Immunology 1998 **95** 585–590

38 000 MW antigen-specific major histocompatibility complex class I restricted interferon-γ-secreting CD8$^+$ T cells in healthy contacts of tuberculosis

R. J. WILKINSON,*† X. ZHU,* K. A. WILKINSON,* A. LALVANI,‡ J. IVANYI,* G. PASVOL,† & H. M. VORDERMEIER* **Tuberculosis and Related Infections Unit, Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, London, †Wellcome Centre for Clinical Tropical Medicine, Northwick Park Hospital, Imperial College School of Medicine, Harrow, and ‡Molecular Immunology Group, Institute of Molecular Medicine, Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford, UK*

SUMMARY

CD8$^+$ T lymphocytes are required to protect mice against *Mycobacterium tuberculosis*, although in early infection the mechanism appears not to be via perforin or granzyme-mediated lysis of the infected target, and may be via interferon-γ (IFN-γ) production. We therefore investigated whether CD8$^+$ T cells specific for the immunoprotective 38 000 MW antigen of *M. tuberculosis* could be detected in infected humans. Using a recombinant vaccinia virus expressing the 38 000 MW antigen of *M. tuberculosis* (rV38) and a control vaccinia virus (rVras) we demonstrated that both viruses stimulated IFN-γ production from freshly isolated peripheral blood mononuclear cells (PBMC) in a 36-hr enzyme-linked immunospot assay. Cell depletion and antibody blockade established that the bulk of the 38 000 MW antigen-specific IFN-γ response was mediated by CD8$^+$, major histocompatibility complex class I-restricted T cells, whereas the anti-vaccinia virus response was predominantly mediated by CD4$^+$ T cells. In further evaluations PBMC from all seven healthy tuberculosis-exposed contacts had a 38 000 MW antigen-specific IFN-γ response, whereas seven patients with untreated sputum-positive pulmonary tuberculosis had very low levels of 38 000 antigen-specific IFN-γ-producing cells. These preliminary observations demonstrate the utility of recombinant vaccinia viruses in restimulating freshly isolated CD4$^+$ and CD8$^+$ T cells. The bias towards a higher frequency of IFN-γ-producing CD8$^+$ T cells in contacts rather than patients may indicate a protective role for CD8$^+$ cells in human tuberculosis.

INTRODUCTION

Tuberculosis can only be partially prevented by the vaccine bacillus Calmette–Guérin (BCG) and remains an important cause of death.[1] The first step towards improving vaccine-induced protection is to define the immune correlates of protection. In the murine model a need for CD8$^+$ T cells has been identified,[2] although the mechanism in early infection does not appear to require perforin or granzyme-induced lysis of the infected target,[3] and may be via the production of interferon-γ (IFN-γ).[4] In humans the picture is less clear, although children with a mutation in the IFN-γ receptor succumb to atypical mycobacterial infection,[5] the best available evidence for an important role for this cytokine in humans. Several studies have examined the hypothesis that there is a deficiency of CD4$^+$-mediated IFN-γ production in patients with active tuberculosis with conflicting results.[6,7]

In contrast to CD4$^+$ cells, the role of CD8$^+$ T cells in human disease is relatively poorly defined. A patient with an isolated deficiency of CD8$^+$ cells had recurrent attacks of tuberculosis despite chemotherapy.[8] CD8$^+$ cells are selectively increased in broncho-alveolar lavage from patients with pulmonary or miliary disease[9,10] and in tuberculous cervical adenitis nearly as many CD8$^+$ cells as CD4$^+$ are present.[11] Major histocompatibility complex (MHC) class I-restricted CD8$^+$ T-cell clones proliferate in response to mycobacterial antigen;[12] and the lysis of BCG-infected monocytes by CD8$^+$ cells has been demonstrated,[13,14] although the antigenic specificity and restriction of the responding cells was not determined. More recently it has been shown that CD8$^+$ αβ double-negative T-cell clones, responding to mycobacterial mycolic acid in the context of the relatively non-polymorphic CD1b molecule, produce IFN-γ and can reduce the intracellular growth of *Mycobacterium tuberculosis in vitro*.[15] However, the

Received 21 May 1998; revised 31 August 1998; accepted 31 August 1998.

Abbreviations: ATCC, American Type Culture Collection; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; ELISPOT, enzyme-linked immunospot assay; ESAT-6, early secreted antigenic target; LREC, local research ethics committee; NBT, nitroblue tetrazolium; PPD, purified protein derivative.

Correspondence: Dr R. J. Wilkinson, Division of Infectious Diseases, Case Western Reserve University, Biomedical Research Building, 10900 Euclid Avenue, Cleveland, Ohio 44106-4984, USA.

analysis of CD1b-restricted T cells so far has been restricted to T-cell clones and lines and the crucial estimation of precursor frequency *in vivo* has not been reported.

The focus of this study was the MHC class I-restricted $CD8^+$ T-cell response. The detection of human $CD8^+$ responses in tuberculosis requires antigen to be processed endogenously, possibly at odds with the intraphagosomal location of the mycobacteria.[16] A further difficulty is that the conventional read-out assay (the release of ^{51}Cr from labelled targets) is relatively insensitive for detecting low responder frequencies. The required *in vitro* expansion of $CD8^+$ cells for detection by ^{51}Cr release also complicates direct estimation of the frequency of antigen-specific T cells. The release of IFN-γ detected by the interleukin enzyme-linked immunospot (ELISPOT) assay correlates with cytotoxicity as determined by ^{51}Cr release.[17–19] We used the IFN-γ ELISPOT assay in combination with peptide pools based on published human leucocyte antigen (HLA) class I motifs to define an HLA-B52-restricted T-cell epitope within the ESAT-6 antigen of *M. tuberculosis*.[19] However, as this predictive method relies on published algorithms it may miss potential epitopes, and a range of pooled peptides containing motifs for a large number of class I alleles has to be used. In this study we have also detected class I-restricted, $CD8^+$ *M. tuberculosis* antigen-specific T cells by the IFN-γ ELISPOT assay. However, endogenous presentation of antigen was achieved by a different method: we infected fresh peripheral blood mononuclear cell (PBMC) cultures at low multiplicity (to avoid excessive cytopathic effects) with a recombinant vaccinia virus expressing the 38 000 MW molecule, another important immunoprotective antigen of *M. tuberculosis*.[20,21] In this way we were able to demonstrate frequencies of 38 000 MW antigen-specific $CD8^+$ T cells as high as 1/6410 $CD8^+$-enriched freshly isolated PBMC.

MATERIALS AND METHODS

Subjects

Twenty- to thirty-millilitre samples of citrated peripheral blood was drawn from purified protein derivative (PPD)-positive patients with untreated sputum- and culture-positive pulmonary tuberculosis attending the Department of Infection and Tropical Medicine, Northwick Park Hospital, Harrow, UK. Healthy tuberculin PPD-positive donors (Heaf Grade ≤ 2) with close occupational or family exposure to infectious cases of *M. tuberculosis* but normal chest radiography were recruited from the tuberculosis contact clinic at the same hospital. In both patients and healthy contacts a history of previous BCG or vaccinia virus vaccination was sought both by enquiry and by inspection of likely scar sites. Where ascertainment was equivocal the subjects' status was assigned 'Not known' (NK). Ethical permission for the study was given by the Harrow Local Research Ethics Committee (LREC; Reference number 1646).

Isolation of PBMC and immunomagnetic depletion of T-cell subsets

Whole citrated blood was diluted 1/2 in phosphate-buffered saline (PBS; Gibco, Paisley, UK) and the PBMC were separated over a Ficoll gradient (Pharmacia, Uppsala, Sweden). Immunomagnetic depletion of either $CD4^+$ or $CD8^+$ T-cell subsets was performed using Dynabeads® (Dynal, Merseyside, UK) according to the manufacturer's instructions. Briefly Dynabeads washed twice in washing buffer [PBS/2% fetal calf serum (FCS)] were added to 5×10^6 PBMC/ml in washing buffer at 4° using the ratio five Dynabeads per PBMC. The suspension was then gently mixed at 4° on an orbital shaker for 45 min. Taking care not to let the temperature of the solution rise above 4° the solution was immediately subjected to two rounds of immunomagnetic capture of the undesired T-cell subset before counting, washing and resuspension of uncaptured cells in RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate and 10 mM HEPES buffer (Gibco). Fluorescence-activated cell sorter (FACS) analysis (Becton Dickinson, Santa Fe, CA) showed that immunomagnetic depletion as described consistently resulted in $\geq 90\%$ reduction of the chosen T-cell subset.

Construction, and infection with, the recombinant vaccinia virus (rV38) expressing the 38 000 MW antigen

A recombinant vaccinia virus expressing the 38 000 MW antigen (rV38) was produced as previously described.[21] Briefly, the coding sequence of the *M. tuberculosis* 38 000 protein was inserted into the *Sma*I site of the vaccinia virus recombinant plasmid pSC11. The coding fragment was obtained by subcloning into the *Sma*I site a 1·2-kilobase *Nde*I–*Bam*HI fragment derived from the plasmid pMS10.4 that encoded the 38 000 MW protein.[22] Recombinant vaccinia viruses were produced by transfection of the plasmid into the osteosarcoma cell line 143 coinfected with wild-type vaccinia virus followed by selection for recombinant viruses. The resulting recombinant virus, designated rV38, was plaque purified by repeated plating (four times) and protein expression was confirmed by Western blot analysis of infected 143 cells using the monoclonal antibody (mAb) TB71.[21,23] A recombinant vaccinia virus expressing the human Ras protein, designated rVras[24] was used as control. To determine the conditions under which rV38 would stimulate the production of IFN-γ by $CD8^+$ T cells, a healthy tuberculin-positive donor with high occupational exposure to tuberculosis was chosen and various numbers of CD4-depleted PBMC/well (62 500–500 000) and multiplicities of viral infection (0·1–10) were tested. An optimal response was obtained at a multiplicity of infection (MOI) of 1 and using 250 000 cells (data not shown) and was adopted as standard protocol in all further experiments.

IFN-γ ELISPOT assay

Sterile immobilon-P microtitre plates (Millipore Corp., Bedford, MA) were coated overnight at 4° with 100 μl/well of anti-IFN-γ mAb 1-DIK at 15 μg/ml (Mabtech AB, Nacka, Sweden) in sterile PBS. The plates were washed once in sterile PBS then blocked by incubating at 37° for 1 hr in 100 μl RPMI-1640 plus 20% blood group AB^+ human serum. Cells suspended in 100 μl RPMI ± recombinant virus ± blocking antibody were then added directly to duplicate wells to give a final serum concentration of 10%. Concanavalin A (Con A; 5 μg/ml) -stimulated wells were included as positive controls in all experiments. To establish restriction a pan anti-HLA class I antibody (Pharmingen, San Diego, CA) at concentrations of 0·1–10 μg/ml; an anti-CD8 antibody OKT8 at dilutions of 1/50–1/5000; the pan-anti-HLA-DR antibody

L243 [American Type Culture Collection, Rockville, MD (ATCC); HB-55, mouse IgG2a] at 10 μg/ml; or the same concentrations of isotype control (murine anti-mouse I-A^{b+k}, ATCC HB-163), were used. The plates were incubated for 36 hr at 37° in 5% CO_2. The wells were then washed up to nine times (to remove all the cells) in 0·45 μM filtered PBS/0·05% Tween-20. The wells were then incubated for 2 hr at room temperature with 100 μl of biotinylated detection mAb 7B-61 anti-IFN-γ at 1 μg/ml (Mabtech) in PBS/3% bovine serum albumin (BSA)/0·05% Tween-20. The wells were washed five times and then incubated at room temperature for 30 min in 100 μl 1:750 streptavidin alkaline phosphatase (Sigma, St. Louis, MO) in PBS/3% BSA/0·05% Tween-20. Following six final washes, the substrate BCIP/NBT (Biorad, Hercules, CA) was added at a concentration of 2% of each in substrate buffer. The colour development was stopped by holding the plate under a running tap and after drying, the spots were enumerated using a stereomicroscope. Apart from antibody-blocking experiments, duplicate wells were tested and the variability between duplicates was ±2·8%.

RESULTS

We first determined whether the IFN-γ response was directed towards the 38 000 MW antigen or to vaccinia virus antigens. The responses of total PBMC, as well as $CD4^+$- and $CD8^+$-depleted fractions were assayed. Undepleted PBMC responded to rV38 and to a lesser extent to rVras (Fig. 1). Cell depletion established that most of the 38 000 MW antigen-specific response was in the fraction enriched for $CD8^+$ cells. Conversely the bulk of the anti-vaccinia virus response was in the $CD4^+$-enriched fraction. A similar predominance of $CD4^+$ response to rVras was seen in other donors vaccinated with vaccinia virus (Table 1). By contrast, the numbers of rVras-specific IFN-γ-producing T cells in donors unequivocally not vaccinated with vaccinia virus was very low.

To characterize the effector population within the $CD8^+$-enriched PBMC fraction, we performed antibody-blocking experiments using mAb known to be effective in blocking either class I or class II responses. Anti-HLA class I antibody at concentrations of 0·1–10 μg/ml; an anti-CD8 antibody OKT8 at dilutions of 1/50–1/5000; the pan-anti-HLA-DR antibody L243 at 10 μg/ml; or the same concentrations of isotype control were used. These antibodies had no effect on the number of Con A-stimulated IFN-γ spot-forming cells (SFC) which remained too numerous to reliably count (>1000 SFC/125 000 cells). Anti-class I antibody caused a dose-dependent decrease in response to rV38, maximal at 10 μg/ml (Fig. 2a). The same dose of anti-class II or isotype control antibody had no effect. In a further experiment using $CD8^+$-enriched PBMC mAb blockade of CD8 also reduced the response in a dose-dependent manner, and the combination of anti-CD8 and anti-class I antibodies completely abolished it (Fig. 2b). Taken together, these results demonstrate that the 38 kDa-specific effectors are class I-restricted $CD8^+$ T cells.

We evaluated the ELISPOT technique further in seven healthy tuberculin-positive healthy contacts (all male, average age 39±4·5 years) and seven patients with untreated sputum- and culture-positive pulmonary tuberculosis (five female, two male, 39±8·8 years). In all cases there was a strong response to Con A 5 μg/ml (data not shown). Amongst all the healthy

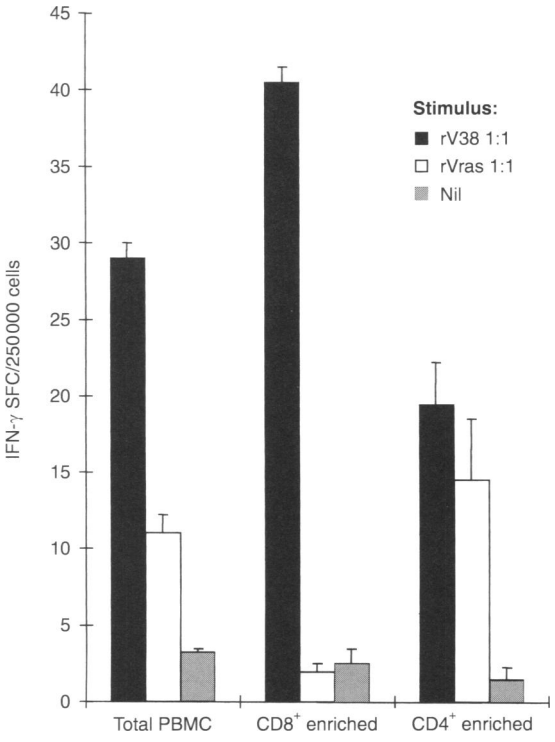

Figure 1. Number of IFN-γ spot-forming cells (SFC)/250 000 cells in cultures of PBMC from a healthy contact of tuberculosis stimulated at a multiplicity of infection of 1 for 36 hr with rV38 or rVras. The $CD4^+$ or $CD8^+$ T cells were depleted using Dynabeads®. Both viruses stimulated IFN-γ production. Cell depletion established that the bulk of the 38 000 MW antigen-specific response was in the fraction enriched for $CD8^+$ cells, whereas the anti-vaccinia virus component was almost entirely abolished by this depletion. Error bars indicate the SEM of duplicate or quadruplicate wells.

contacts we were able to elicit a 38 000 MW antigen-specific response from either PBMC or $CD8^+$-enriched PBMC, although in some cases (e.g. C6, C7) the magnitude of response was low (Table 1). By comparison the 38 000 MW-specific response in patients was diminished, even the best response (P1) showed only 4/250 000 rV38 specific IFN-γ spots in $CD8^+$-enriched PBMC. Although the bulk of the anti-rVras response was in undepleted PBMC or the $CD4^+$-enriched cells, there was evidence in four cases (C6, C7, P2 and P5) of low frequency vaccinia virus-specific IFN-γ SFC in the $CD8^+$-enriched compartment at a frequency of 1/22 700–1/83 333 (Table 1). Although the vaccinia virus status of three of these four donors could not be unequivocally determined by the presence or absence of a characteristic scar, all were of sufficient age (56, 28, 33 and 86 years, respectively) to have received vaccinia virus vaccine.

DISCUSSION

We have demonstrated that the infection of fresh PBMC cultures with vaccinia virus at low multiplicity leads to the activation of T cells as detected by the production of IFN-γ by the ELISPOT assay. Both $CD4^+$ and $CD8^+$ cells were restimulated. Interestingly, the bulk of the antirVras response was mediated by $CD4^+$ cells whereas the $CD8^+$ cell response

Table 1. Numbers of IFN-γ spot-forming cells per 250 000 cells in cultures stimulated at a MOI of 1 for 36–40 hr with recombinant vaccinia viruses expressing either the 38 000 MW antigen of *M. tuberculosis* or the proto-oncogene *ras*

			Vaccination			PBMC			CD8-enriched PBMC		
Subject	Sex	Origin and ethnicity	Age	BCG	vaccinia	rV38	rVras	38 000-specific	rV38	rVras	38 000-specific
Contacts											
C1	M	'UK, White'	35	Yes	Yes	29	11	18	41	2	39
C2	M	'Nigeria, African'	34	Yes	NK	10	3	7			
C3	M	'Germany, White'	38	No	Yes	36	17	19	15	1	14
C4	M	'Kenya, Asian'	53	No	NK	47	33	14			
C5	M	'Uganda, Asian'	29	No	No				8	0	8
C6	M	'Kenya, Asian'	56	No	NK	12	8	4	5	6	−1
C7	M	'Guyana, Afro-caribbean'	28	No	NK				11	8	3
Patients											
P1	F	'Indian, Asian'	22	No	No	3	4	−1	4	0	4
P2	M	'Nigeria, African'	33	No	Yes	57	70	−13	2	3	−1
P3	F	'Djibouti, African'	35	Yes	Yes	3	3	0	3	2	1
P4	F	'Russia, White'	23	Yes	No	0	0	0	3	1	2
P5	M	'Indian, Asian'	86	No	NK				2	5	−3
P6	F	'UK, Afro-caribbean'	22	Yes	No				3	2	1
P7	F	'Somalia, African'	52	No	NK				0	0	0

Seven healthy recent contacts and seven untreated patients with sputum- and culture-positive pulmonary tuberculosis were studied. IFN-γ spot-forming cells in the absence of viral stimulation were low. The frequency of IFN-γ-producing $CD8^+$ T cells specific for the 38 000 MW antigen was calculated by subtracting the rVras response from the rV38. NK, not known; blank spaces indcated instances when an assay was not performed.

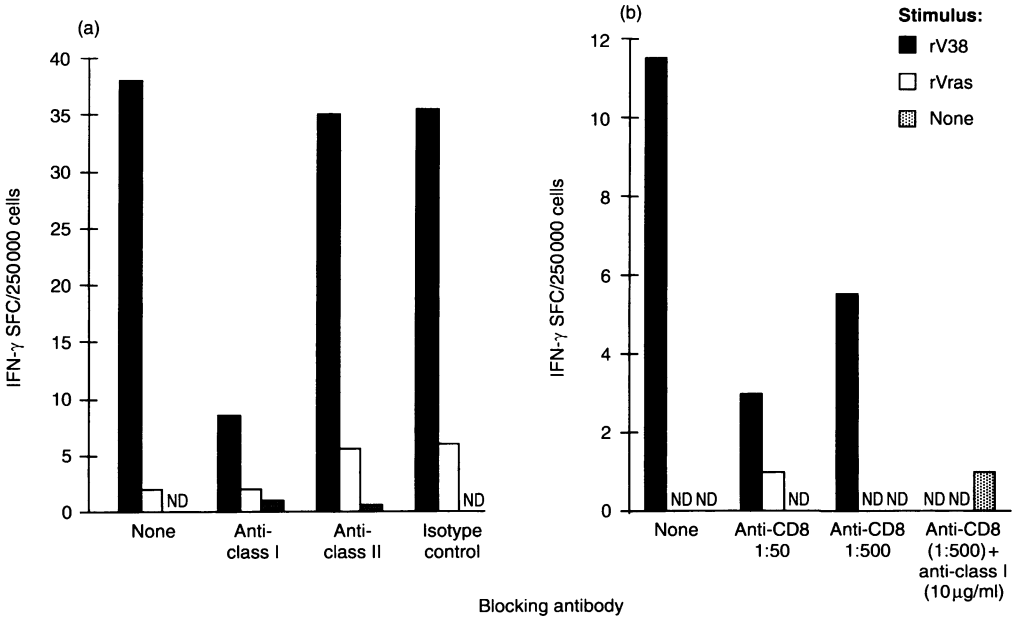

Figure 2. The 38 000 MW antigen-specific IFN-γ-producing cells are $CD8^+$ and HLA class I-restricted. In both (a) and (b): 250 000 $CD8^+$-enriched PBMC from two healthy donors were stimulated at a multiplicity of infection of 1 for 36 hr with rV38 or rVras. (a) The effect of anti-HLA class I or class II mAb on the number of IFN-γ spot-forming cells. Anti-class I antibody (10 μg/ml) was able to substantially diminish the *M. tuberculosis*-specific response, whereas anti-Class II (10 μg/ml) and isotype control antibodies had no effect. (b) The effect of anti-CD8 mAb and combined antibody blockade on the number of IFN-γ spot-forming cells. ND = no IFN-γ SFC detected.

was better stimulated by a vaccinia virus expressing the 38 000 MW antigen of *M. tuberculosis*. This component of the response was Class I-restricted and the frequency of 38 kDa specific $CD8 \pm T$ cells was higher in contacts, rather than patients with, tuberculosis. Both $CD8^+$ T cells and IFN-γ are required for protection against tuberculosis in mice[2,4,25,26] and vaccination using either a plasmid construct expressing the 38 000 MW antigen or using the same rV38 vaccinia virus is

protective by a mechanism involving both $CD8^+$ T cells and the production of IFN-γ.[20,27] We therefore speculate that the 38 000 antigen-specific $CD8^+$ IFN-γ-producing T cells we detected may also protect humans.

Although our primary objective in this preliminary investigation was the *M. tuberculosis* specific response, the anti-vaccinia virus response was also of interest. The donor in the example shown in Fig. 1 had been vaccinated against smallpox in 1967. Our observation that $\approx 1/12\,500$ $CD4^+$ cells were vaccinia-specific is similar to the conventional limiting dilution analysis of the vaccinia virus-specific memory $CD4^+$ T-cell response from a donor immunised with vaccinia virus 35 years earlier.[28] These findings suggest that a significant proportion of memory to vaccinia virus resides in the IFN-γ-secreting $CD4^+$ compartment. However, the exact precursor frequency cannot be determined using our system due to lytic and cytopathic effects of the virus. In addition this effect may be different in depleted and undepleted cultures as the greater $CD4^+$-mediated rVras-specific IFN-γ production in undepleted cultures may reduce cytopathic effects of the virus leading to a greater net IFN-γ response. In some donors we also detected a low frequency of $CD8^+$ vaccinia virus-specific $CD8^+$ IFN-γ-producing cells. The existence of a population of long-lived antiviral memory $CD8^+$ T cells that exhibit effector function without needing to divide and differentiate over several days has recently been demonstrated by Lalvani and colleagues[18] and our observations are consistent with this hypothesis.

In conclusion, we have demonstrated *M. tuberculosis* 38 000 MW antigen-specific, MHC class I-restricted $CD8^+$ T cells able to produce IFN-γ on contact with endogenously processed antigen expressed via a recombinant vaccinia virus. The technique of using low-dose recombinant vaccinia expressing mycobacterial antigen(s) and the ELISPOT assay could be readily extended to larger studies of other important antigens of *M. tuberculosis* and other pathogens, even in suboptimal conditions such as field studies in the tropics. Although the intraphagosomal location of *M. tuberculosis* might tend to mitigate against entry of proteins into the MHC class I-processing antigen pathway our work is further evidence in favour of the hypothesis that this does occur.[29] As the 38 000 antigen is a phosphate-binding protein secreted under conditions of phosphate depletion[30,31] it may be secreted into the cytosol. Our data also indicate that the frequency of such IFN-γ-producing $CD8^+$ T cells is higher in subjects infected with but healthy, when compared to patients with active pulmonary tuberculosis. Although an analysis of 38 000 MW-specific $CD8^+$ cells from disease sites might show a higher frequency of response,[10,32] it is also possible that the preponderance of MHC class I-restricted IFN-γ-producing $CD8^+$ cells in healthy infected subjects indicates that these cells contribute to protection in humans.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council of the UK. We are grateful to Drs G. Lombardi and H. Stauss of the Department of Immunology, Hammersmith Hospital for the gifts of the OKT8-blocking antibody and rVras, respectively. Dr W. H. Boom of Case Western Reserve University is thanked for his critical review of the manuscript. RJW is a Wellcome Trust Fellow in Clinical Tropical Medicine.

REFERENCES

1. Colditz G.A., Brewer T.F., Berkey C.S. *et al.* (1994) Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature, *J Am Med Assoc* **271,** 698.
2. Flynn J., Goldstein M., Triebold K., Koller B. & Bloom B.R. (1992) Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *Proc Natl Acad Sci USA* **89,** 12013.
3. Cooper A.M., D'Souza C., Frank A.A. & Orme I.M. (1997) The course of *Mycobacterium tuberculosis* infection in the lungs of mice lacking expression of either perforin-or granzyme-mediated cytolytic mechanisms. *Infect Immun* **65,** 1317.
4. Tascon R., Stavropoulos E., Lakacs K.V. & Colston M.J. (1998) Protection against *Mycobacterium tuberculosis* infection by $CD8^+$ T cells requires the production of Interferon-γ. *Infect Immun* **66,** 830.
5. Newport M.J., Huxley C.M., Huston S. *et al.* (1996) A mutation in the interferon-γ receptor gene and susceptibility to mycobacterial infection. *N Engl J Med* **335,** 1941.
6. Hirsch C.S., Hussain R., Toossi Z., Dawood G., Shahid F. & Ellner J.J. (1996) Cross-modulation by transforming growth factor beta in human tuberculosis: suppression of antigen-driven blastogenesis and interferon gamma production. *Proc Natl Acad Sci USA* **93,** 3193.
7. Surcel H.-M., Troye-Blomberg M., Paulie S. *et al.* (1994) T_h1/T_h2 profiles in tuberculosis based on proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. *Immunology* **81,** 171.
8. Bothamley G.H., Festenstein F. & Newland A. (1992) Protective role for CD8 cells in tuberculosis. *Lancet* **339,** 315.
9. Faith A., Schellenberg D.M., Rees A.D. & Mitchell D.M. (1992) Antigenic specificity and subset analysis of T cells isolated from the bronchoalveolar lavage and pleural effusion of patients with lung disease. *Clin Exp Immunol* **87,** 272.
10. Ainslie G.M., Solomon J.A. & Bateman E.D. (1992) Lymphocyte and lymphocyte subset numbers in blood and in bronchoalveolar lavage and pleural fluid in various forms of human pulmonary tuberculosis at presentation and during recovery. *Thorax* **47,** 513.
11. Randhawa P.S. (1990) Lymphocyte subsets in granulomas of human tuberculosis: an *in situ* immunofluorescence study using monoclonal antibodies. *Pathology* **22,** 153.
12. Rees A., Scoging A., Mehlert A., Young D.B. & Ivanyi J. (1988) Specificity of proliferative response of human CD8 clones to mycobacterial antigens. *Eur J Immunol* **18,** 1881.
13. Ottenhof T.H.M. & Mutis T. (1995) Role of cytotoxic cells in the protective immunity against and immunopathology of intracellular infections. *Eur J Clin Invest* **25,** 371.
14. Turner J. & Dockrell H.M. (1996) Stimulation of human peripheral blood mononuclear cells with live *Mycobacterium bovis* BCG activates cytolytic $CD8^+$ T cells *in vitro*. *Immunology* **87,** 339.
15. Stenger S., Mazzacarro R.J., Uyemura K. *et al.* (1997) Differential effects of cytolytic T cell subsets on intracellular infection. *Science* **276,** 1684.
16. Clemens D.L. & Horwitz M.A. (1995) Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med* **181,** 257.
17. Di Fabio S., Mbawuike I.N., Kiyono H., Fujihashi K., Couch R.B. & McGhee J.R. (1994) Quantitation of human influenza virus-specific cytotoxic T lymphocytes: correlation of cytotoxicity and increased numbers of IFN-gamma producing CD8+ T cells. *Int Immunol* **6,** 11.
18. Lalvani A., Brookes R., Hambleton S., Britton W.J., Hill A.V.S. & McMichael A.J. (1997) Rapid effector function in CD8+ memory T cells. *J Exp Med* **186,** 859.
19. Lalvani A., Brookes R., Wilkinson R.J. *et al.* (1998) Human

cytolytic and Interferon-γ secreting $CD8^+$ T lymphocytes specific for *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **95,** 270.

20. Zhu X., Venkataprasad N., Thangaraj T. *et al.* (1997) Functions and specificity of T cells following nucleic acid vaccination of mice against *M. tuberculosis* infection. *J Immunol* **158,** 5921.
21. Zhu X., Venkataprasad N., Ivanyi J. & Vordermeier H.M. (1997) Vaccination with recombinant Vaccinia viruses protects mice against *Mycobacterium tuberculosis* infection. *Immunology* **92,** 6.
22. Singh M., Andersen Å.B., McCarthy J.E.G. *et al.* (1992) The *Mycobacterium tuberculosis* 38 kDa antigen: overexpression of the gene in *Escherichia coli* and purification and characterisation of the recombinant product. *Gene* **117,** 53.
23. Young D., Kent L., Rees A., Lamb J. & Ivanyi J. (1986) Immunological activity of a 38-kilodalton protein purified from *Mycobacterium tuberculosis*. *Infect Immun* **54,** 177.
24. Skipper J. & Stauss H.J. (1993) Identification of two cytotoxic T lymphocyte-recognised epitopes in the RAS protein. *J Exp Med* **177,** 1493.
25. Flynn J., Chan J., Triebold K., Dalton D., Stewart T. & Bloom B. (1993) An essential role for Interferon-γ in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* **178,** 2249.
26. Cooper A., Dalton D., Stewart T., Griffin J., Russell D. & Orme I. (1993) Disseminated tuberculosis in Interferon-γ gene-disrupted mice. *J Exp Med* **178,** 2243.
27. Zhu X., Stauss H., Ivanyi J. & Vordermeier H. (1997) Specificity of CD8+ T cells from subunit-vaccinated and infected H-2b mice recognizing the 38 kDa antigen of *Mycobacterium tuberculosis*. *Int Immunol* **9,** 1669.
28. Demkowicz W.E. Jr, Littaua R.A., Wang J. & Ennis F.A. (1996) Human cytotoxic T-cell memory: long-lived responses to vaccinia virus. *J Virol* **70,** 2627.
29. Mazzaccaro R.J., Gedde M., Jensen E. *et al.* (1996) Major histocompatibility Class I presentation of soluble antigen facilitated by *Mycobacterium tuberculosis* infection. *Proc Natl Acad Sci USA* **93,** 117861.
30. Lefèvre P., Braibant M., De Wit L. *et al.* (1997) Three different putative phosphate transport receptors are encoded by the *Mycobacterium tuberculosis* genome and are present at the surface of *Mycobacterium bovis* BCG. *J Bacteriol* **179,** 2900.
31. Chang Z., Choudhary A., Lathigra R. & Quicho F.A. (1994) The immunodominant 38-kDa lipoprotein antigen of *Mycobacterium tuberculosis* is a phosphate-binding protein. *J Biol Chem* **269,** 1956.
32. Tan J.S., Canaday D.H., Boom W.H., Balaji K.N., Schwander S.K. & Rich E.A. (1997) Human alveolar T lymphocyte responses to *Mycobacterium tuberculosis* antigens. Role for $CD4^+$ and $CD8^+$cytotoxic cells and relative resistance of alveolar macrophage to lysis. *J Immunol* **159,** 290.