Effect of Contaminating Red Blood Cells on OKT3-Mediated Polyclonal Activation of Peripheral Blood Mononuclear Cells

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Received 7 September 2001/Returned for modification 23 October 2001/Accepted 4 March 2002

Erythrocytes are typically present as impurities in the majority of peripheral blood mononuclear cell (PBMC) preparations. This study was undertaken to investigate the effects of contaminating red blood cells (RBC) on the ability of OKT3 to activate CD4+ and CD8+ T cells. Surprisingly, the levels of gamma interferon, tumor necrosis factor alpha, and interleukin-1β (IL-1β) produced by PBMC upon stimulation by OKT3 were increased (P < 0.05) in a dose-dependent manner when increasing amounts of autologous RBC (RBC-to-PBMC ratios of 2:1, 10:1, and 50:1) were spiked into PBMC preparations. The OKT3-driven induction of the IL-2 receptor (CD25) and the proliferation of T lymphocytes in response to phorbol myristate acetate were not affected by the addition of RBC.

Lymphocytes are among the most extensively studied cells of the hematopoietic system because of their central role in the generation of immune responses. Information provided from the study of T lymphocytes is important not only in understanding the basic concepts of immune function but also in enabling the development of lymphocyte-based adoptive immune therapies. Lymphocytes can be collected from the peripheral blood, lymphoid tissues, and certain internal organs. In most cases, lymphocytes are initially isolated from the peripheral blood compartment and purified by Ficoll density gradient centrifugation. However, regardless of the method used to isolate T cells or peripheral blood mononuclear cells (PBMC), there always exists a low level of contaminating red blood cells (RBC). In addition, when PBMC are isolated on a large scale, as with most ex vivo adoptive immunotherapy approaches, the level of contaminating RBC increases even further. It has been shown previously that lymphocytes in whole blood stimulated with mitogen produce more interleukin-2 (IL-2) than Ficoll-Hypaque-purified lymphocytes in culture (5). What remains unknown is the effect of varying levels of contaminating RBC on the ability of well-characterized T-cell stimulants to activate lymphocytes under normal cell culture conditions.

A unique form of outpatient adoptive immunotherapy referred to as autolymphocyte therapy (ALT) for the treatment of patients with metastatic renal cell carcinoma has been developed (6, 6a). Patients are infused monthly with ~10^9 T lymphocytes activated ex vivo in a conditioned medium containing a mixture of OKT3 (mouse monoclonal anti-CD3 antibody) and a broad panel of autologous cytokines. The cytokine mixture is generated by stimulation of patient PBMC ex vivo with 25 ng of OKT3/ml for 3 days during the first cycle of the therapy (8). During the secondary cycles, i.e., monthly, of therapy, patient PBMC are cultured with the autologous cytokine mixture from the first cycle of therapy for 5 days and then infused back into the patient.

For this procedure, lymphopheresis is performed for each cycle to collect large numbers of PBMC. The resulting apheresis cell products (ACP) are highly enriched in white blood cells and contain various amounts of RBC, platelets, plasma, etc. The various ACP can vary greatly in their RBC content depending on the leukopheresis machine used, the skill of the apheresis technician, the clinical status of the patient, etc. In addition to the effects RBC could have on the preparation of cells on adoptive immunotherapy, RBC could also change immune parameters used in vitro to monitor immune responses in PBMC ex vivo during diseases or treatment trials.

Since it is difficult to generate large volume preparations of 100% pure PBMC, it would be desirable to know the potential effects of these contaminants on various critical parameters (cell phenotype, cell proliferation, and cytokine production) associated with the in vitro culture of human PBMCs. Herein, we report the results of a series of experiments in which the effect of increasing amounts of RBC on OKT3-mediated activation of PBMC was measured following the culture procedure used for outpatient adoptive immunotherapy, ALT.

MATERIALS AND METHODS

Cell sources. ACP from nine normal donors were used as sources of PBMC and RBC in this study, and they were collected using three different apheresis machines (three each from Haemonetics V-50, Fenwal CS-3000, and Cobe Spectra apheresis machines). The ACP were shipped overnight from multiple collection sites to the cell processing laboratory in thermally insulated boxes at room temperature. Previous studies had shown that viability (>70%) and CD3/CD25 expression (>50% of preculture values) were acceptable after 3 days of culture when cells were processed within 24 to 48 h. Cells held for 72 h before processing did not meet these specifications.

Cell separation. ACP were divided into two equal volumes, one aliquot to be used for isolation of PBMC and another for isolation of RBC. To isolate PBMC, 15-ml aliquots of ACP were diluted to 50 ml with saline. To remove platelets, the diluted ACP were centrifuged at 200 × g in a Sorvall RT 6000B centrifuge for 15 min. The supernatants, which contained platelets, were discarded. The cell pellets were resuspended with 35 ml of 0.9% saline (Baxter I.V. System, catalog no. 2B1323Q). The cell suspensions were underlaid with 14 ml of Lymphoprep
RBC were treated with 0.5 ml of Optilyse, vortexed, and incubated at room temperature for 30 min by following the manufacturer's instructions. Derived from the third day of primary cultures were analyzed for CD3/CD25 (IL-2R), major histocompatibility complex class II (MHC-II), and CD45RO levels utilizing enzyme-linked immunosorbent assay (ELISA) kits obtained from R&D systems (Minneapolis, Minn.).

Cell yield. Cell yield was determined using a Coulter ZM counter. Cell counts were taken immediately before adding the cells into the culture bags and immediately after harvesting the cells. Cell yields in the 3-day cultures varied from 83 to 98% of the starting cell population and in the 5-day cultures from 124 to 134% of the starting cell population. There was no statistically significant difference in cell yield when RBC were added to the PBMC.

Primary culture—cytokine production. To determine the effect of RBC on PBMC cytokine production, primary cultures were stimulated with OKT3 for 3 days. Cytokine production from PBMC increased with the addition of increasing amounts of RBC (Fig. 1A, B, and C). The levels of cytokine production from unstimulated cells were 17.7 ± 9.4 pg/ml for IL-β, 37.9 ± 6.5 pg/ml for TNF-α, and 74.8 ± 19.2 pg/ml for IFN-γ. IL-1β (Fig. 1A) production increased from 328 ± 109 pg/ml in the PBMC-alone group to 462 ± 154 pg/ml in the 10:1 mixtures of RBC and PBMC (P = 0.02) and to 624 ± 171 pg/ml in the 50:1 mixtures (P = 0.003). The IL-1β production in the 2:1 group was increased compared to that in the PBMC-alone group, but the difference was not significant statistically (P = 0.07). TNF-α (Fig. 1B) production increased from 1,174 ± 391 pg/ml in the PBMC-alone group to 1,377 ± 459 pg/ml in the 2:1 mixture (P = 0.02), to 1,624 ± 541 pg/ml in the 10:1 mixture (P = 0.02), and to 1,946 ± 266 pg/ml in the 50:1 mixture (P = 0.02). IFN-γ (Fig. 1C) production increased from 2,925 ± 975 pg/ml in the PBMC-alone group to 4,181 ± 1,394 pg/ml in the 2:1 mixture (P = 0.03), to 6,850 ± 2,283 pg/ml in the 10:1 mixture of RBC and PBMC (P = 0.01), and to 8,733 ± 1,309 pg/ml in the 50:1 mixtures of RBC and PBMC (P = 0.0003). The increases in cytokine production at higher RBC-to-PBMC ratios varied for the different cytokines. IFN-γ production showed the largest increase, threefold in the 50:1 mixture of RBC and PBMC relative to the PBMC-alone group. TNF-α production had a 1.7-fold increase, followed by IL-1β, which had a 1.9-fold increase.

There was also a variation in the cytokine response among donor. Some donors (R8, R9, and R15) produced more cytokine than others (R7, R10, R12, R13, R14, and R16). Generally speaking, if PBMC from a given donor produced low levels of one cytokine, all cytokine levels were reduced (data not shown). However, the effects of RBC additions were consistent.
When PBMC were cultured alone, 62% of CD3+ T cells (Table 1). Ratios, the difference was not statistically significant. The level of CD3+ T cells was almost equivalent whether or not RBC were added. CD3+ T cells expressed the CD45RO marker; once again, the addition of RBC had very little effect. Unlike CD45RO and CD25, the percentage of CD3/MHC-II-positive T cells decreased at higher RBC-to-PBMC ratios (P < 0.05) (Table 1). Thus, the only marker measured that was significantly affected by the addition of RBC was MHC-II.

**Secondary culture—phenotype.** Autologous PBMC were cultured with or without RBC in CM containing OKT3 for 5 days. Cells were then analyzed for the expression of a panel of differentiation and activation markers. The level of CD3+ T cells was 83.9% ± 4.9% when PBMC were cultured alone (Table 1). The addition of RBC to the PBMC population had very little effect on the level of CD3+ T cells present at the end of the culture. In addition, there was little effect on the levels of CD4+ and CD8+ T cells. The ratio of CD4+ T cells to CD8+ T cells was approximately 1:1 whether or not RBC were added to the PBMC at the beginning of the culture. The percentage of CD3/CD25-positive T cells generated when PBMC were cultured alone was 17.3 ± 2.5. Although there was an increase in CD3/CD25+ T cells at higher RBC-to-PBMC ratios, the difference was not statistically significant (Table 1). When PBMC were cultured alone, 62% ± 4.2% of the CD3+ T cells expressed the CD45RO marker; once again, the addition of RBC had very little effect. Unlike CD45RO and CD25, the percentage of CD3/MHC-II-positive T cells decreased at higher RBC-to-PBMC ratios (P < 0.05) (Table 1). Thus, the only marker measured that was significantly affected by the addition of RBC was MHC-II.

**Secondary culture—proliferation.** To determine if the function of PBMC was affected by the addition of RBC in the secondary culture, the PBMC from the secondary culture were evaluated for proliferative ability. After the initial 5-day culture, the PBMC were further stimulated with a low dose of PMA (1 ng/ml) for two additional days and proliferation was evaluated. There was no statistically significant difference (P > 0.5) in proliferation between PBMC with and without addition of RBC. The data (RBC-to-PBMC ratio, mean counts per minute ± standard deviation) were as follows: 0:1, 173,887 ± 54,887; 2:1, 197,117 ± 17,393; 10:1, 194,764 ± 17,030; 50:1, 202,937 ± 320,505.

**DISCUSSION**

Erythrocytes are a major cellular component of blood, and the normal RBC-to-PBMC ratio is approximately 600:1. RBC are also the major cellular impurity in ACP, which was used to isolate PBMC and generate therapeutic T cells. The range of the RBC-to-PBMC ratio in the apheresis products before PBMC isolation was from 1:4:1 to 105:0:1, with a large variation dependent upon which commercially available apheresis machine (Cobe, Fenwall, Haemonetics) was used. Here, RBC at a similar ratio range were added back to purified PBMC and the effects on T-cell activation were evaluated.

There were no significant differences in total cell yields whether or not RBC were added. CD3+ T cells expanded preferentially in both primary (data not shown) and secondary activation culture, composing about 80% of the cultured cells. CD4+ and CD8+ cells each made up about 50% of the T cells. Compositions of CD3+, CD4+, and CD8+ T cells were not changed with addition of RBC to the culture.

In the primary culture, 66.8% of CD3+ T cells expressed CD25 (IL-2 receptor) on their cell surfaces, while only 21.4% of CD3+ T cells in the secondary culture expressed CD25. Although CD25 expression on the T cells in the secondary culture was lower than that in the primary culture, RBC did...
not influence CD25 expression on T cells in either culture. In secondary cultures, CD3+ CD45RO+ T cells were 62% of the T cells in the PBMC-alone group; 35% would be observed for resting T cells (1, 2), indicating that the CM could stimulate the T cells. However, RBCs had no effect on the percentages of cells expressing CD45RO in the culture. Unlike CD45RO and IL-2R expression, the percentages of MHC-II-positive cells in the RBC groups were lower than in the PBMC-alone group. However, RBC could have a confounding effect on immunological assays based on serial or prognostic cytokine measurements. For serial measurements of cytokine from PBMC, RBC contamination should be kept to a minimum.

### ACKNOWLEDGMENTS

We thank Mary Lynne Headley and Robert Urban for their critical evaluation and support of the manuscript.

### REFERENCES


