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Comparison of the direct antiglobulin rosetting reaction (DARR) and direct immunofluorescence (DIF) for demonstration of sIg-bearing lymphocytes in pigs, sheep and cattle

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Summary. Tests with untreated and trypsin-treated red cells (RBC) from a variety of species showed that anti-Ig-coupled pig RBC are good indicator cells for the study of ruminant blood sIg^+ lymphocytes by the DARR test; coupled donkey and rabbit RBC are suitable for investigating pig lymphocytes. The different species showed the following percentages of sIg^+ lymphocytes ($M \pm SE$) by direct immunofluorescence (DIF) and the direct antiglobulin rosetting reaction (DARR) respectively: pigs $9{\cdot}2 \pm 0{\cdot}7\%$ and $16{\cdot}3 \pm 1{\cdot}2\%$; sheep $20{\cdot}2 \pm 1{\cdot}2\%$ and $33{\cdot}1 \pm 1{\cdot}6\%$; Cattle $13{\cdot}5 \pm 1{\cdot}4\%$ and $28{\cdot}9 \pm 3{\cdot}5\%$. The mean ratio of sIg^+ lymphocytes shown by the two tests (DARR/DIF) for each species was $1{\cdot}80 \pm 0{\cdot}08$ for pigs, $1{\cdot}73 \pm 0{\cdot}7$ for sheep and $2{\cdot}15 \pm 0{\cdot}18$ for cattle. Preincubation of pig and sheep lymphocytes at 37° for 1 h did not alter the proportion of sIg^+ lymphocytes detected by either test. Thus the DARR test reveals a further population of sIg^+ lymphocytes in addition to that detected by immunofluorescence, whose number is proportional to the B population as measured by DIF and whose sIg is intimately associated with the membrane.

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INTRODUCTION

Definition of lymphocyte subpopulations in domestic animals is becoming important for the investigation of natural and acquired disease resistance. We have used the mixed antiglobulin rosetting reaction (Binns, Feinstein, Gurner & Coombs, 1972a) and direct immunofluorescence (Binns, Symons & White, 1972b; Binns & Symons, 1974) with specific anti-immunoglobulin sera to reveal B cells in foetal and post-natal pigs. Foetal and post-natal sheep B cells have been counted using direct immunofluorescence (Symons & Binns, 1975). The direct antiglobulin rosetting reaction (Parish & Hayward, 1974; Haegert & Coombs, 1976) has proved a sensitive alternative to direct immunofluorescence for revealing B lymphocytes in rats, humans, rabbit and guinea-pigs (Coombs, Wilson, Eremin, Gurner, Haegert, Lawson, Bright & Munro, 1977). Rosetting methods have the added advantage of allowing subsequent isolation of rosetted lymphocytes by centrifugation on a density 'step'. This communication defines conditions for sensitive detection of pig and ruminant B lymphocytes using the DARR reaction and compares the sensitivity of DARR and DIF tests in detection of surface Ig in these species.

MATERIALS AND METHODS

Animals

Studies of sIg^+ lymphocytes were made on the follow-

ing blood samples: thirty-one samples from twenty-five Large White pigs aged 3·6 weeks to 6·3 years with twenty-four samples taken between 13 and 19 weeks; thirty-two samples from twenty-seven Clun Forest (CF) or CF cross sheep aged 5 months to 10·8 years (four sheep > 2 years old); and nine samples from nine Jersey cows aged 3–13·5 years.

Red blood cells

Indicator red cells were obtained from the standard Jersey cow T4, Donkey 'Rosetta', two young Large White pigs 6906 and 6911, a New Zealand White rabbit and a human O, Rh+ donor. All blood was taken into acid citrate dextrose except that of the donkey which was taken into Alsever's solution. The red cells were normally coupled with antibody within 3 days and in most cases were fresh.

Lymphocyte preparation

Lymphocytes were prepared from defibrinated blood, taken from the anterior vena cava of pigs and jugular vein of sheep and cows by methods detailed elsewhere (Binns, Pallares, Symons & Sibbons, 1977; Binns, 1978). Briefly, after carbonyl iron treatment, sheep and cow blood erythrocytes were removed by incubating with 5 volumes of glycerol PBS for 30 min at room temperature followed by centrifuging and rapid resuspension in 5 volumes of PBS, when erythroid cells lyse. Four per cent glycerol in PBS (v/v) was used for sheep blood and 5% for cow blood. The resulting lymphoid cell pellet was washed four times, suspended in PBS at 10^7/ml and kept on ice until used. Erythrocytes were removed from pig blood by a 20–30 min incubation with a 1/4 volume of 'Dextraven 150' at 37°. After erythrocyte sedimentation, the supernatant was removed, centrifuged and the cell pellet resuspended in 2 ml PBS, layered and centrifuged on Ficoll/Triosil. The resulting lymphocyte layer was washed and resuspended as for ruminant lymphocytes.

Preparation of fluorescent anti-IgG and DARR indicator red cells

Each lymphocyte preparation was divided, one sample for study of sIg by direct immunofluorescence (DIF) using fluorescein conjugated IgG antibody and the other for study of sIg by the direct antiglobulin rosetting reaction (DARR) using the same IgG antibody (unconjugated) linked to red cells by chromic chloride treatment. The methods are detailed elsewhere for DIF on pig (Binns & Symons, 1974) and sheep lymphocytes (Symons & Binns, 1975). The method for preparation and standardization of DARR indicator cells using untreated or trypsin-treated (TT) red cells is as described for study of human and other species' lymphocytes (Coombs et al., 1977). Surface Ig was studied using IgG preparations from antisera containing anti-light and heavy chain antibodies. Pig anti-ruminant IgG_2 (5824/BO) was used for sheep and cattle lymphocytes and sheep anti-pig IgM (V455/4, a reagent with only minimal reactivity with pig IgG shown by reverse passive haemagglutination) for pig lymphocytes.

A variety of red cells were tested for use in the DARR test: for ruminant lymphocytes, untreated and trypsin-treated bovine and pig red cells were tried; for pig lymphocytes we used untreated human, rabbit, donkey and bovine and trypsin-treated bovine and donkey red cells (red cells which form E rosettes were avoided). After coupling, each red cell batch was tested (i) for undesirable spontaneous agglutination, (ii) for agglutination by anti-IgG directed against the IgG coupled onto the red cell surface and (iii) for reverse passive haemagglutination by the same species Ig as was being studied on the lymphocyte surface. The titre in the reverse passive haemagglutination test is related to the sensitivity of the indicator red cell in the detection of sIg by rosette formation. Coupled red cells were normally used within 1 week.

Rosette formation and counts of sIg^+ lymphocytes

The methods used in centrifugation and reading of rosettes were similar to those used for other lymphocyte markers in pigs (Binns et al., 1977) with the difference that centrifugation of rosettes was at ~225 *g* and 4°. The rosettes were counted after 1 h incubation in the pellet at 4°. After initial tests the number of lymphocytes and indicator red cells were reduced from 1 drop lymphocytes at 4×10^6/ml with 2 drops 1% RBC to 1 drop lymphocytes at 2×10^6/ml with 1 drop 1% RBC without affecting the sensitivity of the test. In this way the whole pellet could be mounted and rosettes counted.

Each lymphocyte sample was read two to five times in the DARR test counting rosettes per ~300 lymphocytes. DIF tests were read one to three times. Data are shown as mean and where appropriate ± standard error (M ± SE).

Preincubation of lymphocytes in Ig-free medium

Investigations were made of the effect of incubation of lymphocytes at 37° on the proportion of sIg^+ lympho-

cytes using the two methods. Lymphocytes at 10^7/ml in MEM (Minimal Essential Medium with Hanks's salts, with 25 mM HEPES and without L-glutamine) 5% foetal calf or presuckled piglet serum in siliconized tubes were incubated for 1 h at 37°, and on ice as a control, and then washed three times. The first wash was at room temperature and subsequent washes at 4°. (Tubes were siliconized by rinsing with silicone fluid MS 1107 (Hopkins & Williams Ltd.) diluted to 2% in ethyl acetate following by drying in a hot air oven. The tubes were rinsed with deionized water before use.) Counts were made before and after treatment to assess cell loss.

RESULTS

Examination of several red cells as indicator cells in the DARR test on pig and sheep lymphocytes (Table 1)

In initial tests for study of sIg on ruminant lymphocytes the standard trypsin-treated (TT) bovine RBC method used for other species (Coombs *et al.*, 1977) was tried, in spite of the possibility that contaminating bovine Ig in the RBC suspension might cause spontaneous agglutination. Trypsin-treated pig RBCs were also used. These RBCs are of the same species as the antiserum-donor (pig anti-ruminant IgG). Table 1, Experiment 1 shows that DARR proved more sensitive than DIF and that both TT bovine RBC and TT pig RBC produced good strong rosettes in similar numbers in testing sheep lymphocytes. TT bovine RBC, however, showed a tendency to spontaneous haemagglutination as anticipated making resuspension and reading of rosettes difficult. These trypsin-treated red cells also tended to form weak rosettes with a small proportion of granulocytes. Similar specific rosettes (not shown in the Table) were also formed with bovine lymphocytes (DIF, 10·0%; DARR with TT bovine RBC, 22·6%; with TT pig RBC, 19·8%). In control tests no rosettes were formed (i.e. $<0{\cdot}5\%$) by anti-ruminant Ig coated red cells with pig lymphocytes (previously incubated in MEM/foetal calf serum and washed). Likewise in further control tests no rosettes

Table 1. Comparison of different red cells as carrier indicator cells in the DARR test for demonstration of sIg^+ lymphocytes from sheep and pig blood

Expt No.	Lymphocyte origin	No. of samples	DIF or carrier RBC for DARR	sIg^+(%) M±SE	Standardization of coupled indicator red cells by: Spontaneous agglutination	Antiglobulin* reaction	Reverse passive* haemagglutination for Ig antigen
1	Sheep	1	DIF	29·8			
			TT.Bov.†	40·1	+		
			TT.Pig	37·9	–	>500	>500
2	Sheep	7	DIF	21·9±2·9			
			TT.Pig	40·0±3·5	–	40	>500
			Pig	37·9±2·6	–	80	>500
3	Pig	3	DIF	10·4±1·2			
			TT.Bov.	20·9±1·2	±	10	160‡
			Donkey	20·8±1·3	–	10	160
			Rabbit	22·1±1·0	–	10	80
			Human	14·7±2·0	–	40	10
4	Pig	3	DIF	14·4±2·5			
			TT.Donk.	22·0±1·4	–	20	500
			Donkey	24·7±2·4	–	40	500

Experiment 2 employed seven samples from four sheep tested over 5 days.
Experiments 3 and 4 were on six samples from five pigs; each day three pigs were bled, one on both days.
* Titration end point; dilution in thousands.
† TT, trypsin treated.
‡ Tests using bovine, donkey, rabbit and human serum revealed no agglutination of coupled donkey red cells through cross-reaction with sheep anti-pig Ig.

were formed around sheep, cow or pig lymphocytes with indicator red cells coated with normal pig IgG. In all subsequent tests control Ig coated indicator red cells formed no rosettes with the lymphocytes tested ($<1\%$). Accordingly these results are not detailed.

In Experiment 2, TT pig RBCs were compared with untreated pig red cells as indicator red cells after coupling. Seven lymphocyte preparations from four sheep were tested over 5 days. As shown in Table 1 pig RBCs are as sensitive as TT pig RBCs both in rosette formation and in detection of sheep Ig by reverse passive haemagglutination. The untreated red cells also had even less tendency to rosette with granulocytes. In all subsequent tests on ruminant lymphocytes, untreated pig red cells coupled to pig anti-ruminant IgG were used.

Similar studies were made to select the indicator red cell for investigating pig lymphocytes. In Experiment 3 (Table 1) TT bovine and untreated donkey and rabbit red cells (none of which form rosettes spontaneously with pig lymphocytes either in saline or dextran, Binns, 1978) coupled to sheep anti-pig Ig proved equally sensitive indicator cells both for rosette formation and in reverse passive haemagglutination with pig Ig. TT bovine RBC, however, again showed a tendency to form weak rosettes with some granulocytes. In contrast, rosettes with human red cells coupled with sheep anti-pig IgM were weaker and the number of lymphocytes rosetting were only marginally higher than revealed in the DIF test. The coupled human RBC were also less sensitive to reverse passive haemagglutination by pig Ig. Experiment 4 (Table 1) showed that coupled untreated donkey RBCs were as sensitive as TT donkey RBCs both in rosette formation and in reverse passive haemagglutination by pig Ig. Coupled untreated red cells from our consistent donkey 'Rosetta' were used in all subsequent tests with pig lymphocytes.

Enumeration of sIg$^+$ lymphocytes from pig, sheep and cattle: a comparison of the DIF and DARR test for revealing B lymphocytes

Table 2 summarizes the results obtained from pig, sheep and cattle lymphocytes. As was indicated in Table 1 the DARR test revealed substantially more sIg$^+$ lymphocytes than the DIF test, the average ratio DARR/DIF being 1·80, 1·73 and 2·15 respectively for the pig, sheep and cow. This ratio was significantly ($P<0·1\%$) greater than unity for each species. Figure 1 details the proportions of sIg$^+$ lymphocytes revealed by the two methods. Thus for each species DARR revealed nearly twice the number of sIg$^+$ lymphocytes, independent of the size of the B cell pool.

The possibility that the extra population of positive lymphocytes had acquired immunoglobulin passively

Table 2. Summary of the proportions of lymphocytes bearing sIg in pig, sheep and cattle blood shown by the DIF and DARR techniques

	Species		
	Pig	Sheep	Cattle
Number of samples	31	32	9
Age Mean	41·8w	2·3y	6·6y
Range	(3w – 6·3y)	(0·46 – 10·6y)	(2·9 – 13·5y)
DIF (%)			
Mean (SD)	9·2 (3·9)	20·2 (7·0)	13·5 (4·1)
Range	(2·7 – 18)	(6·5 – 39·6)	(7·6 – 20·1)
DARR (%)			
Mean (SD)	16·3 (6·7)	33·1 (8·9)	28·9 (10·6)
Range	(4·4 – 30)	(19·8 – 53·6)	(14·7 – 43·6)
DARR/DIF*			
Mean ± SE	1·80 ± 0·08	1·73 ± 0·07	2·15 ± 0·18
Range	(1·19 – 2·89)	(1·13 – 3·18)	(1·44 – 3·25)

* DARR/DIF is significantly different from 1 for all species ($P<0·1\%$).

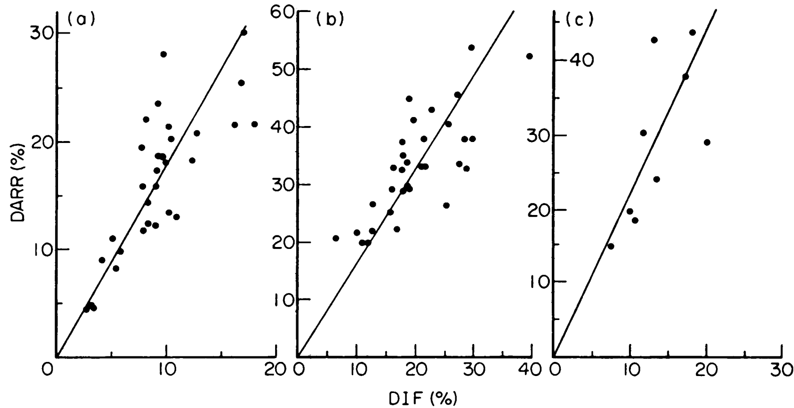

Figure 1. The proportions of sIg^+ lymphocytes in (a) pig, (b) sheep and (c) cattle blood detected by the DARR and DIF techniques. The lymphocytes preparations are from thirty-one pig, thirty-two sheep and nine cattle blood samples. Each point plots the mean DIF and mean DARR from a lymphocytes preparation. The DARR technique always revealed more sIg^+ lymphocytes than DIF. The relationship between the numbers of sIg^+ detected by the two methods was examined statistically. Since both variables are subject to random variation, conventional linear regression techniques are not appropriate and a more realistic alternative method was chosen, by which a line was drawn for each species which minimized the sum of the squared perpendicular distances to the line of all points (Kendall & Stuart, 1961). Since the intercepts were not significantly different from zero, the linear relationship finally taken was of the form $y = bx$ (i.e. a straight line through the origin). This relationship for the three species was: pigs, $y = 1{\cdot}778x$; sheep, $y = 1{\cdot}623x$; and cattle, $y = 2{\cdot}218x$. The coefficient 'b' was significantly ($P < 0{\cdot}1\%$) greater than unity for each species.

Table 3. The effect of pre-incubation in medium at 37° on the number of sIg^+ pig and sheep lymphocytes

Animal	DIF ± SE (%)		DARR ± SE (%)	
	4°	37°	4°	37°
Pig 1	10·3±0·9 (2)*	9·7±2·7 (2)	21·5±0·8 (5)	19·1±0·9 (4)
Pig 2	18·0±0·6 (3)	17·5±0·6 (3)	21·5±1·1 (5)	21·0±1·4 (5)
Sheep 1	17·8±0·4 (3)	16·8±0·4 (3)	32·5±0·3 (5)	32·7±1·6 (5)
Sheep 2	15·9±0·6 (3)	15·8±0·8 (3)	24·5±1·5 (5)	25·5±0·5 (5)

* Number of estimations (i.e. two to five counts on different slides)

Lymphocytes incubated in siliconized tubes at 10^7/ml medium with 5% presuckled serum for 1 h either on ice or at 37° followed by washing three times. In pigs 1 and 2 and sheep 1 the first wash was with medium at room temperature and in sheep 2 with medium at 37°. Subsequent washes used cold medium.

Pig 1 and 2 and sheep 1 lymphocytes were incubated in medium with foetal calf serum and sheep 2 in medium with presuckled pig serum. Counts before and after incubation showed no loss of cells.

that could be eluted by incubation at 37° was tested on pig and sheep lymphocytes. Table 3 shows that there was no reduction in sIg^+ lymphocytes detected by either method after incubation at 37° in medium free of the Ig of the species of lymphocyte under test (see footnote Table 3). Thus both tests reveal surface Ig which is intimately associated with the lymphocyte membrane and apparently not passively acquired. The cattle were the only species with a uniform distribution of ages, and these showed a decrease in percentage of sIg^+ lymphocytes detected by both methods with increasing age, similar to that seen for sheep using DIF (Symons & Binns, 1975). In the other species the mass of data were obtained from young animals, pigs mainly 13–19 weeks and sheep 5–20 months.

DISCUSSION

This study shows that the direct antiglobulin rosetting reaction provides a sensitive alternative to direct immunofluorescence (Binns & Symons, 1974; Symons & Binns, 1975) for the quantitation of surface Ig bearing lymphocytes in pigs, sheep and cattle. Like other rosette methods, however, choice of the species of the indicator red cells is important. Use of red cells which

form 'spontaneous' rosettes with the lymphocytes under test would lead to complications in the interpretation of the DARR rosettes. Sheep and goat red cells form rosettes with pig and ruminant T lymphocytes (Escajadillo & Binns, 1975; Binns, 1978). Therefore we have avoided the use of these species of RBC in spite of the fact that their use rules out a second possible complication: namely that Ig contaminating the red cells may agglutinate the indicator red cells coupled to sheep anti-pig Ig by reverse passive haemagglutination.

Our initial results with trypsin-treated bovine red cells, which are suitable for DARR tests on human, rat, rabbit and guinea-pig lymphocytes (Coombs *et al.*, 1977), showed that these red cells tend to agglutinate after coupling with pig anti-ruminant Ig even if previously washed six times. Although they formed good rosettes, the tendency to 'spontaneous' agglutination led to variability of results and difficulty in counting. On the other hand our finding that untreated pig red cells are satisfactory proved fortunate not only in that the anti-bovine Ig serum was raised in a pig but also because trypsin-treatment has inherent problems. We are thus able to avoid the variability of indicator red cells that results from trypsinisation and the problem we encountered of weak rosette formation of Ig coupled TT RBC with polymorphs. In the same way untreated donkey and rabbit RBC were superior to trypsin-treated bovine RBC, forming good rosettes round pig lymphocytes when coupled with antibody, without non-specific rosette formation when coupled with control Ig. Our choice of donkey over rabbit RBCs allowed us to have a single consistent donor and avoided the great care which has to be taken when rabbit red cells are used. Although both these red cells gave good rosettes in numbers similar to TT bovine RBC, it is of interest that human RBC coupled at the same time were inferior both in rosette formation and in reverse passive haemagglutination with Ig even though they proved more agglutinable by anti-Ig in the antiglobulin reaction.

Our results on pig, sheep and cattle lymphocytes confirm the findings of Haegert, Hurd & Coombs (1978) on human lymphocytes that the DARR test is more sensitive than DIF for revealing sIg^+ lymphocytes. Whereas our DIF results are consistent with previous findings in pigs (Binns & Symons, 1974) and sheep (Symons & Binns, 1975), the DARR test always revealed more sIg^+ lymphocytes. Nevertheless there is a broad correlation between numbers shown by both tests with nearly twice as many sIg^+ lymphocytes being revealed by the DARR test in each species in spite of the variation in numbers of B lymphocytes. The exact nature of the DARR positive, DIF negative population is of the greatest interest. It would appear that the cells shown by DARR alone have not acquired their immunoglobulin passively since incubation at 37° of both pig and sheep lymphocytes did not alter the proportion of sIg^+ lymphocytes detected by either DIF or DARR. The extra population was not forming rosettes through the Fc receptor since indicator red cells coupled with normal IgG form $<0{\cdot}5\%$ rosettes with lymphocytes. Thus the DARR test was apparently able to detect populations of lymphocytes with less sIg than was the DIF. The nature of the extra subpopulations of sIg^+ lymphocyte will be the subject of further studies.

The development of this test should prove useful in the analysis of lymphocyte subpopulations in these important domestic animals. The strong specific rosette formation provides a more sensitive, easy and highly reproducible analytical tool and should be valuable for the preparative isolation of sIg bearing lymphocytes on density gradients in the study of the biology of B cells in these species in health and disease.

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