Human red blood cells have an enhancing effect on the relative expansion of CD8⁺ T lymphocytes *in vitro*

B. Porto*, A. M. Fonseca†, I. Godinho‡, F. A. Arosa†
and G. Porto‡

*Laboratory of Cytogenetics and ‡ Laboratory of Molecular Pathology and Immunology, Abel Salazar Institute for the Biomedical Sciences (ICBAS), and †Laboratory of Molecular Immunology, Institute for Molecular and Cell Biology (IBMC), University of Porto, Porto, Portugal

Received 18 May 2001; revision accepted 23 May 2001

Abstract. The present study was designed to analyse the effect of red blood cells on T-cell proliferation and expansion. A comparative study was done in peripheral blood cell cultures stimulated with phytohemagglutinin, with or without red blood cells. The presence of red blood cells had a consistent enhancing effect on T lymphocyte proliferation, as determined by an increase in both the mitotic index and thymidine uptake. Phenotypic characterization of T cell blasts by flow cytometry revealed that, in the presence of red blood cells, expanding cells were preferentially CD8⁺ cells. Accordingly, proliferation of CD8⁺ lymphocytes from two patients with CD8⁺ hyperlymphocytosis was dependent on the presence of red blood cells. In contrast, proliferation of CD4⁺ lymphocytes from two patients with CD4⁺ hyperlymphocytosis was strongly inhibited by the presence of red blood cells. This is the first reported evidence that human red blood cells have an enhancing effect on the expansion of CD8⁺ lymphocytes *in vitro*.

INTRODUCTION

Routine cytogenetic studies can be successfully achieved using either one of the two types of phytohemagglutinin (PHA)-stimulated peripheral blood cultures: whole blood microcultures and macrocultures of separated leucocytes. The actual procedures (Verma & Babu 1995) have suffered little or no modification from the ones originally described (Moorhead *et al.* 1960; Arakaki & Sparkes 1963) and no particular criteria seem to underlie the choice to use one or another method. The two main differences between these methods are the presence or absence of red blood cells (RBC) and the optimal inoculum size necessary to obtain an adequate number of metaphases. This inoculum is increased for cultures of separated leucocytes (the term macroculture) when compared with the inoculum for whole blood cultures (the term microculture). This difference suggests that RBC may have some kind of enhancing effect on lymphocyte proliferation *in vitro*. Indeed, earlier studies demonstrated that human and sheep RBC enhance B- and T-cell responses after mitogenic stimulation *in vitro* (Tarnvik 1970; Johnson *et al.* 1972; Silva

et al. 1981; Sigfusson & Souhami 1984; Wilkinson & Morris 1984; Ebert 1985; Rugeles *et al.* 1987; Shau & Golub 1988). More recent studies suggest that the presence of RBC upregulate the expression of interleukin-2 (IL-2) receptor and the secretion of several cytokines by human T lymphocytes, which may result from the interaction of CD2 with its leucocyte function-associated (LFA)-3 ligand expressed by the RBC (Makgoba *et al.* 1987; Virella *et al.* 1988; Kalechman *et al.* 1993). Despite the accumulated evidence that RBC enhance lymphocyte proliferation, the possible physiological significance of this effect on immunological function(s) remains uncertain. Furthermore, the question of which subset of T lymphocytes could be influenced by RBC in culture has never been addressed. The main purpose of the present work was to study the effect of RBC on the mitogen-induced proliferation of T lymphocytes *in vitro* and, in particular, on the relative expansion of the CD4⁺ and CD8⁺ T-cell subsets.

MATERIALS AND METHODS

Subjects

A population of 16 randomly selected healthy blood donors from the Haematology Service of the Santo António General Hospital in Porto was used to perform cell cultures in the presence or absence of RBC. In addition, two patients with CD8⁺ hyperlymphocytosis and two patients with CD4⁺ hyperlymphocytosis were tested in order to examine the effect of RBC differentially in CD4⁺ or CD8⁺ cells. These were selected from a population of patients routinely evaluated by cytogenetic analysis in the course of the diagnosis and follow-up of a lymphoproliferative disorder, at the same hospital.

One of the patients was recruited to perform long-term cultures. This patient is a 58-year-old apparently healthy female with a clonal proliferation of CD8⁺ T cells detected on a routine examination. Follow-up of this patient for 10 years did not show any evidence of progression of disease, which was therefore considered a benign lymphoproliferative disorder.

All procedures were done with the informed consent of the participants.

Cells and cell cultures

For determination of the mitotic index samples of whole blood or blood depleted of RBC (total leucocytes) by gravity sedimentation were used. For determination of [³H]TdR uptake and cell phenotype, peripheral blood mononuclear cells (PBMC) were obtained from whole blood after separation on Lymphoprep (Density: 1.077 ± 0.001 g/mL, Nycomed, Oslo, Norway). RBC were separated from whole blood after centrifugation at 400 *g* and removal of the buffy coat and plasma.

Whole blood or RBC-depleted leucocytes were cultured at a white blood cell concentration of 0.5 × 10⁶ cells/mL, in RPMI complete medium supplemented with 15% fetal calf serum (FCS) and antibiotics. Cultures were stimulated with 5 µg/mL of PHA and placed in an incubator at 37 °C with a 5% CO₂ atmosphere.

PBMC were plated in triplicate in round-bottomed 96-well plates (Nunc) at 6 × 10⁴ cells/well in a final volume of 200 µL of RPMI complete medium supplemented with 10% FCS in the presence or absence of autologous RBC at a RBC : PBMC ratio of 100 : 1, stimulated with 5 µg/mL of PHA and placed in an incubator at 37 °C, 5% CO₂ and 99% humidity.

In some experiments CD4⁺ and CD8⁺ T cells were purified as described (Arosa *et al.* 1998). Briefly, T cells were obtained by depleting non-T cells (monocytes and B cells) after rosetting of PBMC with sheep red blood cells (ProBiologica, Lisboa, Portugal) yielding T lymphocytes

that were > 95% CD3⁺, CD4⁺ and CD8⁺ T cells were obtained by negative selection by indirect conventional panning techniques using rabbit anti-mouse surface immunoglobulin-coated plates and mouse anti-human CD4 and CD8 antibodies (DAKO, Denmark). The CD4⁺ and CD8⁺ selected populations usually contained < 10% contaminating CD8⁺ and CD4⁺ cells, respectively, as determined by flow cytometry.

Determination of lymphocyte proliferation: mitotic index and ³H-thymidine uptake

After 3 and 6 days of culture, cells in whole blood or RBC-depleted leucocyte cultures were harvested after 1 h incubation with colchicine (4 µg/mL) followed by hypotonic treatment with 75 mM KCl and fixed in a 1 : 3 solution of acetic acid : methanol. In view of the predicted low number of cell divisions in some cultures, a method was used intended to give accurate scores in the calculation of the mitotic index. After determining the cell concentration in the final suspensions, 50 µL were dropped on very well cleaned glass slides, air dried and stained with 4% Giemsa for 7 min. The mitotic cells (arrested metaphases during the one hour colchicine treatment) on the whole slide were counted using a light microscope (100× magnification). The mitotic index was calculated by dividing the total number of mitotic cells by the known total number of cells on the slide (× 100). Using this procedure it was possible to score mitotic cells in a population of 10⁴–10⁵ cells and determine mitotic indexes as low as 0.01%.

After 3 or 5 days of culture, PBMC cells were harvested on glass fibre filters using a semi-automatic cell harvester (Skatron, Norway) after a 4-h incubation with 0.74 kBq or 0.2 µCi of [methyl-³H]-thymidine (specific activity 185 GBq or 5.0 Ci/mmol, Amersham Pharmacia Biotech, Amersham, UK). The incorporated radioactivity was measured in a Beckman liquid scintillation counter. Proliferation index was defined as follows: c.p.m. in the presence of RBC/ c.p.m. in the absence of RBC.

Flow cytometry

For phenotypic characterization, 1 × 10⁶ PBMC were cultured in 5 mL round tubes (Costar, USA) in 1 mL of complete RPMI medium supplemented with 10% FCS and stimulated with PHA in the presence or absence of autologous RBC at a RBC : PBMC ratio of 100 : 1 for 5 days. PBMC were stained at day 0 (resting cells) and 5 days after mitogenic stimulation (activated cells) as described previously (Arosa *et al.* 1997). The following conjugated monoclonal antibodies were used: CD3–fluorescein isothiocyanate (FITC), CD4–phycoerythrin (PE) and CD8–PE (Dakopatts, Glostrup, Denmark) or CD8–PerCP (Becton Dickinson, San Jose, CA, USA). Five-day activated lymphocytes were harvested and red blood cells lysed in lysis solution (10 mM Tris, 150 mM NH₄Cl, pH 7.4). Cultures without RBC were also treated with lysis solution. After three washes with Hanks' balanced salt solution (HBSS) blast-activated lymphocytes were stained for 30 min at 4 °C in staining buffer (phosphate-buffered saline (PBS), 0.2% bovine serum albumin (BSA), 0.1% NaN₃) in round-bottomed microtitre plates (Greiner, Germany) with ~0.5 × 10⁶ cells/well. After staining the cells were washed three times with staining buffer and assessed within 24 h in a FACScan (Becton Dickinson). For each sample 10 000 viable lymphocytes, as determined by propidium iodide exclusion, were acquired, using forward and side scatter characteristics, and subsequently analysed using Lysys II software.

Statistical analysis

Group means for the MI and [³H]dT uptake in cultures with and without RBC were compared by the paired *t*-test.

Figure 1. Effect of RBC on the proliferation of PHA-stimulated cultures of peripheral blood cells from normal individuals. (a) Comparison between the mitotic index on the third day of whole blood cultures (RBC⁺) and cultures of blood depleted of red cells (RBC⁻) in 11 separate subjects. (b) Comparison between the ³H-thymidine uptake (c.p.m.) on the third day of PBMC cultures with (RBC⁺) and without red blood cells (RBC⁻) in five separate subjects.

RESULTS

Effect of RBC on the proliferation of PHA-stimulated cultures

Cultures of total leucocytes stimulated with PHA in the presence of autologous RBC (whole blood samples) proliferated more vigorously than cultures depleted of RBC. A consistent enhancing effect of RBC on the PHA response was shown by a significantly higher ($P < 0.0005$) mitotic index (MI) at day 3 in the presence of RBC (Fig. 1a). To better ascertain the effect of RBC on T cell proliferation, cultures of PBMC were stimulated with PHA in the presence or absence of autologous RBC. In agreement with the results obtained with leucocytes, [³H]TdR incorporation was significantly higher ($P < 0.005$) in cultures of PBMC with RBC when compared with cultures of PBMC without RBC (Fig. 1b).

Effect of RBC on the phenotype of proliferating lymphocytes

To ascertain whether the enhancing effect of RBC in the mitogen-induced proliferation of lymphocytes was due to the preferential expansion of CD4⁺ or CD8⁺ T cells, or both, we characterized the phenotype of the proliferating cells by two colour flow cytometry. As shown in Fig. 2, the presence of RBC enhanced the expansion of the CD3⁺ CD8⁺ T-cell subset 5 days after activation when compared with cultures without RBC. An increase in CD3⁺ CD8⁺ T cells induced by the presence of RBC was observed in the majority of subjects studied ($n = 7$) and was statistically significant (37.5 ± 7.0 without RBC versus 47.2 ± 11.1 with RBC, $P < 0.001$). In marked contrast, the percentage of CD3⁺ CD4⁺ T cells at day 5 was not significantly influenced by the presence of RBC (46.2 ± 5.5 versus 45.0 ± 8.8). To further characterize this effect we

Figure 2. Effect of RBC on the phenotype of proliferating lymphocytes. Peripheral blood mononuclear cells (PBMC) were stimulated with 5 µg/mL of PHA in the presence or absence of RBC for 5 days as indicated in the Methods section. Cells were stained before (resting cells) and five days after stimulation (activated cells) for CD3, CD4 and CD8 as indicated in the Methods. The percentage of CD8⁺ T cells at day 0 (resting PBMC) and 5 days after stimulation in the absence (PHA) or presence (PHA + RBC) of red blood cells among CD3⁺ gated T cells (left dot plots) are illustrated on the right histograms. Results are representative of seven separate experiments.

studied highly purified populations of CD4⁺ and CD8⁺ T cells. As illustrated in Fig. 3, the inability of CD4⁺ and CD8⁺ T cells to proliferate in response to PHA, was restored by the presence of RBC. Noteworthy, the presence of RBC preferentially favoured proliferation of the CD8⁺ T-cell subset, as evidenced by the higher proliferation index (Fig. 3).

Effect of RBC in PHA-stimulated cultures of CD8⁺ and CD4⁺ cells

The above results suggested that the enhancing effect of RBC in the response of mitogen-stimulated lymphocytes resulted in the preferential expansion of CD8⁺ T lymphocytes. To further corroborate this effect, cultures of peripheral blood cells from two patients with CD8⁺ T hyperlymphocytosis were studied (Table 1). In one case (patient 1) three replicate cultures were done. In another case (patient 2) two replicate cultures were done. In each culture the MI was determined at days 3 and 6. The two patients differed in terms of peak of proliferation (day 6

Figure 3. Effect of RBC on the proliferation of PHA-stimulated cultures of CD4⁺ and CD8⁺ T lymphocytes. Total PBMC, CD4⁺ or CD8⁺ T cells were stimulated with 5 µg/mL of PHA in the presence or absence of RBC for 5 days as indicated in the Methods section. T cell proliferation was determined on the fifth day of cultures after addition of 0.2 µCi of [³H]TdR for the last 4 h of culture. [³H]thymidine uptake (c.p.m.) and proliferation index (PI) in each culture condition is shown (mean ± SD of triplicates). Results are representative of two separate experiments.

Table 1. Effect of RBC in the mitotic index of cultured PHA-stimulated T lymphocytes from two patients with CD8⁺ hyperlymphocytosis and two patients with CD4⁺ hyperlymphocytosis

	Day 3		Day 6	
	RBC ⁻	RBC ⁺	RBC ⁻	RBC ⁺
Hyperlymphocytosis				
CD8⁺				
Patient 1				
Experiment 1	0.14	0.21	0.29	1.43
Experiment 2	0.26	0.37	0.53	1.22
Experiment 3	0.01	0.22	0.42	0.73
Patient 2				
Experiment 1	1.70	2.60	0.00	0.40
Experiment 2	0.00	1.54	0.00	0.21
CD4⁺				
Patient 3				
Experiment 1	0.65	0.03	0.22	0.03
Patient 4	0.34	0.02	1.01	0.02

The mitotic index was calculated as indicated in Materials and Methods.

RBC⁻: cultures of blood depleted of RBC.

RBC⁺: cultures of whole blood.

for patient 1 and day 3 for patient 2). A great variability was seen for the MI, in both patients, in replicates of RBC-depleted cultures at day 3. Remarkably, this variation was not seen in replicates of whole blood cultures. In spite of the variation described, the MI was, in general, consistently and significantly higher in whole blood cultures than in RBC-depleted cultures ($P = 0.003$). A similar result was obtained for [³H]TdR uptake in PBMC cultures with and without RBC (data not shown). In contrast, an opposite effect of RBC was seen in cultures of peripheral blood cells from the two patients with CD4⁺ hyperlymphocytosis (Table 1). The MI was consistently and significantly lower in whole blood cultures than in RBC-depleted cultures ($P = 0.029$).

Time in culture (weeks)		0	1	2	3	4	5	6	7	8	9
Total cell no. ($\times 10^6$)	+ RBC	1.26	6.30	10.85	8.54	3.92	8.96	5.53	3.78	5.25	8.12
	- RBC	2.59	6.16	3.78	2.38	3.78	1.89	2.45	2.73	2.59	1.33

Figure 4. Effect of the addition of red blood cells (RBC) in maintaining CD8⁺ cells in long-term PHA-stimulated culture. 'Whole blood' corresponds to starting whole blood culture. The times at which RBC or PHA were added are indicated by arrows or +, respectively. RBC were added only to starting whole blood cultures. The Mitotic Index calculated weekly is indicated. Total cell numbers in each culture studied weekly (+ RBC, starting whole blood culture; - RBC, culture of blood depleted of RBC) is indicated. One of three similar experiments.

Effect of RBC in long-term CD8⁺ T-cell cultures

Next we investigated whether the enhancing effect of RBC can be maintained for longer periods of time. For that purpose, peripheral blood cultures from one of the patients with CD8⁺ hyperlymphocytosis were set up. The culture medium was substituted weekly by fresh medium containing 5 $\mu\text{g/mL}$ of PHA. Fresh heterologous RBC, obtained from peripheral blood of normal donors of opposite sex, were added to the cultures of the patient with CD8⁺ hyperlymphocytosis in the proportion 100 : 1, after previous removal of old RBC by cell lysis followed by two washes with RPMI, on the 3rd, 6th and 8th weeks after the starting of the culture. Heterologous RBC have a similar enhancing effect as autologous RBC on T-cell proliferation (data not shown). As shown in Fig. 4, whenever fresh RBC, but not PHA, were added to the culture a new peak of proliferation occurred, coinciding with an increase in the MI, followed by an increase in total cell number. Karyotyping of the arrested metaphases confirmed the chromosomal constitution of the patient. Moreover, regular phenotyping during the time of culture revealed that the cells maintained in culture were CD8⁺ T cells (ranging from 90 to 97%).

DISCUSSION

Human peripheral whole blood cultures stimulated with PHA are the simplest and most widely used for routine cytogenetic purposes. With this method a minimum inoculum size is needed

to produce an efficient proliferation of lymphocytes. Because the presence of RBC is classically considered to be detrimental to the culture, due to their role in metabolizing vital nutrients in the medium, this problem is overcome by using a large volume of medium and a relatively small volume of blood (hence the term microculture). The cell preparations obtained from macrocultures of separated leucocytes contain less cell debris, which appears to be related to the relative absence of RBC and platelets in those cultures. The disadvantage of this method is the large volume of blood required to obtain an adequate dividing cell population. In fact, fewer mononuclear cells are needed to produce mitotic activity when they are introduced into the culture in the form of whole blood. This difference on the inoculum size between the two methods suggests that the presence of RBC may induce an enhancing effect on the proliferative response of lymphocytes to PHA. In fact, an influence of autologous RBC on human lymphocyte stimulation by PHA was first reported by Carstairs in 1962, who wrote that 'the presence of some red cells seems to benefit the cultures'. This idea was confirmed later by several authors who demonstrated a potentiating effect of human and sheep RBC on lymphocyte proliferation (Tarnvik 1970; Johnson *et al.* 1972; Silva *et al.* 1981; Sigfusson & Souhami 1984; Wilkinson & Morris 1984; Ebert 1985; Rugeles *et al.* 1987; Shau & Golub 1988).

In the present study we confirm that the presence of autologous RBC in cultures of human peripheral blood lymphocytes has a consistent enhancing effect on the proliferative response of lymphocytes to PHA. Importantly, we have demonstrated that the presence of RBC favours the expansion of T lymphocytes belonging to the CD8⁺ subset. Indeed, the percentage of CD8⁺ but not CD4⁺ T cells doubled after mitogenic stimulation of PBMC in the presence, but not in the absence, of RBC. Although previous reports have shown the enhancing effect of RBC on lymphocyte proliferation, characterization of the expanded T cells has never been addressed. Our results suggest that RBC may preferentially favour the proliferation of PHA-activated CD8⁺ T cells. The fact that peripheral blood CD8⁺ cells from a patient with CD8⁺ hyperlymphocytosis were able to proliferate over a 9-week period without the addition of growth factors or feeder cells but with the sole addition of PHA and fresh RBC strongly supports this assumption. On the contrary, RBC were seen to inhibit the proliferation of CD4⁺ T cells in two patients with CD4⁺ hyperlymphocytosis. However, this effect was not observed when isolated CD4⁺ T cells from normal individuals were used.

All these findings have important practical implications since they clearly show that according to the culture conditions there will be a differential modulatory effect on T-cell proliferation. They should be taken into account whenever cytogenetic or proliferative studies are performed in patients who may have alterations of the relative proportion of CD4⁺ and CD8⁺ T cells, like in cases of hyperlymphocytosis or T-cell chronic leukaemia. The higher MI and lower variability in whole blood cultures from patients with CD8⁺ hyperlymphocytosis makes this approach more suitable for routine cytogenetic analysis. On the contrary, in CD4⁺ hyperlymphocytosis patients RBC-depleted cultures should be used for cytogenetic studies. In addition, the present results may constitute an advance in our understanding of the regulation of the two major T-cell populations. The finding of a modulating effect of RBC on the relative expansion of the lymphoid sets *in vitro* may reflect a physiological regulatory function with important implications both in health and disease. Further studies are needed in order to characterize the mechanism(s) by which RBC enhance T lymphocyte proliferation. We have recently demonstrated that they could be related with the reduction of intracellular oxidative stress in activated human T cells (Fonseca *et al.*, 2001).

REFERENCES

- ARAKAKI DT, SPARKES RS (1963) Microtechnique for culturing leukocytes from whole blood. *Cytogenetics (Basel)* **2**, 57.
- AROSA FA, IRWING C, MAYER L, DE SOUSA M, POSNETT DN (1998) Interactions between peripheral blood CD8 T lymphocytes and intestinal epithelial cells (iEC). *Clin. Exp Immunol.* **112**, 226.
- AROSA FA, OLIVEIRA L, PORTO G, DA SILVA BM, KRUIJER W, VELTMAN J, DE SOUSA M (1997) Anomalies of the CD8⁺ T cell pool in haemochromatosis: HLA-A3 linked expansions of CD8⁺CD28⁻ T cells. *Clin. Exp Immunol.* **107**, 548.
- CARSTAIRS K (1962) The small lymphocyte; its possible pluripotential quality. *Lancet* **1**, 829.
- EBERT EC (1985) Sheep red blood cells enhance T-lymphocyte proliferation. *Clin. Immunol. Immunopathol.* **37**, 203.
- FONSECA AM, PORTO G, UCHIDA K, AROSA FA (2001) Red blood cells inhibit activation induced cell death and oxidative stress in human peripheral blood T lymphocytes. *Blood* **97**, 3152.
- JOHNSON, RA, SMITH, TK, KIRKPATRICK, CH (1972) Augmentation of phytohemagglutinin mitogenic activity by erythrocyte membranes. *Cell Immunol.* **3**, 186.
- KALECHMAN Y, HERMAN S, GAFTER U, SREDNI B (1993) Enhancing effects of autologous erythrocytes on human or mouse cytokine secretion and IL-2R expression. *Cell Immunol.* **148**, 114.
- MAKGOBA MW, SHAW S, GUGEL EA, SANDERS ME (1987) Human T cell rosetting is mediated by LFA-3 on autologous erythrocytes. *J. Immunol.* **138**, 3587.
- MOORHEAD PS, NOWELL PC, MELLMAN WJ, BATTIPS DM, HUNGERFORD DA (1960) Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp. Cell Res.* **20**, 613.
- RUGELES MT, LA VIA M, GOUST JM, KILPATRICK JM, HYMAN B, VIRELLA G (1987) Autologous red blood cells potentiate antibody synthesis by unfractionated human mononuclear cell cultures. *Scand. J. Immunol.* **26**, 119.
- SHAU H, GOLUB SH (1988) Modulation of natural killer-mediated lysis by red blood cells. *Cell Immunol.* **116**, 60.
- SIGFUSSON A, SOUHAMI R (1984) The effects of erythrocyte contamination on pokeweed mitogen induced immunoglobulin synthesis in man. *J. Immunol. Methods.* **72**, 167.
- SILVA A, LOPEZ-BOTET M, DE ALVAREZ J, LANDAZURI MD (1981) Enhancement of the functional activities of human T cells after their interaction with SRBC. *J. Immunol.* **126**, 393.
- TARNVIK A (1970) A role for red cells in phytohemagglutinin-induced lymphocyte stimulation. *Acta Pathol. Microbiol. Scand. (Sect B)*. **78**, 733.
- VERMA RS, BABU A (1995) Tissue culture techniques and chromosome preparation. In: Verma RS, Babu A, eds. *Human Chromosomes. Manual of Basic Techniques*, 2nd edn, p. 8. New York: Pergamon Press.
- VIRELLA G, RUGELES MT, HYMAN B, LA VIA M, GOUST JM, FRANKIS M, BIERER BE (1988) The interaction of CD2 with its LFA-3 ligand expressed by autologous erythrocytes results in enhancement of B cell responses. *Cell Immunol.* **116**, 308.
- WILKINSON M, MORRIS AG (1984) Role of the E receptor in interferon- γ expression: sheep erythrocytes augment interferon- γ production by human lymphocytes. *Cell. Immunol.* **86**, 109.