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Cytotoxic T-cell responses to *Mycobacterium bovis* during experimental infection of cattle with bovine tuberculosis

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SUMMARY

Cytotoxic T-cell responses are thought to play a significant role in the host defence against mycobacterial infections. Little is understood about such responses of cattle to *Mycobacterium bovis*, the causative agent of bovine tuberculosis. The work described in this report demonstrates the activity of cytotoxic cells during experimental infection of cattle with *M. bovis*. The cytotoxic cells were found to have the ability to specifically lyse macrophages infected with *M. bovis* and were detected in peripheral blood lymphocytes after *in vitro* re-exposure to *M. bovis*. Cytotoxic activity was detected 4 weeks after experimental infection with *M. bovis*; a similar level of activity was maintained during the infection and it was mediated by both $WC1^+$ γδ and $CD8^+$ T cells. In addition, inhibition of the growth of *M. bovis* within infected macrophages was detected when they were exposed to cultures containing *M. bovis*-specific cytotoxic cells. The ability to detect cytotoxic cells after infection of cattle with *M. bovis* will allow their activity to be measured during vaccination trials. Correlation of cytotoxic activity with disease outcome may aid in the design of new vaccines and vaccination strategies.

INTRODUCTION

Tuberculosis presents an important health problem to humans and cattle and is an economic problem in countries where bovine tuberculosis is endemic. In order to develop a more successful vaccine it is important to understand the protective immune response of the host against the causative agent. In recent years, small animal models, particularly mice, have been used to determine which components of the immune system are involved in protective immunity against mycobacteria. Studies in humans infected with *Mycobacterium tuberculosis* have shown that many of the results obtained using experimental infection of mice also apply to humans. It is well established that cell-mediated responses are important in protection, and several subpopulations of T cells appear to be involved. Protective immunity to mycobacteria is considered to be mediated predominantly by T helper (Th)1 $CD4^+$ and $CD8^+$ T-cell secretion of interferon-γ (IFN-γ)[1,2] but other T-cell populations, such as γδ T cells[3] and natural killer (NK) T cells,[4] are probably also involved. Results from vaccine trials, using experimental challenge of cattle with *M. bovis*, have shown that vaccines which stimulate fast and strong IFN-γ responses following infection are protective, whereas those which stimulate antibody responses are not.[5] Therefore, the Th1 paradigm holds for bovine tuberculosis and all the major T-cell subsets appear to be involved in the response to infection.[6–10]

IFN-γ and other Th1 cytokines upregulate the antimicrobial function of macrophages (Mϕ) to kill the bacilli which they harbour.[11] Bactericidal and bacteriostatic functions of activated Mϕs can be demonstrated *in vitro* but it is clear that virulent mycobacteria are hard to kill. There may be mechanisms, other than those involving Mϕs, that are engaged in the killing of virulent mycobacteria. Cytotoxic T lymphocytes (CTL) have the ability to kill human[12] and mouse Mϕs[13] infected with *M. tuberculosis*. Bovine CTL have been shown to kill cells infected with other intracellular pathogens.[14,15] At least *in vitro*, the direct killing of mycobacteria by CTL has been demonstrated[16] and the involvement of molecules, such as granulysin, in the cytotoxic granules of CTL, have been implicated in this killing.[17,18] CTL may also have a role in more efficient antigen presentation, so that intracellular mycobacteria released from infected cells can be taken up by more proficient antigen-presenting cells.

Studies with putative DNA and subunit vaccines support the idea that CTL should be considered in the design of

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new-generation vaccines for tuberculosis.[19–21] Protection can be mediated by the adoptive transfer of T-cell clones, specific for *M. tuberculosis* hsp65, in the mouse, and the most protective clones not only produced high levels of IFN-γ when stimulated with mycobacterial antigens, but also displayed the highest cytotoxic activity.[22] Vaccines that stimulate CTL responses may be useful as both prophylactic and immunotherapeutic agents.[23,24]

T-cell clones from *M. bovis*-infected cattle can cause the release of mycobacteria from infected Mϕs,[25] but CTL activity has not been measured by more conventional methods. In this report, a cytotoxicity assay, based on the release of ^{51}chromate (^{51}Cr) was used to follow cytotoxic responses in cattle experimentally infected with *M. bovis*.

MATERIALS AND METHODS

Animals

Friesian-cross female calves (5–6 months old) were obtained from tuberculosis-free accredited herds from an area of New Zealand where both farmed and feral animals are free of tuberculosis. Prior to the start of the experiments, the cattle tested negative for reactivity to purified protein derivative from *M. bovis* (bovine PPD) in the whole-blood IFN-γ assay.[26] The cattle were grazed on pasture in an isolation unit, and all procedures involving the use of animals had the approval of the Wallaceville Research Centre Animal Ethics Committee (Upper Hutt, New Zealand).

Mycobacteria

A virulent *M. bovis* strain, WAg 202 (previously designated 83/6235), used in previous cattle studies,[27] was used to infect cattle, stimulate CTL activity *in vitro* and infect Mϕs. It was originally isolated from a tuberculous lesion of a brushtail possum (*Trichosurus vulpecula*). *M. bovis* was grown to mid-log phase in Tween albumin Dubos base broth (Difco Laboratories, Detroit, Michigan) supplemented with 0·006% (vol/vol) alkalinized oleic acid, 0·5% (wt/vol) albumin fraction V and 0·25% (wt/vol) glucose. The numbers of bacteria were estimated by the degree of turbidity. Dilutions were made, in Tween albumin broth, to obtain the appropriate doses for inoculating cattle, and the remainder were frozen and stored at −20° in 1-ml aliquots for *in vitro* infections. The number of colony-forming units (CFU) inoculated into cattle was determined retrospectively by plating 10-fold dilutions onto Middlebrook 7H11 agar (Difco) supplemented with 0·5% (wt/vol) glucose and 1% (wt/vol) sodium pyruvate; the numerical value thus obtained was used to calculate the number of CFU in frozen stocks that were used for *in vitro* infection of cells.

M. bovis *infection of calves*

Animals were infected with 5×10^3 CFU *M. bovis* by the intratracheal route, and killed and necropsied 17–21 weeks after inoculation, as described previously.[27] *M. bovis* was cultured from the lungs or lymph nodes of all animals.

Generation of CTL in vitro

Cattle peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by centrifugation at 1400 ***g*** for 35 min over Lymphoprep (density 1·0777) (Nycomed, Oslo, Norway). PBMC were washed three times with phosphate-buffered saline (PBS) and cells were resuspended in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco), 2 mM glutamine (Sigma, St Louis, MO), 1 mM non-essential amino acids (Gibco), 100 U/ml penicillin G (Sigma), 0·05 mM 2-mercaptoethanol (Sigma) and 110 mg/l sodium pyruvate (Sigma) (tissue culture medium). To generate CTL, PBMC were cultured, at a density of 3×10^6/ml, in 10 ml of tissue culture medium in T25 flasks (Nunc, Roskilde, Denmark) containing 6×10^5 CFU *M. bovis*, unless stated otherwise. In some experiments, *M. bovis*-infected Mϕs or bovine PPD (300 μg/ml; CSL Ltd, Parkville, Victoria, Australia) were used to stimulate CTL *in vitro*. Infected Mϕs, for *in vitro* stimulation of CTL, were prepared as follows. PBMC were cultured at 3×10^6/ml for 4 hr, non-adherent cells were removed by careful washing (three times) with tissue culture medium at 37°, and adherent cells were infected with *M. bovis* at a multiplicity of infection (m.o.i.) of 2 : 1. After 18 hr, the cells were washed and fresh PBMC (at 3×10^6/ml) were added. Cultures were incubated in humidified 5% CO_2 in air for 7 days.

Cytotoxicity assay

The method follows one described previously, with modifications.[13] Mϕ target cells were prepared by culturing 1×10^6 PBMC in tissue culture medium in round-bottomed 96-well plates (Nunc) and then, after an 18-hr incubation, non-adherent cells were removed. After a further 5 days of culture, target cells were either infected with *M. bovis* (at an m.o.i. of 2 : 1) or uninfected, and then labelled with 3 μCi/well of ^{51}Cr (Amersham, Bucks., UK) overnight (18 hr). Prior to use as targets, they were washed three times with prewarmed (37°) tissue culture medium. Effector cells from PBMC stimulated *in vitro* were centrifuged over Lymphoprep to remove dead cells and washed three times. *In vitro*-stimulated or fresh PBMC were serially diluted and added to the infected and uninfected target cells. After 18 hr, supernatants were removed to measure ^{51}Cr release. Any remaining ^{51}Cr in the targets was released by lysis with 5% Triton-X-100 (Sigma) so that the total uptake could be calculated. Target cells incubated without CTL effectors were included to measure the spontaneous release of ^{51}Cr. Mycobacteria were killed by adding glutaraldehyde (2% final concentration). Radioactivity was determined using Optiphase scintillant in a Trilux liquid scintillation counter (Wallac, Turku, Finland). Specific lysis, in counts per minute (c.p.m.) was calculated as follows:

$$\text{Specific lysis} = [(\text{c.p.m. in test supernatant} \div \text{c.p.m. in total test sample}) - (\text{c.p.m. in control supernatant} \div \text{c.p.m. in total control sample})] \times 100.$$

The standard error of the mean (SEM) of triplicate determinations did not usually exceed 4·5% specific lysis.

IFN-γ assay

Heparinized blood, in 1·5-ml aliquots, was dispensed into a 24-well plate within 4 hr of collection, and 100 μl of bovine PPD

(final concentration 20 μg/ml) or 100 μl of PBS was added. The whole-blood cultures were incubated at 37° for 18 hr and the IFN-γ levels in the plasma supernatants were measured, as described previously,[26] using the BOVIGAM sandwich enzyme-linked immunosorbent assay (ELISA) kit (CSL Ltd). The amount of IFN-γ was calculated from a standard curve prepared with recombinant bovine IFN-γ.[28]

Phenotying of CTL

T-cell subset depletion was carried out using the Mini-Macs system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). For $CD8^+$ depletion, CTL effector cells were harvested and incubated with antibody specific for CD8 (CC63; Serotec, Oxford, UK) at 4° followed by goat anti-mouse immunoglobulin G (IgG) magnetic beads and passed through a Mini Macs column, according to the manufacturers' instructions. $WC1^+$ γδ effector cells were similarly depleted using WC1-specific antibody (CC15; Serotec) and the Mini-Macs system. Depleted cells were washed twice in tissue culture medium prior to being assayed for CTL activity. Depletion of the specific subset was >95%. In one experiment, $CD8^+$ depletion was carried out using complement-mediated lysis following antibody treatment. CTL effector cells were harvested and incubated with antibody specific for CD8 (CC63; Serotec) at 37° for 30 min followed by guinea-pig complement (Behring Diagnostics GmbH, Marburg, Germany) at 37° for 30 min. Cells were centrifuged over Lymphoprep and washed twice in tissue culture medium before being assayed for CTL activity.

Mycobacteria growth-inhibition assay

PBMC that had been restimulated *in vitro* were incubated with *M. bovis*-infected Mϕs for 5 days. Growth of *M. bovis* within the Mϕs was determined, as described previously, using uptake of [^{3}H]uracil.[29] Briefly, Mϕ cultures were lysed with 0·1% saponin (Sigma), pulsed with $3{\cdot}7 \times 10^4$ Bq [^{3}H]uracil (specific activity 1·85 TBq/mmol; Amersham) per well and harvested 24 hr later. Mycobacteria were heat killed and the incorporated radioactivity was counted in a Trilux liquid scintillation counter (Wallac). Growth inhibition was expressed as follows:

$$\text{Growth inhibition (\%)} = [(\text{c.p.m. in restimulated cultures} \div \text{c.p.m. in control cultures}) \times 100].$$

Statistical analysis

To determine significant differences, the results from the CTL and IFN-γ assays were $\log_{10}$ transformed to achieve homogeneity of variance and were then analysed by analysis of variance (ANOVA).

RESULTS

Cytotoxic activity of PBMC from experimentally infected cattle

To determine conditions whereby specific cytotoxic cells could be generated, the PBMC of two experimentally infected cattle were restimulated *in vitro* under various conditions. After 7 days of culture they were tested for the ability to lyse their own *M. bovis*-infected or uninfected Mϕs by the ^{51}Cr-release assay. PBMC from one animal, cultured for 7 days without restimulation, did not display cytotoxic activity (Fig. 1a). PBMC that were cultured for 7 days with live *M. bovis* at 6×10^5 or 3×10^6 CFU per 3×10^7 PBMC, specifically lysed *M. bovis*-infected Mϕs (Fig. 1b, 1c, respectively). When the dose of *M. bovis* was increased to $1{\cdot}5 \times 10^7$ CFU, *M. bovis* cytotoxic activity was almost undetectable (Fig. 1d). The other animal was used to compare the ability of bovine PPD, live

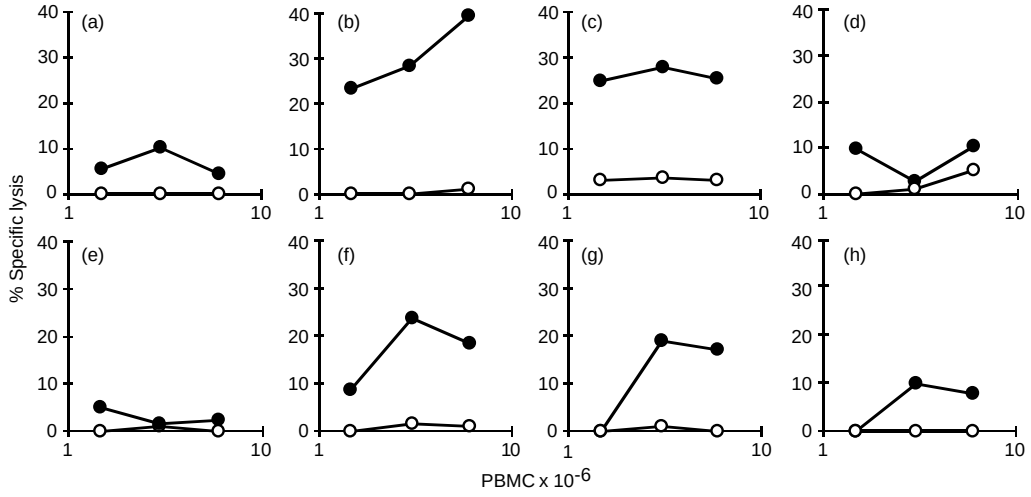

Figure 1. Lysis of *Mycobacterium bovis*-infected macrophages (Mϕs) by cytotoxic cells. Peripheral blood mononuclear cells (PBMC) from two calves (no. 1 and no. 2), experimentally infected with *M. bovis* 11 weeks previously, were restimulated *in vitro* and assayed for cytotoxic T-lymphocyte (CTL) activity on *M. bovis*-infected (●) or uninfected (○) Mϕs. PBMC from calf no. 1 were cultured alone (a), with 6×10^5 colony-forming units (CFU) of *M. bovis* (b), with 3×10^6 CFU of *M. bovis* (c), or with $1{\cdot}5 \times 10^7$ CFU of *M. bovis* (d). PBMC from calf no. 2 were cultured with bovine purified protein derivative (PPD) (e), with 6×10^5 CFU of *M. bovis* (f), or with *M. bovis*-infected Mϕs (g) or uninfected Mϕs (h).

M. bovis (6×10^5 CFU) and *M. bovis*-infected or -uninfected Mϕs to stimulate CTL *in vitro* from PBMC. PPD did not stimulate CTL activity (Fig. 1e). Adding 6×10^5 CFU *M. bovis* directly to PBMC cultures (Fig. 1g), gave almost the same response as adding Mϕs previously infected with *M. bovis* (Fig. 1f). When uninfected Mϕs were used there was a barely detectable response (Fig. 1h). Similar numbers of viable lymphocytes were recovered from each culture and comparable levels of IFN-γ were detectable in cultures that had been re-exposed to mycobacterial antigens (data not shown). In subsequent experiments, 6×10^5 CFU *M. bovis* were used for *in vitro* restimulation of PBMC to generate CTL.

Effector activity of PBMC after *in vitro* restimulation with *M. bovis* may reflect activity derived from non-effector CTL precursors or memory cells present in the blood. In order to measure CTL effector-cell activity in fresh blood, PBMC from the two calves used in the first experiment, and an additional experimentally infected calf, were assayed for cytotoxicity on infected or uninfected Mϕs without prior *in vitro* restimulation. For comparison, PBMC, from the same blood sample, were restimulated *in vitro* with live *M. bovis* for 7 days (Fig. 2). There was minimal cytotoxic activity in fresh PBMC against *M. bovis*-infected Mϕs (<10% specific lysis; Fig. 2a) but fresh PBMC from one calf (Fig. 2a, no. 2) contained cytotoxic cells that were able to lyse uninfected Mϕs. *M. bovis*-specific cytotoxic activity was detected in all three animals after *in vitro* stimulation (Fig. 2b).

A further experiment was carried out to follow the generation of CTL activity during the course of experimental infection with *M. bovis*. PBMC were prepared immediately before infection and at various time-points after infection, restimulated *in vitro* and assayed for cytotoxic activity. The zero time-point, immediately prior to infection, where cytotoxic activity was ≤5% specific lysis, using either infected or uninfected target cells, acted as the negative control. Cytotoxic activity was

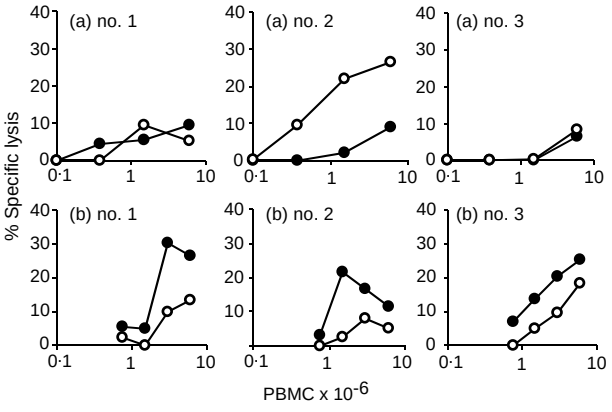

Figure 2. Comparison of cytotoxic activity in fresh and cultured peripheral blood mononuclear cells (PBMC). Freshly isolated PBMC from three calves (no. 1, no. 2 and no. 3), experimentally infected with *Mycobacterium bovis* 13 weeks previously, were assayed for cytotoxic activity (a) or were restimulated *in vitro* with *M. bovis* for 7 days prior to assay (b). The mean percentage-specific lysis of *M. bovis*-infected macrophages (Mϕs) (●) or uninfected Mϕs (○) of triplicate determinations are shown.

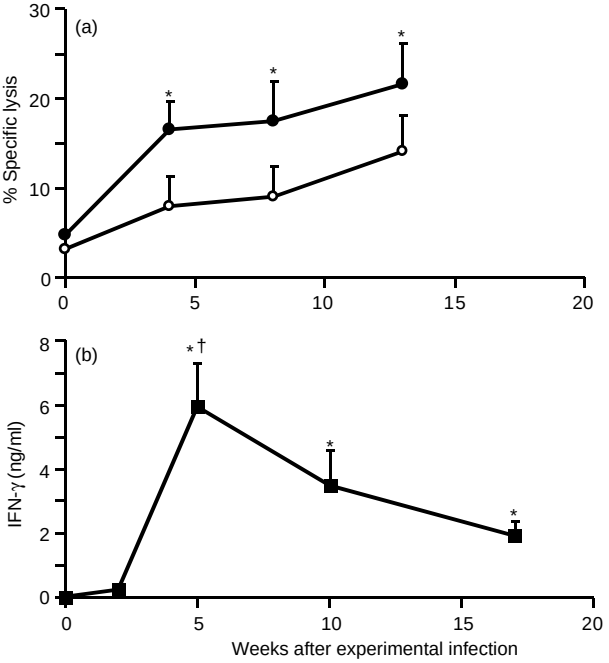

Figure 3. Cytotoxic activity and interferon-γ (IFN-γ) responses during experimental infection with *Mycobacterium bovis*. Six calves (no. 4, no. 5, no. 6, no. 7, no. 8 and no. 9) were experimentally infected with *M. bovis*; during the course of the infection, peripheral blood mononuclear cells (PBMC) were assayed for *in vitro* cytotoxic responses to *M. bovis* [mean response ± standard error of the mean (SEM); Fig. 3a), or plasma from whole blood stimulated with bovine purified protein derivative (PPD) was assayed for IFN-γ (mean response ± SEM; Fig. 3b). Cytotoxic activity generated from 6×10^6 PBMC was determined in triplicate using *M. bovis*-infected (●) or -uninfected (○) macrophage targets. *Significantly different from 0 weeks (preinfection; $P < 0{\cdot}05$); †significantly different from week 17 ($P < 0{\cdot}05$).

detected at 4 weeks after challenge, and responses, although not significantly different during the course of infection, were significantly different at 4, 8 and 13 weeks postchallenge compared with 0 weeks ($P < 0{\cdot}05$; Fig. 3a). For comparison, whole-blood IFN-γ responses to bovine PPD during the course of infection are shown (Fig. 3b). IFN-γ responses peaked 4 weeks after infection; the mean values at 5, 10 and 17 weeks postchallenge were significantly different from those at 0 weeks ($P < 0{\cdot}05$) and there was a significant decrease between weeks 5 and 17 postinfection ($P < 0{\cdot}05$).

$WC1^+$ γδ and $CD8^+$ T-cell depletion

The effect of depleting $WC1^+$ γδ T cells (using Mini Macs columns) from CTL cultures of PBMC, taken from four calves 13 weeks following an experimental infection with *M. bovis*, was determined. The results are shown in Fig. 4. Untreated cultures displayed good CTL activity (Fig. 4a), whereas after γδ depletion the activity was reduced in all four animals. This was particularly evident as effector cells were diluted out to lower concentrations (<10^5 PBMC). For comparison, PBMC, taken from two of the calves (no. 10 and no. 11), 2 weeks

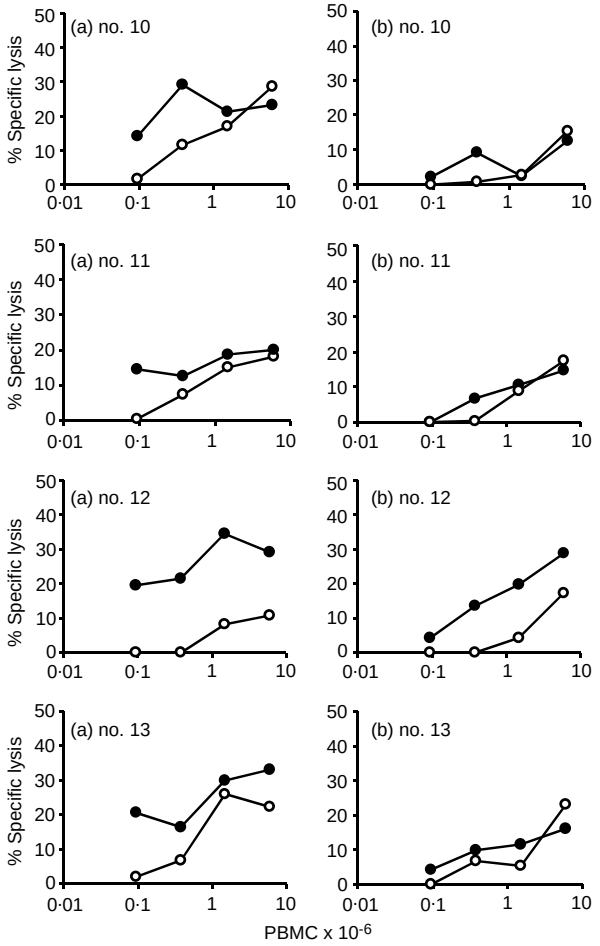

Figure 4. Effect of $WC1^+$ γδ depletion of effector cells on cytotoxic activity. Cytotoxic effector cells generated *in vitro* from four calves (no. 10, no. 11, no. 12 and no. 13), experimentally infected with *Mycobacterium bovis* 13 weeks previously, were assayed for cytotoxic activity (a) or treated to remove γδ cells prior to assay (b). The mean percentage-specific lysis from triplicate assays, using *M. bovis*-infected (●) or uninfected (○) macrophage targets are shown.

previously at 11 weeks after infection, were stimulated *in vitro* and depleted of $CD8^+$ T cells in a similar manner. Another calf (no. 14) had $CD8^+$ T cells depleted by antibody and complement treatment 11 weeks after infection ((Table 1). There was reduced CTL activity in all three animals after $CD8^+$ T-cell depletion compared with non-depleted controls.

Growth inhibition of mycobacteria

To determine whether cell cultures with cytotoxic activity could also inhibit the growth of mycobacteria, PBMC from four animals were stimulated *in vitro* with *M. bovis* to generate CTL activity and tested after 24 hr and 6 days for mycobacterial growth-inhibitory properties on *M. bovis*-infected Mϕs. CTL activity of the cultured cells was determined. There was no inhibition of growth of *M. bovis* after 24 hr of incubation (data not shown) but this was detected in three of four animals when

Table 1. Effect of depleting $CD8^+$ T cells from the effector population

Animal number	Method of anti-$CD8^+$ depletion	Untreated	Anti-CD8 depleted
10	Mini-Macs column	9·4 ± 0·7	6·3 ± 1·7
11	Mini-Macs column	16·4 ± 4·5	7·8 ± 1·4
14	Anti-CD8 antibody + complement	22·5 + 2·0	9·4 ± 2·3

Mean (±SEM) percentage-specific lysis of *Mycobacterium bovis*-infected macrophages by 3×10^6 peripheral blood mononuclear cells restimulated *in vitro* from calves infected with *M. bovis* 11 weeks previously.

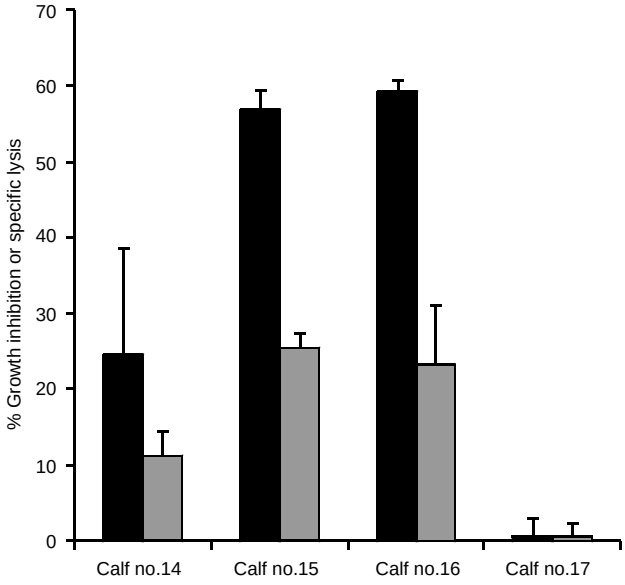

Figure 5. Comparison of effector function. Effector cells from 6×10^6 peripheral blood mononuclear cells (PBMC) of four calves (no. 14, no. 15, no. 16 and no. 17), experimentally infected with *Mycobacterium - bovis* 20 weeks previously, were assayed for their ability to inhibit the growth of *M. bovis* within infected macrophages (Mϕs) (black bars) or to lyse *M. bovis*-infected Mϕs (grey bars). Results are shown as mean percentage growth inhibition and mean percentage specific lysis (± standard error of the mean).

CTL cultures were incubated with infected Mϕs for 6 days. The same three animals (no. 14, no. 15, and no. 16) gave a CTL response, and the culture that displayed the highest level of growth-inhibiting activity also gave the strongest CTL response (Fig. 5). All four animals had macroscopic lesions in the lung and lymph nodes, and the lesioned samples were culture positive for *M. bovis*.

DISCUSSION

It is clear, from studies using mouse models of tuberculosis and from human patients, that cytotoxic cells are generated during infections with *M. tuberculosis*.[12,28] In this work, we show that cytotoxic cells, which specifically lyse *M. bovis*-infected Mϕs,

contribute to the immune response during an experimental *M. bovis* infection of cattle. The cytotoxic response was maintained as the disease progressed, and cytotoxic activity could be assigned to $WC1^+$ γδ and $CD8^+$ T cells. Although $CD4^+$ T cells can also function as CTL, they were not studied in this work. This is the first report of such cytotoxic activity in cattle infected with bovine tuberculosis.

The method used to evaluate the killing of mycobacteria-infected Mϕs is based on an assay used in human PBMC[30] and mice.[13] The specific CTL responses were weak, like those detected during *M. tuberculosis* infections of mice.[28] They could be detected when PBMC were exposed to lower doses of *M. bovis* (6×10^5 or 3×10^6 CFU) *in vitro*, but not when a higher dose ($1{\cdot}5 \times 10^7$ CFU) was used. Mycobacteria are known to possess agents that modify immune responses, and the balance of the concentration of activating and inhibitory molecules may be important in stimulating CTL activity. *M. bovis*-infected Mϕs also stimulated *in vitro* CTL responses but, as expected, bovine PPD did not, and *M. bovis*-specific CTL activity could not be detected in fresh blood. The responses varied from animal to animal. This variation could have been caused, in part, by previous exposure of the animals to environmental mycobacteria or as a result of using an outbred population of animals. The responses were maintained during the course of the experimental infection and did not decline as did the IFN-γ responses. When animals were killed at 21 weeks postinfection, mediastinal lymph node cells displayed CTL activity after *in vitro* stimulation with *M. bovis* (data not shown). The dose–response relationship between the concentration of effector cells and the percentage-specific lysis did not always follow the characteristic relationship generally observed in ^{51}Cr-release assays, suggesting that more than one population of cells was involved. Depletion studies showed that both $WC1^+$ γδ T cells and $CD8^+$ T cells were involved in cytotoxic activity.

The importance of cytotoxic responses in protective immunity to tuberculosis is not clear. All the major T-cell subsets – $CD4^+$, $CD8^+$ and γδ – have been shown to kill Mϕs expressing mycobacterial antigens.[7,31,32] In this work, the focus has been on $CD8^+$ and γδ T cells, as their function in protective immunity to mycobacterial infections is not so well defined. Depletion studies showed that both $WC1^+$ γδ and $CD8^+$ T cells had the capacity, when re-exposed to *M. bovis in vitro*, to kill infected Mϕs during experimental infections with *M. bovis*. $WC1^+$ γδ T-cell numbers increase in the blood of cattle early during infection, with other T-cell subsets increasing in number at later time-points.[6] In this work, the depletion of $WC1^+$ γδ T cells *in vitro* at 13 weeks postinfection resulted in reduced CTL activity, and $CD8^+$ T-cell depletion at a similar time-point (11 weeks after infection) also resulted in reduced CTL activity; however, it is possible that different CTL populations are generated at different time-points during the course of infection. $CD8^+$ T cells recognize conventional protein mycobacterial antigens, whereas $WC1^+$ γδ T cells have been shown in cattle, as have γδ T cells in other species, to respond to both protein and non-protein phosphate-rich antigens of mycobacteria.[33] γδ T cells from *M. bovis*-infected cattle have been shown to exert immunomodulatory effects on αβ T cells, inhibiting their proliferation,[10] and it is possible that they could even kill them. γδ T cells from humans can be autoreactive and lyse autologous lymphocytes.[34] Some indication of self-reactivity was detected in PBMC directly after isolation from blood, but the cells responsible for this activity were not investigated in this study. After *in vitro* restimulation, cytolytic cells lysed both uninfected, as well as infected, Mϕs, an observation that may reflect the cross-reactivity between mycobacterial and self-antigens, for example heat-shock proteins. This type of non-specific lysis has long been established as a feature of CTL responses to intracellular pathogens and has been attributed to conventional CTL, NK cells and lymphokine-activated killer cells.[35,36]

Cattle have high numbers of circulating γδ T cells compared to mice and humans, and these cells may play a more significant role in bovine tuberculosis. However, depletion of γδ T cells in both mice[3] and cattle[37] does not appear to markedly change the course of disease. $CD8^+$ T cells have been shown to release mycobacteria from Mϕs.[25] One proposed concept is that the released mycobacteria are taken up by more efficient antigen-presenting cells, but CTL have also been shown to reduce the viability of intracellular and extracellular *M. tuberculosis*.[16–18] In this report, we have shown that the cytotoxic activity of lymphocytes from *M. bovis*-infected cattle appears to correlate with the ability of these lymphocytes to inhibit the number of mycobacteria within infected Mϕs. This inhibitory effect was observed after 6 days of incubation, suggesting that it was not mediated by IFN-γ.[38] However, it is yet to be proven whether some other mediator, for example tumour necrosis factor-α (TNF-α), is involved.

Vaccination of cattle remains an option for the control of bovine tuberculosis,[39] but a better vaccine or vaccination strategy is required. Many of the experimental problems associated with the development of protective vaccination against bovine and human tuberculosis are similar, and bovine tuberculosis models are being viewed as a suitable model for evaluating human tuberculosis vaccines and vaccination strategies.[40] Vaccine trials in cattle provide an opportunity to follow immune responses after vaccination and correlate them with disease or protection.[5,41,42] Whole-blood IFN-γ, interleukin-2 and antibody responses have been studied in previous work and future work is aimed at following cytotoxic responses in the same way.

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