In Vitro Stimulation of Bovine Peripheral Blood Lymphocytes: Comparison of Round- and Flat-Bottom Microtiter Plates for Detection of Tuberculin Hypersensitivity

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Lymphocytes from *Mycobacterium bovis*-sensitized and normal cattle were cultured in round- and/or flat-bottom microtiter plates and stimulated with *M. bovis* purified protein derivative (PPD) tuberculin. Blastogenic responses of lymphocytes from *M. bovis*-sensitized cattle to PPD cultured in round-bottom plates were significantly greater than those of lymphocytes cultured in flat-bottom microtiter plates. Normal lymphocytes of nonsensitized cattle were not stimulated by PPD in either round- or flat-bottom microtiter plates. Kinetics of lymphocyte responses in round-bottom plates are presented.

Miniaturization of lymphocyte culture techniques has been a major advance in immunology in recent years (9). The use of microtiter culture plates and automatic cell harvesters has greatly increased the practicality and reproducibility of lymphocyte transformation tests (8). Lymphocyte stimulation is widely used to measure (i) histocompatibility by mixed leukocyte culture (16); (ii) immune competence by stimulation of lymphocytes with phyto mitogens (3); and (iii) exposure to infectious agents by stimulation of lymphocytes with specific antigens (2).

Previous studies have been concerned with variables involving lymphocyte culture techniques such as: (i) the effect of age, season, and pregnancy on mitogenic responses of cattle lymphocytes to phytohemagglutinin (PHA-P) and concanavalin A (ConA) (F. F. Soper, C. C. Muscoplat, and D. W. Johnson, manuscript in preparation); (ii) the effect of storage time and temperature of blood before lymphocyte stimulation with antigens and mitogens (D. R. Senogles, C. C. Muscoplat, J. M. Kaneene, C. O. Thoen, and D. W. Johnson, *Am. J. Vet. Res.*, in press); and (iii) determination of optimum parameters and factors affecting lymphocyte blastogenic responses of cattle lymphocytes (12). The purpose of the present investigation was to determine the effect of culturing *Mycobacterium bovis*-sensitized and normal lymphocytes from cattle in round-bottom microtiter plates and stimulating these cells with purified protein derivative (PPD) and mitogens compared with that of the traditional procedure conducted in flat-bottom plates.

MATERIALS AND METHODS

Animals. Normal male and female Holstein-Friesian cattle, ages 3 months to 1 year, from the dairy herds of the University of Minnesota, were used for this study. Six cattle were maintained as nonsensitized controls whereas five cattle were sensitized to *M. bovis* by subcutaneous injection of 0.1 ml of 5% (wt/vol) heat-killed *M. bovis* oil emulsion. *M. bovis*-sensitized animals were used as cell donors at least 6 weeks after *M. bovis* inoculation.

Preparation of lymphocyte cultures. Lymphocyte cultures were set up according to standard procedure (12). Briefly, blood was obtained via jugular venipuncture and placed into tubes containing heparin (Upjohn Co., Kalamazoo, Mich.). Heparinized blood was diluted with 2 parts of 0.15 M NaCl solution and subsequently layered over a Ficoll-Hypaque solution (Ficollpaque, Pharmacia, Piscataway, N.J.). The blood was centrifuged at 400 × *g* for 35 min after which the mononuclear cell layer was aspirated with a Pasteur pipette. Cells were washed twice with Hanks balanced salt solution and suspended in RPMI 1640 medium supplemented with bovine fetal serum (15% vol/vol, Flow Laboratories, Rockville, Md.), penicillin (100 U/ml), streptomycin (100 µg/ml), and HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethane-sulfonic acid) buffer (25 mMol). Cells were adjusted to appropriate concentrations and dispensed into either round- or flat-bottom microtiter culture plates (Linbro, New Haven, Conn.). Gamma radiation was the method of sterilization for both flat- and U-shaped bottom, tissue culture-treated polystyrene microtiter plates. Each culture contained 0.2 ml of cell suspension, and each determination was tested in triplicate. PHA-P (Difco Laboratories, Detroit, Mich.), ConA (Miles-Yeda, Rehovot, Israel), pokeweed mitogen (PWM, Gibco, Grand Island, N.Y.), and PPD (*M. bovis* origin, obtained from the Central Veterinary Laboratory, Wey-

bridge, Surrey, England) were added to each culture with an automatic microliter dispenser in concentrations listed below. Cultures were incubated at 37°C in a 5% CO₂, humidified air atmosphere for periods of from 3 to 7 days. Sixteen to 18 h before termination, cultures were labeled with 1.0 μ Ci (specific activity, 6.0 Ci/mmol) of [³H]thymidine (Research Products Int., Elk Grove Village, Ill.).

All cultures were terminated by cooling to 4°C. Cultures were harvested onto filter papers and processed for liquid scintillation counting as described previously (8).

RESULTS

Calculation and expression of results.

Results are expressed as the mean counts per minute from triplicate determinations and as the stimulation index. The stimulation index is calculated by dividing the mean counts per minute of stimulated cultures by the mean counts per minute of nonstimulated control cultures. Statistical analysis was performed and all data were screened for significance at the $P < 0.05$ level.

Comparison of antigen dose response titrations in round- and flat-bottom microtiter plates. Lymphocyte cultures from *M. bovis*-sensitized cattle stimulated with PPD in round plates demonstrated a higher stimulation index than similar cultures in flat plates (Fig. 1). Cells of nonsensitized animals were not stimulated by PPD in either round- or flat-bottom plates. PPD lymphocyte stimulation in *M. bovis*-sensitized cattle indicates that 10 μ g/ml is the optimal concentration for flat plates ($P < 0.05$) as well as for round plates ($P < 0.005$). Round plates produced significantly greater blastogenic responses ($P < 0.025$) than did flat plates using 10 μ g/ml of PPD. Both round and flat plates produced significantly greater responses to PPD in *M. bovis*-sensitized animals than was observed in control nonsensitized animals.

Determination of optimum culture duration for lymphocyte blastogenic responses to PPD in round- and flat-bottom microtiter plates. Lymphocytes from sensitized cattle cultured in round-bottom plates were stimulated significantly more by PPD ($P < 0.05$) than were comparable cultures in flat plates (Fig. 2). This significant difference remained for 3 to 7 days. Cultures in flat plates were optimally stimulated after 6 days in culture. Control animals were not stimulated by PPD in either round or flat plates.

Determination of optimum cell concentration for lymphocyte blastogenic responses to PPD in round- and flat-bottom microtiter plates. Figure 3 shows results from experiments to determine cell concentrations that produce optimal lymphocyte stimulation to PPD. It was determined that with flat plates the only significant ($P < 0.05$) stimulation with PPD

FIG. 1. Comparison of the effect of antigen concentration with *M. bovis* PPD in round- and flat-bottom plates.

FIG. 2. Comparison of the effect of kinetics with *M. bovis* PPD in round- and flat-bottom plates.

FIG. 3. Comparison of the effect of cell concentration with *M. bovis* PPD in round- and flat-bottom plates.

of lymphocytes from *M. bovis*-sensitized cattle compared with that of control cattle occurred at 1.5×10^6 cells/ml. However, with round plates, significantly greater stimulation occurred with 1.0×10^6 cells/ml compared with that of flat plates, and all concentrations of cells from *M. bovis*-sensitized lymphocytes cultured in round plates were significantly greater ($P < 0.05$) than lymphocyte blastogenic responses of control cattle to PPD.

Comparison of lymphocyte mitogenic responses to PHA-P, ConA, and PWM in round- and flat-bottom microtiter plates. Table 1 shows blastogenic responses of peripheral blood lymphocytes to three mitogens, PHA-P, ConA, and PWM. Results of cultures from six animals show that there is no significant difference between mitogenic responses of lymphocytes cultured in either round- or flat-bottom microtiter plates. Blastogenic responses in round-bottom plates tended to be higher than those of flat-bottom plates; however, statistical analysis did not reveal any significant difference between the two different types of culture plates.

DISCUSSION

The use of in vitro lymphocyte stimulation for the detection of delayed hypersensitivity and/or cell-mediated immunity has become widespread in recent years. The immunological status of patients recovered from herpes simplex (15), rabies (17), Epstein-Barr virus infection (6), parainfluenza virus (11), *Brucella abortus* (J. M. Kaneene, D. W. Johnson, R. K. Anderson, R. D. Angus, D. E. Pietz, and C. C. Muscoplat, Am. J. Vet. Res., in press), *Candida albicans* (1), several species of mycobacteria (13, 14), and several others (4, 5, 7) has been assessed by lymphocyte stimulation. Additionally, lymphocyte stimulation has become one of the main tools to aid in the study of histocompatibility and tissue typing (9, 16).

Due to the advent of microtiter techniques (9) and automated cell harvesting devices (8), lymphocyte culturing has become a mainstay in clinical immunology laboratories throughout the world. Initially, most concerns with blastogenic responses were related to phytomitogens such as PHA-P, ConA, and PWM. The results reported here revealed that there was little or no difference in mitogenic response between culturing lymphocytes in round- or flat-bottom microtiter plates. However, there are few studies comparing blastogenic responses of lymphocytes to

antigens with histocompatibility determinants using round-bottom plates. One recent study has shown that round-bottom microtiter plates produce greater blastogenic responses in the human mixed-lymphocyte culture (10).

The present study has shown that lymphocytes cultured with specific antigens in round-bottom microtiter plates resulted in greater levels of stimulation than when cultured in flat-bottom plates. Additionally, greater responses were detected earlier when the number of cells cultured was lower. The use of round-bottom plates, therefore, can significantly improve lymphocyte blastogenic responses in at least one experimental system, namely PPD stimulation of *M. bovis*-sensitized cattle lymphocytes.

The mechanism of increased lymphocyte stimulation in round plates is at present unknown. Mechanisms involving increased macrophage-lymphocyte interaction as well as increased concentrations of lymphokines are currently being investigated.

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TABLE 1. Comparison of the effect of mitogenic response with PHA-P, ConA, and PWM in round- and flat-bottom microtiter plates^a

Mitogens ^c	[³ H]thymidine uptake (cpm) ± SE ^b	
	Flat	Round
PHA-P (100 U/ml)	63,625 ± 11,596	80,359 ± 15,555
ConA (1,000 µg/ml)	131,410 ± 29,248	152,980 ± 26,244
PWM	24,454 ± 5,047	42,808 ± 10,797
Control	951 ± 511	1,430 ± 824

^a Six animals used to determine the mean of the triplicates.

^b SE, Standard error of the mean; cpm, counts per minute.

^c A 2-µl portion of PHA-P, ConA, and PWM added to each well.

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