ACTA VET. BRNO, 60, 1991: 231–236

A SIMPLE AND EFFICIENT VARIANT OF THE E ROSETTE TEST FOR THE DETECTION OF T LYMPHOCYTES IN PIGS

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Received January 11, 1991

Abstract

Knotek, Z., P. Vojtíšek, Petra Mládková, P. Hořín, F. Kovářů, P. Mádr, J. Drábek: *A Simple and Efficient Variant of the E rosette Test for the Detection of T Lymphocytes in Pigs*. Acta vet. Brno, 60, 1991: 231–236.

An efficient method detecting lymphocytes of T lineage in pigs was developed. The influence of different sheep red blood cells/pig lymphocytes ratio, their common preincubation, addition of dextran to the medium, different cell culture media were tested. In the optimized variant of the test the percentage of E rosette forming cells from the peripheral blood lymphocytes of two groups of 25 young pigs (LW × × LA) and 122 adult sows (LW × LA) were 71.29 ± 5.32 % and 76.12 ± 6.08 % respectively. The specificity of this test was assessed by using rabbit anti–T serum, monoclonal antibodies and by determining the distribution of E rosette forming cells in lymphoid tissues.

E rosette forming lymphocytes, blood, lymphoid tissue, distribution

The determination of the number of T and B lymphocytes in the peripheral blood is a pre-requisite for developing schemes of evaluating immunocompetence of man and animals by functional tests (Buschmann and Pawlas 1980). Lymphocytes B are characterized by their capacity to adhere on nylon wool fibers, by the presence of membrane immunoglobulins and the Fc receptors, although the latter are not specific only for B cells (Vojtíšková and Franěk 1989). Lymphocytes T form rosettes with xenogeneic erythrocytes (especially with ovine erythrocytes — SRBCs). The rosettes are formed by SRBCs that bind to a specific receptor. The binding depends upon origin of SRBCs from different individuals and upon culture conditions in vitro (Johansen et al. 1974). Lymphocytes T may be identified also using polyclonal anti-T sera directed against T cell specific antigens, by monoclonal antibodies (MoAbs), or by detecting T cell specific receptors for certain lectins (Jarošková 1977).

From the practical point of view, the rosette test is more suitable for routine screening for immunocompetence in farm animals than is the use of MoAbs, which is limited by their availability and methods of detection. However, since different laboratories use different modifications of E rosette test, the results are not comparable and they also show that not all peripheral T lymphocytes (PBTLs) are always detected. Therefore on the basis of the techniques actually used in pigs (Escajadillo and Binns 1975; Binns 1978; Buschmann and Pawlas 1980) and cattle (Grewal et al. 1976; Higgins and Stack 1977) we attempted to elaborate a simple variant of E rosette test in pigs which would detect the majority of PBTLs.

Materials and Methods

1. Lymphocyte isolation

Two categories of Czech Large White × Landrace crossbred pigs (LW × La) were investigated: pigs of 25–30 kg body mass ("young pigs") and adult sows.

Lymphocytes were isolated from peripheral blood collected by venipuncture of vena jugularis on the density gradient. Briefly, whole heparinized blood (10 UI/ml) was layered on the 17 % Verografin (1.077 g . cm³, Verografin 60 % Spofa, ČSFR), centrifuged at 750 g for 35 min. and lymphocytes were removed from the interphase, washed three times and a suspension with 4.10^6 cells/ml Hanks (Ca^{2-} and Mg^{2-}) was prepared. The viability of the lymphocytes was always higher than 95 % as assessed by the trypan blue exclusion test.

2. Sheep red blood cells

Erythrocytes from only one ram (Friesian breed) were used throughout the experiment. Blood was collected from vena jugularis into a syringe containing an equal volume of Alsever's solution. Cells were stored at 4 °C and they were used two weeks maximally. They were washed three times in Hanks (Ca^{2-} and Mg^{-2}) and resuspended in the appropriate medium just before testing.

3. The primary variant of the E rosette test

Medium (100 μl), lymphocyte suspension (100 μl — 4.10^6 cells/ml of media) and SRBCs suspension (100 μl — $1.2 . 10^8$ cells/ml of media) were mixed in polystyrene tubes (Koh-i-noor Hardmuth, ČSFR). The tubes were centrifuged at 125 g 5 min. and then stored at 4 °C. The cells were gently resuspended in the tubes before counting. At least 200 cells were counted. Lymphocytes with at least 3 bound erythrocytes were considered as RFCs.

4. Optimization of the test

The effect of following factors on the formation of rosettes by pig lymphocytes was evaluated: proportion of the number of SRBCs and lymphocytes, incubation of lymphocytes with SRBCs before centrifugation, addition of dextran in the medium and different culture media (See also Table 1). Different E rosette test variants were used:

Table 1

Numbers of pig E rosette forming cells (E — RFCs) in various incubation milieu.
Values are expressed as mean ± standard deviation

Culture media	n	x ± SD (%)
Hank's solution	10	36.11 ± 5.13
MEM	10	40.26 ± 9.67
FCS	8	50.20 ± 8.71
Dextran T 70 FCS 1 : 1	6	56.03 ± 2.16
Dextran T 70 (SRBCs) Hank's solution (PBLs)	8	65.59 ± 5.96
Dextran T 70 (SRBCs, PBLs)	5	60.68 ± 11.52
Dextran T 70 (SRBCs) MEM (PBLs)	10	74.06 ± 3.49

PBLs — peripheral blood lymphocytes
SRBCs — sheep red blood cells

- three different concentrations of SRBCs were used ($4 . 10^7$, $1.2 . 10^8$ and $2 . 10^8$ cells/ml media) which gave three different SRBCs/pig PBLs ratios (10 : 1, 30 : 1 and 50 : 1)
- preincubation of PBLs with SRBCs was performed in two variants: 10 and 30 minutes at 37 °C respectively, followed by an incubation at 4 °C
- Hanks (Ca^{2-} and Mg^{2-}), MEM (ÚSOL Praha, ČSFR), Fetal calf serum (Bofes — University of Veterinary Science, Brno, ČSFR), Dextran 6 % (Dextran T 70, T 550 and D 2000, Pharmacia, Uppsala, Sweden) and mixture of dextran with FCS 1 : 1 were used as media. Both incubation of SRBCs and/or PBLs in dextran were tested. Final concentrations of dextran was 4 % or 6 % and from the results obtained the optimum variant of the test was deduced.

5. The specifity of the test

The specifity of the E rosette formation in the final optimum variant of the test was verified by following procedures:

- RFCs and sIg positive B lymphocytes detected by the monoclonal antibody LIG — 4 (Dvořák et al. 1986) by means of indirect immunofluorescence from at least 200 cells were counted in the same sample of peripheral blood
- the rosette formation by lymphocytes previously labelled by LIG — 4 was evaluated

Table 2

The specificity of the E rosette test (percentage of PBLs)

T lymphocytes			B lymphocytes	E – RFCs
E – RFCs	anti–T$^+$	MAC – 83$^+$	LIG – 4$^+$	LIG – 4$^+$
71.20	78.77	75.61	11.50	0.83

PBLs – peripheral blood lymphocytes
E – RFCs – pig E rosette forming cells
n = 1

Table 3

Percentage of MAC-83 positive (MAC-83$^+$) cells after separation of rosette test samples

Lymphocytes from:	
The non – rosetting interphase	The E–RFCs sediment
0	97.63

E – RFCs – E rosette forming cells
n = 1

- a parallel detection of T lymphocytes forming E rosettes by specific anti-T serum (ATS, Kovářů et al. 1985) and by anti-T monoclonal antibody reacting with pig T lymphocytes MAC-83 (Lunney and Pescovitz 1988) was performed. In all the tests with ATS or MoAbs a fluorescein conjugated rabbit Ig to sheep Ig (DAKO, Denmark) and/or SwARa/FITC (ÚSOL Praha, ČSFR) was used
- the tissue distribution of RFCs in lymphoid organs was investigated. These cells were counted in suspensions of thymocytes, spleen cells and bone marrow cells isolated on 17 % Verografin density gradients from 3–4 weeks old piglets
- the effect of addition of anti-T and anti-B antibodies on the E rosette formation was investigated. Lymphocytes were incubated at 37° (for 1 h in: 1) rabbit anti pig anti-T serum diluted 1 : 100; 2) rabbit heat inactivated serum (control); 3) MoAb MAC-83 diluted 1 : 1 000; 4) MoAbs LIG-4 undiluted. After three washing PBLs were used in the optimum variant of the E rosette test. A minimum of 200 cells was evaluated
- the separation of RFCs on a density gradient and their characterization: E rosette test was performed in 3 ml amounts in each tube. After 3 hrs of incubation supernatants were discarded and the sediment from tubes was resuspended in RPMI 1640 (ÚSOL Praha, ČSFR) medium. The suspension (4 ml) was layered on Ficoll- Paque (Pharmacia, Sweden) and centrifuged at 125 g for 15 minutes. The sediment (RFCs) was submitted to haemolysis by concentrated MEM supplemented with 10 % FCS. These cells and the cells from the interphase were checked for the presence of T antigen using the MoAb MAC-83.

Results

1. The primary variant of the E rosette test

The number of RFCs detected by this variant was 27.34 $\pm$ 3.07 % (mean $\pm$ $\pm$ SD) in the peripheral blood of young pigs when the rosettes were counted immediately, and 36.11 $\pm$ 5.13 % after 2 or 18 hours. The difference in the number of RFCs after 2 and 18 hrs was not statistically different as determined by the paired t-test ($p > 0.05$; $T = 1.946$; $n = 25$).

Table 4

Percentage of E rosette forming cells (E — RFCs) after incubation of pig (n = 1) peripheral blood lymphocytes in antisera

Control (IRS 1 : 100)	ATS (1 : 100)	LIG – 4 (1 : 100)	MAC – 83 (1 : 1 000)
67.00	0.66	70.92	5.64

IRS – inactivated rabbit serum

Table 5

Distribution of E rosette forming cells (E — RFCs) and MAC 83 — positive (MAC-83$^+$) lymphocytes in lymphoid tissues

	Bone marrow	Spleen	Thymus
E – RFCs (%) n = 3	8.08 ± 5.45	59.29 ± 3.18	92.51 ± 2.00
MAC – 83$^+$ (%) n = 1	12.81	61.62	88.14

2. The optimum variant

The best results were obtained for the SRBCs/PBLs ratio being 30 : 1 (36.11 ± ± 5.13 % RFCs). The preincubation of SRBCs and PBLs at 37 °C or 4 °C was not shown to have any effect (29.90 — 40.50 % RFCs as compared with 41.08 % RFCs in the control — without preincubation). The formation of rosettes was maximal in the milieu of dextran with MEM. Dextran T 70 (MW 70 000) was the most efficient (74.06 ± 3.49 % RFCs) as compared with dextrans of molecular weight 500 and 2 000 (50.50 ± 1.70 % and 59.30 ± 4.81 % RFCs respectively). Moreover, agglutination of erythrocytes was observed in the presence of high molecular weight dextrans. Therefore, the following variant of the E rosette test was adopted: Lymphocytes isolated and washed in Hanks are adjusted to a concentration of $4 \cdot 10^6$ cells/ml of MEM. SRBCs washed in Hanks are adjusted to a concentration $1.2 \cdot 10^8$ cells/ml of 6 % Dextran T 70. Dextran (100 μl) + PBLs in MEM (100 μl) + SRBCs in 6 % dextran (100 μl) are gently mixed in a polystyrene tube, centrifuged at 125 g 5 min and then stored in 4 °C for 2 hrs. Immediately before counting the cells are gently resuspended and one drop of the suspension is transferred into the counting chamber. At least 200 cells are counted and the percentage of RFCs is evaluated.

3. Specificity of the test

The results are summarized in the Tables 2—4. The distribution of RFCs in lymphoid tissues is in Table 5. In a group of 25 LWxLA hybrids of 25 kg of body mass, the mean percentage of RFCs in the peripheral blood lymphocytes was 71.29 ± 5.32 % and in 122 adult sows there was 76.12 ± 6.08 % RFCs.

Discussion

From 10 to 30 % of peripheral blood lymphocytes in pigs are B cells as assessed by immunofluorescence, EAC rosette tests and other methods (see Müller 1984 for a review): 70—80 % are lymphocytes of T lineage as determined by

monoclonal antibodies (Pescovitz et al. 1984), although it is not clear whether this lineage involves only „classical" T lymphocytes or whether it is not a common name for two or more populations of the same origin, including for example null cells according to Duncan et al. (1989). It is thus obvious that the primary variant of the spontaneous E rosette test does not detect all peripheral blood T lymphocytes. In our task to optimize the test, we have not confirmed the favorable effect of the common preincubation of SRBCs and PBLs as reported by Buschmann and Pawlas (1980). Our results are in agreement with Escajadillo and Binns (1975). The use of 2—amino—ethylisothiouronium bromide (AET) that improves the rosette formation for instance in cattle (Paul et al. 1979) was not efficient in our hands (data not shown). We have confirmed the essential effect of the incubation milieu, especially of dextran, as described by Binns (1978). The number of RFCs as determined by the optimal variant are in agreement with numbers of T lymphocytes detected in the pig blood by E rosette test (Binns 1978), monoclonal antibodies (Pescovitz et al. 1984) and they are confirmed by our parallel tests with ATS and MoAbs. We cannot exclude the possibility that under the conditions of the optimal variant of the test some of RFCs are null cells. However, the effect of ATS on the E rosette formation, the distribution of RFCs in different lymphoid organs, the concordance of the number of RFCs with the number of MAC — 83 positive lymphocytes, together with the fact that less than 1 % of LIG — 4 positive (sIg positive) cells formed rosettes lead to the conclusion that the formation of rosettes is specific for cells of T lineage. Since the test is simple and does not require special reagents or chemicals, except for dextran, we believe that this method could be useful especially for routine scale screening for the number of peripheral blood T lymphocytes in pigs. It is quite clear, however, that the population of RFCs is heterogenous and it is possible to characterize its different subpopulations for special, namely research purpose by using MoAbs. Nevertheless, even rosette tests may detect some functionally defined populations e.g. IL—2 productive lymphocytes as described in cattle (Zelarney and Belden 1988). From this point of view, functional characterization of subpopulation of lymphocytes detected in the primary variant of the test should be useful.

Detekce T lymfocytů v periferní krvi a lymfoidních tkáních prasat pomocí jednoduché a účinné varianty E rozetového testu a monoklonálních protilátek

Byla vypracována účinná metoda k detekci T lymfocytů u prasat. Byl ověřen vliv poměru ovčích erytrocytů k prasečím lymfocytům, společné inkubace suspensí těchto buněk, přidání dextranu do kultivačního média, respektive použití různých kultivačních médií na tvorbu celkových E rozet. Optimalizovanou metodou bylo v periferní krvi 25 běhounů a 122 dospělých prasnic (kříženci plemen bílé ušlechtilé a landrace) stanoveno 71,29 ± 5,32 % a 76,12 ± 6,08 % lymfocytů tvořících E rozety.

Детектирование Т-лимфоцитов в периферической крови и лимфоидных тканей свиней с помощью простого и действенного варианта Е - розеткового теста и моноклонических антител

Определили эффективный метод детектирования Т-лимфоцитов у свиней. Проверили влияние соотношения эриртоцитов овец и лимфоцитов свиней, совместную инкубацию суспензий данных клеток, добавление декстрана в культуральную среду или использование разнообразных культурных сред в образовании общих Е розеток. Оптимизированным методом определили в периферической крови 25 подсвинок и 122 взрослых свиноматок (помесей белой породистой породы и ландрас) 71,29 ± 5,32 % и 76,12 ± 6,08 % образующих Е розетки лимфоцитов.

Acknowledgements

The authors express their thanks to Dr. F. Klima, Academy of Sciences, Berlin, for providing Hanks (Ca^{2-} and Mg^{2-}) and Mrs. Květa Rybinová and Mrs. Vlasta Nováková for their excellent technical assistance. The monoclonal antibody MAC-83 was a generous gift of D. Richard M. Binns, ARC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge.

References

BINNS, R. M.: Sheep erythrocyte rosettes in pigs, cattle and goats demonstrated in the presence of dextran. J. Immunol. Methods, **21,** 1978: 197–210

BUSCHMANN, H.–PAWLAS, S. S.: A study of porcine lymphocyte populations. I. Separation of porcine lymphocyte subpopulations. II. Characterization of porcine lymphocyte population. Vet. Immunol. Immunopathol., **1,** 1980: 215–241

DUNCAN, I. A.–BINNS, R. M.–DUFFUS, W. P. H.: The null T cell in pig blood is not an NK cell. Immunology, **68,** 1989: 392–395

DVOŘÁK, P.–DVOŘÁKOVÁ, D.–HRUBAN, V.–HORÁK, V.–STANĚK, R.: Monoclonal antibodies specific for pig serum and cell surface IgM. Res. Vet. Sci., **41,** 1986: 356–360

ESCAJADILLO, C.–BINNS, R. M.: Rosette formation with sheep erythrocytes – A possible T-cell marker in the pig. Int. Arch. Allergy Appl. Immunol., **48,** 1975: 261–275

GREWAL, A. S.–ROUSE, B. T.–BABIUK, L. A.: Erythrocyte rosettes A marker for bovine T cells. Can. J. Comp. Med., **40,** 1976: 298–305

HIGGINS, D. A.–STACK, M. J.: Bovine lymphocytes: Recognition of cells forming spontaneous (E) rosettes. Clin. exp. Immunol., **27,** 1977: 348–359

JAROŠKOVÁ, L.: Membrane receptors on lymphocytes. Ontogenic studies in foetal pig. Acta vet. Brno, **46,** 1977: 55–78

JOHANSEN, K. S.–JOHANSEN, T. S.–TALMAGE, D. W.: T cell rosette formation in primates, pigs and guinea pigs. J. Allergy. Clin. Immunol., **54,** 1974: 86–93

KOVÁŘŮ, F.–KOVÁŘŮ, H.–FIŠAR, Z.: Ontogenic analysis of some surface markers on pig lymphocytes using fluorescence-activated cell sorter. Fol. Microbiol., **30,** 1985: 277–290

LUNNEY, J. K.–PESCOVITZ, M. D.: Differentiation antigens of swine lymphoid tissues. In: Differentiation antigens in lymphohemopoietic tissues. Miyasaka M., Trnka Z. (eds), Marcel Dekker Inc., N. York–Basel, 1988, 421–454

MÜLLER, G.: Zur Bestimmung von B und T Lymphozyten bei Rind und Schwein. Arch. Exp. Vet. Med., **38,** 1984: 554–564

PAUL, P. S.: BROWN, T. T.–MILLER, J. M.–Van Der MAATEN, M. J.: Enhancement of rosette formation between sheep erythrocytes and bovine T-lymphocytes by AET and dextran. Immunol. Lett. **1,** 1979: 93–96

PESCOVITZ, M. D.–LUNNEY, J. K.–SACHS, D. H.: Preparation and characterization of monoclonal antibodies reactive with porcine PBL. J. Immunol., **133,** 1984: 368–375

VOJTÍŠKOVÁ J.–FRANĚK, F.: Isolation and characterization of pig lymphocyte Fc receptor. Fol. Biol. Praha, **35,** 1989: 273–288

ZELARNEY, P. T.–BELDEN, E. L.: Bovine interleukin 2: Production by an E-rosette defined lymphocyte subpopulation. Vet. Immunol. Immunopathol., **18,** 1988: 297–305