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MITOGENIC RESPONSES OF SHEEP PERIPHERAL BLOOD T-LYMPHOCYTES TO PHA AND LPS IN A HANGING DROP MICROASSAY

By

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MLANGWA, J. E. D.: *Mitogenic responses of sheep peripheral blood T-lymphocytes to PHA and LPS in a hanging drop microassay.* Acta vet. scand. 1984, *25*, 548—560. — Culture conditions for studying sheep PBL responses to mitogens (PHA and LPS) in a hanging drop micro-assay were investigated. Using those conditions, the responses of putative sheep T-cells (vide infra) to the two mitogens were examined.

The results show that sheep PBL at a cell density of $1—2 \times 10^6$/ml responded optimally to LPS (50 μg/ml) and PHA (3.125 μg/ml) when cultured in RPMI-1640 in the presence of 2ME, 5 % FCS for 48 to 146 h. The cells were cultured as hanging drops (20 μl) in Terasaki plates. Lamb serum failed to support responses to LPS.

T-cells prepared by the nylon wool technique and Helix pomatia-Sepharose-6MB column chromatography responsed vigorously to PHA, but responses to LPS were weak.

mitogen responses; techniques; immunology.

Previous work (*Mlangwa* 1983) has shown that sheep T-cells possess receptors for Helix pomatia agglutinin (HP), and that is was essential to treat the lymphocytes with neuraminidase in order to expose HP receptors. It was also shown that HP-Sepharose-6MB columns (Pharmacia, Sweden), and nylon wool columns can be used to fractionate ovine lymphocytes to obtain a T-cell enriched sub-population.

Sheep T-cells undergo mitosis when stimulated by phytohaemaglutinin (PHA), a T-cell mitogenic lectin (*Cahill et al.* 1978, *Fahey* 1980 and *Outteridge et al.* 1981), and are not significantly stimulated by lipopolysaccharide (LPS), a B-cell mitogen (*Cahill et al.* 1978).

In this article a microassay for studying sheep peripheral blood lymphocytes (PBL) responses to PHA and LPS in hanging drops (*O'Brien et al.* 1979, *Ferranti et al.* 1980) is described. Furthermore, the effect of phagocytic cell depletion by the method used to prepare PBL on mitogen responses of ovine PBL, and the responses of HP eluted cells and nylon wool non-adherent sub-populations to PHA and LPS are reported.

MATERIALS AND METHODS

Animals, cell preparations, nylon wool and HP affinity chromatography fractionation and identification were as previously described (*Mlangwa* 1984). PBL were prepared by the carbonyl iron technique (*Mlangwa* 1984).

Mitogens

Lymphocyte responses to PHA (Type VS, Sigma) and LPS (phenol extract) of Escherichia coli 128: B 12 (Sigma, St. Louis, Mo., U.S.A.) were examined. The same batch of each mitogen was used throughout the investigations.

Lymphocyte culture and stimulation

Sheep leukocyte preparations were suspended in RPMI-1640 containing foetal calf serum (FCS) (Gibco Biocult, U.K.) supplemented with 100 I.U./ml penicillin, 100 μg/ml streptomycin, 1 % glutamine (Gibco, U.K.) buffered with HEPES (25 mmol/l) and 0.2 % sodium bicarbonate buffers and 2×10^{-5} mmol/l of 2-mercaptoethanol (2ME) where indicated. This medium will be referred to as RPMI-complete.

20 μl of leukocyte suspension were added to Terasaki plate wells (Nunc, Copenhagen, Denmark). Varying dilutions of mitogens in RPMI-complete were added to the wells in 1 μl volumes. The cultures were set up in triplicates.

The plates were then inverted without lids and placed on a support (4 small Widal tube rubber stoppers placed at the corners of an inverted Terasaki plate lid) approximately 0.8 cm above a bath of sterile saline in a container fitted with a loosely fitting lid. The whole assembly was incubated in a humidified water jacket incubator, gassed with 5 % CO_2 in air at 37°C.

Thymidine incorporation

At 18 h (except where indicated) before terminating the cultures, 0.02 μCi of methyl-(^{3}H)-thymidine (specific activity 45 Ci/mmole) (Radio-Chemical Centre, Amersham, U.K.) in 1 μl of RPMI-1640 was added to each well (*Trail et al.* 1981). The cultures were further incubated as hanging drops until harvesting.

Cell harvesting

Cell harvesting was done according to *O'Brien et al.* (1979) using a Titertek Microharvester (Flow Laboratories, U.K.). The filter discs were washed with 0.15 NaCl solution, 10 % trichloroacetic acid and 70 % methanol, in that order. The dry filters were placed in counting vials and 4.5 ml of scintillation liquid (Aqua Luma, Amersham, U.K.) were dispersed into each vial and counted for 5 min in a beta-counter (Beckman Instruments, Irvine, Ca., U.S.A.).

Data presentation

The data have been presented in 3 different ways: —

(a) log (cpm): the log of the mean of test cultures cpm minus control cultures cpm.
(b) cpm ± s: Mean counts per min ± standard deviation.
(c) Stimulation index (SI): mean counts per min of test culture/mean counts per min of control culture.

Control culture = culture incubated without mitogen.

RESULTS

Effect of mitogen dose. PBL were exposed to different concentrations of PHA and LPS (0—200 μg/ml). PHA gave optimal responses at a concentration of 3.125 μg/ml (Fig. 1), whereas no clear cut optimal concentration for LPS could be found, but LPS concentrations between 25 and 100 μg/ml gave high responses. A concentration of 50 μg/ml was arbitrarily chosen, and was used in experiments in which a single LPS concentration was used. All responses were depressed at doses above 100 μg/ml of both mitogens. LPS stimulation resulted in lower levels of (^{3}H) thymidine incorporation compared to PHA stimulation.

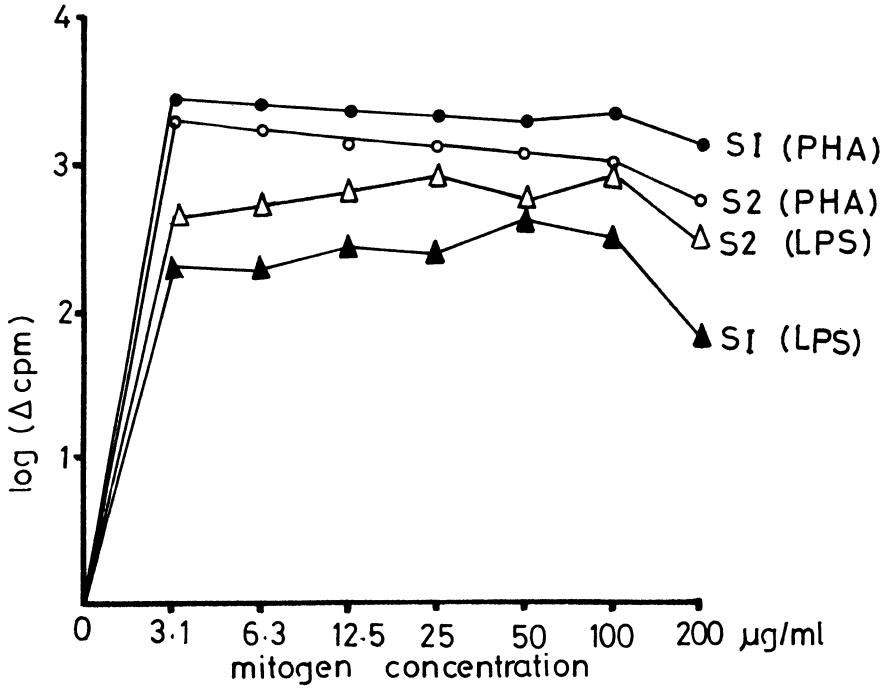

Figure 1. Effect of mitogen concentration on thymidine uptake (log (cpm)) by ovine PBL cultured in the presence of PHA and LPS. Thymidine incorporation measured during the last 18 h of a 66 h culture period. Cells were cultured in 15 % FCS at a density of 20×10^3/ml. S1 and S2 refer to preparations from two different animals.

Effect of time course on mitogen-induced (^{3}H)-thymidine incorporation. To determine the optimal time of maximum thymidine incorporation, different cell densities (10 to 80×10^3 cells/well) of the same PBL preparation were exposed to PHA and incubated for up to 146 h. At all cell concentrations used, adequate responses were obtained from 48 to 146 h of total incubation (Fig. 2). With time, the rate of thymidine incorporation appears to be inversely related to the cell density.

Effect of serum source and concentration. PBL were cultured in RPMI-1640 containing antibiotics and either lamb serum (LS) (Gibco, U.K.) or FCS (both inactivated at 56°C for 30 min) at various serum concentrations (0—20 %)

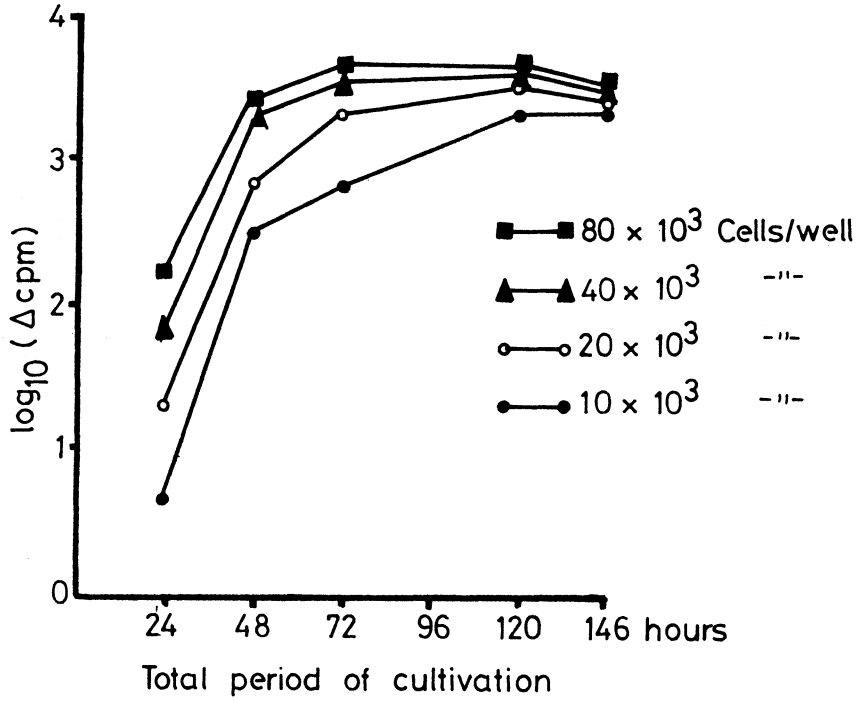

Figure 2. The effect of different total time of cultivation and cell concentration on thymidine uptake of ovine PBL cultured in 15 % FCS with 3.125 μg/ml PHA. The cultures were pulsed with thymidine during the last 24 h of the cultivation period.

in the absence or presence of 5×10^{-5} M — 2ME (Sigma Chemical Co., St. Louis, Mo., U.S.A.).

Only foetal calf serum supported ovine PBL responses to LPS in the presence of 2 ME (data not shown). LS failed to do so, whether 2 ME was incorporated into the culture media or not. In the absence of 2 ME, PBL responses to LPS were negligible.

The influence of 2 ME on thymidine uptake by cultured PBL in the presence of PHA and either LS or FCS was not clearcut. However, in all experiments 5×10^{-5} M — 2ME were incorporated into the media since ovine PBL gave detectable responses to LPS only in the presence of 2 ME.

FCS was found to enable optimal ^{3}H-thymidine incorporation by PHA at a 5 % concentration, whereas LS showed the highest stimulating effect at 2.5 %. These optimal concentrations were unaffected by the inclusion or exclusion of 2 ME in the

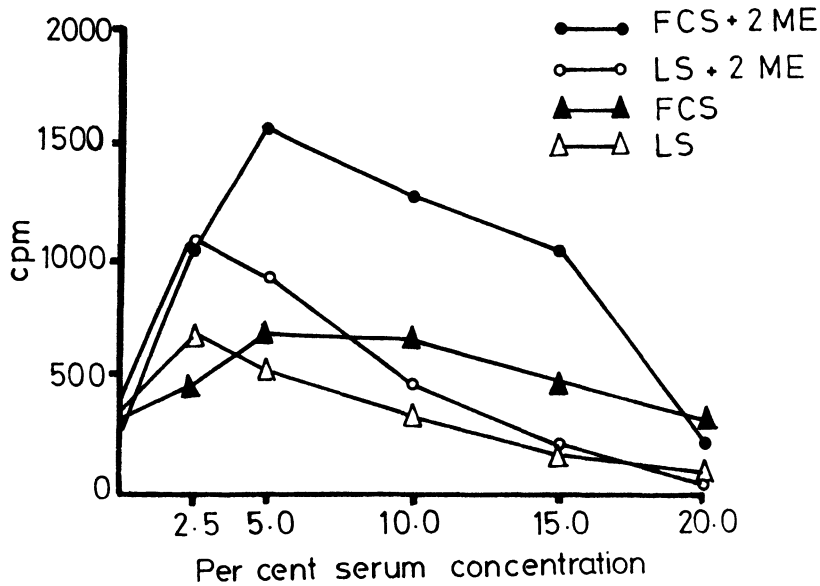

Figure 3. The effect of serum source and concentration on thymidine uptake by ovine PBL stimulated with optimal dose of PHA in either the presence or absence of 2-mercapto-ethanol. Cells were cultured at a density of 20×10^3 cells/ml and pulsed with thymidine during the last 24 h of a 66 h cultivation period.

culture media. At their respective optimal concentrations, FCS supported higher ^{3}H-thymidine incorporation than LS by ovine PBL cultured in the presence of PHA. Concentrations below optimal caused stronger inhibition of responses than the inhibition due to higher serum concentrations (Fig. 3).

Effect of cell concentration. The influence of cell concentration on the response of PBL to PHA and LPS were investigated by exposing differing numbers of PBL ($2.5—80\times10^3$/well) to the mitogens. Employing optimum PHA and LPS doses, cell densities between 20 and 80×10^3/well, gave high responses, and at lower densities the variation was greater than at higher densities (Fig. 4). Optimal cell concentration was not deducible from the results, although a cell concentration of either 20×10^3/well or 40×10^3/well ($1—2\times10^6$ cells/ml) was adopted in experiments in which fixed cell concentrations were used.

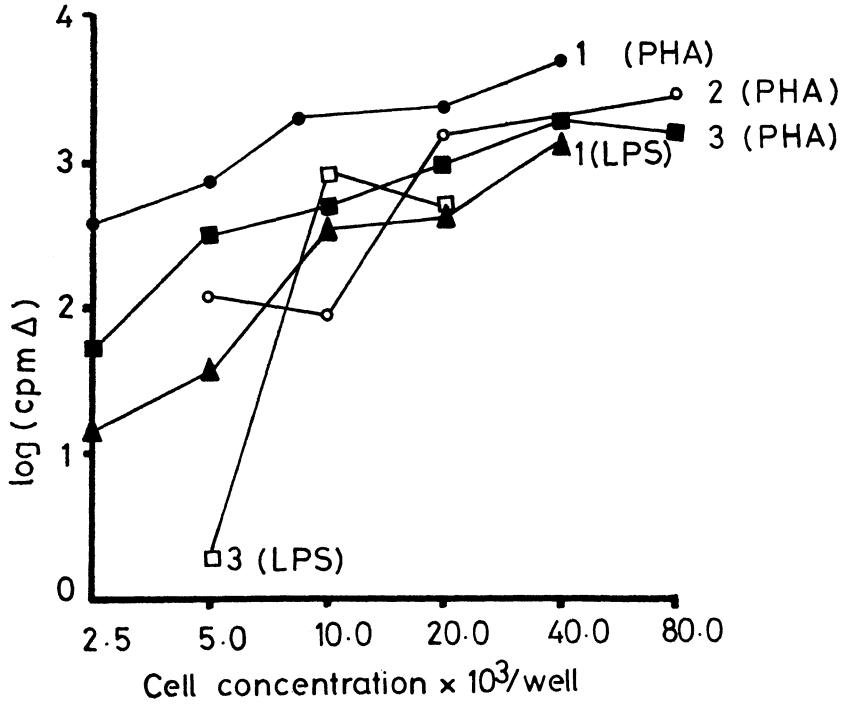

Figure 4. The effect of cell density on thymidine uptake by ovine PBL cultured with optimal doses of PHA and LPS in 5 % FCS. Thymidine incorporation measured during the last 18 h of a 66 h cultivation period. The numbers (1, 2 and 3) refer to preparations from 3 different animals.

Effect of depletion of phagocytic cells on ovine PBL responses to PHA and LPS. Responses of PBL (monocyte depleted cells) to PHA and LPS were greater than responses of peripheral blood mononuclear (PBM) cells (Table 1).

Mitogen reactivity of sheep lymphocytes after fractionation on nylon wool columns. PBL were filtered through nylon wool columns and the mitogenic responses of unpassed PBL and the non-adherent (NAD) fraction were examined using optimal doses of mitogens (LPS and PHA). Table 2 shows that the NAD fraction was responsive to PHA, whereas its responsiveness to LPS was markedly reduced compared to that of the unseparated cells. Cell recovery (NAD) was

Table 1. Effect of phagocytic cell depletion on thymidine uptake by ovine PBL incubated in the presence of PHA and LPS.

Animal No.	Mitogen	Responding cell population			
		PBM cpm[a]		PBL cpm	
1	Control	12.3	(3)	92.3	(51)
	PHA	1356.0	(263)	5706.0	(1090)
	LPS	201.0	(80)	2780.0	(849)
	PØ	8.9		0.9	
2	Control	97.3	(44)	644.7	(30)
	PHA	3137.6	(297)	4109.7	(276)
	LPS	934.0	(212)	3400.3	(343)
	PØ	8.1		0.3	
3	Control	405.5	(280)	75.7	(36)
	PHA	2952.7	(33)	3679.8	(175)
	LPS	740.0	(110)	2550.2	(405)
	PØ	5.7		1.2	

[a] Values represent the mean cpm triplicate culture ± standard deviation in brackets.

[b] PØ = percentage of cells ingesting — 3 latex particles PHA and LPS were used at 3.125 and 50 µg/ml, respectively. 40×10^3 cells were incubated for 66 h and pulsed with thymidine 18 h before terminating the cultures.

Table 2. Responses of nylon wool non-adherent (NAD) cells to PHA and LPS.

Experiment	Cell population	Control cpm (no mitogen)	PHA (3.125 µg/ml) cpm	LPS 50 µg/ml) cpm
1	PBL	95.8 ± 38.4[a]	2455.6 ± 157.4	1356.0 ± 122.2
	NAD	177.8 ± 44.7	2881.0 ± 360.3	321.7 ± 182.3
2	PBL	177.7 ± 39.3	3460.4 ± 509.1	346.4 ± 94.1
	NAD	29.7 ± 15.6	2542.4 ± 91.8	15.4 ± 1.9
3	PBL	11.0 ± 1.5	4373.5 ± 541.3	486.4 ± 50.8
	NAD	13.4 ± 3.3	3367.8 ± 525.1	106.5 ± 36.3

[a] Values represent the mean cpm (counts per min) of triplicate cultures ± standard deviation of 40×10^3 cells incubated for 66 h with optimal mitogen doses and pulsed with thymidine 18 h before terminating the culture.

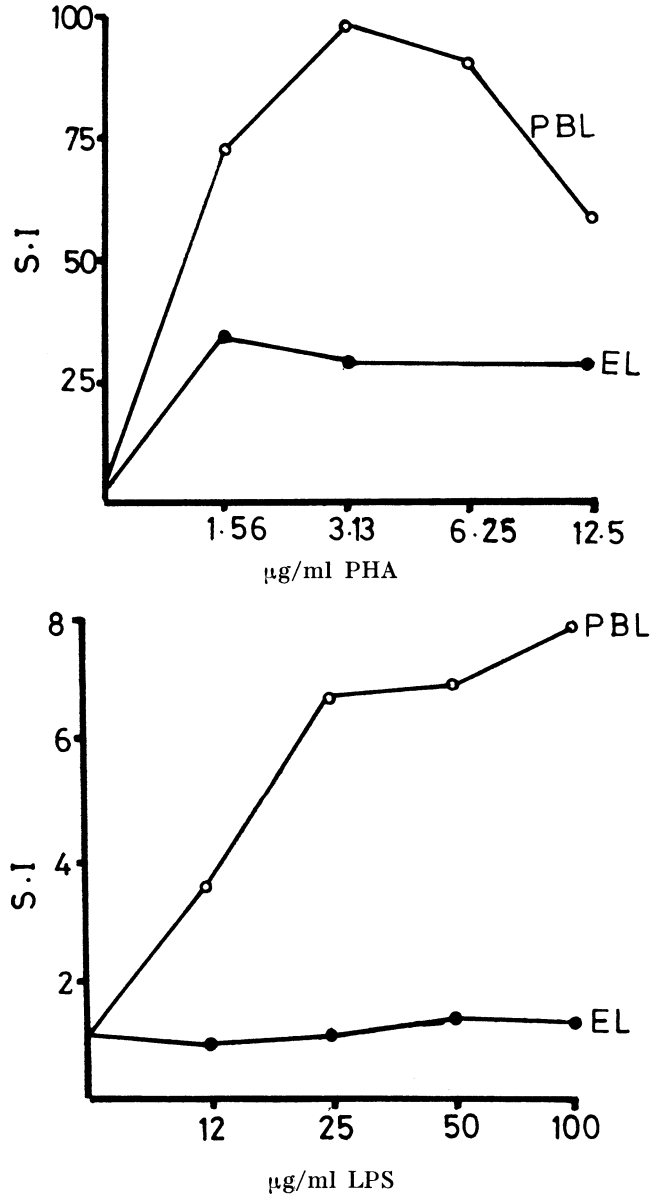

Figure 5. Stimulation indices (S.I) of 40×10^3 PBL and cells binding to Helix pomatia lectin (EL) to PHA (above) and LPS (below). The cells were cultured for 56 h and pulsed with 3H thymidine during the last 8 h.

Table 3. Summary of culture conditions suitable for studying ovine PBL responses to PHA and LPS in-vitro by the hanging drop micro-assay.

Parameter	Optimal condition	Comments
Culture media	RPMI-1640[a]	
FCS	5 %	2 ME essential for responses to LPS
LS	2.5 %	Does not support responses to LPS
Cell density	$1—2 \times 10^6$/ml	Used as a matter of convenience, optimal cell density could not be determined from the results
Mitogen concentration		
PHA	3.125 µg/ml	
LPS	50.00 µg/ml	
Total culture period	56—72 h	Chosen for convenience, incubation period of 48—146 h gave adequate responses
Thymidine pulsing time	Last 8—24 h[a]	[a]used successfully but not compared with other alternatives
Methyl ^{3}H-thymidine amount	0.02 µCi/well[a]	

36 ± 3.5 % ($\bar{x} \pm s$) in 3 experiments. In experiment 1, immunofluorescence revealed that 12 % and 75 % of PBL were SIg^+ and PNA^+, respectively, whereas NAD consisted of < 2 % SIg^+ cells and 93 % PNA^+ cells.

Mitogenic responses of PBL fractionated on HP-Sepharose-6MB column: The cell fraction (1), which was washed out of the column with PBS-BSA-NaN_3, responded to both LPS and PHA with stimulation indices of 36.5 and 238.6 being maximal for LPS and PHA, respectively.

Helix pomatia eluted cells. Compared to PBL, the responses of eluted (Fraction II) cells were lower (Fig. 5 a and 5 b). Whereas the responses to LPS were more or less abrogated, the mitogenic responses to PHA had stimulation indices greater than 20 at all mitogen concentrations employed.

DISCUSSION

This study indicates that the hanging drop microassay (HDMA) can be used to investigate PBL responses to both T- and B-cell mitogens. The results and optimal conditions (Table 3) are similar to those obtained by the usual micro-method (*Burrels & Wells* 1977, *Cahill et al.* 1978, *Staples et al.* 1981). Of the two assays, the DHMA is more economic because smaller quantities of reagents are needed, enabling the examination of many parameters simultaneously (*Ferranti et al.* 1980, *O'Brien et al.* 1979).

Lamb serum failed to support proliferation of PBL incubated with LPS, extending the findings of *Burrels & Wells* (1977) on autologous sheep serum. This may be due to the absence of synergistic stimulatory factors or presence of inhibitory factors in the sera.

Although the PBL were depleted of monocytes they responded vigorously to PHA. Therefore the carbonyl iron technique does not interfere with sheep lymphocyte responses to mitogens.

In guinea pigs (*Rosenstreich* 1976), mice and man (*Unanue* 1981), such a depletion reduces responses to PHA markedly. *Usinger et al.* (1981) reported no reduction of responses to mitogens after monocyte depletion and concluded that adherent cells were probably not required as accessory cells. But *Mastro & Sniezek* (1983/84) clearly showed that rigorous removal of monocytes diminishes responses to mitogens, affecting responses to Con A more than to PHA. The behavior of sheep PBL in this study is probably due to the residual contaminating monocytes. There may also exist other accessory cells which are not avidly phagocytic or adherent in ruminants, e.g. dendritic cells (*Kristensen et al.* 1982) which occur as a minor population but are extremely efficient as accessory cells.

PBL that were nylon wool non-adherent responded to PHA, but their responses to LPS, were markedly reduced. Similar results were obtained by *Cahill et al.* (1978) using lymph-borne cells. This finding, together with the fact that non-adherent cells are T-lymphocytes (*Mlangwa* 1984, *Fahey* 1980, *Fahey et al.* 1980, *Outteridge et al.* 1981) confirms and extends the findings of *Cahill et al.* (1978) that sheep T-cells respond to PHA and not LPS.

The Helix pomatia eluted cells (Fraction II) responded to PHA but their responses to LPS were markedly reduced. These

findings taken together with those of surface markers (*Mlangwa* 1984) indicate that the eluted cells are indeed T-cells or a cell population composed mostly of T-cells.

In Helix pomatia separation experiments, fraction I cells responded to both LPS and PHA. This was not unexpected because surface marker studies (*Mlangwa* 1984) revealed that fraction I cells, although enriched in B-cells, still had a substantial number of HP^+ cells.

It is probably the B-cell fraction of PBL that responded to LPS, since B-cell depletion (Fig. 5 b and Table 2) markedly reduced responses to LPS. Indeed, *Cahill et al.* (1978) showed that it is the B-cells that responds to LPS.

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SAMMENDRAG

Mitogenrespons for T-lymfocyter fra perifert blod hos får for phytohæmagglutinin (PHA) og lipopolysakkarid (LPS) ved mikroanalyse i hængende dråbe.

Dyrkningsforhold for studiet af lymfocyter hos får og deres respons til mitogener (PHA og LPS) i hængende dråbe mikroanalyse er undersøgt. Ved denne teknik undersøgtes responsen af formodede T-celler hos får til de to mitogener.

Resultaterne viste, at lymfocyter gav det største respons til LPS (50μg/ml) og PHA (3.125 μg/ml), når celletætheden var $1—2 \times 10^6$/ml, og når dyrkning blev foretaget i RPMI-1640 medium tilsat 2-mercaptoethanol og 5 % føtalt kalveserum, over en periode af 48—146 timer. Cellerne blev dyrket på Terasaki skåle i hængende dråbe kulturer (20 μl). Lammeserum kunne ikke benyttes ved LPS stimulation.

T-celler, fremstillet efter nylonvat teknikken og Helix pomatia Sepharose-6MB søjlekromatografi, gav kraftig respons til PHA, mens LPS udvirkede svag response.

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