In vivo study on dual-signal hypothesis and its correlation to immune response using mycobacterial antigen

Aravindhan Vivekanandhan* and Sulochana Das,†**

*Department of Biochemistry and Molecular Biology, University of Madras, Guindy, Chennai 600 025, India

**Department of Immunology, Tuberculosis Research Centre, Chetput, Chennai 600 031, India

Antigens have the innate ability to trigger both mitosis and apoptosis simultaneously (dual-signal hypothesis). The fate of the cell depends on the dosage and duration of the trigger. In the present study, dual-signal hypothesis was tested in a murine model using a mycobacterial antigen (purified protein derivative–PPD). As a positive control, *E. coli*-lipopolysaccharide (LPS) was used. The results were correlated with the humoral immune response. Our results showed that PPD had induced apoptosis in thymus only (after 7 days), while LPS had induced apoptosis in both thymus and bone marrow (after 18 h). Further, the plasma from PPD-treated animals had higher levels of *Mycobacterium tuberculosis*-specific antibodies and also recognized a few *M. tuberculosis*-specific antigens on immunoblot. To conclude, PPD has induced thymic apoptosis and has also elicited a potent antibody response.

CELL division and cell death are coupled phenomena. Mitogens/antigens have the innate ability to trigger both proliferation and apoptosis simultaneously (dual-signal hypothesis), which is very much dependent upon the dosage and duration of the trigger and on various *in vivo* conditions[1–7]. The apoptotic programme is not executed due to the presence of survival factors and once these factors are exhausted, proliferating cells undergo apoptosis[8]. During an immune response, lymphocytes which are specific for a particular antigen are activated and undergo proliferation (clonal selection). These activated lymphocytes bring about the elimination of the pathogen, offering protection to the body[9]. Peripheral deletion of these activated lymphocytes in the secondary lymphoid organs (spleen and lymph nodes) curtails the immune response[10].

Several bacteria and their antigens have already been shown to induce apoptosis in the immune system[11]. *Mycobacterium tuberculosis* and its antigen, purified protein derivative (PPD), have been shown to induce apoptosis in monocytes/macrophages under *in vitro* conditions[3,12]. *M. tuberculosis*-induced apoptosis of the infected macrophages reduces the viability of the bacilli and may offer protection against tuberculosis[13]. On the other hand, necrosis of the infected macrophages may exacerbate the disease by facilitating bacterial dissemination. *M. tuberculosis*-induced proliferation of T lymphocytes helps in eliminating the bacilli[6,7,9,10]. The end result of *M. tuberculosis*-induced proliferation and apoptosis is seen as the immune response mounted against the bacterium. Even though *M. tuberculosis* elicits both humoral immunity (HI) and cell-mediated immunity (CMI) under *in vivo* conditions, the exact role played by these two components in curtailing the disease is not clearly known[9,10].

In the present study, dual-signal hypothesis was tested under *in vivo* conditions in a murine model using PPD and lipopolysaccharide (LPS) and was correlated to the immune response. Activation-induced apoptosis was estimated using DNA fragmentation assay and flow cytometric analysis. Activation-induced proliferation was studied using *in vivo* proliferation assay. The humoral immune response was estimated using ELISA and IMMUNOBLOT. The link between proliferation, apoptosis and immune response may provide new insights into the pathogenesis of tuberculosis. Our study addresses PPD-induced proliferation, apoptosis and immune response simultaneously under *in vivo* conditions.

Animals (BALB/c mice; 4–6 weeks old; 25 g from the internal animal breeding house, TRC, Chennai) were maintained under controlled condition. The sample size was 4 to 5 animals for each group. The animals were injected intraperitoneally with LPS (100 µg/animal; *E. coli*-LPS, Sigma Chemicals Co., St. Louis, MO) or PPD (100 µg/animal; PPD, Central Veterinary Lab, Weybridge, UK) and were sacrificed after 18 h or 7 days respectively. The dosage and duration for LPS were determined based on cited literature[1]. Lymphoid organs (thymus, bone marrow and spleen) and blood were collected under aseptic conditions. The mononuclear cells were separated by Ficol-Hypaque density gradient centrifugation[14]. The number of viable cells was determined by trypan blue staining.

Antigen/mitogen-induced DNA fragmentation was studied as previously described, with certain modifications[2]. Briefly, the cell pellet was suspended in 500 µl of lysis buffer (1 M Tris, 1 M NaCl and 0.5 M EDTA) containing proteinase K (Amersham Corporation, Arlington heights, IL) and SDS. After overnight incubation, DNA was precipitated and checked in 1.5% agarose gel with standard molecular weight marker. In order to correlate apoptosis with proliferation, *in vivo* proliferation assay was performed as previously described with certain modifications[15,16]. In short, the animals were injected with 500 µl of ^{3}H thymidine (50 µCi, BARC, Mumbai) 1 h before sacrifice and the DNA was extracted. The concentration of DNA was quantified with a spectrophotometer using the formula:

$$\text{Concentration of DNA} = \frac{\text{O.D.}_{260\text{ nm}} \times 50 \times X}{1000},$$

where X is the dilution factor.

†For correspondence. (e-mail: trcicmr@md3.vsnl.net.in)

The amount of radioactivity was quantified using liquid scintillation counter (WALLAC 1490). The proliferation index (cpm/μg of DNA) was calculated for all the samples.

Quantification of apoptosis was done using flow cytometry, as mentioned previously[17]. Briefly, 1×10^6 cells were fixed in 80% alcohol and were stored at 4°C. Before acquisition, the cells were stained with 500 μl of flurochrome solution (200 μg RNase A [Amersham Corporation, Arlington heights, IL] and 100 μg PI [Sigma Chemicals Co., St. Louis, MO] in Ca^{2+} and Mg^{2+}-free PBS) and were incubated at 37°C for 1 h. Acquisition was done with FACsort (BECTON DICKINSON, CA) and analysis was done using the software, Cell Quest. Initial identification of cells was made using FSC/SSC plots. The debris was excluded based on its low FSC and SSC signals. The apoptotic and normal cells were gated and analysed for their fluorescence property.

The antibody titre in the plasma of control and treated animals was determined by ELISA[18]. The ELISA plates were coated with mycobacterial antigens (Culture filtrate antigen, CFA–5 μg/ml and PPD–5 μg/ml) and were blocked with 5% BSA. The source of the primary antibody was the plasma (1 : 20 dilution). The secondary antibodies were peroxidase conjugated anti-mouse isotypes (dilution IgG – 1 : 1000; IgM – 1 : 500). The substrates were orthophenylenediamine (OPD; Sigma Chemicals Co., St. Louis, MO) and H_2O_2. The antibody titre was expressed as optical density (O.D.), which was estimated in an ELISA reader (SPECTRA MAX 250) at 490 nm.

The antigen-binding pattern of the plasma of control and treated animals was studied using immunoblot[18]. CFA (200 μg/cm) was resolved electrophoretically, transferred to nitrocellulose paper, blocked and the blots were cut into strips. The strips were incubated overnight at 4°C with mouse plasma (1 : 20 dilution). The bands were visualized using peroxidase conjugated anti-mouse immunoglobulin, diaminobenzidine (DAB; Sigma Chemicals Co., St. Louis, MO) and H_2O_2. Standard molecular weight marker was run and developed using amido black staining.

Data were expressed as the mean ± SD. Statistical significance was determined using unpaired Student's *t*-test. Results were considered significant when $P < 0.05$.

In the present study both PPD and LPS-induced DNA fragmentation is in the thymus (Figure 1). Quantification of thymic apoptosis by flow cytometry revealed a basal level of 28% in control animals, which was significantly increased ($P < 0.05$) by the action of PPD and LPS to 60% and 77% respectively (Figures 2 and 3). Since an increase in apoptosis is accompanied by a decrease in proliferation, the proliferation index in thymus was quantified using *in vivo* proliferation assay. As expected, there was a decrease in the proliferation index in thymus of PPD-treated animals (Figure 4). The basal level of apoptosis and proliferation was much higher in the thymus when compared to other lymphoid organs, due to both positive and negative selection of the thymocytes that takes place in the thymus of young mice[10,19]. It has been estimated that only 5% of immature thymocytes that enter the thymus gland leave the thymus as mature T cells, while the remaining cells die by apoptosis[15]. There exists a delicate equilibrium between apoptosis and proliferation in the thymus gland which had been disturbed by PPD and LPS stimulation. Our results were in good accordance with a previous study, where Ozeki *et al.*[2] have reported murine thymic atrophy under *in vivo* conditions, after the administration of *M. tuberculosis* cord factor.

Interestingly, in the bone marrow only LPS brought about DNA fragmentation (Figure 1). The flow cytometric data showed 10% of basal level of apoptosis, which was significantly increased ($P < 0.05$) by the action of LPS to 51% (Figure 3). PPD triggered neither apoptosis nor proliferation in the bone marrow. The *in vivo* proliferation assay showed no difference in the proliferation index between the control and PPD-treated mice (Figure 4). The basal level of apoptosis seen in the bone marrow is due to positive and negative selection of immature B cells in the bone marrow[10]. The negative selection was increased by the action of LPS. No DNA fragmentation was detected in the spleen and blood of both LPS- and PPD-treated animals, indicating the unresponsiveness of mature leukocytes to undergo activation-induced apoptosis (Figure 1). Even though flow cytometric analysis and *in vivo* proliferation assay indicated a slight increase in apoptosis and decrease in proliferation index from the basal level in spleen and blood of treated animals, it was not statistically significant (Figures 3 and 4).

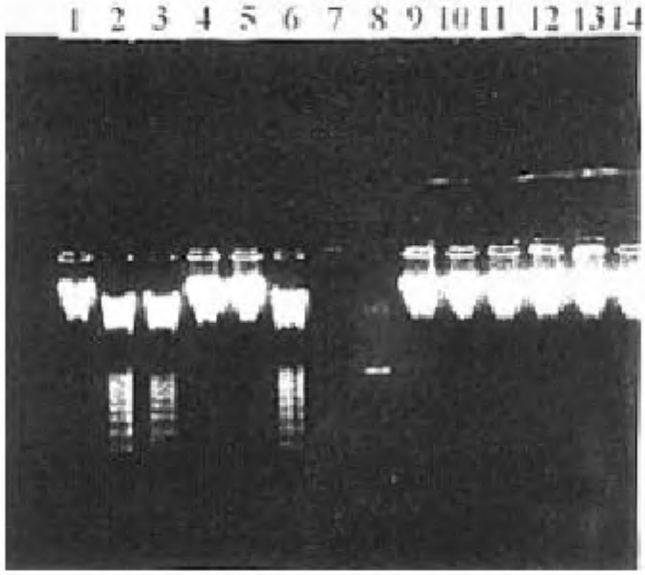

Figure 1. PPD- and LPS-induced DNA fragmentation in the lymphoid organs. Lanes 1–3, Thymus; lanes 4–6, bone marrow; lanes 9–11, spleen; lanes 12–14, PBMC. Lanes 1, 4, 9 and 12, control group; lanes 2, 5, 10 and 13, PPD group; lanes 3, 6, 11 and 14, LPS group. Lane 8, Molecular weight marker containing integer multiples of 123 bp fragments.

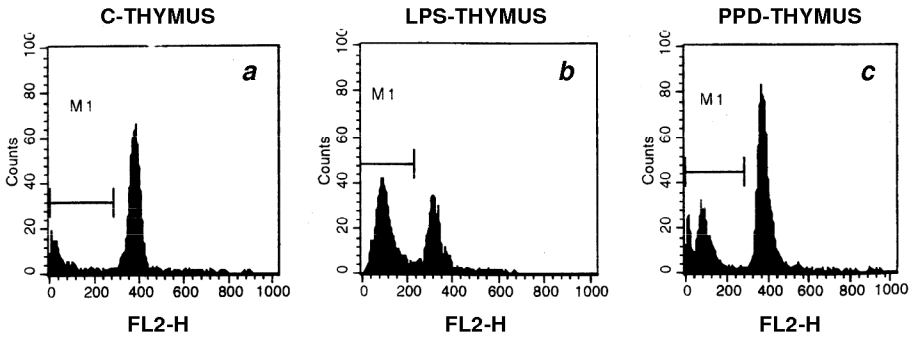

Figure 2. DNA histogram analysis of thymocytes. Fixed cells were stained with PI and were acquired by flow cytometry. Cells which show < 2 N DNA content are the apoptotic cells (M1 marker). *a*, Control group; *b*, LPS group, and *c*, PPD group.

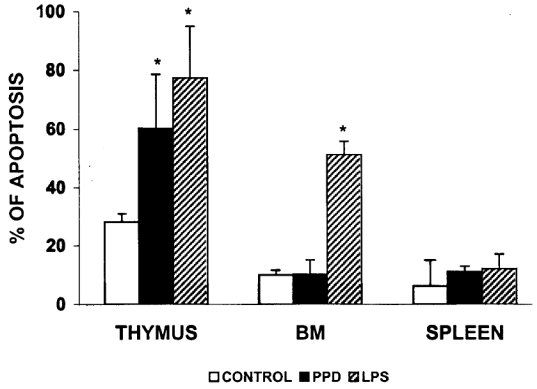

Figure 3. Estimation of apoptosis in the primary and secondary lymphoid organs by flow cytometry. Animals were treated with PPD (100 µg) or LPS (100 µg) and were sacrificed at different time points (PPD, 7 days; LPS, 18 h). There was a significant increase *($P < 0.05$) in the percentage of apoptosis in the thymus of both PPD and LPS groups and in the bone marrow of the LPS group.

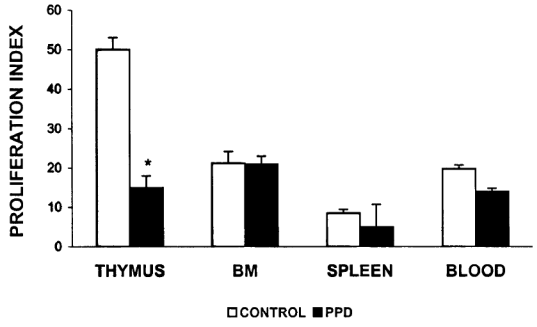

Figure 4. Estimation of proliferation by *in vivo* proliferation assay. Animals were injected with ^{3}H thymidine and DNA was extracted. The proliferation index (cpm/µg) was estimated in the lymphoid organs and PBMC in control and PPD group. Thymus from the PPD groups showed a significant decrease *($P < 0.05$) in the proliferation index.

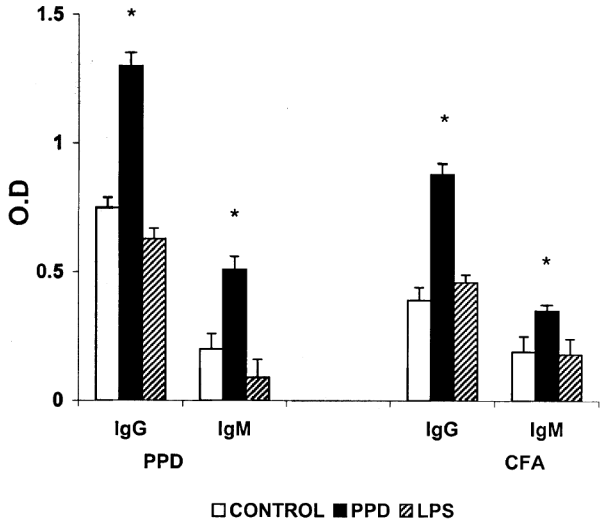

Figure 5. Isotype profile against *M. tuberculosis* antigens. PPD- and CFA-specific IgG and IgM were estimated by ELISA in the control, PPD and LPS groups. PPD-treated animals had significantly higher *($P < 0.05$) level of PPD- and CFA-specific isotypes (IgG and IgM).

In order to correlate the pathophysiological effect of PPD to the humoral immune response elicited by PPD, the isotype profile in plasma was studied using ELISA, against PPD and CFA antigens. There was a significant increase ($P < 0.05$) in PPD- and CFA-specific IgM and IgG levels in the plasma of PPD-treated animals (Figure 5). This indicated that PPD, apart from triggering apoptosis in the thymus, also elicits a potent humoral immune response against the *M. tuberculosis* antigens. The LPS group was also included in this experiment because LPS is a B cell mitogen and can bring about non-specific activation of polyclonal B cells[20,21]. Hence plasma from LPS-treated animals was tested for heterologous antigen recognition (CFA and PPD). No statistically significant difference in the antibody titre (IgG and IgM) against CFA and PPD was seen between the control and LPS groups (Figure 5). The antigen recognition pattern against CFA was studied using immunoblot. Immunoblot results showed minimal recognition of antigens by the control and PPD groups. However, PPD-treated animals recognized few *M. tuberculosis*-specific antigens in the low-

molecular weight region (results not shown). The immunoblot results match well with the ELISA results.

To conclude, an *in vivo* defect in T cell proliferation associated with enhanced level of T cell apoptosis was noted. T cell death by apoptosis may represent one of the important components of the ineffective immune response against *M. tuberculosis*-induced immunopathology in susceptible hosts[14,22]. Thus high concentration of *M. tuberculosis* antigens may incapacitate CMI in young susceptible mice by inducing thymic atropy.

1. Zhang, Y., Takahasi, K., Jiang, G., Kawai, M., Fukada, M. and Yokochi, T., *Infect. Immunol.*, 1993, **61**, 5044–5048.
2. Ozeki, Y., Kaneda, K., Fujiwara, N., Morimoto, M., Oka, S. and Yano, I., *Infect. Immunol.*, 1997, **65**, 1793–1799.
3. Hayashi, T., Catanzaro, A. and Rao, S. P., *Infect. Immunol.*, 1997, **65**, 5262–5271.
4. Jenkinson, E. J., Kingston, R., Smith, C. A., Williams, G. T. and Owen, J. T. T., *Eur. J. Immunol.*, 1989, **19**, 2175.
5. Hueber, A. and Evan, G. I., *Trends Genet.*, 1998, **14**, 364–367.
6. Anderson, P., Askgaard, D., Liungqvist, L., Bentson, M. W. and Heron, I., *Infect. Immunol.*, 1991, **59**, 1558–1563.
7. Esin, S., Butoni, G., Kallenius, G., Granes, H., Campa, M. and Svenson, S. B., *Clin. Exp. Immunol.*, 1996, **140**, 419.
8. Duke, R. C. and Cohen, J. J., *Lymphokine Res.*, 1986, **5**, 289.
9. Kaufmann, S. H. F., *Annu. Rev. Immunol.*, 1993, **11**, 129–163.
10. Duhe, R. C. and Cohen, J. J., *Annu. Rev. Immunol.*, 1992, **10**, 267–293.
11. Wang, S. D., Huang, K. J., Lin, Y. S. and Lei, H. Y., *Immunology.*, 1994, **152**, 5014.
12. Placido, R. *et al.*, *J. Pathol.*, 1997, **181**, 31–38.
13. Oddo, M., Reuno, T., Attinger, A., Bakker, T., MacDonald, H. and Meylan, P., *J. Immunol.*, 1998, **160**, 5448.
14. Das, G., Vohra, H., Saha, B., Agrewala, J. N. and Misra, G. C., *Clin. Exp. Immunol.*, 1999, **115**, 324.
15. Kornblum, H., Loughlin, S. E. and Leslie, F. M., *Dev. Brain Res.*, 1987, **31**, 45.
16. Egerton, M., Scollay, R. and Shortman, K., *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 2579–2582.
17. Noguchi, P. D., in *Current Protocol in Immunology* (eds Coligan, J. E. *et al.*), Library of Cataloging in Publication Data, USA, 1997, vol. 1.
18. Harlow, E. and David, L., in *Antibodies – A Laboratory Manual*, Cold Spring Harbor Lab.
19. MacDonald, H. R. and Kees, R. K., *Nature*, 1990, **343**, 642–644.
20. Morrison, D. C. and Ryan, J. L., *Adv. Immunol.*, 1979, **28**, 293–450.
21. Reed, N. D., Manning, J. K. and Rudback, J. A., *Infect. Dis., Suppl.*, 1973, **28** S70–S74.
22. Kremer, L., Estaquier, J., Wolowczuh, I., Biet, F., Ameisen, J. and Locht, C., *Infect. Immunol.*, 2000, **68**, 4264–4273.

ACKNOWLEDGEMENTS. We thank Dr P. R. Narayanan, Director, Tuberculosis Research Centre, (TRC), Chennai, for his continuous enthusiasm and support. We also thank Dr V. Kumarasami, Head, Department of Immunology and Dr Alamelu Raja, Deputy Director, TRC, Chennai, for their support and encouragement. We thank members of the animal house department for their help.

Received 18 October 2000; revised accepted 18 April 2001

Diversity and abundance of higher marine fungi on woody substrates along the west coast of India

K. Prasannarai*,† and K. R. Sridhar*,‡

*Department of Biosciences, Mangalore University, Mangalagangotri, Mangalore 574 199, India
†Present address: Department of Botany, St. Philomena College, Puttur 574 202, India

The diversity of marine fungi on intertidal wood collected from 13 locations in the west coast of India (beaches, islands and harbour locations) was assessed. Out of 3327 wood samples scanned, 72% possess sporulating marine fungi. Altogether 88 species belonging to 47 genera were encountered. *Torpedospora radiata* was the only fungus common to all locations. Five fungi were more frequent (> 10%): *Antennospora quadricornuta*, *Clavatospora bulbosa*, *Crinigera maritima*, *Periconia prolifica* and *Torpedospora radiata*. Twenty-four species were rare (0.04–0.5%); notable rare species were: *Amylocarpus encephaloides*, *Arenariomyces majusculus*, *Calathella mangrovei*, *Carbosphaerella leptosphaerioides*, *Cirrenalia pseudomacrocephala*, *Crinigera maritima*, *Dryosphaera navigans*, *Dryosphaera tropicalis*, *Nimbospora bipolaris* and *Nimbospora effusa*. Twenty-two species reported in the present study are new records for the Indian Peninsula. *A. encephaloides* was recorded from the tropical location, so also *A. majusculus* and *C. pseudomacrocephala* from the Indian Ocean. The species richness and diversity was highest in islands than in beaches and harbour locations. It has been predicted that islands adjacent to the west coast of India provide critical habitat for marine fungi.

MARINE fungi are the important intermediaries of energy flow from detritus to higher trophic levels in the marine ecosystems[1]. They require sea water for the completion of their life cycle. More than 500 species of marine fungi have been described[2]. Higher marine fungi constitute Ascomycotina, Basidiomycotina and Deuteromycotina. Majority of them being ascomycetes, their spores show adaptation to the marine ecosystem in the production of appendages, which facilitate buoyancy in water, entrapment and adherence to substrates. Marine filamentous fungi have been reported on a variety of detritus: decaying wood, leaves, seaweeds, seagrasses, calcareous and chitinous substrates. Studies on marine fungi were initiated at the temperate parts of the world. Subsequently, tropical locations were the centres of interest to understand the abundance and diversity[2,3]. Tropical regions of Atlantic and Pacific Oceans were investigated more intensively than the Indian Ocean[3]. A few quantitative studies

‡For correspondence. (e-mail: sirikr@yahoo.com)