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Improved methods for purification and depletion of monocytes from bovine peripheral blood mononuclear cells

Functional evaluation of monocytes in responses to lectins

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We have compared different techniques for the enrichment and depletion of monocytes from bovine peripheral blood mononuclear cells. Adherence to plasma-coated gelatin was the most efficient and reproducible method for enrichment of monocytes (80% monocytes), whereas depletion of peripheral blood mononuclear cells of monocytes (0.3% monocytes and less) was best achieved by defibrination of the blood from which the PBM were separated. In both instances, purity of the cell population could be improved further by an additional step, namely, FACS sorting with a monocyte-specific monoclonal antibody to purify monocytes (97% monocytes and more), and adherence to polystyrene to remove residual monocytes from defibrinated PBM (0.1% monocytes and less).

Depletion of monocytes abolished the response of PBM to concanavalin A and phytohaemagglutinin. The lectin-induced response could be restored by adding gelatin/plasma purified monocytes. This activity of monocytes could be replaced by 2-mercapthoethanol.

Key words: *Bovidae; Monocyte depletion; Monocyte enrichment; Lectin stimulation; Monoclonal antibody*

Introduction

Cells of the monocyte/macrophage lineage have been shown to participate in immune responses (Rosenstreich and Oppenheim, 1976) by processing and presenting antigens to lymphocytes (Unanue et al., 1984), by producing growth factors necessary for proliferative responses (Mizel et al., 1978; Larsson, 1980) and by modulating these responses (Laughter and Twomey, 1977; Hamilton et al., 1983). In order to study cellular interactions in the immune responses of cattle, it is necessary to have reliable methods for depleting peripheral blood mononuclear cells (PBM) of monocytes and for obtaining highly enriched populations of monocytes. In man and mouse, several methods have been developed for depletion and enrichment of mononuclear phagocytes (Mosier, 1967; Ly and Mishell, 1974; Bevilacqua et al., 1981; Feige et al., 1982). In cattle, methods based on the binding of monocytes to polystyrene and Sephadex G-10, have been used for depletion and enrichment of monocytes from PBM (Usinger et al., 1981; Bielefeldt Ohmann et al., 1983). However, these methods did

Abbreviations: PBM, peripheral blood mononuclear cells; A-PBM, peripheral blood mononuclear cells obtained from blood collected in Alsever's solution; D-PBM, peripheral blood mononuclear cells obtained from defibrinated blood; ConA, concanavalin A; PHA, phytohaemagglutinin; 2ME, 2-mercaptoethanol; FBS, foetal bovine serum; ANAE, α-naphthyl acetate esterase; EDTA, ethylenediaminetetraacetate; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; MAb, monoclonal antibody; PBS, phosphate-buffered saline.

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not completely deplete PBM of monocytes and, in our experience, were not reproducible. In addition, the methods did not provide significant enrichment of monocytes.

In the studies presented in this paper we compared several different techniques for both enrichment of bovine monocytes and also their depletion from PBM. Based on this comparative study we identified a simple and reproducible method for depleting bovine PBM of monocytes and a reliable method for obtaining highly enriched populations of monocytes from PBM. The proliferative responses of PBM stimulated by the mitogens concanavalin A (ConA) and phytohaemagglutinin (PHA) were used to assess functionally the efficacy of the methods employed and to evaluate some properties of bovine monocytes in these reactions.

Materials and methods

Donor cattle

The cattle used in these studies, were Boran (*Bos indicus*), Friesian or Hereford (*Bos taurus*), females or castrated males aged between 6 and 36 months. The animals were reared indoors from 2 to 3 days of age and were clinically normal when used.

Peripheral blood mononuclear cells (PBM) and plasma

Blood was collected from the jugular vein, either into a conical flask containing glass beads (5 mm diameter) and defibrinated by gently swirling for 10 min, or into an equal volume of Alsever's solution. Aliquots of 30 ml of defibrinated or anticoagulant-treated blood were layered onto 20 ml Ficoll/diatrizoate sodium solution of density 1.077 g/ml (Ficoll-Paque, Pharmacia Fine Chemicals, Uppsala, Sweden) in 50 ml polypropylene conical tubes (Falcon, Becton Dickinson, Oxnard, CA, U.S.A.) and centrifuged at $900 \times g$ for 30 min at 23°C. Mononuclear cells from defibrinated blood (D-PBM) or anticoagulant-treated blood (A-PBM) were aspirated by pipette from the interphase, diluted with an equal volume of unsupplemented RPMI 1640 medium in the case of D-PBM, or Alsever's solution in the case of A-PBM, and pelleted by centrifugation (10 min at $450 \times g$, 23°C). The A-PBM were washed three times in Alsever's solution by repeated suspension and centrifugation (10 min at $180 \times g$, 23°C) to remove platelets and avoid clotting, while D-PBM were washed only once in unsupplemented RPMI 1640 medium as most platelets had already been removed during defibrination of the blood. Both cell populations were resuspended in RPMI 1640 medium containing 10% heat-inactivated foetal bovine serum (FBS; Gibco, Uxbridge, U.K.), 2 mM L-glutamine and 50 μg/ml of gentamycin (culture medium). The batch of FBS was selected by screening for support of cell proliferation in mixed leukocyte reactions.

Autologous plasma was obtained from heparinised blood after centrifugation at $3500 \times g$ for 30 min at 23°C. The plasma was filtered through a 0.45 μm filter (Millipore, Molsheim, France) to sterilise and remove any residual cells or platelets.

Detection of non-specific esterase

The presence of α-naphthyl acetate esterase (ANAE) activity was used as a means of identifying monocytes in PBM. Cytospin smears were fixed with acetone/formaldehyde and stained for ANAE activity as described previously (Yang et al., 1979). Only those cells with diffuse cytoplasmic staining, as opposed to single dot-like staining of some lymphocytes, were considered to be monocytes (Kajikawa et al., 1983) and are referred to in the text as $ANAE^+$ cells.

Separation of monocytes by adherence to polystyrene

The method used was that of Mosier (1967). Fifteen ml of a suspension of A-PBM in culture medium at a concentration of 5×10^6 cells/ml, were placed in a 75 cm^2 culture flask (Costar, Cambridge, MA, U.S.A.) and incubated for 2 h at 38°C. After incubation, flasks were gently shaken and non-adherent cells were removed by pipetting and rinsing the flask twice with warm (38°C) medium. The adherent cells were detached by incubation for 10 min at 23°C with Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution containing a mixture (Gibco) of trypsin 0.5 mg/ml (activity 1 : 250) and 0.6 mM ethylenediaminetetraacetate (EDTA). Adherent cells were pelleted (10 min at

$180 \times g$, 4°C), washed in unsupplemented RPMI 1640 medium by resuspension and centrifugation (10 min at $180 \times g$, 4°C) and resuspended in culture medium. After removal of the monocytes from the polystyrene flask, all procedures were carried out at 4°C in 10 ml polycarbonate conical tubes (Nunc, Roskilde, Denmark) to avoid loss of monocytes by binding to polystyrene (Grinnell et al., 1972).

Separation of monocytes by adherence to Sephadex G-10

The method employed was that of Jerrels et al. (1980). Sephadex G-10 (Pharmacia Fine Chemicals), preswollen and washed in distilled water, was autoclaved and stored at 4°C. Before use, the resin was incubated twice with an excess of culture medium buffered with Hepes for 30 min at 38°C. A sterile 20 ml glass syringe, with a nylon mesh (30 μm pore size) at the bottom of the barrel, was loaded with the resin to a final bed volume of 8 ml. A cell suspension of 1×10^8 A-PBM in 2 ml culture medium buffered with Hepes was allowed to run into the column, which was then incubated for 45 min at 38°C. Following incubation, the non-adherent cells were eluted from the column with 30 ml of culture medium at 38°C. To detach adherent cells, 5 ml of culture medium at 38°C, containing 0.9% lidocaine (Xylocaine 2%, Astra, Sodertalje, Sweden) was allowed to run into the column, which was then incubated for 10 min at 38°C. The adherent cells were eluted from the column with 30 ml of culture medium containing 0.9% lidocaine. Adherent cells were pelleted, washed and resuspended as described for the polystyrene-adherent cells.

Separation of monocytes by centrifugation on a hypotonic Percoll density gradient

The procedure followed was that according to Feige et al. (1982) except that a discontinuous density gradient was used. Two hypotonic (260 mmol/kg) solutions of Percoll (Pharmacia Fine Chemicals) in RPMI 1640 medium with different densities were prepared. Two ml of a Percoll solution with a density of 1.054 g/ml, were layered on top of 4 ml of a Percoll solution with a density of 1.060 g/ml, in a 10 ml polycarbonate conical tube. A cell suspension of 1×10^8 A-PBM, prewashed twice in 50 ml RPMI 1640 medium which was made hypotonic (260 mmol/kg), was layered on top of the Percoll gradient and centrifuged at $600 \times g$ for 45 min at 23°C. Cells at the interface of the two Percoll solutions were aspirated with a pipette, diluted with an equal amount of isotonic RPMI 1640 medium, pelleted and washed twice in isotonic RPMI 1640 medium before resuspending in culture medium at 4°C. The specific gravity of the solutions was determined at 20°C with density hydrometers (Assistent, Sondheim/Rhon, F.R.G.). Osmolality was measured with a vapor pressure osmometer (model 5100C; Wescor, Logan, UT, U.S.A.).

Separation of monocytes by adherence to gelatin/plasma

The method used was that of Freundlich and Avdalovic (1983) with some minor modifications. 75 cm^2 flasks (Costar) containing 10 ml of a suspension of 2% gelatin (BDH Chemicals, Poole, U.K.) in distilled water, were incubated at 38°C for 2 h. The gelatin solution was then removed by pipette and the flasks allowed to dry at 38°C. 10 ml of autologous plasma was added to the dry gelatin-coated flask and incubated for 1 h at 38°C. The plasma was removed and the flask rinsed twice with unsupplemented RPMI 1640 medium. 15 ml of a cell suspension containing 5×10^6 A-PBM/ml of culture medium were incubated in the gelatin/plasma-coated flask for 1 h at 38°C. Non-adherent cells were aspirated with a pipette and the flask rinsed gently with culture medium at 38°C. To remove adherent cells, 10 ml of 10 mM EDTA in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution were added and the flask incubated for 5 min at 23°C. The flask was shaken and adherent cells removed by pipette. The adherent cells were pelleted, washed and resuspended in culture medium at 4°C as for the polystyrene-adherent cells.

Preparation of bovine fibronectin and fibronectin-free serum

A 48 ml column was prepared from 15 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) to which 250 mg of calf dermal gelatin (BDH Chemicals) was coupled (Engvall and Ruoslahti, 1977). 160 ml of defibrinated bovine

plasma was dialysed for 12 h at 23°C against 2 litres of equilibrating buffer, consisting of PBS pH 7.4 with 10 mM sodium citrate and 1 M urea, and loaded on the affinity resin. The column was washed with the equilibrating buffer and the absorbed protein was eluted in one step with the equilibrating buffer containing 4.5 M urea. The eluted protein was dialysed against 2 litres of 10 mM Tris-HCl pH 8.0 with 4.5 M NaCl, for 12 h at 23°C and subjected to anion exchange chromatography on a 2×36 cm column containing diethylaminoethyl (DEAE-52) cellulose (Whatman, Maidstone, U.K.). The only major protein peak was eluted using a gradient of NaCl between 10–12.5 mS. To assess homogeneity of the isolated protein, 50 μg of protein was subjected to gel electrophoresis performed according to Laemmli (1970) on 8 mm slab gels containing 0.1% SDS. Bovine fibronectin had a subunit molecular weight of 225000 and appeared to consist of a doublet similar to that described previously for human (Chen et al., 1977) and horse (Chiquet et al., 1979) plasma fibronectin.

The gelatin-Sepharose affinity column was used to deplete serum completely of fibronectin. For this purpose the equilibrating buffer was PBS with 10 mM sodium citrate, without urea. The unbound protein was collected and recycled once through the column. It was then reconstituted with PBS to the original volume of the sample that had been applied on the column.

Analysis of cells

Indirect immunofluorescence staining and analysis of cells was performed using the fluorescence-activated cell sorter (FACS II; Becton-Dickinson, Sunnyvale, CA, U.S.A.) as described elsewhere (Lalor et al., 1986). To avoid loss of monocytes during the staining procedure, staining and sorting of monocytes was done in Ca^{2+}-, Mg^{2+}-free Hanks' balanced salt solutions supplemented with 5% heat-inactivated immunoglobulin-free horse serum (Gibco) using 10 ml polycarbonate tubes. Three monoclonal antibodies (MAb) were used (Lalor et al., 1986): MAb R1 reacts with B cells and monocytes in PBM and is believed to recognise a monomorphic determinant on bovine class II MHC molecules; MAb P5 recognises T cells and monocytes in PBM; MAb P8 recognises monocytes in PBM. A FITC-conjugated goat anti-bovine IgM antibody was also used to identify B cells by direct immunofluorescence staining.

Proliferative responses of PBM to lectins

PBM suspended in culture medium were assayed in 96-well flat-bottomed culture plates (Costar) for proliferative responses to the lectins concanavalin A (ConA, type IV; Sigma, St. Louis, MO, U.S.A.) and phytohaemagglutinin (PHA-P; Difco, Detroit, MI, U.S.A.). ConA and PHA were used at a final concentration in the well of 5 and 10 μg/ml respectively. Preliminary experiments using 2.5×10^5 PBM/well had shown that these lectin concentrations induced optimal responses in A-PBM. Responses of PBM were compared over a range of cell concentrations as described previously (Baldwin et al., 1985). Where indicated, 2-mercaptoethanol (2ME; Merck, Schuchardt, F.R.G.) was added to a final concentration of 50 μM. The final volume in the well was 200 μl. Cultures were incubated in an atmosphere of 5% CO_2 in air and proliferation was assayed after 3 days by the incorporation of [^{125}I]iododeoxyuridine (0.5 μCi/well for 8 h; Amersham International, Amersham, U.K.) by multiplying cells. Cells were harvested on glass microfibre filters (GF/C; Whatman) and radioactivity counted using a Beckman 5500 Gamma counter. All microwell cultures were set up in triplicate. Results were expressed as counts per min (cpm) of radioactivity per well or cpm per unit number of cells depending on the experiment.

Results

Characterisation and number of monocytes in populations of PBM

Monocytes in PBM were characterised by the presence of ANAE activity and/or by the expression of the determinant for MAb P8. The number of $ANAE^+$ cells in A-PBM from cattle varied between individual animals, ranging from 5% to 15%. In each animal, there was a close correlation between the number of $ANAE^+$ cells and the number of cells staining with MAb P8. This was also true for the various populations of PBM that

TABLE I

COMPARISON OF THE PERCENTAGES OF BOVINE CELLS WITH ANAE ACTIVITY WITH THOSE EXPRESSING THE DETERMINANT RECOGNISED BY MAb P8 IN DIFFERENT POPULATIONS OF PBM

Animal	Cell population	% Cells	
		ANAE$^+$ [a]	MAb P8$^+$ [b]
B630	A-PBM	6.6	5.8
C165	A-PBM	10.2	10.6
B734	A-PBM	11.0	11.5
B171	A-PBM	10.6	8.2
B166	Polystyrene adherent	50.0	50.4
	Polystyrene non-adherent	1.5	2.2
	Gelatin/plasma adherent	82.0	78.8
	Gelatin/plasma non-adherent	2.0	2.2
B166	Gelatin/plasma adherent	90.6	93.5
B166	Sorted for P8$^+$ cells [c]	97.2	nd
	Sorted for P8$^-$ cells [c]	2.3	nd
C210	Sorted for P8$^+$ cells [c]	98.0	98.0

[a] Five hundred cells examined.
[b] FACS analysis on 10000 cells.
[c] Gelatin/plasma adherent cells were stained with MAb P8 and sorted with the FACS for MAb P8$^-$ and MAb P8$^+$ cells.

were either enriched for, or depleted of, ANAE$^+$ cells (Table I).

Comparison of methods for enrichment of monocytes

Four different methods were used to enrich PBM for monocytes: hypotonic density gradient centrifugation on Percoll, adherence to Sephadex G-10, adherence to polystyrene and adherence to gelatin/plasma (Table II).

By centrifugation of A-PBM on a hypotonic discontinuous density gradient, a population containing about 33% monocytes could be recovered, although in some instances recoveries as high as 50% were obtained (Table II). Separating monocytes by adherence to Sephadex G-10 was only slightly more successful, yielding enriched populations containing 38% monocytes (Table II). Both methods were found to be cumbersome and time consuming.

Adherence to polystyrene surfaces or plasma-coated gelatin surfaces consistently gave higher levels of enrichment for monocytes than the two methods described above. These methods also proved to be easier to perform than either hypotonic density gradient centrifugation or adherence to Sephadex G-10. More than 60% of the polystyrene-adherent cells were monocytes while the cells obtained by adherence to gelatin/plasma comprised 75% or more (upto 93%) monocytes (Tables I and II). Removal of adherent cells from plasma-coated gelatin was easier and more complete than from polystyrene surfaces: while gelatin/plasma-adherent cells could be removed by a 5 min incubation with 10 mM EDTA, a 10 min incubation with trypsin was needed to remove the polystyrene-adherent cells. Monocyte recovery from PBM incubated on the gelatin/plasma was between 40 and 50% of the total monocytes present.

Purified bovine fibronectin (200 μg/ml) could be used instead of plasma to coat the gelatin surfaces, giving yields of cells and percentages of monocytes similar to those obtained with plasma (Table III). When plasma was substituted by FBS a similar percentage of the adherent cells com-

TABLE II

COMPARISON OF METHODS USED FOR ENRICHING PBM FOR MONOCYTES [a]

Method	Non-adherent/monocyte-reduced		Adherent/monocyte-enriched	
	% Cell recovery [b]	% Monocytes [c]	% Cell recovery [b]	% Monocytes [c]
Percoll	nd	nd	10.7 ± 8.8 (2)	33.4 ± 9.9 (9)
Sephadex G-10	67.6 ± 6.9 (8)	3.6 ± 0.6 (4)	7.1 ± 6.6 (6)	38.6 ± 8.7 (4)
Polystyrene	50.6 ± 9.1 (3)	5.3 ± 5.1 (3)	8.0 (1)	63.7 ± 13.5 (3)
Gelatin/plasma	67.0 ± 4.2 (2)	1.8 ± 0.3 (3)	5.4 ± 1.4 (2)	78.0 ± 10.3 (10)

[a] Results are expressed as the mean ± SD of several experiments, with the number of experiments in parenthesis. nd = not done.
[b] % Cells recovered from the initial population.
[c] % Monocytes present in the recovered population; monocytes were identified by ANAE and/or MAb P8 (see Table I).

TABLE III

COMPARISON OF THE CAPACITY OF MONOCYTES TO ADHERE TO GELATIN PREINCUBATED WITH FBS, AUTOLOGOUS SERUM OR PLASMA, UNSUPPLEMENTED MEDIUM OR PURIFIED FIBRONECTIN

Exp.	Gelatin coated with:	Adherent cells		Non-adherent cells	
		% Cell recovery [a]	% ANAE$^+$ [b]	% Cell recovery [a]	% ANAE$^+$ [b]
1	10% FBS	1.8	88.3	70.0	13.8
	50% plasma	4.4	79.3	64.0	1.5
	20% fibronectin	4.5	75.7	68.0	1.5
2	10% autologous serum [c]	nd [d]	83.0	nd	13.6
	50% autologous plasma	nd	80.2	nd	2.2
3	RPMI 1640 medium	nd	92.2	nd	10.6
	10% FBS [c]	nd	97.0	nd	9.2
	50% autologous plasma	nd	91.2	nd	1.4

[a] % Cells recovered from the initial population.
[b] % Monocytes present in the recovered population; 500 cells examined.
[c] Additionally depleted for fibronectin by passage over a gelatin column.
[d] nd = not done.

prised monocytes. However, the yield of adherent cells was markedly reduced and was reflected by the presence of large numbers of monocytes in the non-adherent population (Table III). Similar observations were made when plasma was replaced with autologous serum or unsupplemented RPMI 1640 medium (Table III).

Purification of monocytes using MAb P8

Highly purified populations of monocytes could easily be obtained in a reproducible manner when cells harvested by adherence to gelatin/plasma were stained by immunofluorescence with MAb P8 and sorted for MAb P8$^+$ cells using the FACS. Sorted cell populations comprised 97% or more monocytes, as evaluated either by the MAb P8 or the presence of ANAE activity (Table I).

Depletion of monocytes

Substantial numbers (2–5%) of monocytes were still present in the non-adherent cell populations obtained from Sephadex G-10, polystyrene and gelatin/plasma (Table II). However, defibrination of blood yielded PBM (D-PBM) which were markedly depleted for monocytes (0.2–1% monocytes remaining). Incubation of D-PBM for 2 h on polystyrene resulted in a further reduction of the number of monocytes so that depletion to levels

TABLE IV

STAINING OF A-PBM, D-PBM AND MONOCYTE-DEPLETED D-PBM WITH MONOCLONAL ANTIBODIES THAT REACT WITH DIFFERENT POPULATIONS OF CELLS WITHIN PBM [a]

Immunofluorescence staining	% Stained cells in		
	A-PBM	D-PBM	D-PBM depleted on polystyrene
MAb P5	70.6 ± 11.7 (5)	65.7 ± 7.6 (3)	80.0 ± 8.5 (2)
MAb R1	31.2 ± 7.4 (5)	30.5 ± 13.4 (2)	19.0 ± 11.3 (2)
Anti-bovine IgM	19.8 ± 5.4 (5)	18.3 ± 3.4 (6)	14.0 ± 2.8 (2)
MAb P8	10.5 ± 3.5 (10)	nd	0.5 ± 0.7 (2)
ANAE activity [b]	11.7 ± 3.5 (14)	0.3 ± 0.3 (16)	0.1 ± 0.1 (4)

[a] Results are expressed as the mean ± SD of several experiments, with the number of experiments in parenthesis. nd = not done.
[b] For each experiment 500 cells were examined.

below 0.2% could be achieved reproducibly. To determine whether these procedures altered the representation of B and T lymphocytes, A-PBM, D-PBM and D-PBM further depleted on polystyrene were stained by immunofluorescence with goat anti-bovine IgM and MAbs R1, P5 and P8. There was no appreciable alteration in the proportion of B and T lymphocytes in D-PBM, as compared to A-PBM (Table IV). However, D-PBM depleted on polystyrene showed a 20–30% decrease in the B cell population (Table IV).

Functional evaluation of monocyte-depleted PBM and enriched monocytes in responses of PBM to lectins

Both ConA and PHA induced strong proliferative responses in A-PBM cultured at cell concentrations of $0.16–2.5 \times 10^5$ cells per well (Fig. 1). Maximum proliferation per unit number of cells was obtained with 1.25×10^5 cells per well. Monocyte-depleted D-PBM did not respond to ConA or PHA at these cell concentrations (Fig. 1). However, monocyte-depleted D-PBM did respond when autologous monocytes obtained from the gelatin/plasma-adherent population, were added to the culture: proliferation was detectable with 1% monocytes and reached maximal levels with 8% monocytes (Fig. 2). Gamma-irradiated monocytes (5000 rads from a ^{137}Cs source) had a similar

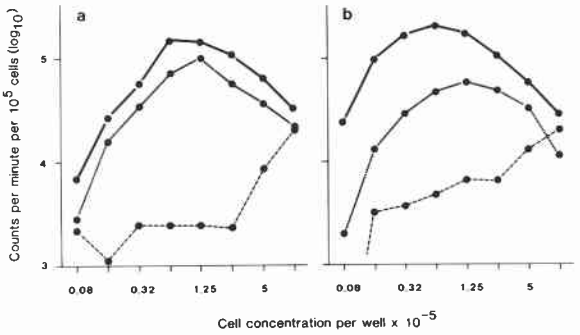

Fig. 1. Influence of cell concentration per well on proliferative responses of PBM to (*a*) ConA and (*b*) PHA. Counts per minute per well were adjusted to counts per minute per 10^5 cells. Responder cells are monocyte-depleted D-PBM (●— — —●), A-PBM (●· · · · · ·●) and monocyte-depleted D-PBM plus 2ME (●———●). Background proliferation (no lectin) was in all instances below 712 cpm per 10^5 cells; A-PBM contained 10.2% monocytes.

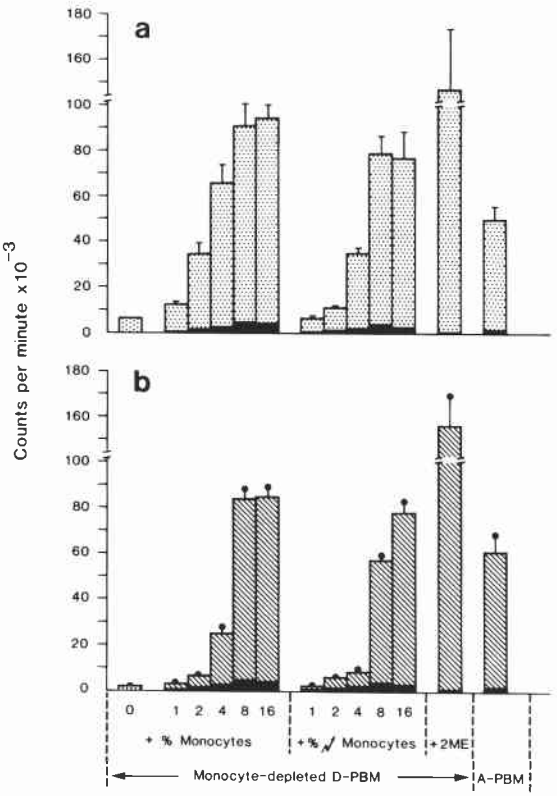

Fig. 2. Histogram of the proliferative response of 2.5×10^5 PBM to (*a*) PHA and (*b*) ConA. Responses in the absence of lectins are represented in black. Different cell numbers of a population containing 80% monocytes obtained by adherence to plasma-coated gelatin, were added to the responder cells to give 1, 2, 4, 8 or 16% monocytes per well. Monocytes were characterised by ANAE and/or MAb P8.

beneficial effect although they gave slightly lower maximal responses than non-irradiated monocytes (Fig. 2).

A similar, if not greater, effect to that achieved by addition of monocytes, was obtained on the proliferative response of monocyte-depleted D-PBM when 2ME was added to the culture (Figs. 1 and 2). In the presence of 2ME, monocyte-depleted D-PBM gave maximum proliferation per unit number of cells with $0.64–1.25 \times 10^5$ cells per well. Monocyte-depleted D-PBM in the presence of 2ME and A-PBM without 2ME had parallel response curves when incorporation of [^{125}I]-iododeoxyuridine per unit number of cells was plotted against cell concentration per well (Fig. 1).

Discussion

The studies outlined in this paper have provided comparative information on the methods of enrichment and depletion of bovine monocytes from PBM. On the basis of these results, we conclude that adherence to plasma-coated gelatin is the most efficient and reproducible method for enrichment of monocytes, whereas depletion of PBM of monocytes can best be achieved by defibrination of the blood from which the PBM are separated. In both instances the purity of the cell population can be improved further by an additional step, namely, FACS-sorting with the MAb P8 to purify monocytes and adherence to polystyrene to remove residual monocytes from defibrinated PBM. The finding that in all of these populations of cells, there was a close correlation between the numbers of cells staining for ANAE and those staining with the MAb P8 confirms previous observations (Lalor et al., 1986) that the P8 determinant is a reliable marker for monocytes in bovine PBM.

Adherence of monocytes to plasma-coated gelatin yielded adherent populations containing 80% and sometimes more than 90% monocytes. 40–50% of the monocytes present in PBM could be recovered, without subjecting the cells to harsh treatment. From this enriched population, monocytes could be purified to more than 97% by FACS with MAb P8. The substance in plasma thought to be responsible for monocyte adherence is fibronectin (Bevilacqua et al., 1981). This glycoprotein, which is present in substantial amounts (300 ± 100 μg/ml) in human plasma, has an affinity for gelatin and is recognised and bound by receptors on the cell membrane of monocytes in a Mg^{2+}-dependent process. We were able to replace plasma by purified bovine fibronectin but not by serum or RPMI 1640 medium. The finding that a small percentage of the total number of monocytes did adhere to the uncoated gelatin is probably due to the presence of gelatin-binding domains of fibronectin on the surface membrane of some monocytes enabling them to adhere to the fibronectin-free gelatin.

Defibrination of blood yielded populations of PBM containing an average of 0.3% monocytes. Further depletion of D-PBM on polystyrene reduced the proportion of monocytes below levels of 0.2%, although this resulted in a reduction of the B cell population.

The requirement for monocyte/macrophages in lymphocyte responses to ConA or PHA has been demonstrated in mouse (Habu and Raff, 1977; Ahmann et al., 1978), guinea pig (Rosenstreich et al., 1976), human (Schmidtke and Hatfield, 1976; De Boer et al., 1981) and goat (De Martini et al., 1983). In addition, work in cattle stressed the need for macrophages with lymph node cells (Mastro and Sniezek, 1983), or monocytes with thymocytes (Kumar et al., 1980) and PBM (Bielefeldt Ohmann et al., 1983), in order for these cell populations to respond to ConA or PHA.

The removal of monocytes by defibrination and subsequent polystyrene depletion, abolished the capacity of PBM to respond to ConA or PHA at cell concentrations of 2.5×10^5 or less per well. However, at higher cell concentrations per well, low levels of proliferation were observed in monocyte-depleted D-PBM, comparable to those of A-PBM at 50 times lower cell concentrations per well. As monocyte-depleted PBM contained 50–100 times less monocytes than intact PBM, this suggests that proliferation of monocyte-depleted PBM may occur when residual monocytes reach critical threshold levels per well.

The capacity of PBM to respond to ConA and PHA could be restored by adding gelatin/plasma enriched populations of monocytes to the monocyte-depleted D-PBM. Reconstituting monocytes to their normal concentrations in PBM resulted in proliferative responses comparable to those of A-PBM. This activity delivered by monocytes in proliferative reactions to ConA and PHA could be substituted by the reducing agent 2ME, suggesting that monocytes act in these responses by conditioning the medium rather than by physical contact with other cells. Previous studies have shown that 2ME (or macrophages) can activate a serum factor which induces proliferation of T cells (Opitz et al., 1978), and that 2ME can induce proliferation of lymphocytes by enhancing the entry of cystine (Burger et al., 1982). Contrary to these and other results (Bielefeldt Ohmann et al., 1983), Usinger et al. (1981) found no requirement for monocytes or 2ME in responses of bovine PBM to ConA and PHA. This observation may be due to

the use of human plasma which had been preincubated with bovine blood cells, as a supplement in their medium; the human plasma might have been conditioned by monocytes present in the bovine blood cells.

The availability of reliable methods for depleting PBM of monocytes and separating functionally active monocytes, will enable further studies to be conducted in vitro on the role played by monocytes in cellular and humoral immune responses in cattle.

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