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Erythrocytes modulate cell cycle progression but not the baseline frequency of sister chromatid exchanges in pig lymphocytes

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ABSTRACT

The effect of co-culturing varying concentrations of pig and human red blood cells (RBCs) on the baseline frequency of sister chromatid exchanges (SCEs) and cell-cycle progression in pig plasma (PLCs) and whole blood leukocyte cultures (WBCs) was studied. No variation in SCE frequency was observed between pig control WBC and PLC. Addition of pig and human RBCs to pig PLCs did not modify the baseline frequency of SCEs. On the other hand, cell proliferation was slower in PLCs than in WBCs. The addition of pig or human RBCs to PLCs accelerated the cell-cycle progression of pig lymphocytes. When RBCs were added to PLCs the concentration and time sequence of RBC incorporation affected the cell-cycle progression of swine lymphocytes. When doses of pig or human RBCs equivalent to those present in WBCs were added immediately after PLC stimulation, the cell-cycle kinetics were similar to those of WBCs. Shorter co-incubation periods or a reduction in the dose of RBCs made cell-cycle progression intermediate between PLC and WBC values. Thus, pig and human RBCs modulated the *in vitro* cell-cycle progression of pig lymphocytes in a time- and dose-dependent manner, and the low baseline frequency of SCEs of pig lymphocytes is independent of the presence or absence of erythrocytes in culture.

INTRODUCTION

Since 1974, the analysis of sister chromatid exchange (SCE) frequency in peripheral circulating lymphocytes cultured *in vitro* has been used as an assay for monitoring the exposure of humans and other mammals to clastogenic agents (Kato, 1977; Latt *et al.*, 1980; Perry, 1980; Gebhart, 1981). Notwithstanding, the use of SCE bioassays to test chemical and physical agents requires standardization of the protocols in order to permit comparisons. Whole blood (WBCs), plasma leukocyte (PLCs) and mononuclear leukocyte cultures (MLCs) are routinely used for SCE assays. However, differences in baseline SCE frequencies and cell-cycle progression of the same cell among these three types of cultures have been reported to occur in lymphocytes from humans (Ray and Altenburg, 1978; Larramendy and Reigosa, 1986; Larramendy *et al.*, 1990, 1993, 1995, 1996), pigs (Larramendy and Reigosa, 1986; Larramendy *et al.*, 1990, 1993, 1995, 1996) and rats (Kligerman *et al.*, 1982; Wilmer *et al.*, 1983, 1984).

We previously reported that human, but not porcine PLCs and MLCs exhibited nearly a two-fold increase in the baseline frequency of SCEs compared

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with that observed for WBCs. The incorporation of either human or pig red blood cells (RBCs) in human PLCs and MLCs produced a RBC dose-dependent decrease in the frequency of SCEs (Larramendy and Reigosa, 1986). Additionally, in both human and pig RBC-free cultures (PLCs and MLCs) the proliferation of lymphocytes is slower than in WBC. We also observed that co-culturing of RBCs and leukocytes modulates the cell-cycle progression of both human and porcine lymphocytes, whereas the baseline frequency of SCEs is affected only in human cells (Larramendy *et al.*, 1990, 1993).

In the present study we investigated the proliferative response and the SCE baseline frequency of pig lymphocytes with regard to the time of co-culturing of different concentrations of human and pig RBCs in the culture medium.

MATERIAL AND METHODS

Blood samples

Pig blood samples (50-70 ml) were drawn from the tail veins of 8-10-month old male Duroc Jersey pigs (El Trébol Breed, Santa Fe, Argentina) bred under optimal sanitary conditions and veterinary control. Human blood samples were obtained from healthy male volunteers (20-40 years old) selected according to the previously described recommendations (Bianchi *et al.*, 1979). From each donor, 50 ml blood was drawn by venipuncture (Blood Bank of Buenos Aires Province, La Plata).

Whole blood cultures

Pig WBCs were set up (Larramendy and Reigosa, 1986). Briefly, 1.0 ml of whole blood was seeded in 9.0 ml of complete culture medium (80% Ham's F10 (Gibco, Grand Island, NY), 20% fetal calf serum (Gibco), 0.3 ml of phytohemagglutinin M (Gibco), 100 units penicillin/ml (Gibco), 100 µg streptomycin/ml (Gibco)). Culture medium was supplemented with 0.2 mg L-glutamine/ml (BDH Chemicals, Poole, UK) and 0.13 mg L-arginine/ml (BDH), as recommended elsewhere (Lezana *et al.*, 1978; Bianchi *et al.*, 1981). The final concentration of cells was approximately 1.7×10^6 leukocytes/ml and 8.0×10^8 RBCs/ml. During the last 3 h of culture, the cells were treated with 0.1 µg colchicine/ml (Sigma Chemical Co., St. Louis, MO). At 48 h from seeding, the cells were harvested, exposed to a hypotonic solution (0.075 M KCl, 37°C, 15 min), and fixed in methanol-acetic acid (3:1). Chromosome spreads were obtained by the air-drying technique. Cultures were established in duplicate for each sample from each animal, and at least two different animals were used for each experiment.

Plasma leukocyte cultures

Pig PLCs were set up (Larramendy and Reigosa, 1986). Briefly, after gravity sedimentation of whole blood (approximately 30-40-ml sample) for 1-2 h at room temperature, 1.0 ml of plasma leukocyte suspension was added to 9.0 ml of complete culture medium. The final concentration of leukocytes was approximately 3.4×10^6 cells/ml. Cell treatment, culture and harvesting conditions were as described for WBCs.

Effect of time of addition of erythrocytes to culture medium on mitogenic stimulation of lymphocytes

Human and pig RBCs were obtained from erythrocyte pellets of Ficoll-Hypaque gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) according to Böyum (1968). Cells were washed twice in culture medium and diluted in such a way that the addition of 1.0 ml of RBCs suspension to cultures gave the desired concentration ($2.2\text{-}4.5 \times 10^8$ RBCs/ml and $4.0\text{-}8.0 \times 10^8$ RBCs/ml of culture medium for human and pig cells, respectively). Human and pig RBCs were introduced into pig WBCs and PLCs at 12-h intervals from 0 h up to 36 h of culture initiation. Cell treatment, culture and harvesting conditions were as described for WBCs.

Fluorescence-plus-Giemsa (FPG) method for sister chromatid differentiation

Chromosome spreads were stained using the FPG technique for sister-chromatid differentiation reported in detail elsewhere (Larramendy and Knuutila, 1990).

Sister chromatid exchange analysis and cell-cycle progression

All slides were coded and a single observer scored SCE frequencies in 50 diploid metaphases per sample. A minimum of 200 metaphase cells per sample were scored to determine the percentages of cells which had undergone one, two, three or more mitoses. All those metaphases showing differential staining of sister chromatids in less than one fourth of the chromosomal complement were considered to be in at least the fourth

cell-cycle. Tukey's test for comparisons was used to determine the significance of differences between the mean SCE frequencies. A χ^2 test was used for cell-cycle progression data. The level of significance chosen was 0.05, unless indicated otherwise.

RESULTS AND DISCUSSION

No differences in SCE frequencies were found among control WBCs and PLCs, nor in PLCs in which different concentrations of human and porcine RBCs were added at varying intervals after stimulation (Tables I and II). These results demonstrate that, regardless of the donor, the low SCE baseline frequency of pig lymphocytes is not modulated by the lapse between lymphocyte stimulation and the addition of RBCs to the cultures, nor by the concentration of the erythrocytes seeded in the medium. We conclude that the low baseline frequency of SCEs of the porcine lymphocytes is totally independent of the presence or absence of RBCs during culture. This finding confirms previous observations reported by us (Larramendy and Reigosa, 1986; Larramendy *et al.*, 1990, 1995). It is puzzling that pig erythrocyte-free cultures do not show an elevated SCE frequency, as we know that human (Larramendy and Reigosa, 1986; Larramendy *et al.*, 1990, 1995) and rat leukocytes (Wilmer *et al.*, 1983, 1984) exhibit this phenomenon, and that human and pig RBCs affect the frequency of exchanges of human cultures (Larramendy and Reigosa, 1986; Larramendy *et al.*, 1990). We demonstrated that human as well as pig RBCs have a direct effect on human lymphocytes, preventing the induction of a heightened basal frequency of SCEs by releasing a "corrective" factor present after hemolysis in the erythrocyte ghosts. The corrective factor is not dialyzable, and it is not species specific, since pig RBCs are nearly as efficient as human erythrocytes in decreasing the high SCE frequencies of human PLC and MLC (Larramendy and Reigosa, 1986). To date, the nature of this factor, however, remains unknown. The information we possess is not enough to elucidate whether this corrective factor present in RBCs is the same found by other authors (van Buul *et al.*, 1978; Bartram *et al.*, 1979, 1981; Rüdiger *et al.*, 1980; Shiraishi *et al.*, 1981; Leroux *et al.*, 1984). Its isolation and purification would be a fundamental step in our understanding of an important biological process, and thus

Table I - Effect of the addition of pig red blood cells (RBCs) at different intervals after starting pig plasma leukocyte cultures (PLCs) on sister chromatid exchange (SCE) frequency (a).

Culture type	Pig RBC concentration (x 10^8 RBCs/ml)	Pig RBCs in culture (h)	Pigs		
			Animal 1 (b)	Animal 2 (b)	Animal 3 (b)
WBC	8	48	5.00 ± 0.40	4.85 ± 0.37	5.85 ± 0.64
PLC	0	0	5.20 ± 0.49	5.15 ± 0.41	6.30 ± 0.72
WBC + RBCs-0 h	4	48	5.30 ± 0.54	5.05 ± 0.46	6.45 ± 0.53
WBC + RBCs-0 h	8	48	4.65 ± 0.47	5.15 ± 0.48	5.40 ± 0.48
PLC + RBCs-0 h	4	48	Failed	5.35 ± 0.56	5.35 ± 0.53
PLC + RBCs-0 h	8	48	5.00 ± 0.50	5.00 ± 0.42	6.00 ± 0.49
PLC + RBCs-12 h	4	36	5.45 ± 0.42	4.60 ± 0.48	5.65 ± 0.57
PLC + RBCs-12 h	8	36	Failed	5.15 ± 0.44	6.60 ± 0.59
PLC + RBCs-24 h	4	24	4.95 ± 0.42	5.15 ± 0.65	6.10 ± 0.71
PLC + RBCs-24 h	8	24	5.05 ± 0.46	5.15 ± 0.59	6.50 ± 0.46
PLC + RBCs-36 h	4	12	5.10 ± 0.42	4.70 ± 0.32	6.45 ± 0.54
PLC + RBCs-36 h	8	12	5.25 ± 0.32	5.20 ± 0.37	5.75 ± 0.43

(a) Pig RBCs were introduced into pig PLC at 12-h intervals after culture initiation.
(b) Mean ± standard error of the mean.
WBC, Whole blood culture.

Table II - Effect of the addition of human red blood cells (RBCs) at different intervals after starting pig plasma leukocyte cultures (PLCs) on sister chromatid exchange (SCE) frequency (a).

Culture type	Human RBC concentration (x 10^8 RBCs/ml)	Pig RBCs in culture (h)	Pigs		
			Animal 4 (b)	Animal 5 (b)	Animal 6 (b)
WBC	4.5	48	5.20 ± 0.40	6.04 ± 0.43	5.24 ± 0.29
PLC	0	0	5.36 ± 0.44	5.16 ± 0.41	4.80 ± 0.23
WBC + RBCs-0 h	2.25	48	5.88 ± 0.32	4.92 ± 0.26	5.16 ± 0.26
WBC + RBCs-0 h	4.50	48	5.16 ± 0.31	5.04 ± 0.31	5.40 ± 0.27
PLC + RBCs-0 h	2.25	48	5.60 ± 0.36	5.72 ± 0.35	5.28 ± 0.28
PLC + RBCs-0 h	4.50	48	4.12 ± 0.35	4.44 ± 0.24	4.84 ± 0.24
PLC + RBCs-12 h	2.25	36	5.28 ± 0.37	5.48 ± 0.38	Failed
PLC + RBCs-12 h	4.50	36	4.76 ± 0.36	6.28 ± 0.40	5.24 ± 0.27
PLC + RBCs-24 h	2.25	24	5.44 ± 0.41	5.04 ± 0.25	4.44 ± 0.27
PLC + RBCs-24 h	4.50	24	5.08 ± 0.42	5.52 ± 0.36	5.48 ± 0.29
PLC + RBCs-36 h	2.25	12	5.04 ± 0.27	6.06 ± 0.37	4.08 ± 0.21
PLC + RBCs-36 h	4.50	12	5.72 ± 0.28	6.96 ± 0.43	4.04 ± 0.23

(a) Human RBCs were introduced into pig PLC at 12-h intervals after culture initiation.
(b) Mean ± standard error of the mean.
WBC, Whole blood culture.

would allow a better understanding of the mechanism(s) leading to baseline SCEs.

To determine whether the concentration and the time sequence of RBCs addition to PLCs modify the proliferation rate of lymphocytes, the percentage of cells in their first, second, third or subsequent division was analyzed in the samples presented in Tables I and II. In control cultures, an increase in the percentage of cells in first mitosis (animals 1-6), and a decrease in the proportion of cells in second (animals 4, 5) and third division (animals 1-6), or a decrease in the amount of third mitosis, maintaining approximately the same percentage of second mitosis (animals 2, 3, 6) were observed (Figures 1 and 2). These observations have been previously reported by us, confirming that pig lymphocytes in PLCs proliferate more slowly than in parallel WBCs, though variations among donors are evident (Larramendy *et al.*, 1990, 1993, 1995). Similar observations have also been previously found for human (Mehnert *et al.*, 1984; Larramendy *et al.*, 1990, 1993, 1996) and for rat lymphocytes (Wilmer *et al.*, 1983, 1984). They all agree that mononuclear leukocytes isolated by centrifugation in Lymphodex (Mehnert *et al.*, 1984) and Ficoll-Hypaque (Wilmer *et al.*, 1983, 1984), or cultured in PLCs (Larramendy *et al.*, 1990, 1993, 1996) exhibit a slower cell-cycle progression than WBCs. Whether this observation is restricted to human, pig and rat white cells, or is common behavior of mam-

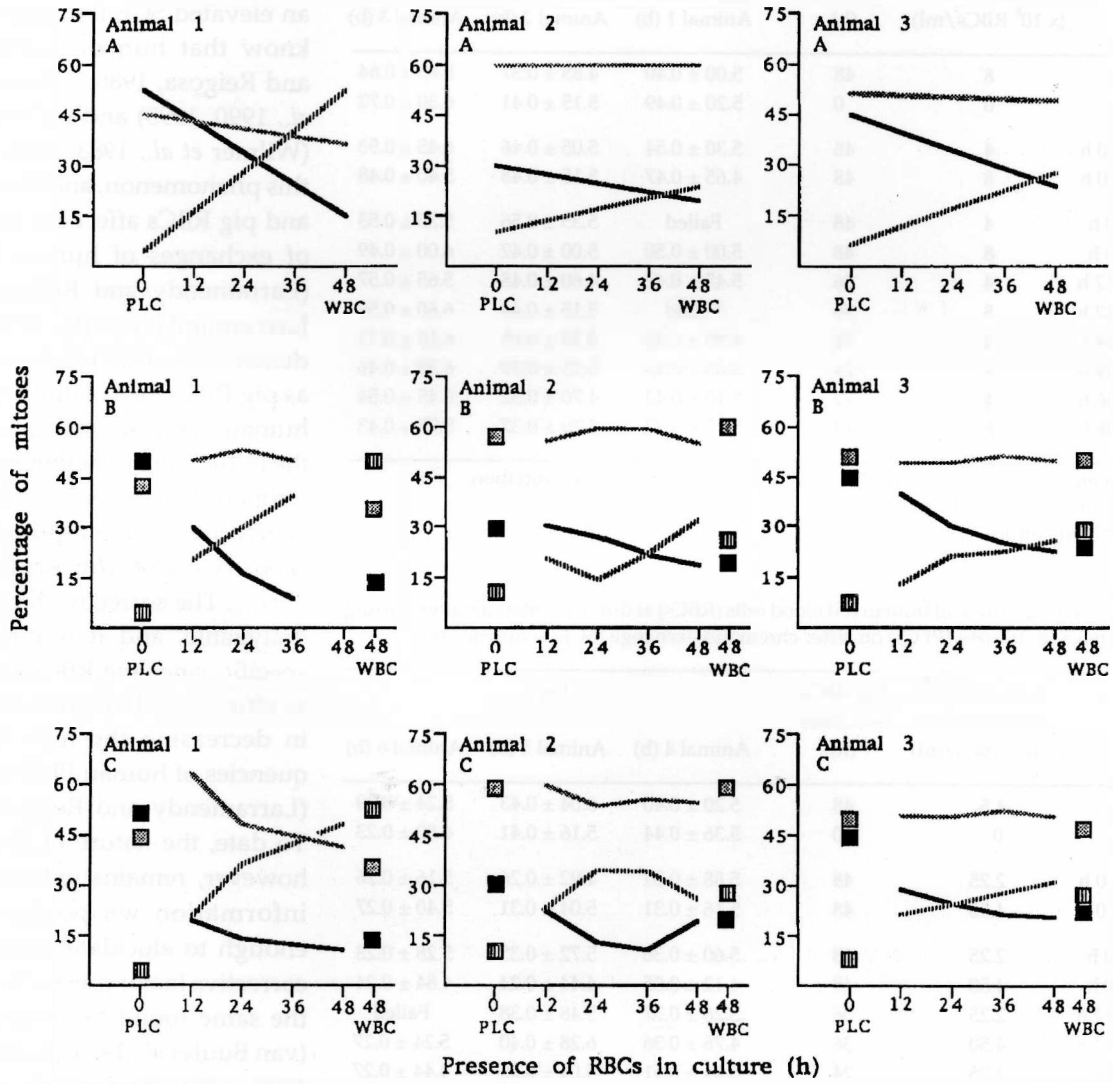

Figure 1 - Effect of the addition of pig red blood cells (RBCs) at different intervals after starting whole blood (WBCs) and plasma leukocyte cultures (PLCs) on proliferation kinetics of pig lymphocytes. In each graph the percentage of cells in first (solid line), second (dotted line), third and subsequent divisions (stripped line) (ordinate) are plotted against the time of permanence of RBCs in the culture. A, Control WBCs and PLCs; B, PLCs in which 4.0×10^8 RBCs/ml were introduced at 12-h intervals from 0 h (48 h of culture) up to 36 h from seeding (12 h of culture); C, PLCs in which 8.0×10^8 RBCs/ml were introduced at 12-h intervals from 0 h (48 h of culture) up to 36 h from seeding (12 h of culture). In B and C, solid black squares, dotted squares, and stripped squares represent the frequency of first, second, and third and subsequent mitoses, respectively.

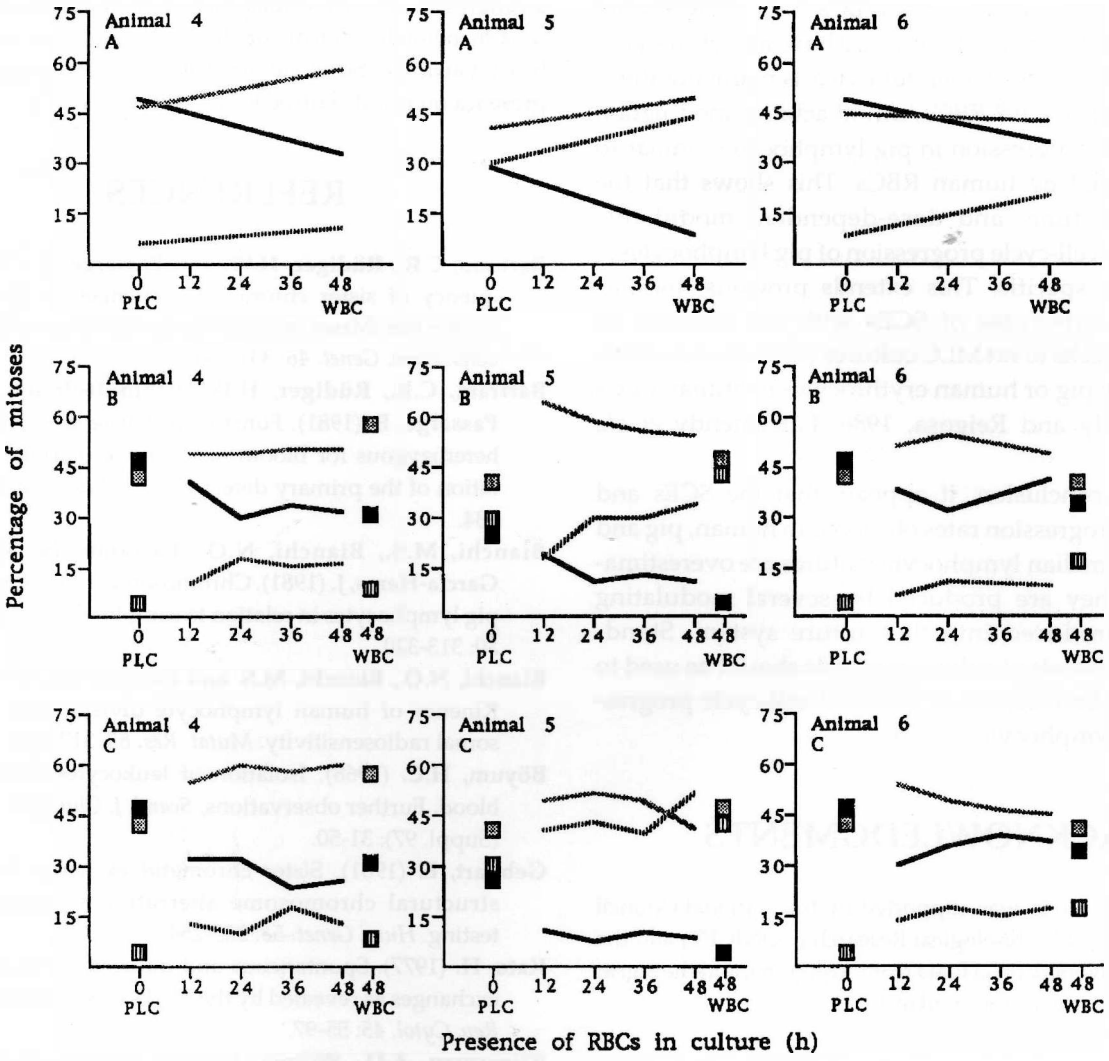

Figure 2 - Effect of the addition of human red blood cells (RBCs) at different intervals after starting whole blood (WBCs) and plasma leukocyte cultures (PLCs) on proliferation kinetics of pig lymphocytes. In each graph the percentage of cells in first (solid line), second (dotted line), third and subsequent divisions (stripped line) (ordinate) are plotted against the time of permanence of RBCs in the culture. A, Control WBCs and PLCs; B, PLCs in which 2.2.0 x 10^8 RBCs/ml were introduced at 12-h intervals from 0 h (48 h of culture) up to 36 h from seeding (12 h of culture); C, PLCs in which 4.0 x 10^8 RBCs/ml were introduced at 12-h intervals from 0 h (48 h of culture) up to 36 h from seeding (12 h of culture). In B and C, solid black squares, dotted squares, and stripped squares represent the frequency of first, second, and third and subsequent mitoses, respectively.

malian lymphocytes, due to the absence of erythrocytes *in vitro* should be investigated further.

The concentration and time sequence of addition of pig and human RBCs to PLCs considerably affects the cell-cycle kinetics of the porcine lymphocytes. When either pig (Figure 1) or human (Figure 2) RBCs were added at 0 h after stimulation of PLCs, the frequency of first, second and third mitoses was not different from the values observed in WBCs. Following the addition of RBCs at different periods, varying from 12 to 36 h, the cell-cycle progression gradually slowed, reaching PLC values. These findings clearly demonstrate that the time-dependent modulating effect of RBCs on the cell-cycle progression of pig lymphocytes is not only exerted by pig erythrocytes but also by human erythrocytes. The time-dependent modulative effect produced by both pig and human RBCs on the cell-cycle progression of pig lymphocytes is dose-dependent. Figures 1 and 2 show that, independent of source, the frequency of cells at first mitosis of PLCs at different harvesting times after being co-cultivated with RBCs for less than 36 h was always smaller in cultures where the highest concentration of RBCs was introduced than in PLCs supplemented with the lowest RBCs concentration. These results demonstrate that pig lymphocytes in the presence of the lowest pig and human RBCs concentration have a lengthened cell cycle compared to PLC co-cultivated with the highest concentration of RBCs. The maximal normalization of cell-cycle progression was detected at numbers of RBCs

equivalent to those present in WBCs (8.0×10^8 RBCs/ml and 4.5×10^8 RBCs/ml, for pig and human erythrocytes, respectively). However, about twice as many pig RBCs are required (8×10^8 RBCs/ml) to achieve modulation in cell-cycle progression in pig lymphocytes similar to that provided by human RBCs. This shows that the erythrocyte time- and dose-dependent modulative effect of the cell-cycle progression of pig lymphocytes is not species specific. This extends previous findings reporting a decrease of SCEs with the addition of human red cells to rat MLC cultures (Wilmer *et al.*, 1983, 1984), or by pig or human erythrocytes to human PLCs (Larramendy and Reigosa, 1986; Larramendy *et al.*, 1990).

In conclusion, it appears that the SCEs and cell-cycle progression rates observed in human, pig and other mammalian lymphocyte cultures are overestimations, as they are produced by several modulating factors introduced into the culture system. Standardized protocols of culture methods should be used to obtain a valid measure of SCE and cell-cycle progression from lymphocytes *in vitro*.

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RESUMO

Foi estudado o efeito de co-culturas com concentrações variadas de células sangüíneas vermelhas (RBCs) suínas e humanas na freqüência basal de trocas de cromátides irmãs (SCEs) e na progressão do ciclo celular em culturas de plasma de porco (PLCs) e culturas leucocitárias do sangue total (WBCs). Não foi observada nenhuma variação na freqüências de SCEs entre os controles de WBC e PLC em porcos. A adição de RBCs de suínos e humanos a PLCs não modificou a freqüência basal de SCEs. Por outro lado, a proliferação celular foi mais lenta em PLCs que em WBCs. A adição de RBCs humanas ou suínas a PLCs acelerou a progressão do ciclo celular de linfócitos suínos. Quando RBCs foram adicionadas a PLCs, a concentração e a seqüência temporal da incorporação de RBC afetaram a progressão do ciclo celular de linfócitos suínos. Quando adicionadas doses de RBCs suínas ou humanas equivalentes àquelas presentes em WBCs, imediatamente após estimulação da PLC, a cinética do ciclo celular foi similar à das WBCs. Períodos mais curtos de co-incubação ou uma redução na dose de RBCs causaram uma progressão do ciclo celular intermediária entre os valores de PLCs e WBCs. Assim, RBCs suínas e humanas modularam a progressão do ciclo celular *in vitro* de linfócitos suínos em uma maneira dependente da dose e do tempo, e a freqüência basal baixa de SCEs de linfócitos suínos independe da presença ou não de eritrócitos na cultura.

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