大韓獸醫學會誌(1992) 第32卷 第2號
Korean J Vet Res (1992) 32(2) : 175~179

Comparison of sheep erythrocytes and Korean native goat erythrocytes-rosette forming rate of pig peripheral blood mononuclear cells

Young-jin Kim, Hee-jong Song, Jong-myeon Kim, Myeong-dai Kang, Chang-yong Yoon, Tae-joong Kim

College of Veterinary Medicine, Chonbuk National University, Chonju, 560-756, Korea

(Received April 30, 1991)

돼지 말초혈액 단핵세포의 면양 및 재래산양 적혈구 rosette 형성능 비교

김영진 · 송희종 · 김종면 · 강명대 · 윤창용 · 김태중
전북대학교 수의과대학

(1991. 4. 30. 접수)

초록 : 돼지(n=22) 순환혈액내 단핵세포(PB-MNCs)중 T 및 B 림프구를 정량하고자 rosette 형성기법을 적용하였다. T 림프구는 여러 농도의 2-aminoethylisothiouronium bromide(Aet)나 dextran (Dex) 용액 또는 Aet와 Dex로 조합 처리된 면양(SRBC) 및 재래산양(GRBC)의 적혈구를 사용한 E-rosette 법으로, B 림프구는 erythrocyteantibody(EA) 및 erythrocyte-antibody-complement(EAC) rosette 법으로 각각 정량하였다. 한편 rosette 형성세포의 냉장정치시간에 따른 판독결과를 비교하여 적정판독시간대를 구명한 바 아래의 결과를 얻었다.

1. PB-MNCs와 SRBC/GRBC와의 자연 rosette형성율(RFR)은 32.9± 7.9%/31.3± 9.4%인데 비하여 Aet 또는 Dex 처리군에서는 모두 증가되었고 특히 0.15M Aet(40.2± 4.6%/37.0± 3.3%) 및 8% Dex(71.5± 4.5%/69.9± 5.8%) 처리군에서 각각 높게 나타났다. 한편 0.1M Aet 처리후 8% Dex 첨가군에서는 67.8± 7.4% 및 69.8± 8.5%로 나타나 복잡한 Aet와 Dex를 복합처리하기 보다는 8% Dex 단독처리가 간편함을 알 수 있었다.

2. Rosette 형성세포의 냉장보관중 최적판독시간대는 10~20시간의 범위이었다.

3. EA 및 EAC의 RFR은 SRBC의 경우 39.1± 10.2%, 27.6± 7.0%, GRBC의 경우 32.6± 6.1%, 21.0± 3.2%로 나타났다.

이러한 결과는 돼지 PB-MNCs내의 T 및 B 림프구를 rosette 형성법으로 분리할 수 있으며 rosette assay에서 SRBC 뿐아니라 GRBC의 이용이 가능함을 제시한다.

Key words : Rosette assay, sheep erythrocytes, Korean native goat erythrocytes, peripheral blood mononuclear cells, pig.

Introduction

Lymphocytes are the major cells involved in immune defence. Lymphocytes have been divided into thymus-derived and bursa derived cells or T and B lymphocytes.

Cell surface receptors, which serve to dichotomous mammalian lymphocytes into either T or B lymphocy-

tes, have often been associated with different functional repertoires of these cells. Unambiguous detection of unique cell surface structures marking these lymphocyte populations is fundamental to understanding their interactions.[1~10]

In pig, there has not been revealed the function of these lymphocytes, but these cells can be separated into T and B lymphocytes, respectively by the rosette assay using heterogeneous erythrocytes.[1,11~15] T lymphocyte rosettes with sheep erythrocytes(SRBC) spontaneously, and its function is inhibited in the presence of anti-thymocytes serum(ATS) but is not affected with rabbit anti-porcine immunoglobulin.[16] ATS is cytotoxic over a range of dilutions($\log_2$ 4) for approximately 60% PB-MNCs and virtually all thymocytes. While B lymphocyte, which has cell surface markers for antibody and complement, rosettes with heterogenous erythrocytes in the presence of antibody and/or complement.[16~25]

SRBC was used for the separation of T and B lymphocytes from peripheral blood mononuclear cells(PB-MNCs) in pig. But it was required to develope the new agents, because the breeding of sheep is difficult in Korea. In this experiment, Korean native goat was applied as a new source of erythrocytes(GRBC) instead of sheep to separate lymphocyte subpopulations from peripheral blood in pigs. And we compare SRBC and GRBC rosette forming rate of PB-MNCs in pig.

Materials and Methods

Animals : Clinically healthy pigs were selected randomly from a local slaughter house in Samnye, Wanju, weighing 60~90kg. Matured female sheep and Korean native goat, breeding at College of Veterinary Medicine, Chonbuk National University, was used as a source of indicator cells.

Separation of PB-MNCs : Pig blood was collected in heparinized(20 IU/mℓ) syringe by jugular venipuncture. Buffy coat cells were separated by centrifugation at 400×g for 30 minutes and layered over the same volume of Ficoll-Hypaque(F-H, Sigma, d=1.077) density gradient. After centrifugation, peripheral blood mononuclear cells(PB-MNCs) at the F-H interface were collected and washed with phosphate buffered saline(PBS, pH 7.2). Then, PB-MNCs were resuspended in RPMI-1640 containing 10% fetal calf serum(10% FCS-RPMI 1640, pH 7.4 ; rosette medium) at concentration of $3-4\times10^6$ cells/mℓ.

RBC and polymers : SRBC or GRBC was diluted with the same volume of Alsever's solution(pH 6.1) and used within 2 weeks. Prior to use, WBC layer was excluded, then washed 3 times with PBS. In order to increase rosettes, RBC were treated with polymers such as 2-aminoethylisothiouronium bromide(Aet, Sigma) and dextran(Dex, Sigma, MW=147,000) as described previously.[18]

Rosette formation : Rosette formation was performed by E-, EA-, and EAC- rosette method.[18] All tests were done in duplicate. Trypan blue(0.2%) was used to viability test for lymphocytes. At least 200 survival cells were counted in each preparation. Lymphocytes which had 5 or more RBC attached, were regarded as rosette forming cells. Rosette forming rate(RFR) was calculated as follows :

$$\%\text{ of rosette forming rate} = \frac{\text{No. of rosette forming cells}}{\text{Total lymphocytes counted} \times \text{Viability}} \times 100$$

Results

Effect of incubation time on rosette formation : In order to determine the optimal incubation time for rosettes, PB-MNCs were mixed with Dex-treated erythrocytes(E-Dex). The mixture was centrifuged and incubated at the various time.

Table 1. Effect of varying concentration of Aet on rosette formation of pig peripheral blood lymphocytes

Treatment of RBC with	% of RFC(M± S.D.)[a]	
	SRBC[b]	GRBC[c]
Untreated	32.9± 7.9	31.3± 9.4
0.05M Aet[d]	36.5± 4.1	32.8± 5.7
0.1 M Aet	37.8± 3.3	33.8± 8.7
0.15M Aet	40.2± 4.6	37.0± 3.3
0.2 M Aet	ND[e]	30.9± 3.6

a) RFC : Rosette forming cells with five or more adherent RBC
b) SRBC : Sheep red blood cells
c) GRBC : Korean native goat red blood cells
d) Aet : 2-aminoethylisothiouronium bromide
e) ND : Not done

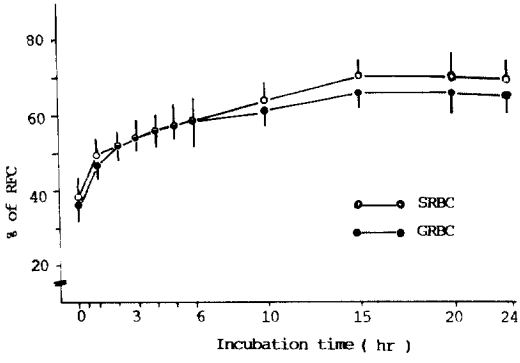

Fig 1. Kinetics of rosette formation of pig PBL with E Dex.

PBL were incubated with E Dex at 37 C for 20 minutes, centrifuged at 200×g, and then incubated for varying periods of time. Results are expressed as mean RFC from 10 animals± S.D.

Table 2. Effect of varying concentration of Dex on rosette formation of pig peripheral blood lymphocytes

% of Dextran	% of RFC(M± S.D.) SRBC	GRBC
2	40.0± 5.8	39.6± 2.5
4	56.7± 6.9	56.1± 8.9
6	65.4± 7.2	67.5± 7.7
8	71.5± 4.5	69.9± 5.8
10	58.9± 7.3	55.1± 0.8

As shown in Fig 1, rosette formation with SRBC and GRBC was 39.8± 9.9%/37.6± 5.7% at 0 time incubation and 70.0± 7.3%/65.4± 7.3% at 15 hours incubation. However RFR was slightly decreased thereafter.

Effect of polymers on rosette formation : When RBC were treated with Aet, RFR was significantly increased than that of untreated control. And the highest RFR was revealed at the concentration of 0.15M Aet (Table 1).

As shown in Table 2, Dex treatment also increased RFR and optimal concentration of Dex was 8%. However, when RBC was treated with optimal or suboptimal concentration of Aet(0.1M or 0.15M) and Dex(6% or 8%) in combination, RFR was rather decreased than that of Dex alone(Table 3).

Comparison of SRBC and GRBC in EA- and EAC-rosette formation : EA- and EAC-rosette forming rate to SRBC was 39.1± 10.2% and 27.6± 7.0%, respectively. And that of GRBC was 32.6± 6.1% and 21.0± 3.2%, respectively(Table 4).

Table 3. Effect of Aet plus Dex treatment on rosette formation of pig PB-MNCs

RBC treated with	% of RFC(M± S.D.) SRBC	GRBC
0.1 M Aet+6% Dex*	53.1± 6.3	51.7± 10.9
0.1 M Aet+8% Dex	67.8± 7.4	69.8± 8.5
0.15M Aet+6% Dex	52.2± 3.1	50.9± 5.7
0.15M Aet+8% Dex	49.9± 5.9	51.1± 5.4

*Dex : Dextran.

Table 4. EA-and EAC-rosette formation rate of pig PB-MNCs with SRBC and GRBC

Rosette assay	% of RFC(M± S.D.) SRBC	GRBC
EA	39.1± 10.2	32.6± 6.1
EAC	27.6± 7.0	21.0± 3.2

Discussion

T lymphocyte numbers can be determined in peripheral blood and in lymphoid organs. This is readily achived by rosetting techniques, based upon the fact that T lymphocytes possess sheep erythrocyte receptor (CD2 molecules).[11,22] However, this techniques could not be applied in every animal species, for this reason that CD2 molecules are not present on T lymphocytes of some animal species(eg ; mouse)and that, even though T lymphocytes have receptors for heterogenous erythrocytes, binding affinity of receptors are too weak to use this technique for the quantitation of T lymphocytes(eg ; pig).[12,17]

The present study was undertaken in an effort to develope a new effective indicator cells and to investigate the suitable conditions for the enumeration of T lymphocytes in peripheral blood of pigs. Sheep erythrocytes(SRBC) and Korean native goat erythocytes (GRBC) were used as indicator cells, and polymers such as Aet and Dex were also applied.

In this experiment, RFR of PB-MNCs with polymer-untreated SRBC and GRBC was 32.9% and 31.3%, respectively. These data were approximately same level as RFR of pig lymphocytes with SRBC which were previously reported by other investigators[11,12,15~17,19,20], and these results indicated that rosette assay using intact erythrocytes was insufficient for the enumeration of T lymphocytes in pigs and that GRBC had al-

most same efficiency to SRBC in binding affinity.[18] However, when polymer-treated erythrocytes were adopted as an indicator cells, rosette were significantly increased to the sufficient level for use of this rosette method as enumeration of T lymphocytes from PBMNCs in pigs.[11,18] Among the polymers which was applied to improve the binding affinity of indicator cells against T lymphocytes, Dex was more excellent than Aet, and optimal concentration of Dex was 8% and 10 hours-incubation was adequate time to firm the rosettes.

Although EA- and EAC-rosette method and immunofluorescent antibody method have been used for isolation of B lymphocytes[24], the rosette-technique is more useful for the culture of these isolated cells because immunofluorescent antibody method is inadequate to obtain viable cells. However, isolation of B lymphocytes by rosette technique which is dependent on the various conditions such as the way of antibody-absorption, complement-binding and individual specificity is unreliable.

Thus in these experiments, we tried to develope more excellent E-, EA- and EAC-rosette techniques. We could found the optimal conditions to obtain the maximal rosettes, and also found GRBC had same efficiency to SRBC in rosettes as an indicator cells.

Taken together, in the present study, we could confirm the maximal condition adequate for isolation of T and B lymphocytes in peripheral blood of pigs, and we showed evidences that GRBC could be applied for rosette techniques as an indicator cells instead of SRBC.

Summary

To develope the methods for isolation and enumeration of lymphocyte subpopulations in pigs, we carried out the rosette-assay using sheep erythrocytes(SRBC) and Korean native goat erythrocytes(GRBC) as a target cells.

To enumerate T lymphocytes, E-rosette methods were applied with RBC treated with various concentration of polymers such as Aet and Dex, singly or in combination. And to enumerate B lymphocytes, EA- and EAC-rosette assay was adopted. The results were as follows ;

1. E-RFR with polymer-untreated SRBC and GRBC was 32.9± 7.9% and 31.3± 9.4%, respectively. On the other hand, RFR with 0.1M Aet plus 8% Dex treated SRBC and GRBC was increased about two-fold(67.8± 7.4% and 69.8± 8.5%), respectively.

2. EA-RFR with SRBC and GRBC were 39.1± 10.2% and 32.6± 6.1%, respectively.

3. EAC-RFR with SRBC and GRBC were 27.6± 7.0% and 21.0± 3.2%, respectively.

These results showed that both SRBC and GRBC could be recommanded as a binding cells for rosette-assay to isolate of lymphocyte-subpopulations in pigs.

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