to obtain MHC-unrestricted lysis of macrophages by T cell clones, assays were performed to assess whether lysis of BCG-infected macrophages had any inhibitory effect on released bacteria. As above, BCG viability and growth were assessed by both ^3H-uridine incorporation and microcolony CFU determination (Table 1). In this series of experiments, 6-day-old macrophage targets were infected for 16 h (16 experiments) with BCG and then cultured in the presence and absence of effector T cells for 6 h. Although marked macrophage cytolysis (approximately 40%) was achieved after 6 h culture with T cells in the presence of Con A, neither ^3H-uridine incorporation nor CFU determination revealed any significant difference in the survival or growth of the released bacteria, compared with those obtained from infected macrophages which had not been subjected to T cell-mediated cytolysis. Indeed, in some experiments a modest increase in bacterial metabolism was obtained in the presence of T cell clone and Con A, as determined by ^3H-uridine labelling. No corresponding increase in the percentage of surviving bacteria, however, was detected by CFU estimation. Con A itself was shown not to affect either bacterial viability or survival at the concentration used to obtain lectin-mediated cytotoxicity (data not shown). Thus the larger series of experiments which could be performed by using lectin-mediated cytotoxicity gave results which were in agreement with those in which antigen-specific cytolysis had occurred, since in both cases cytolysis had no adverse effect on the intracellular bacteria.

Additional experiments were performed to examine the effects of longer periods of co-cultivation of T cells and infected macrophages on bacterial survival. Nine separate experiments were performed using three different macrophage donors (Table 2). When the duration of the effector-target incubation was increased from 6 h to 16 h (nine experiments), the percentage of Con A-stimulated cytolysis increased on average by approximately 10% above that achieved at 6 h (i.e. to 50%), whereas background release in the absence of Con A increased only marginally from 9% to 13%. Although the longer period of co-cultivation achieved consistently higher levels of cytolysis, there was again no significant inhibitory effect on mycobacterial growth and survival. Indeed, increases in bacterial growth were observed in the presence of Con A and clones by both ^3H-uridine and CFU estimates (Table 2). In a further series of experiments T cell clones were co-cultured with infected macrophages for 48 h (six experiments), with similar results (Table 2).

Experiments were also performed using infected monocytes rather than macrophages as targets of T cell-mediated cytolysis (Table 3). For this purpose 2 h blood-derived monocytes from three different donors were utilized (four experiments). Once more, although substantial cytolysis (35–55%) was readily obtained, there was no significant inhibitory effect on the subsequent growth of BCG released from the monocytes.

Effect of $CD4^+$ T cell-mediated lysis of IFN-γ-activated, BCG-infected human macrophages on BCG survival

The possibility that the combination of macrophage activation with IFN-γ and T cell-mediated cytolysis might act synergistically to promote the inhibition of killing of intracellular bacteria was investigated (Fig. 2). Six-day-old human macrophages were pre-activated with 1000 U/ml human recombinant IFN-γ for 24 h before infection with BCG and subsequent addition of cloned T cells. Macrophages from two donors were tested using two different T cell clones. Untreated macrophages were included in each experiment as controls. Figure 2 shows that pretreatment of macrophages with IFN-γ did not result in an inhibitory effect on growth or survival of BCG released following lysis of the infected macrophages by T cells.

DISCUSSION

The role of $CD4^+$ cells in acquired resistance against tuberculosis is well established [14], but has been previously ascribed to their ability to bring about cytokine-mediated activation of macrophages. Current hypotheses, however, supported by murine and human studies, would suggest that both cytolytic

$CD4^+$ and $CD8^+$ T cells are important in protection against mycobacterial infections [14]. *In vitro* experiments using both murine [15] and human cells [16] have reported that co-incubation of antigen-specific CTL with *Myco. bovis*–BCG infected macrophages resulted in inhibition of mycobacterial growth. Moreover, in our laboratory we have previously reported that mycobacterial-specific $CD4^+$ T cells, generated from the blood of BCG-vaccinated subjects and tuberculosis patients, can lyse autologous mycobacterial-infected macrophages [4,8]. In patients with tuberculosis, however, the level of cytolytic activity demonstrated by peripheral blood-derived $CD4^+$ T cells was found to correlate with the presence of pulmonary cavitation or tuberculosis abscess formation [8], suggesting that CTL may contribute more to the tissue damage observed in tuberculosis patients than to protective antimycobacterial immunity.

The results reported in this study also demonstrate that although $CD4^+$ T cell clones could elicit significant antigen-specific or lectin-mediated cytolysis of BCG-infected macrophages, there was no detectable reduction in survival or growth of the phagocytosed intracellular bacteria.

Since the $CD4^+$ T cells never achieved complete lysis of the target macrophage population, it could be argued that those bacteria detected after CTL activation were persisting in a macrophage population resistant to CTL activity. This is not thought to occur, because we have previously determined by CFU estimation that the majority of intracellular BCG are released into the cell supernatant following incubation with mycobacteria-specific $CD4^+$ clones [15]. Moreover, that this accurately reflects released intracellular BCG and not merely extracellular bacterial growth is supported by the fact that BCG grows poorly in RPMI 1640 medium, and that extracellular growth of mycobacteria is known to be inhibited in the presence of human serum at concentrations $>5\%$ [16]. In addition, ZN-stained cytospin preparations of the (40–60%) unlysed target population have revealed these to comprise principally non-infected cells (results not shown), indicating that the macrophages which are not subject to CTL-mediated lysis are not especially active in phagocytosing or harbouring released bacteria. Two possible explanations may account for the existence of a non-infected macrophage population; first, it has been observed that the monocyte-derived macrophage cultures comprise morphologically diverse forms, possibly reflecting different stages of cell differentiation, which may differ in their phagocytic capabilities and hence in their susceptibility to infection with BCG. Alternatively, the non-infected, non-lysed target population, remaining following incubation with the CTL, also tend to occur predominantly on the upper rim of the wells of the round-bottomed microtitre culture plates used in these assays. Therefore they may avoid to some degree both BCG infection and consequently the cytolytic effects of the CTL through lack of sufficient contact with the BCG.

In vivo, however, it can be imagined that in the microenvironment of the granuloma, where the host–bacterial interaction is focused, lysis by CTL of infected macrophages which are incompetent to kill intracellular bacteria may still have a beneficial effect by rendering such released bacteria vulnerable to destruction by freshly migrating, cytokine-activated monocytes, an idea originally expounded by Kaufmann *et al.* [18]. However, in our experiments we also found that clone-mediated cytolysis of 4-h-old infected monocytes demonstrated the same lack of effect on bacteria viability as was seen when 4-day-old macrophages were used. Experiments are currently in progress in our laboratory to further explore the Kaufmann hypothesis by examining mycobacterial viability following exposure to repeated cycles of specific CTL-mediated release and phagocytosis by activated monocytes/macrophages.

The discrepancy in the results obtained in this study and that previously reported for human CTL [17] can be ascribed to a number of possible factors. In the Kaleab et al. [17] study, 7-day post-stimulation $CD4^+$ Dynabead-enriched polyclonal effector populations were utilized, undoubtedly containing a wide spectrum of T cells in terms of their cytokine profiles and antigen specificities. Furthermore, those experiments were performed on only a small number of normal individuals with highly variable results. For example, of four donors tested, only two showed any inhibition of bacterial viability, and conversely, these elicited only poor levels of specific cytolysis. Moreover, the assay system for determining mycobacterial growth incorporated a mixture of antibiotics (VCNT), which was claimed not to inhibit mycobacterial growth. In our experience, however, the addition of any antibiotics to the cytotoxicity assay system does have a marked inhibitory effect on bacterial viability following CTL-mediated lysis, and this effect is completely abolished in the absence of antibiotics (data not shown). Antibiotics therefore may have a greater effect on bacteria released from an intracellular environment than that on bacteria cultured extracellularly. Indeed, it was specifically for this reason that in this study both the clones and macrophage targets were cultured, and cytotoxicity experiments performed, in antibiotic-free media.

Both radiolabelling and CFU determination methods of assessing bacterial survival and growth were employed because each determined a different parameter of bacterial viability. Radiolabelling with ^{3}H-uridine shows the metabolic state of actively dividing bacteria over a short time course, whereas CFU determination depicts actual bacterial survival and growth. The combined results of both assay methods indicate that $CD4^+$ T cell-mediated lysis of infected macrophages neither alters the metabolic state of phagocytosed bacteria, nor reduces the viability of the bacteria. It is pertinent to these results that in the only previously reported example of the inhibition of bacterial viability by cytolytic T cells, using a murine *in vitro* model system [7], bacterial viability was assessed only by ^{3}H-uracil incorporation [7]. In our opinion, however, the results reported in this latter paper would be more consistent with bacteriostasis than killing of bacteria.

In some experiments the presence of cytolytic T cell clones actually enhanced BCG growth; this could be explained if the accumulated products of lysed cells provide a nutrient-enriched environment beneficial to bacterial proliferation. This idea is in contrast to previous reports in which it has been claimed that toxic products are released on cell lysis, and that these may contribute to the extracellular killing of mycobacteria that occurs in granulomatous lesions [19]. Likewise, accumulation of dead polymorphs in TB granulomata was proposed to produce such an effect in infected mice [20]. However, inhibition of bacterial growth in granuloma tissue may be more dependent upon the deprivation of oxygen to bacteria which occurs in such sites rather than to any build up of toxic cellular

products. The finding that rupture of granulomata into alveolar spaces results in explosive bacterial growth is consistent with this, and in the presence of oxygen the growth may actually be fuelled by granuloma-derived cellular debris.

The findings confirm similar results obtained in this laboratory using polyclonal T cell lines specific for PPD (predominantly $CD4^+$). MHC class II-restricted cytolysis of infected macrophages was observed, but again there was no effect on the viability of the intracellular bacteria [20]. Similarly, Molloy *et al.* [22] reported that effective lysis of BCG-infected human monocytes by IL-2-generated lymphokine-activated killer cells (LAK cells) had no detrimental effect on bacterial viability, but resulted only in the release of viable organisms.

Since protective immunity to mycobacteria is likely to require several effector mechanisms working together, we tested for the possibility of synergy between IFN-γ-activated macrophages and T cell-mediated cytolysis. IFN-γ is known to be a potent activator of murine macrophages, promoting killing of intracellular mycobacteria [2,15,23]. In human cells, however, the reverse effect has been reported, with IFN-γ enhancing intracellular mycobacterial growth [24]. In this study, no antibacterial synergy was observed with CTL and IFN-γ, but this may not be surprising since no cytokine has yet been shown to be effective in activating human macrophages to kill *Myco. tuberculosis*; this is in clear contrast to the important role of IFN-γ in the mouse. Data are emerging, however, that inhibitory cytokines such as transforming growth factor-beta (TGF-β) and IL-10 may also be important in modulating the priming effects of IFN-γ on macrophage antibacterial activity, via down-regulation of tumour necrosis factor-alpha (TNF-α) and IL-12 production [25,26]. Experiments are currently in progress to assess the levels of induction of both positive (TNF-α, IFN-γ and IL-12) and negative (TGF-β and IL-10) cytokines in human and murine macrophages following BCG infection, to assess this hypothesis.

The immune mechanisms of resistance to mycobacterial infection appear to be more complex in humans than in mice, and have proved difficult to reproduce *in vitro*. It is probable that multiple mechanism pathways lead to the final expression of acquired resistance to tuberculosis in man. Progress in unravelling such complex interactions may require similarly complex experimental systems, such as organ cultures, or the use of severe combined immunodeficient (SCID) or T cell-deficient mice reconstituted with human cells, to reproduce the granuloma environment. The latter could allow study of granuloma formation by human effector cells in response to mycobacterial infection *in vivo*. Further research is then required to identify the way in which human macrophages check, or fail to check, the intracellular growth of mycobacteria. Elucidation of the protective component(s) of the T cell immune response involved in resolving such infections may also prove beneficial in developing new therapeutic strategies.

ACKNOWLEDGMENTS

N.F. was supported by a Pakistan Ministry of Education Scholarship E.G. no. 2227. D.A.L. was supported by a Glaxo Fellowship Grant no. G-838B.

REFERENCES

1 Kaufmann SHE. $CD8^+$ T lymphocytes in intracellular microbial infections. Immunol Today 1988; **9**:168–74.

2 Flesch I, Kaufmann SHE. Mycobacterial growth inhibition by interferon γ activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. J Immunol 1987; **138**:4408–13.

3 Douvas GS, Looker DL, Vatter AE, Crowle AJ. γ Interferon activates human macrophages to become tumouricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria. Infect Immun 1985; **50**:1–8.

4 Kumararatne DS, Pithie AD, Drysdale P *et al.* Specific lysis of mycobacterial antigen-bearing macrophages by class II MHC-restricted polyclonal cell lines in healthy donors or patients with tuberculosis. Clin Exp Immunol 1990; **80**:314–23.

5 Ottenhoff THM, Kaleab B, van Embden JDA, Thole JER, Kiessling R. The recombinant 65 kD heat shock protein of *M. bovis* (BCG)/*M. tuberculosis* is a target molecule for $CD4^+$ cytotoxic T lymphocytes that lyse human monocytes. J Exp Med 1988; **168**:1947–52.

6 Kaplan G, Sheftel G, Job CK, Mathur NK, Nath I, Cohn ZA. Efficacy of the cell-mediated reaction to the purified protein derivative of tuberculin in the disposal of the *Mycobacterium leprae* from human skin. Proc Natl Acad Sci USA 1988; **85**: 5210–4.

7 De Libero G, Flesch I, Kaufmann SHE. Mycobacteria reactive Lyt 2^+ T cell lines. Eur J Immunol 1988; **8**:59–66.

8 Pithie AD, Rahelu M, Kumararatne DS *et al.* Generation of cytolytic T cells in individuals infected by *Mycobacterium tuberculosis* and vaccinated with BCG. Thorax 1992; **47**:695–701.

9 Hancock GE, Cohn ZA, Kaplan G. The generation of antigen-specific, major histocompatibility complex-restricted cytotoxic T lymphocytes of the $CD4^+$ phenotype. J Exp Med 1989; **169**:909–14.

10 Lorgat F, Mustapha MK, Lukey PT, Ress SR. Evidence for *in vivo* generation of cytotoxic T cells. Am Rev Resp Dis 1992; **145**:418–23.

11 Gaston JSH, Life PF, Jenner PJ, Colston MJ, Bacon PA. Recognition of a mycobacteria-specific epitope in the 65-kD heat shock protein by synovial fluid-derived T-cell clones. J Exp Med 1990; **171**:831–6.

12 Gaston JSH, Life PE, Jenner PJ, Colston MJ, Bacon PA. Epitope specificity and MHC restriction of rheumatoid arthritis a mycobacterial 65 kDa heat shock protein. Int Immunol 1991; **3**:695–72.

13 Fazal N, Bartlett R, Lammas DA, Kumararatne DS. A comparison of the different methods available for determining BCG–macrophage interactions *in vitro*, including a new method of colony counting in broth. FEMS Microbiol Immunol 1992; **105**:355–62.

14 Kaufmann SHE. Immunity to intracellular bacteria. Ann Rev Immunol 1993; **11**:129–63.

15 Pithie AD. A study of cytolytic T cells in tuberculosis patients and BCG vaccinated subjects. MD Thesis University of Birmingham 1993.

16 Rook GAW, Champion BR, Steele J, Varey AM, Stanford JL. I-A restricted activation by T cell lines of anti-tuberculosis activity in murine macrophages. Clin Exp Immunol 1985; **59**:414–20.

17 Kaleab B, Kiessling R, Van Embden JDA *et al.* Induction of antigen-specific $CD4^+$ HLA-DR-restricted cytotoxic T lymphocytes as well as nonspecific nonrestricted killer cells by the recombinant mycobacterial 65-kDa heat-shock protein. Eur J Immunol 1990; **20**:369–77.

18 Kaufmann SHE, Rodewald HR, Hug E, De Libero G. Cloned *Listeria monocytogenes* specific non-MHC-restricted $Lyt2^+$ T cells with cytolytic and protective activity. J Immunol 1988; **140**:3173–9.

19 Dubos R. Properties and structures of tubercle bacilli concerned in their pathogenecity. Symp Soc Gen Microbiol 1955; **5**:103–6.

20 Silva MT, Silva MNT, Appelberg R. Neutrophil–macrophage cooperation in the host defence against mycobacterial infection. Microb Pathog 1964; **6**:369–72.

21 Pithie AD, Lammas DA, Fasal N, Rahelu M, Bartlett JS, Hill G, Kumararatne DS. $CD4^+$ cytolytic T cells can destroy autologous and MHC-matched macrophages but fail to kill intracellular *Mycobacterium bovis*–BCG. Submitted to FEMS Microbiol Immunol 1994.

22 Molloy A, Meyn PA, Smith KD, Kaplan G. Recognition and destruction of Bacillus Calmette–Guérin-infected human monocytes. J Exp Med 1993; **177**:1691–8.

23 Walker L, Lowrie DB. Killing of *Mycobacterium microti* by immunologically activated macrophages. Nature 1981; **293**:69–70.

24 Douvas GS, Looker DL, Vatter AE, Crowle AJ. γ interferon activates human macrophages to become tumouricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria. Infect Immun 1985; **50**:1–8.

25 Bermudez LE. Production of transforming growth factor-β by *Mycobacterium avium*-infected human macrophages is associated with unresponsiveness to IFN-γ. J Immunol 1993; **150**:1838–45.

26 Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of Th1 $CD4^+$ T cells through IL-2 produced by Listeria induced macrophages. Science 1993; **260**:547–9.