Enhancement of Human Lymphokine-activated Killer Cell Cytolysis and a Method for Increasing Lymphokine-activated Killer Cell Yields to Cancer Patients


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ABSTRACT

In a continued effort to make interleukin-2/lymphocyte-activated killer (LAK) cell therapy safer and more efficacious for cancer patients, we examined methods of increasing the yields of cells obtained as a final product for reinfusion. Previously, the major cell loss occurred in the Ficoll-Hypaque gradient separation procedure used before cell culture. Therefore, we investigated the necessity of this step. Leukapheresis procedures (n = 105) from 40 different cancer patients showed that the resultant cell product is predominantly mononuclear (lymphocytes and monocytes; >97%) before the gradient purification step. The only cells observed to decrease in percentage as a result of the step were red blood cells (RBC:WBC ratio of 17:1 before purification to 1:3 after purification). Our study showed that the cytolytic potential of unpurified leukapheresis products against the LAK-sensitive line Dauid and the natural killer cell-sensitive line K562 was greater and that the patients received significantly more cells at times of reinfusion if the gradient separation step was eliminated. By additional experiments, we determined that autologous red blood cells enhance the generation of cytolytic LAK cells. This enhancement was greater if the red blood cells were in contact with the mononuclear cells during the time of cell culture. The elimination of the Ficoll-Hypaque purification step not only reduces the time and cost of the cell collection procedures, it also allows us to return to the patients greater numbers of cytolytic LAK cells following the activation period.

INTRODUCTION

The therapeutic efficacy of adoptive cellular immunotherapy has recently been shown by both West (1) and Rosenberg (2, 3). These studies employed LAK cells and high dose recombiant IL2 in the treatment of a variety of human cancers. The therapy has been particularly effective against melanoma and renal cell carcinoma. The long term effectiveness of IL2/LAK against other malignancies is not known but is presently being evaluated.

IL2/LAK therapy involves the in vivo administration of IL2 followed by repeated leukapheresis procedures. It is believed that IL2 priming causes the mobilization of LAK precursors into the peripheral circulation while the leukapheresis procedures collect large numbers of these precursors in the mononuclear fraction of cells along with other WBCs, RBCs, and platelets. A Ficoll-Hypaque gradient purification technique has routinely been used to remove contaminating granulocytes and red blood cells. Slow speed centrifugal washes have been used to remove the platelets. The resulting mononuclear cells are then activated by being cultured in a relatively high concentration of IL2 (a concentration which could not be safely achieved in vivo). During the culture period, the LAK precursors become activated to lyse tumor cell targets with less cytolytic activity observed against certain normal cells (7-9).

The numbers of cells collected in a typical leukapheresis procedure from cancer patients on this protocol range from 1.0 x 10^8 to 2.0 x 10^11. Considering that these cells are generally cultured for activation at 1.5-3.0 x 10^6/ml, the large numbers of cells obtained represent a tremendous task if conventional culture techniques in flasks or roller bottles are used. Recently, we have reported on the culturing of human LAK cells in Fenwal PL732 plastic bags (7). This has been confirmed by another lab (10). These gas-permeable bags provide a suitable environment for the activation of mononuclear cells to LAK cells and present a marked improvement for short term culturing of large numbers of cells.

In this report, we demonstrate that elimination of the Ficoll-Hypaque purification step provides several distinct time-saving and cost-cutting advantages without any detrimental effects on the procedure. The advantages of this improvement in the protocol are: (a) a significant increase in the numbers of LAK cells reinfused into the patient, (b) an increase in cytolytic activity of the LAK cells, and (c) a reduction in time of cell processing following the leukapheresis procedure. It appears that this enhancement of LAK cell generation is due to autologous RBCs being included in the cultures during the period of LAK cell activation.

MATERIALS AND METHODS

Mononuclear Cell Collections. Mononuclear cell collections were done as previously described (7). In brief, multiple leukapheresis procedures were performed on cancer patients receiving IL2/LAK cell therapy at the Biological Therapy Institute, Franklin, TN. Following the processing of 10-12 liters of whole blood on the Fenwal CS3000 automated blood cell separator, a sample of the mononuclear cells was removed from the collection chamber. This sample was manually washed to reduce the level of contaminating platelets by centrifuging the cells at 180 x g for 8 min (final platelet:WBC ratio was 12:1). The remaining cells in the collection chamber of the CS3000 underwent a Ficoll-Hypaque purification procedure which we have previously described (7) using the CS3000. The final product was resuspended and washed in the CS3000 with 2 liters of saline to remove Ficoll-Hypaque and residual platelets (final platelet:WBC ratio was 6:1). In both products, unpurified and Ficoll-Hypaque purified, the platelet contamination was in a range which we have previously determined (data not shown) to have no effect on LAK cell generation. In all cases, both Ficoll-Hypaque purified and unpurified cells were established in cell culture by the standard activation procedures as outlined below.

Culture Conditions. The culture vessels employed for the studies were either 1 liter Fenwal PL732 plastic bags (500 ml/bag), T175-cm2 plastic tissue culture flasks (150 ml/flask) or 775-cm2 plastic tissue culture flasks (25 ml/flask). The volumes and cell concentration (3.0 x 10^6/ml) were previously determined to be optimal for cell activation with the IL2 used in these studies (data not shown). The flasks and the PL732 bags were laid supine in a 37°C, 5% CO2 (in air) incubator for a period of 3-5 days. For the RBC studies, autologous RBCs were placed either in the top or bottom chamber of a Costar trans-well culture plate (Costar, Cambridge, MA). In all cases, the leukocytes were placed in the bottom chamber. The RBC:WBC ratio varied from 100:1 to as low as 3:1.

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2 The abbreviations used are: FBS, fetal bovine serum; IL2, interleukin-2; LAK cell, lymphokine-activated killer cell; LU, lytic unit.

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CULTURE MEDIUM
The complete liquid medium used in the experiments was obtained from M. A. Bioproducts (Walkersville, MD). The medium was RPMI 1640 (low endotoxin) containing 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM of L-glutamine, 50 μg/ml of gentamicin sulfate and 2% pooled heat-inactivated human AB serum.

Recombinant Human Interleukin-2. The IL2 employed in this study was provided by the Cetus Corporation (Emeryville, CA). The IL2 had a specific activity of 3.0 \times 10^6 units/mg of protein. The IL2 was reconstituted to a high concentration with sterile H2O and added to the culture medium to give a final concentration of 1000 U/ml.

TARGET CELLS
The human erythroleukemia cell line, K562, and the human B-lymphoblastoid cell line, Daudi, were used as tumor cell targets in standard 4-h \(^{51}\)Cr-release assays. The tumor cell lines which were mycoplasma free as determined by routine screening technique (11) were maintained at densities between 5 \times 10^4 and 1 \times 10^6 cells/ml of RPMI 1640 plus 5% heat-inactivated FBS and 2 mM L-glutamine. For cytotoxicity studies, cells were washed, counted and labeled with \(^{51}\)Cr for 2 h.

\(^{51}\)Cr Release Microcytotoxicity Assay. All \(^{51}\)Cr release assays were performed as previously described (12). Briefly, 2.5 \times 10^3 \(^{51}\)Cr-labeled target cells were incubated with varying numbers of effector cells in a final volume of 150 μl of RPMI 1640 plus 5% FBS. Incubation was carried out in V-bottomed microtiter plates at 37°C. After 4 h, 100 μl of culture supernatant was removed and counted for \(^{51}\)Cr. Maximal \(^{51}\)Cr was determined in the presence of 2 N HCl. Percent specific \(^{51}\)Cr release was determined by the formula:

\[
\text{Experimental cpm released - spontaneous cpm released} \times 100
\]

\[
\text{Maximal cpm released - spontaneous cpm released}
\]

In some cases, cytotoxicity is expressed in terms of LU. The number of LU is obtained by determining the number of effector cells necessary to give 33% lysis of 2.5 \times 10^4 target cells in a 4-h assay and dividing that number into 1.0 \times 10^7 effector cells.

Assessment of Cell Number and Viability. All leukapheresis products and activated cell cultures were assessed for viability by standard trypan blue exclusion analysis. Cell numbers were obtained by manual counts using a hemacytometer and a microscope. All counts were done in duplicate and were repeated if the values were not within 10% of one another.

Preparation of Red Blood Cells. When autologous RBCs were studied for their effect upon the generation of LAK cells, they were obtained from samples of Ficoll-Hypaque purified whole blood. In brief, the granulocyte layer was removed from the RBC pellet by aspiration. The RBCs were then washed three times in Hank’s balanced salt solution, counted, and examined for the presence of leukocytes. In all cases, leukocyte contamination was less than 1%.

RESULTS
Differential Analysis of Mononuclear Cells before and after Ficoll-Hypaque Purification. Cancer patients undergoing IL2/LAK cell therapy at the Biological Therapy Institute, Franklin, TN, underwent multiple leukapheresis procedures on a Fenwal CS3000. The cells were either further purified using a Ficoll-Hypaque purification technique on the CS3000 or were studied without purification. Differential analysis (N = 105) of Wright-stained cells obtained both before and after Ficoll-Hypaque purification showed that the predominant cells in the leukapheresis product were lymphocytes (Table 1). There was no significant difference in the percentage of lymphocytes both before and after the Ficoll-Hypaque purification step (before purification, 89.4 ± 9.2; postpurification, 92.2 ± 4.1). Similarly, there was no difference in the percentage of monocytes (prepurification, 8.2 ± 6.7; postpurification, 6.3 ± 2.3), or granulocytes (prepurification, 3.2 ± 4.1; postpurification, 1.5 ± 1.1). The recovery of mononuclear cells from the Ficoll-Hypaque purification procedure in the current study, based on precoultres, postcounts, volumes processed, and actual cells received in the laboratory was 65 ± 21% (N = 105). Interestingly, although there was a large loss in the number of leukocytes due to the purification procedure, the percentages of different types of leukocytes as determined by morphology were not significantly altered. However, RBCs were reduced significantly in numbers following the purification step (RBC/WBC prepurification ratio of 17:1 to a postpurification ratio of 1:3). In addition, there was a reduction in the numbers of platelets in the postpurification product (platelet:WBC prepurification ratio of 12:1 to a postpurification ratio of 6:1). These results indicate that RBCs were mostly eliminated by the Ficoll-Hypaque procedure and raised the question of whether RBCs had a deleterious effect on the generation of human LAK cells.

Cytotoxicity of LAK Cells. Cytotoxicity data obtained from Ficoll-Hypaque purified and nonpurified cells are presented in Fig. 1. The lytic activity of the nonpurified cells was significantly greater (N = 28, P < 0.001, matched pair Student’s t test) against both K562 and Daudi in the effector to target cell ratio of 100:1 to 0.75:1 after 3–5 days of culture as described in “Materials and Methods.” The data is presented as Lytic units per 10^7 effector cells. The LAK cells were obtained from different leukapheresis procedures (N = 28) of 10 cancer patients receiving LAK/IL2 therapy.
Table 2 Effect of Ficoll-Hypaque purification on cytolytic activity against Daudi with Ficoll-Hypaque purification step in some cases reduces the cytotoxic potential that can be generated in cancer patients' WBCs.

Effect of Purification on Total LAK Cells Generated. Sixteen patients were evaluated in regards to the total numbers of cells recovered and reinfusion following 3–5 day-incubation in the presence of IL2. Eight patients were treated using cells purified with Ficoll-Hypaque and eight patients were treated with unpurified cells. Each treatment was divided into two rounds, each round consisting of four leukapheresis procedures. The mean number of recovered cells was greater in both rounds 1 and 2 from the patients whose mononuclear cells did not undergo Ficoll-Hypaque purification (Table 3). In addition, the total number of cells received by the group of patients whose leukapheresis products were not purified was significantly greater (P < 0.01, Wilcoxon rank sum test) than the patients whose cells were purified (Ficoll-Hypaque purified, 1.06 ± 0.24 × 10^11; unpurified group, 1.91 ± 0.79 × 10^11). Thus, cancer patients whose leukapheresed cells were not further purified generally received increased numbers of LAK cells upon reinfusion.

Effect of Autologous RBCs on Generation of Human LAK Cells. As shown above, there was an increased number of cells available for the cancer patients when their leukapheresis products were not Ficoll-Hypaque purified. Analysis of the morphological data indicated that the only cells reduced in percentage by the Ficoll-Hypaque purification step were the RBCs. Since RBCs were found in greater numbers in the nonpurified preparations, we investigated the possible effect of autologous RBCs on LAK generation. Autologous RBCs were purified from whole blood as described in "Materials and Methods." The RBCs were added to mononuclear cell cultures containing IL2 at RBC:WBC ratios ranging from 100:1 to 3:1. The data presented in Fig. 2 shows that lytic activity was enhanced by the presence of additional RBCs. There was at least a twofold increase in lytic activity in each of three experiments in cultures when the RBC:WBC ratio was 100:1. The RBC preparation alone, at all the ratios, did not lyse Daudi target cells (data not shown). The enhancing effect decreases as the number of RBCs are reduced.

To investigate if the effect of RBCs on LAK generation could be explained simply by the presence of RBCs in the ^31Cr release assay, Ficoll-Hypaque purified cells, activated for 3 days in IL2, were tested for cytotoxicity with autologous RBCs added directly to the mononuclear cells at the time of the cytotoxicity assay (Fig. 3). Thus the RBCs were in contact with the mononuclear cells for only 4 h. The results indicate that RBCs added to primed LAK cells at the time of cytotoxicity assays had no enhancing effect at ratios which clearly enhanced lytic activity at the time of the culture period when LAK precursors become primed. RBCs appear to be exerting their enhancing effect on LAK generation during the time of the culture period when LAK precursors become LAK effector cells. Autologous RBCs do not appear to be having an effect on LAK effector function.

Effect of Contact between RBCs and WBCs during LAK Cell Generation. In order to examine if cell to cell contact was
DISCUSSION

A number of clinical centers are currently treating cancer patients with IL2/LAK cell therapy. This therapy involves IL2 priming of the cancer patients and subsequent leukapheresis procedures. The cells obtained from these procedures are placed in cell culture at low density, with a high concentration of IL2. During this culture period a fraction of the mononuclear cells become activated to lyse tumor cells (4–7) with less reactivity observed against normal cells (7–9). In earlier reports (1–3), leukapheresis products were subjected to Ficoll-Hypaque purification steps before the establishment of cell culture. This step was designed to eliminate granulocytes and red blood cells from the leukapheresis product. We report here that this purification step is unnecessary. In fact, this purification step may provide a cell product which, after incubation in IL2, is not as lytic to relevant LAK targets as one which is not subjected to the purification step. In addition, we observed that the principal cell type being removed from the mononuclear cells, RBCs, appears to enhance the generation of LAK cells during the culture period with IL2.

The original protocols developed for this therapy included Ficoll-Hypaque purification because it was felt that purified mononuclear cells would give the best reinfusion product of activated cells. In this study we show that this protocol probably results in fewer cells available for reinfusion as well as a less lytic LAK cell population. Recently, we developed a procedure for examination of leukapheresis products which facilitated their proper evaluation (13). Careful examination of leukapheresis cell products using this technique showed that products obtained from the CS3000 before purification consisted predominantly of lymphocytes and monocytes. The CS3000 product is usually very low in granulocyte contamination (<5%) and the RBC:WBC ratio is usually 5:1 or less. It is important to determine the cell types present in the cell products for each type of leukapheresis machine being used. Variations do exist in the final product composition due to the type of leukapheresis machine and the parameters set in the collection procedure.

Our data indicate that RBCs have a role in the in vitro enhancement of LAK cell activation. This does not exclude the possibility that LAK precursors are lost in the Ficoll-Hypaque purification step. Studies we have done on normal donors have indicated that, in some cases, LAK precursors are found in the waste product containing RBCs, granulocytes, and platelets (data not shown). This was not a consistent finding, however, but indicates that it might occur in some instances thus accounting for lower cytolytic activity of certain patients’ LAK cell populations.

Other investigators have reported that RBCs cause enhancing effects on the generation of both humoral (14–15) and cell-mediated immune responses (16). IL2 receptor expression on lymphocytes (15) as well as lymphokine release (i.e., γ-interferon, References 17 and 18) was shown to increase when lymphocytes were cocultured with autologous RBCs. In our system, RBCs cocultured with mononuclear cells resulted in an increased cytolytic response of the resulting LAK populations. Contact was required for the enhancement to occur. An effect due to a soluble factor cannot be excluded. However, release of the soluble factor must be the result of contact between the RBCs and the mononuclear cells. Alternatively, we examined whether RBCs were presenting IL2 to the mononuclear cells in a manner which could result in cytolytically enhanced LAK cells. RBCs preincubated with high concentrations of IL2 were washed and added to cultures of PBL. No enhancement or induction of LAK cells occurred (data not shown). Finally, it appears that the enhancement of LAK activity by autologous RBCs is on the induction phase of LAK and not on the effector phase. As shown in this report, large numbers of RBCs added directly to 4-h cytotoxicity assays containing LAK cells derived from Ficoll-Hypaque purified mononuclear cell preparations resulted in no increased cytolytic activity. At the moment, we do not know the exact mechanism which causes RBC enhancement of LAK activity. However, we are examining the phenomenon further in order to elucidate the mechanism and possibly take greater advantage of it in future LAK clinical protocols.

Much effort has been made to reduce patient toxicities and improve clinical responsiveness in the IL2/LAK therapy. Al-

![Fig. 3. Effect of autologous RBCs on a 51Cr-release assay. In this representative experiment (of four experiments performed), RBCs were added directly to LAK cells generated from Ficoll-Hypaque purified mononuclear cells immediately before the cytotoxicity assay was begun at ratios of either 100:1, 50:1, or 25:1 (RBC/LAK). The RBC/LAK cell combinations were then tested against Daudi at different E:T (LAK/target) ratios in a 4-h 51Cr-release assay. The control was LAK cells tested against Daudi with no added RBCs.](image-url)

![Fig. 4. Effect of RBC:WBC contact on the generation of human LAK cells.](image-url)
though previous work proceeded using standard methodologies, we and others have shown a significant decrease in time and money, as well as a reduced risk of contamination, utilizing automated methodologies (7, 10, 19). Along this line, in the present report, we have presented evidence that Ficoll-Hypaque purification of leukapheresis products before initiation of LAK cell cultures is neither required nor advised. We have shown current clinical protocols.

we and others have shown a significant decrease in time and greater numbers of LAK cells with higher lytic activity. We hope this change will increase the efficacy of LAK cells in our current clinical protocols.

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