Gamma-interferon bioassay for detection of bovine tuberculosis in cattle: Kinetics of production and dose response in whole blood culture*

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Stimulation with Mycobacterium bovis PPD sensitised lymphocytes (whole blood or peripheral blood lymphocytes) results in release of gamma-interferon that can be detected by simple bioassay. The optimum concentration of bovine PPD was 20 µg/ml and the optimum incubation period was 24 hr for maximum production of gamma-interferon in whole blood culture (128 units/ml) and peripheral blood culture (64 units/ml).

The accurate diagnosis of infected animal remains a major problem for eradication of bovine tuberculosis. The serological tests are usually not much sensitive and are highly unspecific due to antigenic cross-reactivity between mycobacteria often encountered by all mammalian species. The standard test for detection of tuberculosis in various eradication programmes has been single intradermal tuberculin test. This test suffers from both specificity and sensitivity1,2 and also alters the subsequent immune status of the individual tested3. The lymphocyte transformation test has been used in human and cattle to detect cellular reactivity to bovine PPD antigens4. Gamma-interferon is a cytokine produced by CD4+ and CD8+ T lymphocytes in response to activation by specific antigen or mitogen. Apart from its antiviral properties gamma-interferon is a potent modulator of a wide variety of immune responses. The release of gamma-interferon by T cells in response to specific antigen has been a useful method for measuring cellular responses in bovines and has been used as in vitro cellular bioassay for bovine tuberculosis5.

In present communication we have examined gamma-interferon assay production in whole blood culture. The kinetics of production of gamma-interferon and dose response curve of antigen (bovine PPD) has been established. The production of gamma-interferon in whole blood culture has been compared with that in peripheral blood lymphocyte culture.

Sensitization of animals—Three calves were sensitised by two subcutaneous injections of 10 mg of killed Mycobacterium bovis AN5 in 1 ml of saline.

Preparation of peripheral blood lymphocytes (PBL)—Peripheral blood lymphocytes were separated from peripheral blood by method of Boyum6. Briefly, the blood was collected in heparinized flasks. The blood was then centrifuged at 1000g for 30 min, the buffy coat removed, diluted in phosphate buffered saline (PBS, Ca2+ and Mg2+ free) and overlaid on to Histopaque (d=1.077g/ml) in the ratio of 3:1 in centrifuge tube. After centrifugation at 800g for 30 min the interphase layer was collected and washed twice (400g, 10 min) in PBS. Cell viability and concentration were determined by trypan blue dye exclusion method (0.1%).

Production of gamma-interferon—Whole blood culture—The procedure for whole blood culture as described by Rothel et al.7 was adopted. Briefly, heparinized blood collected from each animal was dispensed in 1.5ml in 24-well tissue culture plates and 100 µl of bovine PPD (at a final concentration of 5, 10, 20, 50 or 100 µg/ml) was added. The whole blood cultures were incubated for 4, 8, 16, 24, 40 or 48 hr according to the requirements of the experiment. After incubation, plasma was removed from above the sediment red cells. The plasma supernatants were stored at -20°C before assay.

Peripheral blood lymphocyte culture—The procedure described by Wood et al.8 was adopted. Briefly isolated lymphocytes were cultured in 24 well...
trays (10⁶ cells/ml) in 1ml per well of GM of RPMI-1640. Different dilutions of bovine PPD were added to the final concentration of 5, 10, 20, 50 or 100 μg/ml. The cultures were incubated at 37°C in 5% CO₂ tension for different hours (4, 8, 16, 24, 40 or 48 hr) according to the need of the experiment. After incubation the supernatant was collected and stored at −20°C before assay.

Bioassay for bovine gamma-interferon—MDBK cells (10⁴) were added to each well of 96-well tray and cultured at 37°C in 5% CO₂ tension for 3 days in DMEM medium containing 10% FCS. When the cell monolayer reached confluence, the medium was removed by suction and replaced with 100 μl of warm maintenance medium (DMEM with 3% FCS only). The 100 μl of samples of gamma-interferon supernatant were added to the top wells and serial 2 fold dilutions were prepared down the tray leaving one row for virus control and one row for the cell control. After 24 hr of incubation medium was removed and replaced with 100 μl (approx. 500 TCID₅₀/100 μl) of BHV-1 (Bovine herpes virus-1) in all the wells except the healthy cell controls in which only 100 μl of MM was added. After a further 48 hr incubation, the cells were fixed and stained with 1% crystal violet in 10% formalin saline. The gamma-interferon titre was expressed as the reciprocal of the highest dilution showing 50% cytopathic effect (CPE) as described by Wood et al.

Effect of concentration of bovine PPD on gamma-interferon production (Table1)—Effect of various concentration of bovine PPD was determined on in vitro production of gamma-interferon in two kinds of culture system. In this all the culture were incubated at 37°C for 24 hr. In whole blood culture system the maximum gamma-interferon activity was found at a concentration of 20 μg/ml of PPD with incubation period of 24 hr. This activity was fairly maintained at 50 and 100 μg/ml concentration. In peripheral blood lymphocyte culture the maximum gamma-interferon activity was found at a concentration of 20 μg/ml of PPD with peak activity of 64 units. This activity was fairly maintained up to 50 μg/ml concentration of PPD, while at 100 μg/ml concentration the activity was reduced. The unsensitised control animal did not show any significant gamma-interferon activity both in whole blood culture and peripheral blood culture.

Kinetics of production of gamma-interferon (Table2)—Kinetics of gamma-interferon production was determined in two kinds of culture systems. In this study the concentration of PPD was kept at 20 μg/ml in all the culture systems. In whole blood culture, significant gamma-interferon activity was detected as early as 8 hr of incubation. In peripheral blood culture significant gamma-interferon activity was detected as early as 16 hr of incubation, with peak level reaching at 24 hr.

Bovine tuberculosis remained a great public interest due to its economic losses and public health importance. The diagnosis of bovine tuberculosis has been facing a problem for many years. The standard test for detection of bovine tuberculosis is the tuberculin test that lacks the specificity and sensitivity. The lack of detectable antibody response in majority of the infected cattle that had good cellular response with mycobacterial infection has been reported. The predominant immune response to M. bovis infection in cattle is cellular than humoral in nature. The release of gamma-interferon by T cells in response to specific antigen has been a useful method for measuring cellular response to bovine tuberculosis, which correlates well with lymphocyte proliferation test. Amongst the three tests, such as

Table 1—Effect of various concentration of antigen (PPD) on gamma-interferon production in whole blood culture and peripheral blood lymphocyte culture

<table>
<thead>
<tr>
<th>No. &amp; status of animal</th>
<th>Titre of gamma-interferon in bioassay (units/0.1ml)*</th>
<th>Concentration of PPD (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Sensitised with M. bovis</td>
<td>0     8   32  64  64  32</td>
<td>0  5 10 20 50 100</td>
</tr>
<tr>
<td>2. do</td>
<td>0     16  32  128 64  64</td>
<td></td>
</tr>
<tr>
<td>3. do</td>
<td>2     8   32  64  64  32</td>
<td></td>
</tr>
<tr>
<td>4. Unsensitised</td>
<td>0     2   0   2  0  0</td>
<td></td>
</tr>
<tr>
<td>Peripheral blood lymphocyte culture</td>
<td>1. Sensitised with M. bovis</td>
<td>2  8  16 32 32 16</td>
</tr>
<tr>
<td>2. do</td>
<td>0     16  16 64 32 16</td>
<td></td>
</tr>
<tr>
<td>3. do</td>
<td>0     8   16 32 32 16</td>
<td></td>
</tr>
<tr>
<td>4. Unsensitised</td>
<td>0     2   4  0  0  0</td>
<td></td>
</tr>
</tbody>
</table>

*The gamma-interferon titre is expressed as the reciprocal of the highest dilution showing 50% cytopathic effect (CPE).
gamma-interferon assay, ELISA and SID the gamma-interferon assay showed highest sensitivity and specificity. Before development of such assay it is essential to standardise the production kinetics, dose response etc.

In contrast to Wood et al., who used monoclonal based ELISA, in the present study a virus inhibition assay has been used for detection of gamma-interferon in plasma supernatant of whole blood culture. BHV-1 was taken as the challenge virus, which is inhibited by only gamma type of interferon thus making the assay system specific for gamma-interferon. Using the assay system gamma-interferon activity was detected in supernatant of whole blood culture and PBL culture. The finding that optimum concentration of PPD is 20 μg/ml is in agreement with the results of Wood et al. However, in PBL culture their results showed highest gamma-interferon activity at concentration of 50-100 μg/ml. Rothel et al. concluded that the concentration of antigen (PPD) in blood culture had little effect on gamma-interferon production if used 20 μg/ml or greater. The reason for this may be that at 20 μg/ml PPD concentration T cell receptors become saturated with antigen molecules, hence, become unresponsive to further increase in antigen concentration. Effect of incubation period on gamma-interferon production was also determined in the present study. Whole blood culture significant gamma-interferon activity was detected as early as 8 hr of incubation with peak activity reaching at 24 hr. These results are in accordance with the findings of Wood et al. who have found that release of gamma-interferon was maximal by 24 hr using whole blood culture. The higher titres of gamma-interferon with whole blood culture may be attributed to the presence of optimum number of APC (antigen presenting cells) required for production of gamma-interferon from stimulated T cells. However, Rothel et al. have shown that peak level of gamma-interferon reaches at 16 hr and does not alter much between 16 and 32 hr. Wood et al. have shown that maximum gamma-interferon is released after 48 hr of incubation. The results clearly indicate that M. bovis stimulates sensitised T cells for the synthesis and release of gamma-interferon, the marker of CMIR, which can be detected by simple bioassay.

References