*Immunology* 1995 **86** 519–524

# Protection against tuberculosis by bone marrow cells expressing mycobacterial hsp65

C. L. SILVA, R. L. R. PIETRO, A. JANUARIO, V. L. D. BONATO, V. M. F. LIMA, M. F. DA SILVA & D. B. LOWRIE* *Department of Parasitology, Microbiology and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil and *Laboratory for Leprosy and Mycobacterial Research, National Institute for Medical Research, London, UK*

## SUMMARY

Although mice acquire only a slight degree of protection against tuberculosis by immunization with *Mycobacterium leprae* (*M. leprae*) hsp65 in incomplete Freund's adjuvant, protection is substantial following immunization by injection with J774 macrophage-like tumour cells that express the protein from the mycobacterial gene *via* a retroviral vector. We here took the same vector, used it to transfect the gene into normal murine bone marrow cells *in vitro*, and then used the transfected cells to reconstitute haematopoiesis in lethally irradiated mice. Bone marrow-cell clonal expansion and production of the protein *in vivo* resulted in specific delayed-type hypersensitivity and protection against challenge with *Mycobacterium tuberculosis* (*M. tuberculosis*) in about half of recipients. Counts of live bacteria in liver at 3 weeks were fivefold lower in delayed-type hypersensitivity (DTH)-positive than in DTH-negative mice. Other mice acquired neither DTH nor protection despite the presence of the protein in peripheral blood.

## INTRODUCTION

Mice implanted with tumour cells expressing the *Mycobacterium leprae* (*M. leprae*) 65kD heat shock protein (J774-hsp65 cells) from a transgene acquire a remarkably high degree of protection against subsequent challenge infection with virulent *Mycobacterium tuberculosis* (*M. tuberculosis*).[1] The protection is because of the generation of T cells specifically recognizing the protein since it can be passively transferred to naive mice by transferring such T cells cloned from the spleens of the protected mice.[2] Historically, it has been difficult to obtain substantial protection against tuberculosis by vaccinating with anything other than live mycobacteria such as bacillus Galmette–Guérin (BCG). Injections of hsp65 protein in adjuvant are ineffective. Why then are the transgenic tumour cells so effective?

Our working hypothesis is that endogenous generation of the protein within antigen-presenting cells is crucial to the generation of high protective immunity. The protective efficacy of $CD8^+/CD4^-$ T-cells[2–4] is consistent with this view. However, additional factors might account for the potency of J774-hsp65 cells as immunogen. For example, tumour antigen is co-expressed and evokes a cytolytic T-cell response that is protective against the parent tumour.[5] Hence the efficient lysis of J774-hsp65 cells by combined anti-tumour and anti-mycobacterial responses might be important to maintain a proper balance between endogenous and exogenous antigen-presentation pathways. Normal antigen-presenting cells such as macrophages may not be so readily lysed.

As a first step in exploring what features are needed *in vivo* by the antigen-presenting cells we have transfected murine bone marrow haematopoietic stem cells with a retroviral vector carrying the mycobacterial gene and asked if these cells are capable of (a) repopulating mouse tissues *in vivo* (b) conferring protection against tuberculosis to recipient mice. Numerous studies have shown that transfected murine bone marrow stem cells can generate persistent populations of differentiated cells expressing transgenes in various tissues for many months.[6–9] For our purpose an advantage of these cells compared with tumour cells is that they are essentially normal and they are capable of differentiating into diverse antigen-presenting cell types.

Received 30 January 1995; revised 20 June 1995; accepted 28 June 1995.

Correspondence: Professor C. L. Silva, Dept of Parasitology, Microbiology and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, 14.049–900, Ribeirao Preto, SP, Brazil.

## MATERIALS AND METHODS

*Mice*

Young adult (7–8 week) female BALB/c mice were obtained from the vivarium of the School of Medicine of Ribeirão Preto, University of São Paulo, Brazil, and maintained under standard laboratory conditions.

*Cell lines*

NIH 3T3 cells and Psi-CRE cells were maintained in

Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO) supplemented with 10% v/v newborn calf serum. WEHI-3 (ATCC TIB68; American Type Culture Collection, Rockville, MD) cells were maintained in RPMI-1640 medium containing streptomycin (100 μg/ml, penicillin (1000 U/ml), L-glutamine (1 mM) and 12% v/v fetal calf serum (RPMI-C).[10]

*Infective recombinant retrovirus*

The *M. leprae* hsp65 gene was present in a 3·6 Kb genomic fragment ligated into the *Bam*H1 site of pZIPNeoSV(X) as previously described.[10] The retroviral packaging cell line psi-CRE was transduced with this DNA (pZIPML65) by calcium-phosphate precipitation and neomycin-resistant cells were selected with G418 (0·3 μg/ml; Sigma, St Louis, MO) as previously described.[10] Virus production by resistant clones was assayed by titrating 24-hr culture supernatants in the presence of polybrene (4 μg/ml) for ability to confer resistance to G418 upon 3T3 cells. Supernatant virus was concentrated by centrifugation (30 000 ***g***, 12 hr) and viral RNA was probed with $^{32}$P-hsp65 DNA to confirm the presence of the gene.[10] Infectious vector without the mycobacterial gene was made in parallel.

*Infection of bone marrow cells with recombinant retrovirus*

Mice were given a single i.v. dose of 5-fluorouracil (140–150 mg/kg bodyweight; Sigma) 4 days before being killed. This increases the yield of primitive stem cells from bone marrow[11,12] and increases the proportion of stem cells that are replicating from around 10 to almost 100%.[13,14] Since retroviral integration into the host cell genome requires cell replication this provides a source of stem cells with an increased susceptibility to stable transfection.[11,15]

Bone marrow donors were killed by cervical dislocation and cells were flushed from femurs and tibias, washed twice and resuspended in DMEM supplemented with streptomycin (100 μg/ml, penicillin (1000 U/ml), L-glutamine (1 mM), HEPES (10 mM), 12% v/v fetal calf serum and 10% v/v WEHI-3-conditioned medium. Bone marrow cells were infected by incubating $2 \times 10^6$ cells in 10-cm tissue culture dishes with $5 \times 10^6$ viral colony-forming units (CFU)/ml and polybrene (4 μg/ml) overnight at 37° in a humidified 5% $CO_2$ incubator. The cells were then washed twice to remove polybrene and excess retrovirus and incubated in supplemented DMEM for 24 hr. The bone marrow cells were alternatively infected by cocultivation with psi-CRE virus-producer cells. Thus a 24-hr monolayer that was established from $5 \times 10^5$ virus-producer cells in a 10-cm dish was seeded with $10^6$ bone marrow cells and cocultivated for 24 hr in DMEM containing polybrene (2 μg/ml), 20% v/v fetal calf serum and 20% v/v WEHI-3 conditioned medium. Non-adherent cells were then harvested, washed and resuspended in medium without polybrene. In the experiments described here, the bone marrow cells were transfected using the former method, i.e. with concentrated virus. Infected cells were selected by incubation for 2 days in the presence of G418 (0·5 mg/ml) and finally washed and resuspended in HEPES-buffered Hanks' balanced salt solution (H-HBSS, pH 7·2). Viability, estimated by trypan blue exclusion, ranged from 20 to 60%. Bone marrow cells transfected with pZIPML65 (BMC-65) or with vector only (BMC-V) were made in parallel.

*Transplantation*

Recipient mice were housed in sterile cages and maintained on sterile food and acidified autoclaved water. Tetracycline was included in drinking water for 3 days before and 2 weeks after the animals were irradiated and injected with bone marrow cells. Irradiation was with 9·5 Gy (cobalt source) of total body exposure during 14 min. Irradiated animals were immediately injected with 0·2 ml H-HBSS containing $2 \times 10^6$ bone marrow cells into a lateral tail vein.

*Probing mouse tissues for the presence and expression of the mycobacterial gene*

Peripheral blood cells were collected at 15 days and splenic lymphoid follicle cells at 30 days after bone marrow transplantation. High molecular weight DNA was prepared from the cells and probed using standard procedures.[16] Cell lysis was in sodium dodecyl sulphate (SDS) buffer containing proteinase K and DNA purification was with phenol : chloroform and ethanol precipitation. DNA was applied to nitrocellulose membranes either directly (slot blot apparatus, 10 μg/well) or after digestion with *Bgl*II restriction endonuclease and electrophoresis in agarose gel alongside molecular weight markers. Membranes were probed with either vector-specific DNA sequence (2·2 Kb *Xho*I fragment containing the neo gene of pZIPNeoSV(X) [neo probe]) or mycobacterium-specific sequence (3·6 Kb *Eco*RI *M. leprae* genomic fragment containing hsp65) that had been labelled for chemiluminescence detection (ECL; Amersham International, Amersham, UK).

Protein was extracted from peripheral blood with nonidet NP-40 buffer (0·5 ml, 30 min on ice) then after removal of cell debris by centrifugation (1000 ***g***, 10 min), lysate (20 μl) was slot-blotted onto nitrocellulose membrane, air-dried and non-specific protein-binding sites were blocked with 3% w/v gelatin in Tris-buffered saline at ambient temperature for 1 hr.[10] Membranes were probed by overnight incubation at ambient temperature with mAb CL44, which is specific for mycobacterial hsp65 (R.W. Stokes, unpublished), then rinsing and staining with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Sigma).

*Immunization of control mice with hsp65 protein*

Groups of mice were injected subcutaneously on day zero and day 7 with 100 μl of emulsion containing 25 μg of recombinant DNA-derived *M. leprae* hsp65 (rML65hsp[1]) in IFA, then i.v. at day 14 with 15 μg antigen in phosphate-buffered saline (PBS).

*Delayed-type hypersensitivity (DTH) tests*

Mice were injected with 5 μg of rML65hsp into the right footpad. Increases in footpad thickness relative to the contralateral footpad were measured with a micrometer guage after 24, 48 and 72 hr. Injection of diluent alone did not induce any increase in thickness at these times.

*Challenge infection with* Mycobacterium tuberculosis

*M. tuberculosis* H37Rv (ATCC) was grown in Dubos broth (Difco, Detroit, MI) supplemented with bovine serum albumin (Sigma) and tween 80 (Sigma), washed, stored at −70°, ultrasonicated and diluted for use in PBS containing 0·05% Tween 80 as previously described.[1] Viability was measured by plating 10-fold serial dilutions on Middlebrook 7H11 agar (Difco) and counting colony-forming units (CFU) after 21 days

**Table 1.** Optimization of transplantation

| No. of injected cells* | | | Nos of mice surviving/ reconstituted† |
|---|---|---|---|
| BMC-65 | BMC-V | BMC-N | |
| $9 \times 10^5$ | – | – | 1/10 |
| $6 \times 10^5$ | $3 \times 10^5$ | – | 0/6 |
| $3 \times 10^5$ | $6 \times 10^5$ | – | 5/6 |
| – | $9 \times 10^5$ | – | 6/6 |
| – | – | $9 \times 10^5$ | 9/10 |

* Lethally irradiated mice were immediately reconstituted i.v. with normal bone marrow cells (BMC-N) or with bone marrow cells that had been infected with pZIPNeoSV(X) vector (BMC-V) and/or with cells transfected with the vector containing the gene for *M. leprae* hsp65 (BMC-65).

† Mouse survival was scored at 30 days post-transplantation.

incubation at 37°. Thirty-six days after bone marrow transplantation, mice were challenged by intravenous injection of $5 \times 10^6$ CFU in 0·2 ml of PBS. After 3 weeks the mice were killed and the livers were removed and homogenized for CFU enumeration as above.

## RESULTS

### Mouse survival after bone marrow transplantation

When lethally irradiated mice were reconstituted by injection of about $10^6$ normal bone marrow cells (BMC-N) they almost all survived for at least 30 days (Table 1). This was also the case if the cells were infected with the retroviral vector, but if they were infected with vector carrying the mycobacterial gene then only one out of 10 survived. However, when the implanted cells comprized a mixture of only one-third BMC-65 cells and two-thirds BMC-V cells then survival was good (five out of six). Accordingly, subsequent tests of the effect of BMC-65 were done with mice reconstituted with such a mixture.

### Persistence and expression of the mycobacterial gene

When peripheral blood samples were taken 15 days after bone marrow reconstitution and probed for mycobacterial and vector DNA (Fig. 1a), 16 of 20 mice receiving BMC-65 plus BMC-V (1 : 2) showed the presence of the mycobacterial DNA in the blood cells (Fig. 1b). For two of the mice (Nos 13 and 16), blood samples did not react with the neo probe (Fig. 1c) despite reacting with the mycobacterial probe, suggesting sequence rearrangement/deletion preceeding clonal expansion.

This interpretation was supported when four of the mice showing circulating mycobacterial DNA were killed 30 days after reconstitution and spleen cell DNA was extracted and probed by Southern blotting after digestion with restriction endonuclease *Bgl*II and electrophoresis. This enzyme cuts once in the mycobacterial and once in the vector DNA of pZIPML65 to excise a 1·35 Kb fragment bearing sequences specific to both the mycobacterial and neo DNA probes (Fig. 1a). The fragment was detected in only two of four mice probed (Fig. 2, lanes 1 amd 4, panel A *cf.* panel B), the other 2 mice (lanes 2 and 3) showed mainly fragments of various sizes

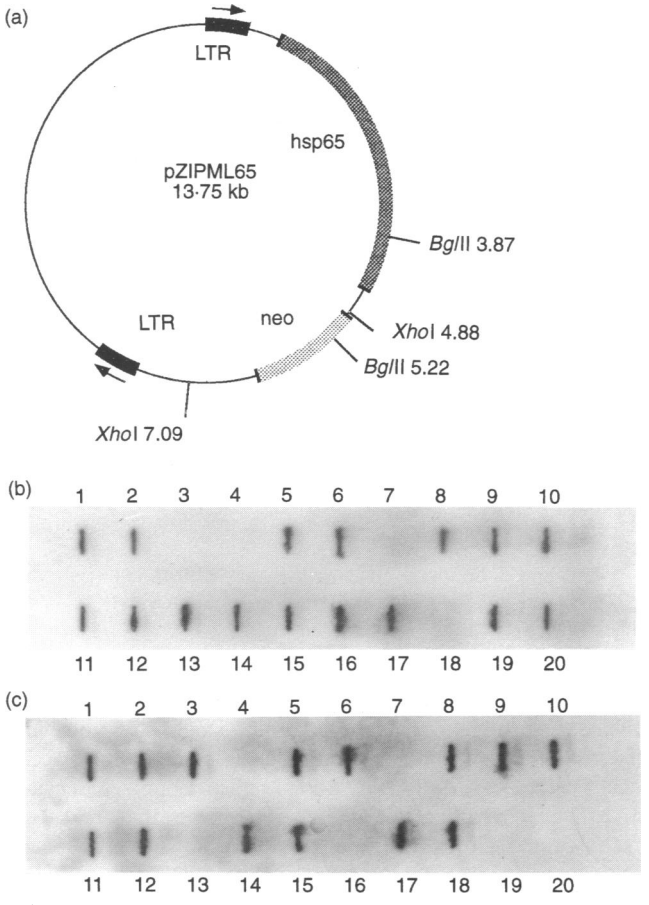


**Figure 1.** DNA probing of peripheral blood cells. Blood was collected from 20 individual mice 15 days after reconstitution with bone marrow cells transfected with pZIPML65 (BMC-65) and extracted DNA was blotted onto nitrocellulose and probed by hybridization with probes labelled for chemoluminescence detection. (a) Map of pZIPML65 showing relevant restriction enzyme sites. (b) Blot probed with *M. leprae* 3·6 Kb *Eco*RI fragment containing hsp65. (c) Blot probed with *Xho*I fragment of the vector (Neo probe).

hybridizing to either the mycobacterial probe (a) or the neo probe (b) but not to both. Integration of the vector into a variety of sites in the mouse genome was evidenced in Southern blots of spleen cells from from control mice that had received BMC-V only. Multiple bands were seen with the neo probe in patterns that varied from mouse to mouse (lanes 6 to 9, Fig. 2b).

The blood samples that had been taken from mice at 15 days post-reconstitution and probed for mycobacterial DNA were also probed with mAb CL44 for the presence of the protein, ML65hsp. Fourteen of the 20 mice showed the presence of the protein in peripheral blood (Fig. 3). Most of these were also positive for the mycobacterial DNA; however, two of them did not have the gene detectable in the blood (nos 4 and 18, Fig. 2 *cf.* Fig. 3), suggesting that the protein might be synthesized in non-circulating cells and/or in cells of erythroid in addition to nucleated lineages.

### Immunization and protection against challenge infection

The nine remaining mice that had shown both mycobacterial

 

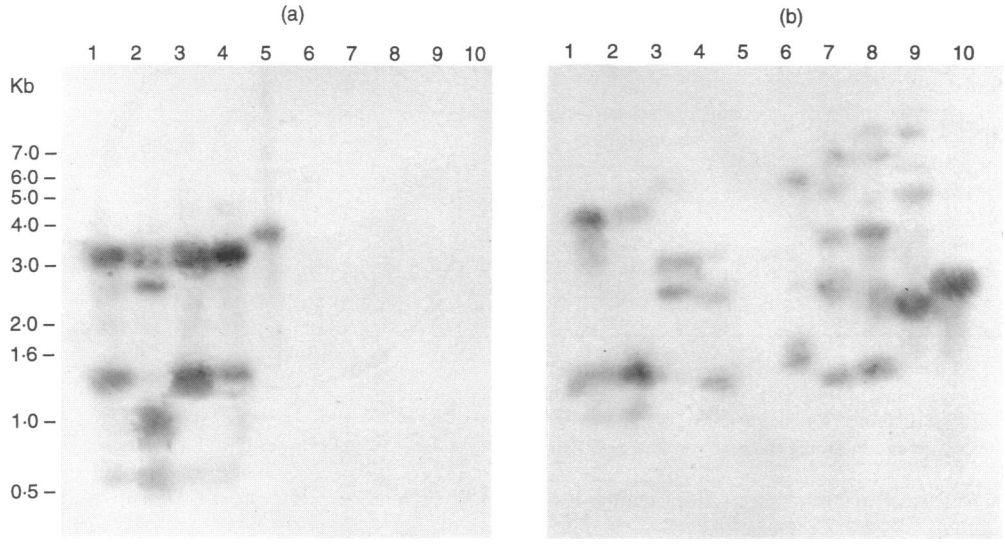


**Figure 2.** Southern DNA analysis of pZIPML65 integration in spleen cells. Lanes contained *Bgl*II-digested DNA from individual spleens collected 30 days after transplantation with BMC-65 (four mice, lanes 1–4) or BMC-V (four mice, lanes 6–9). Controls (lanes 5 and 10) contained either *M. leprae* 3·6 Kb *Eco*RI fragment containing hsp65 (lane 5) or 2·2 Kb *Xho*I fragment of vector (lane 10). Probing was with either the *M. leprae* (a) or the neo probe (b).

DNA and protein in the peripheral blood at 15 days after bone marrow reconstitution were tested at 30 days for specific delayed-type hypersensitivity to rML65hsp. Four were sensitive and five were not (Fig. 4). Control mice that had been reconstituted with BMC-V only and had shown vector-specific DNA in peripheral blood at 15 days were all DTH negative and controls that had been immunized by injection of the protein in incomplete Freund's adjuvant (IFA) were all DTH positive.

After they had been DTH-tested, mice were challenged by infecting them with *M. tuberculosis* and the numbers of live mycobacteria in the liver were determined 3 weeks later. There was no difference between normal mice, those that were reconstituted with BMC-V and those that had been reconstituted with BMC-65 but shown no DTH reaction (Fig. 5). In contrast, the BMC-65-reconstituted mice that had shown positive DTH reactions had about fivefold fewer bacteria in the liver than normal or BMC-V-reconstituted mice and this was highly significant ($P < 0{\cdot}001$). Mice immunized with rML65hsp showed a small but significant protective effect ($P < 0{\cdot}01$).

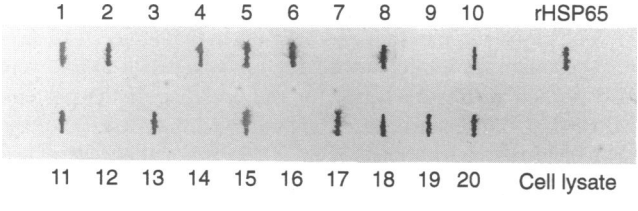


**Figure 3.** Hsp65 protein in peripheral blood of mice transplanted with BMC-65. Protein was extracted from blood samples of 20 individual mice 15 days after transplantation, blotted onto nitrocellulose and probed with mAb CL44. Recombinant DNA-derived protein (rML65hsp, 20 ng) and protein extracted from blood cells of normal mice were used as positive and negative controls. Bound antibody was detected with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin.

## DISCUSSION

The protective effect of hsp65 expressed from bone marrow cells against challenge infection with virulent *M. tuberculosis* establishes that co-expression of tumour antigen, or some other tumour cell property, was not essential for the similar effect that we found using J774 cells.[1] The magnitude of protection with transfected bone marrow cells (about fivefold fewer live bacteria in livers of experimental versus control mice at 3 weeks after challenge) was consistent with the previous effect with hsp65-expressing J774 cells (about 10-fold fewer at the same time point)[1] and greater than the effect of vaccinating with the protein in adjuvant. It is encouraging for vaccine development that in these models a single mycobacterial protein, when expressed in isolation as a transgene, can generate the same high level of protective immunity as the

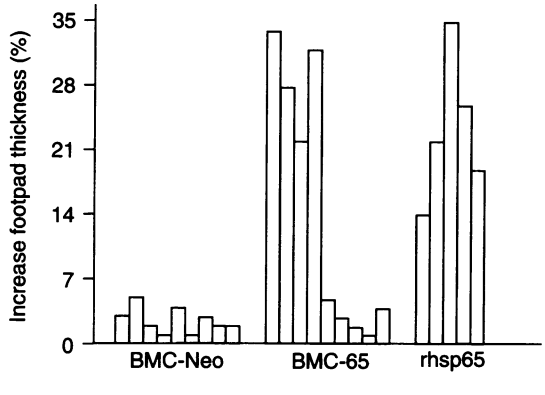


**Figure 4.** Delayed-type hypersensitivity in bone marrow recipients. Thirty days after transplantation of bone marrow cells transfected with pZIPML65hsp (BMC-65) or vector alone (BMC-V), or 30 days after commencing immunization with rML65hsp in IFA, mice received footpad injections of rML65hsp (5 $\mu$g) and local swelling at 48 hr was recorded.

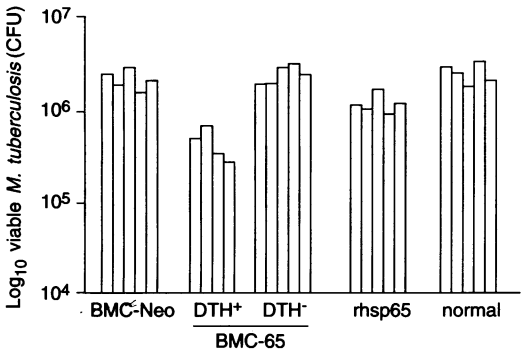


**Figure 5.** The protective immunizing effect of BMC-65 against tuberculosis. Thirty-six days after transplantation of bone marrow cells transfected with pZIPML65hsp (BMC-65) or vector alone (BMC-V), or 36 days after commencing immunization with rML65hsp in IFA, mice were challenged by i.v. injection with $5 \times 10^6$ CFU of *M. tuberculosis* H37Rv. After 3 weeks the numbers of CFU in livers were determined. Normal mice served as non-immunized controls. Results for individual mice are shown and those from BMC-65 transplanted mice that were DTH-positive and DTH-negative at 30 days are distinguished.

live BCG vaccine (unpublished). Non-specific protection, through systemic macrophage activation for example, seems not to be involved since there was no protection against challenge with *Listeria monocytogenes*.[1]

Our findings are consistent with extensive evidence that murine bone marrow cells expressing a foreign gene from a retroviral vector can repopulate mouse tissues.[6–9,11–13] Such observations have provided a promising approach to gene therapy of a number of genetic disorders since cells expressing foreign genes may proliferate and persist for at least 6 months.[7–9] Although we monitored foreign DNA persistence in spleens only for a month, some of the transfected cells are likely to have been stem cells capable of long-term proliferation and differentiation.[11] However, as measured by survival of recipient irradiated mice, bone marrow cells carrying the mycobacterial gene for hsp65 had a diminished capacity to reconstitute the animals when compared with marrow cells bearing the vector alone.

The reasons for the impaired reconstitutive ability of the cells expressing hsp65 have not been explored, but it might result from impaired growth or from immune recognition. When J774 tumour cells express the protein from this vector they have an inherent growth defect that manifests *in vivo* even in the absence of specific cellular immunity.[5] Bone marrow cells that express the mycobacterial gene might suffer a similar proliferative or functional impairment. The long-term persistence of cells expressing the protein is likely also to be limited by the developing immune response in the presence of an intact or reconstituted immune system. J774 tumour cells expressing the protein generate hsp65-specific cytotoxic T cells that lyse tumour cells or macrophages presenting the antigen.[2]

We have not rigorously established that the marrow cells expressing hsp65 multiply *in vivo*. However, it seems likely that any proliferative, functional or immunological deficits are partial or selective. When the survival of irradiated mice was ensured by the additional presence of sufficient numbers of normal (BMC-V) bone marrow cells, Southern blotting of hsp65-specific DNA in spleens at 30 days revealed DNA integration patterns that differed between mice. This is evidence of clonal expansion of cells *in vivo* following integration of the DNA into different sites in the host genome.[11] The frequent dissociation between mycobacterial and vector DNA sequences in the host genome indicates an instability which could be of concern for clinical applications such as gene therapy or DNA vaccination[17] but which was not sufficient to prevent expression in this experimental system. The presence of detectable amounts of mycobacterial protein in the circulation for at least 15 days is consistent with the presence of substantial numbers of functional cells. Taken together these findings suggest that a significant proportion of the cells were able to proliferate and produce hsp65 for several weeks *in vivo*. Whether the majority, and perhaps all, of these cells would eventually be eliminated by the ensuing immune response is a matter for conjecture.

Our finding that only about half of the mice that had the mycobacterial protein in the circulation at 15 days had acquired DTH by 30 days was unexpected. Mycobacterial hsp65 is a powerful immunogen with multiple T- and B-cell epitopes[18–20] and cellular and humoral immune responses are readily acquired in BALB/c mice in response to injection with the protein in IFA or with J774 cells expressing the protein (ref. 10 and R. W. Stokes and P. Jenner, unpublished data). Antibody responses were not measured in the present experiments and so we can not distinguish whether the DTH-negative mice had not responded at all or had selectively failed to mount T-cell-dependent responses. Further experiments will be needed to assess if such mice had become tolerant to the antigen and whether that can be a consequence of presentation by cell clones with a special phenotype. Bone marrow transplantation is an approach which may lend itself to *in vitro* selection of the cell lineage and phenotype used to express the transgene *in vivo*.[6,15]

## ACKNOWLEDGMENT

This study was supported in Brazil by Fundação de Amparo à Pesquisa do Estado de São Paolo (FAPESP), Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) and Financiadora de Estudos e Projetos (FINEP).

## REFERENCES

1. Silva C.L. & Lowrie D.B. (1994) A single mycobacterial protein (hsp65) expressed by a transgenic antigen-presenting cell vaccinates mice against tuberculosis. *Immunology* **82**, 244.
2. Silva C.L., Silva M.F., Pietro R.C.L.R. & Lowrie D.B. (1994) Protection against tuberculosis by passive transfer with T-cell clones recognizing mycobacterial heat-shock protein 65. *Immunology* **83**, 341.
3. Guleria I., Mukherjee R. & Kaufmann S.H.E. (1993) In vivo depletion of CD4 and CD8 T-lymphocytes impairs Mycobacterium-w vaccine-induced protection against M. tuberculosis in mice. *Med Microbiol Immunol* **182**, 129.
4. Flynn J.L., Goldstein M.M., Triebold K.J., 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.
5. Lukacs K.V., Lowrie D.B., Stokes R.W. & Colston M.J. (1993) Tumor cells transfected with a bacterial heat-shock gene lose

tumorigenicity and induce protection against tumors. *J Exp Med* **178**, 343.

6. Dzierzak E.A., Papayannopoulou T. & Mulligan G. (1988) Lineage-specific expression of a human β-globin gene in murine bone marrow transplant recipients reconstituted with retrovirus-transduced stem cells. *Nature* **331**, 35.
7. Osborne W.R.A., Hock R.A., Kaleko M. & Miller A.D. (1990) Long-term expression of human adenosine deaminase in mice after transplantation of bone marrow infected with amphotropic retroviral vectors. *Hum Gene Ther* **1**, 31.
8. Ohashi T., Boggs S., Robbins P. *et al.* (1992) Efficient transfer and sustained high expression of the human glucocerebrosidase gene in mice and their functional macrophages following transplantation of bone marrow transduced by a retroviral vector. *Proc Natl Acad Sci USA* **89**, 11332.
9. Krall W.J., Challita P.M., Perlmutter L.S., Skelton D.C. & Kohn D.B. (1994) Cells expressing human glucocerebrosidase from a retroviral vector repopulate macrophages and central nervous system microglia after murine bone marrow transplantation. *Blood* **83**, 2737.
10. Silva C.L., Palacios A., Colston M.J. & Lowrie D.B. (1992) *Mycobacterium leprae* 65hsp antigen expressed from a retroviral vector in a macrophage cell line is presented to T cells in association with MHC class II in addition to MHC class I. *Microb Pathog* **12**, 27.
11. Lemischka I.R., Raulet D.H. & Mulligan R.C. (1986) Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell 45*, 917.
12. Spain L.M. & Mulligan R.C. (1992) Purification and characterization of retrovirally transduced hematopoietic stem cells. *Proc Natl Acad Sci USA* **89**, 3790.
13. Ross E.A.M., Anderson N. & Micklem H.S. (1982) Serial depletion and regeneration of the murine hematopoietic system. Implications for hematopoietic organization and the study of cellular aging. *J Exp Med* **155**, 432.
14. Van Zant G. (1984) Studies of hematopoietic stem cells spared by 5-fluorouracil. *J Exp Med* **159**, 679.
15. Szilvassy S.J. & Cory S. (1994) Efficient retroviral gene transfer to purified long-term repopulating hematopoietic stem cells. *Blood* **84**, 74.
16. Sambrook J., Fritsch E.F. & Maniatis T. (1989) *Molecular cloning, A laboratory manual*, 2nd edition. Cold Spring Harbor Lab, New York.
17. Robertson J.S. (1994) Safety considerations for nucleic acid vaccines. *Vaccine* **12**, 1526.
18. Anderson D.C., Barry M.E. & T.M. Buchanan (1988) Exact definition of species-specific and Cross-reactive epitopes of the 65-kilodalton protein of *Mycobacterium leprae* using synthetic peptides. *J Immunol* **141**, 607.
19. Brett S.J., Lamb J.R., Cox J.H., Rothbard J.B., Mehlert A. & Ivanyi J. (1989) Differential pattern of T cell recognition of the 65-kDa mycobacterial antigen following immunization with the whole protein or peptides. *Eur J Immunol* **19**, 1303.
20. Thole J.E.R. & Van der Zee R. (1990) The 65 kD antigen: molecular studies on a ubiquitous antigen. In: *Molecular Biology of the Mycobacteria* (ed J. McFadden), p. 37. Academic Press, London.