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From the Department of Veterinary Virology and Immunology, Royal Veterinary and Agricultural University, Copenhagen, Denmark.

SHEEP PERIPHERAL BLOOD-T-LYMPHOCYTES: ISOLATION, SEPARATION AND SURFACE RECEPTORS FOR HELIX POMATIA AGGLUTININ AND PEANUT AGGLUTININ FROM ARACHIS HYPOGAEA

By

J. E. D. Mlangwa

MLANGWA, J. E. D.: *Sheep peripheral blood-T-lymphocytes: Isolation, separation and surface receptors for Helix pomatia agglutinin and peanut agglutinin from Arachis hypogaea.* Acta vet. scand. 1984, *25*, 536—547. — A study was made to develop and evaluate techniques for the isolation of ovine peripheral blood lymphocytes (PBL) and to investigate the suitability of peanut agglutinin (PNA) and Helix pomatia agglutinin (HP) as markers for sheep T-cells. The results show that sheep PBL can be prepared reproducibly by incubating ovine blood with carbonyl iron, centrifuging the blood over Percoll (colloidal silica polyvinylpyrrolidine) separating media (Pharmacia, Sweden) and harvesting the PBL at the interface. PBL so prepared, rarely undergo spontaneous agglutination, which is frequently seen with buffy coat cells and peripheral blood mononuclear cells. PNA and HP can be used as markers for ovine T-lymphocytes, because, under appropriate conditions, these lectins do not bind to B cells. Highly enriched peripheral blood T-lymphocytes were successfully prepared by the nylon wool technique and affinity column chromatography using HP. Highly purified B-cell sub-populations could not be prepared using the HP-Sepharose-6MB chromatography columns.

sheep; lymphocytes; T-cells; surface receptors; lectins; immunology.

The isolation, fractionation and characterization of lymphocytes and their distinct subpopulation is vital in studies of cellular immune mechanisms. In sheep, preparation of peripheral blood mononuclear cells by *Boyums* method (1976) frequently results in cells unsuitable for further fractionation, because of their great tendency to agglutnate (*Outteridge et al.* 1981, personal observation).

There enhanced erythrocyte rosette (Er) technique (*Binns* 1978, *Fahey* 1980, *Outteridge et al.* 1981) is technically cumbersome and probably identifies a subpopulation of T-cells. The availability of other surface markers such as T-cells specific antigens (*Chandra et al.* 1981, *Issekutz & Stoltz* 1983, and *Miyasaka et al.* 1983), and T-cells receptors for lectins such as PNA (*Fahey* 1980), can and have given further insight into the heretogeneity of sheep T-lymphocytes. Helix pomatia agglutinin (HP) is a lectin that has a binding specificity for α-N-acetyl-galactosamine > N-acetyl-glucosamine (*Hammarström* 1974). Neuraminidase treated human (*Hellström et al.* 1976), rat (*Swanborg et al.* 1977) and bovine (*Morein et al.* 1979) T-lymphocytes express HP receptors.

Lectin-ligand interactions are reversible, this property has made it possible to develop cell affinity chromatography techniques for separating HP binding (HP^+) cells from those lacking receptors (HP^-) in the above species (*Hellström et al.* 1976, *Swanborg et al.* 1977, *Morein et al.* 1979 and *Shrempf-Decker et al.* 1980). The purpose of this investigation was to identify a suitable method of isolating peripheral blood lymphocytes and to determine whether HP can be used as a marker for sheep T-cells and, if so, whether sheep lymphocytes could be fractionated to give a population enriched in T-cells. The relationship between cells with receptors for HP and PNA was also investigated.

MATERIALS AND METHODS

Animals

Icelandic ewes fed hay and pellets and kept in isolation stalls were used.

Blood

Blood was drawn from the jugular vein in a sterile manner into glass bottles containing preservative free heparin (Heparin Leo, Denmark) 10—20 I.U/ml final concentration and mixed immediately.

Colloidal silia polyvinylpyrrolidine (Percoll, Pharmacia, Sweden) was diluted in a one-step procedure as per manufacturer's instructions to make a separating medium with a density of 1.083 g/ml (SM). 3 ml of SM were then placed in 10 ml glass test tubes and 7 ml of undiluted blood layered on the

SM (*Boyum* 1976). The tubes were centrifuged at 1000 g, for 25 min at 18°C.

Cells at the interface were harvested, pooled and washed twice with a calcium and magnesium ion free balanced salt solution containing 5 I.U. heparin/ml and 5 % fetal calf serum (FCS).

Lysis of contaminating sheep red blood cells (SRBC)

Any contaminating erythrocytes were lysed, prior to washing. Briefly, the cell harvest was incubated with 0.83 % tris ammonium chloride at 20°C for 5 min after which cold PBS was added and washing carried out.

Preparations of peripheral blood lymphocytes (PBL)

Sephadex G-10 depletion of monocytes from PBM (Peripherial Blood Mononuclear Cells) was carried out as described by *Usinger et al.* (1981).

Plastic adherence depletion of monocytes from PBL was carried out according to *Usinger et al.* (1981), except that the cells were incubated for 30 min at room temperature.

Carbonyl iron ingestion technique. Phagocytic cells were removed according to *Jerrels et al.* (1980) with some modifications. 5—15 mg carbonyl iron (SF Garde, Fluka, Switzerland) per ml of blood was made into a suspension in RPMI-1640 containing 10 % FCS at a concentration of 0.5—1 g/ml. The carbonyl iron suspension was thoroughly mixed with blood and incubated at 37°C for 60 min on a rocking platform. The blood was then aspirated from the tubes while standing on the pole of a magnet and layered on SM. All subsequent steps were as for PBL preparation.

Nylon wool separation. Nylon wool (Leukopack, Fenwall Laboratories, Illinois, U.S.A.) was prepared according to *Greaves et al.* (1976). Separation was carried out according to *Usinger et al.* (1981), with some modifications. Before use, the columns were equilibrated with RPMI-1640 containing 10 % FCS, 25 mmol/l Hepes (Gibco, U.K.) and antibodies for 45 min at 37°C. During equilibration, any air pocket which appeared was removed using a sterile Pasteur pipette. Varying numbers of PBL were loaded into columns, allowed to run into the nylon wool, and in-

cubated at 37°C for 45 min. Non-adherent (NAD) cells were eluted from the column with 20—30 ml of 37°C medium.

Neuraminidase treatment. Cells were treated with neuraminidase, according to *Morein et al.* (1979) with some modifications. Briefly, 15 µl (18.75 µg) neuraminidase (Clostridium perfringens, type V, 0.2—0.87 U/mg (Sigma Chemicals, St. Louis, U.S.A.)) were added to about $25—30 \times 10^6$ PBL in 2 ml PBS and the cell suspension incubated at 37°C for 45 min on a rocking platform. The cells were then washed in PBS-BSA-NaN_3, and viability was about 98 %.

Immunofluorescent staining

HP conjugated with fluorescein isothiocyanate (HP-FITC) (E. Y. Laboratories, San Mateo, Ca., U.S.A.) was used, at a concentration of 1 mg/ml HP. HP^+ cells were detected by direct immunofluorescence. Briefly, to 100 µl cells (10^7 cells/ml) in HBSS-BSA-NaN_3 in 10×75 mm plastic tubes, 10 µl HP-FITC were added mixed thoroughly and incubated at 4°C for 30 min. The cells were then washed once in HBSS-BSA-NaN_3 and centrifuged through a 1 ml layer of FCS once at 4°C.

Wet smears were prepared and examined under U.V. light (Leitz Orthoplan microscope, Germany).

PNA conjugated with FITC (E. Y. Laboratories, San Mateo, Ca., U.S.A.) at a concentration of 1 mg/ml was used to detect PNA^+ cells. Labelling procedure and conditions were the same as for HP-FITC labelling.

B(SIg^+) cells were detected by the same technique using rabbit anti-sheep IgG fraction which was labelled with FITC (Cappel Laboratories, Cochranville, Pa., U.S.A.) at an optimal dilution of 1:10.

Specificity

To test for specificity of HP, neuraminidase treated lymphocytes were incubated with HP-FITC, in the presence of 250 µg of either D-N-acetyl-galactosamine or D-glucose.

The specificity of the rabbit anti-sheep IgG-FITC conjugated IgG was tested by incubating sheep lymphocytes with unlabelled rabbit antiserum to sheep serum, followed by the addition of the labelled conjugate after incubation and washing. Blocked preparations did not show fluorescence for SIg.

HP-Sepharose — MB column cell fractionation

The column was prepared according to the manufacturer's (Pharmacia, Uppsala) instructions with a few modifications. Briefly, about 4 ml of HP-Sepharose (4 mg HP) were poured into a 10 ml plastic syringe fitted with an 80 μm nylon wire mesh (Pharmacia, Sweden) bed support. The column was equilibrated with PBS containing 0.2 % bovine serum albumin (BSA) and 0.02 % Sodium azide (NaN_3). $60—80 \times 10^6$ neuraminidase treated sheep lymphocytes in PBS-BSA-NaN_3 were loaded onto the column, allowed to penetrate the bed and incubated for 15 min at room temperature. Unbound lymphocytes were washed out with 30 ml PBS-BSA-NaN_3 (Fraction I) and bound cells were eluted 0.2 % N-acetyl-D-glucosamine in PBS (Fraction II) after *Shrempf-Decker et al.* (1980).

Morphological examination of cells prepared by the carbonyl iron technique

50 μl of 0.01 % acridine orange solution in PBS were added to 50 μl cell suspension (10^6 cells per ml), mixed, and incubated for 5 min. A drop of the mixture was then placed on a microscope slide, coversliped and examined under incident ultra-violet light. On average, 98.6 % of the cells were lymphocytes (range 98—99.4 %), 0.8 % monocytes (0.3—1 %) and 0.6 % polymorphonucleated cells (0.05—1.1 %).

Viability and cell counts

Cell viability and counts were determined according to *Hudson & Hay* (1980). To improve viability, 5 % FCS was added to the dye solution.

Monocyte identification

Monocytes were identified by their ability to phagocytize 1.01 μm latex particles (Sigma, St. Louis, Mo., U.S.A.). Briefly, 1×10^6 cells in 2 ml RPMI-1640 containing 10 % FCS and antibiotics were mixed with 20 μl of 0.1 % latex suspension, incubated at 37°C for 45 min on a rocking platform. The cells were then layered on 1 ml of FCS twice and centrifugated at 200 g for 10 min at 4°C. Nigrosin solution was added to the cell pellet and wet smears prepared and examined, under oil immersion. At least 200 cells were examined, a cell with ≥ 3 cytoplasmic particles was scored as a monocyte.

RESULTS

Preparation of sheep PBL

Table 1 presents cell recoveries and percentage of cells with ingested latex particles of lymphocytes prepared in 4 different ways from blood. PBM prepared by centrifuging blood on separating media had a greater tendency to undergo spontaneous clumping, followed by cells prepared by plastic adherence. Cells prepared by the carbonyl iron technique had the same degree of latex particle ingesting cells (monocytes) as the cells prepared by passage of PBM through Sephadex G-10 columns. The main drawback of the latter was low cell recovery. Cells prepared by these two methods did not undergo spontaneous agglutination.

Table 1. Preparation of sheep peripheral blood lymphocytes.

Animal No.	Pre-depletion Percoll (PBM)		Post depletion					
			Sephadex-G 10		Plastic adherence		Carbonyl iron	
	Recovery [a]	% Latex	Recovery	% Latex	Recovery	% Latex	Recovery	% Latex
1	2.86	8	1.57	<1	ND	ND	1.52	5[b]
2	2.04	12	0.60	<1	1.37	9	1.16	<1
3	1.78	12	0.55	<1	1.06	7	1.00	<1
4	2.14	16	0.84	<1	ND	ND	1.04	<1
Mean ± s	2.22 ±0.56	12.0 ±3.3	0.89 ±0.47	<1	1.22 ±0.22	8.0 ±1.4	1.07 ±0.08	<1

[a] Recovery: $\times 10^6$ cells/ml of blood.
% Latex: the percentage of cells that had ingested $\geqq 3$ latex particles.

[b] Iron powder was added directly to the blood, incubated for 60 min with occasional shaking. This sample was excluded in calculating the carbonyl iron means.

HP and PNA receptors on ovine PBL

PBL were prepared by the carbonyl iron technique. The binding of HP and PNA to these PBL is presented in Table 2. Only neuraminidase-treated PBL were able to bind HP (64 %) and this binding was inhibited by incubating the cells in the presence of the specific ligand, N-acetyl-D-galactosamine but not with the non-specific sugar D-glucose. PNA bound to PBL (61 %), which were not treated enzymatically. The sum of SIg^+ cells plus either PNA^+ or HP^+ cells never exceeded 100 % in any row.

Table 2. Surface marker studies on sheep peripheral blood lymphocytes.

Animal No.	nHP%	HP%	PNA%	nHP (D Gal. NAC) % a	nHP (D glucose) % a	SIg%
1	64	0.1	66	0.1	69	15
2	73	0.1	63	0.1	72	17
3	56	0.1	53	0.1	57	19
Mean	64.3	0.1	60.7	0.1	66	17
± s	±8.5	—	±6.8	—	±7.9	±2.0

n — neuraminidase treated lymphocytes.
[a] Cells incubated with 250 μg of the sugar (in brackets) during labelling.
PNA — Cells bearing peanut agglutination receptors.
HP — Cells with receptors for Helix pomatia lectin.
SIg — Surface immunoglobulin bearing (B) cells.
D Gal. NAC = D — N acetyl — Galactosamine.

Double labelling experiments

PBL incubated overnight at 37 °C in 5 % CO_2 at mosphere were used. They were labelled with FITC conjugated rabbit anti-sheep IgG and tetramethyl rhodamine isothicyanate labelled lectins (either PNA or HP) simultaneously without or after neuraminidase treatment. Wet smear were made and examined for fluorescence. Almost all SIg^+ cells examined were positive for PNA receptor, in neuraminidase treated PBL, whereas < 1 % of SIg^+ positive cells stained for HP receptors. Untreated SIg^+ cells did not bind PNA.

Table 3. Separation of sheep peripheral blood lymphocytes on nylon wool columns.

Experiment	Amount of nylon wool	Cells loaded ($\times 10^6$)	Before separation			Non-adherent fraction			
			HP%	PNA%	SIg%	Yield%	HP%	PNA%	SIg%
1	600 mg	72	74.4	61.3	16.2	21.9	93.9	88.8	0.5
2	500 mg	100	78.0	68.0	12.0	47.0	93.0	92.0	3.0

HP — Lymphocytes bearing HP receptors.
PNA — Cells possessing peanut agglutination receptors.
SIg — Cells with surface immunoglobulins.

Nylon wool separation

Separation of PBL, prepared by the carbonyl iron technique on nylon wool columns (Table 3), resulted in a non-adherent fraction (NAD) that was highly enriched in HP (93.5 %) and PNA (90.4 %) binding cells. The recovery of NAD cells was 34.5 % of the cells loaded onto the columns.

HP column fractionation

Table 4 shows the results of 2 different experiments. An average of 24 % and 37 % of the loaded cells were recovered in fractions I and II, respectively. The cells eluted with N-acetyl-D glucosamine (Fraction II) were enriched in HP-binding cells (average 93 %) and depleted of most of the SIg-bearing cells (average 4 %). Although Fraction I was enriched in SIg^+ cells (average 34.5 %), this fraction also contained a substantial number of HP-binding cells.

Table 4. Fractionation of neuraminidase-treated lymphocytes on HP-lectin Sepharose 6MB columns.

Experiment	Before separation			Fraction I			Fraction II		
	Input%	HP%	SIg%	Yield%	HP%	SIg%	Yield%	HP%	SIg%
1	100	73	21	24	53	36	50	91	4
2	100	72	13	24	ND	33	24	95	4

HP — Lymphocytes possessing Helix pomatia lectin receptors.
SIg — Lymphocytes bearing surface membrane immunoglobulins.
Fraction I — the cell fraction washed out with PBS-BSA-NaN_3.
Fraction II — the cell fraction eluted with 2 mg/ml D-glucose NAC.
ND — Not done.

DISCUSSION

It was observed that sheep PBM prepared according to a modification of *Boyum*'s (1976) method, frequently underwent spontaneous agglutination as reported by *Outteridge et al.* (1981). The spontaneous agglutination made it difficult to isolate highly enriched lymphocyte sub-populations. In addition, *Fahey* (1980) showed that PNA can be used as a marker of presumptive ovine T-cells, provided phagocytic cells were separated from PBL. It was essential to deplete phagocytes in this study because PNA was used as a surface marker for T-cells. Incuba-

tion of blood with carbonyl iron, followed by separation on percoll appeared to be superior to the other methods (Table 1) of phagocyte removal. Fortunately, monocyte removal abrogated agglutination. It, therefore, appears that the presence of monocytes in PBM and buffy coats, facilitates spontaneous clumping (*Outteridge et al.* 1981). The poor PBL recovery of PBM cells passed through sephadex G-10 columns was probably due to the trapping of cells in clumps. Similarly, the high contamination of monocytes by lymphocytes (*Mlangwa* 1983 and *Fiscus et al.* 1982) prepared according to *Kumagai et al.* (1979), may be attributed to the binding of PBL to the monocytes. Approximately similar percentages of ovine PBL bound HP and PNA (Table 2).

The behaviour of the cells was similar in nylon wool separation experiments. The nylon wool non adherent lymphocytes (NAD) and those eluted out of the Helix pomatia columns (HP^+ cells) were highly depleted of SIg^+ (B) cells. The NAD did bind PNA, a T-cell marker in sheep (*Fahey* 1980) and bovine (*Pearson et al.* 1979, *Usinger & Splitter* 1981 and *Johansson & Morein* 1983) and HP lectin, a bovine T-cell marker (*Morein et al.* 1979 and *Johansson & Morein* 1983). This suggested that both HP^+ and PNA cells were T-cells. Indeed double staining cells, PNA^+/SIg^+, were negligible, confirming the findings of *Fahey* (1980) and extending those of *Morein et al.* (1979) to the sheep. These two lectins, can be used to identify presumptive ovine T-cells. The notion that one is dealing with T-cells, is strengthened by the finding (*Mlangwa* 1984) that NAD and HP^+ cells responded vigorously to a T-cell mitogen (PHA) but weakly to LPS, a B-cell mitogen. The suitability of PNA as a marker for sheep T-cell has been questioned. Unpublished observations of *Miyasaka & Dudler* (*Miyasaka et al.* 1983) revealed that ileal Peyer's patch SIg^+ cells also have PNA receptors. There is, therefore, a need for a T-cell marker that will recognize T-cells only, regardless of the source of the lymphocytes. To this end, the development of anti-T-cell monoclonal antibodies (*Issekutz & Stoltz* 1983 and *Miyasaka et al.* 1983) is encouraging.

The uniformity and potential unlimited availability of monoclonals to different laboratories may allow meaningful comparison of results between different laboratories.

Even when specific anti-T-cell monoclonals are available, however, lectins may be able to shed additional light onto the heterogeneity of lymphocytes with respect to surface markers

and functional properties. Care should be taken when both lectins (PNA and HP) are used as markers of ovine T-cells, because treatment of PBL with neuraminidase, which is required to expose HP receptors on T-cells, also exposes PNA receptor on sheep B-cells.

Helix pomatia A — Sepharose — 6MB columns were used to fractionate sheep PBL to obtain a fraction highly enriched in HP binding cells and depleated of B-cells (Fraction II). These results are similar to those of *Morein et al.* (1979) in bovine and of *Hellström et al.* (1976) in man. Unlike the results in man and bovine (vide supra), a population highly enriched in SIg^+ was not obtained.

The cells that were washed out of the column (Fraction I) contained a relatively large proportion of HP binding cells. This is probably due to the existence of heterogeneity among the HP binding cells, in such a way that those cells with lower total binding capacity (avidity) are washed through the column before carrying out specific elution. The heterogeneity with respect to avidity may reflect either a heterogeneity among the HP binding affinities of receptors or differences in the number of receptors at cellular level or a combination of both.

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SAMMENDRAG

Perifere T-lymfocyter fra føreblod: Isolering, separering og overflade receptorer for Helix pomatia agglutinin (HP) og jordnødde agglutinin (PNA) fra Arachis hypogaea.

Undersøgelsen blev gennemført med henblik på at udvikle og evaluere teknikker til isolering af ovine, perifere blodlymfocyter (PBL) og med henblik på at undersøge egnetheden af jornødde agglutinin (peanut agglutinin, PNA) og Helix pomatia agglutinin (HP) som markør for føre T celler.

Resultaterne viser, at ovine PBL kan præpareres reproducerbart ved at inkubere føreblod med carbonyl jern, centrifugere i Percoll (kolloidt silicium polyvinylpyrrolidin) separeringsmedium og derefter at høste PBL fra interfasen. PBL, der er præpareret efter denne metode, agglutinerer sjældent spontant, hvilket ellers ofte ses med buffy coat celler og perifere blod mononukleære celler.

PNA og HP kan benyttes som ovine T celle markører, fordi disse lektiner under egnede betingelser ikke bindes til B celler. Højt oprensede perifere blod-lymfocyter kunne med godt resultat præpareres ved hjælp af nylon uld teknikken og affinitetssøjle kromatografi med HP, men derimod ikke med HP-Sepharose-6MB kromatografi søjler.

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Reprints may be requested from: J. E. D. Mlangwa, the Department of Veterinary Virology and Immunology, Royal Veterinary and Agricultural University, 13 Bülowsvej, DK-1870 Copenhagen V, Denmark.