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Augmentation of cell number and LAK activity in peripheral blood mononuclear cells activated with anti-CD3 and interleukin-2

Preliminary results in children with acute lymphocytic leukemia and neuroblastoma

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Summary. A wide variety of human cancers currently have no effective treatment and are potential targets for lymphokine-activated killer (LAK) cellular immunotherapy. Relapsed acute lymphocytic leukemia (ALL) and neuroblastoma are two of the major therapeutic challenges in pediatric oncology today. However, one problem which makes LAK immunotherapy in children particularly difficult is obtaining the large numbers of cells required. Present adult therapeutic LAK protocols have utilized short-term (5 day) cultures of interleukin-2 (IL2)-activated cells which are initially obtained from leukopheresis. Since routine use of this procedure in small children is not practical, we have investigated a different approach to obtain increased cell numbers by activation of peripheral blood mononuclear cells with OKT3, a mitogenic anti-CD3 monoclonal antibody, and IL2. Cell growth and LAK activity in OKT3 + IL2-activated cultures were compared to cultures activated with IL2 alone in 2 children with relapsed ALL and 2 children with stage IV neuroblastoma. OKT3 + IL2-activated cultures had marked increases in cell number: after 14 days the OKT3 + IL2-activated cultures yielded an approximately 500-fold increase in cell number compared to a 7-fold increase for cultures activated with IL2 alone. In vitro ^{51}Cr release assays were used to estimate LAK activity of the cultures at 7 and 14 days. When tested against HL60, a natural killer (NK)-resistant tumor cell line, not only were total cytolytic units greatly increased in OKT3 + IL2-stimulated cultures but lytic activity on a per cell basis (lytic units/1×10^6 cells) had also markedly increased on day 14 of culture. Phenotypic analysis demonstrated that 80% to 90% of cells in OKT3 + IL2-stimulated cultures were CD3 + T cells. Variable low percentages of CD16 + NK cells were seen in these cultures. In summary, OKT3 + IL2 activation resulted in a large increase in cell yield and the development of high level LAK activity using peripheral blood mononuclear cells from children with cancer. This approach may facilitate the utilization of increased cell numbers in future adoptive immunotherapy protocols, especially in pediatric patients.

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Introduction

Fresh populations of peripheral blood mononuclear cells are able to kill some tumor cell lines such as K562 without prior activation; cells which express this activity have been designated natural killer (NK) cells. Human NK cells are recognized morphologically as large granular lymphocytes and express antigens including the F_c receptor for IgG, (CD16), and HNK-1 (Leu-19) [7, 11]. It has been repeatedly demonstrated that peripheral blood mononuclear cells cultured with interleukin-2 (IL2) for 3 to 5 days (from here on referred to as short-term culture), develop the ability to lyse fresh tumor and NK-resistant cell lines. This activity has been termed the lymphokine-activated killer (LAK) phenomenon [7, 11, 16, 18].

Recent reports have concluded that LAK activity in short-term cultures is mainly attributed to activated CD16+ CD3− NK cells [7]. However, other groups have demonstrated that appreciable LAK activity can be derived from both CD4+ and CD8+ T cells as well as B cells [3]. Recent studies [4, 7] have indicated that other subpopulations which develop LAK activity include CD3+, CD4−, CD8− Tcells, and Leu-19+, CD2+, CD3−, CD16− NK cells. Finally, a report has detailed the ability of monocytes to develop increased tumor killing potential when cultured in the presence of IL2 [15]. Thus it appears that a variety of cell types including activated NK and possibly activated T cells or monocytes contribute to the LAK phenomenon.

LAK cells in conjunction with in vivo IL2 administration have been associated with significant tumor reduction in a variety of animal immunotherapy models [18, 20]. Results of trials in humans, however, have been variable [5, 19] with complete responses occurring in fewer than 10% of patients. One possible explanation for the marginal anticancer efficacy of LAK therapy in human trials is the variable cell doses used. It has been suggested [16] that at least 2×10^{11} cells would be required to provide an adult human with a cell dosage equivalent to that employed in curative murine adoptive immunotherapy models. Presently it has been difficult to consistently provide these very high cell numbers. Because of the difficulty in obtaining such large quantities of cells by standard short-term (3–5 day) LAK culture techniques, our laboratory has been investigating an approach using an anti-CD3 mono clonal antibody with mitogenic properties, OKT3, to stimulate cell proliferation in LAK cell cultures. This approach has been effective in the generation of not only increased

numbers of cells, but also in obtaining cell populations with reasonable levels of LAK activity [16].

It has been suggested that malignancies which infiltrate the bone marrow may be particularly susceptible to activated NK or LAK attack [2, 14]. Stage IV neuroblastoma involving the bone marrow and relapsed acute lymphocytic leukemia (ALL) are two major therapeutic challenges in pediatric oncology. Stimulation of cell-mediated immunity using IL2 and LAK cells may provide an additional, complementary approach to the treatment of children with poor prognosis neuroblastoma or ALL. However, the logistics of obtaining adequate numbers of functionally active cells for LAK therapy in children are formidable since standard leukopheresis methods to obtain large numbers of peripheral blood mononuclear cell are difficult, if not impossible, to perform in children. In view of these considerations we have investigated OKT3 + IL2 stimulation of peripheral blood mononuclear cells obtained by venipuncture in a small cohort of children with stage IV neuroblastoma and relapsed ALL with minimal marrow involvement.

Materials and methods

Patient material. Two 8-year-old children with ALL in bone marrow relapse were studied. Bone marrow aspirates obtained at the time of blood sampling contained 7% and 10% CD10 (CALLA)+ and TdT+ lymphoblasts; no lymphoblasts were seen in the peripheral blood smears of either child. Neither patient had received chemotherapy within 2 weeks of blood sampling.

Two children with stage IV neuroblastoma age 36 months and 18 months were studied. In both patients iliac crest trephine bone marrow biopsies revealed rare clumps of tumor cells which stained with neuron-specific enolase. Although the catecholamine metabolites, vanillyl mandelic acid (VMA) and homovanillyl acid (HVA), were markedly elevated in the urine at diagnosis, both children had been treated previously with chemotherapy and urinary VMA and HVA were within the normal range at the time of blood sampling. All patients were healthy, afebrile, with normal peripheral blood lymphocyte counts at the time of sample collection.

Heparinized blood was obtained by venipuncture. The volume of whole blood obtained ranged from 12 to 25 ml; all samples were processed in a laminar flow hood in a sterile manner. Blood samples were layered over Ficoll-Hypaque (specific gravity 1.078) and centrifuged at 400 *g* for 30 min. The band of mononuclear cells at the interface was removed and washed twice with phosphate-buffered saline, resuspended in tissue culture medium, and then counted.

Culture conditions. Samples were adjusted to a density of 5×10^5 cells/ml in RPMI 1640 medium supplemented with 25 m*M* HEPES, 2 m*M* L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (GIBCO, Grand Island, NY), 6% pooled heat-inactivated human serum, and 1000 units/ml recombinant IL2 (Cetus Corporation, Emeryville, Calif.).Cultures were incubated at 37° C in a humidified 5% CO_2 atmosphere. Cultures were sampled every 2 days and cell density adjusted to 5×10^5 cells/ml in fresh culture medium.

Anti-CD3 activation. Monoclonal antibody (OKT3, Ortho, Raritan NJ) was added only once at the beginning of the culture and not thereafter. Some experiments were done in the presence of 3-fold dilutions of OKT3 between 0.1 and 100 ng/ml in the presence of 6% heat-inactivated human serum or serum which had been depleted of IgG using Protein A-Sepharose (Pharmacia, Centennial, NJ) as described by the manufacturer. Since 10 ng OKT3/ml reliably and reproducibly activated peripheral blood mononuclear cells in the presence of IgG-containing serum, this concentration was employed in studies using patients' cells.

Tumor cell lines. Tumor cell lines K562 and HL60 were cultured in RPMI 1640 supplemented with 25 m*M* HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 m*M* L-glutamine with 10% fetal calf serum (GIBCO, Grand Island, NY). Cells were subcultured at a density of 5×10^5 cells/ml every 48 h.

Cytotoxicity assay. Tumor cell line targets (2×10^6 in 0.5 ml media) were incubated with 500 μCi $Na^{51}CrO_4$ (5,000 μCi/ml, New England Nuclear Research Products, Boston, Mass.) at 37° C for 90 min then suspended in 5 ml human heat-inactivated serum and centrifuged at 1500 *g* for 5 min. The supernatant was decanted, the pellet washed three times with 5 ml of tissue culture media, and then counted. A portion was resuspended in fresh media to yield 1×10^4 cells/ml. A total of 500 targets were added to each well.

Effector cells were aliquoted in triplicate in v-bottomed 96-well microtiter plates (Costar). Effectors were serially diluted such that effector to target ratios were 30:1, 10:1, 3:1, 1:1, 0.3:1, and 0.1:1. The microtiter plates were then gently centrifuged at 500 rpm for 5 min and incubated at 37° C. Spontaneous release (negative control) samples contained only media and targets; maximal release (positive control) samples contained detergent and targets. After 4 h plates were centrifuged at 1000 rpm for 10 min and 0.1 ml aliquots harvested from each well into glass scintillation vials. Radioactivity was determined by counting with 3 ml aqueous scintillation fluid (Biofluor, New England Nuclear Research Products, Boston, Mass.) in an LKB 1216 liquid scintillation counter. Percent cytotoxicity was determined using the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{experimental mean cpm} - \text{spontaneous release mean cpm}}{\text{maximal mean cpm} - \text{spontaneous mean cpm}} \times 100$$

Definition of cytolytic (lytic) units. Percent cytotoxicity for each effector to target ratio (0.1:1, 0.3:1, 1:1, 3:1, 10:1, and 30:1) was plotted on semilog graph paper. One lytic unit was defined as the number of effector cells required to achieve 40% cytotoxicity against K562 and 20% cytotoxicity for HL60 targets. Total lytic units per culture were estimated by dividing of the total number of cells (in millions) by the number of lytic units/1 $\times 10^6$ cells. Total number of cells was calculated as if all cells had been expanded and recovered at the time of subculture rather than aliquots (5–15 ml) serially subcultured.

Estimation of cell number increase. Cultures were sampled every 2 days; 10 μl culture aliquots were mixed with 10 μl of trypan blue and counted using a standard hemocytom-

eter. Thus estimates of cell proliferation were provided by direct cell enumeration. Viability of cells was also assessed by this procedure.

Immunofluorescent analysis of cell populations. These procedures were done at 4° C in subdued light. Between 5×10^6 and 10×10^6 cells from cultures to be analyzed were suspended in phosphate-buffered saline containing 0.5% fetal calf serum and 0.1% sodium azide (PBS, Sigma, St. Louis, Mo.). Cells (5×10^5 to 2×10^6/well) were then plated in u-bottomed 96-well microtiter plates and washed twice with 0.15 ml PBS buffer. Monoclonal antibodies used for analysis were fluorescein isothiocyanate conjugated (FITC) OKT3, OKT4, OKT6, OKT8 (Ortho, Raritan, NJ) recognizing CD3, CD4, CD1 and CD8 respectively and anti-Leu 11 (Becton Dickenson, Sunnyvale, Calif.) which recognizes CD16. The amount of FITC antibody per test was 10 µl for OKT3, OKT4, OKT6, OKT8, and mouse IgG (control) and 20 µl per test for Leu 11. Diluted FITC antibody was added to washed pellets, then the mixtures were gently agitated. Plates were incubated at 4° C for 30 min and then washed 3 times with PBS. Cells were fixed with PBS containing 2% paraformaldehyde [10], and then analyzed using a Becton Dickenson FACS IV fluorescence activated cell sorter. The combination of a subtractive routine using the negative FITC control and fluorescent intensity (>25 gating) was utilized to obtain the percentage positive fluorescent cells in the sample.

Results

Cell number increase in peripheral blood mononuclear cells stimulated with anti-CD3 (OKT3) antibody and IL2

Peripheral blood lymphocytes from two ALL and two neuroblastoma patients were cultured at 5×10^5 cells/ml in medium containing 1000 units/ml of IL2 and 10 ng/ml of OKT3. Fresh media with IL2 but no OKT3 was added every 48 h while the cell number was adjusted to 5×10^5 cells/ml. The 14-day growth profiles of representative ALL and neuroblastoma cultures activated with IL2 only or OKT3 + IL2 are illustrated in Fig. 1. When peripheral blood lymphocytes from patients with ALL or neuroblastoma were seeded at identical densities in IL2-containing media, marked differences in the rates of proliferation of cells with and without OKT3 stimulation were observed. Only 2 of 4 IL2-stimulated cultures of peripheral blood mononuclear cells without OKT3 showed modest increases in cell number (6- and 18-fold) by day 14; the other two patients had essentially no increase in cell number. Thus, either minimal or very poor proliferation was seen in cultures stimulated with IL2 alone. In marked contrast stimulation with both anti-CD3 monoclonal antibody (OKT3) and IL2 induced a significant increase in cell number in all four patients. The cultures which expanded best with OKT3 + IL2 stimulation also had better proliferation in media supplemented with IL2 alone. One ALL patient had a cell number increase of 800-fold and a child with neuroblastoma had a 735-fold increase in 14 days; the other ALL patient had an increase of 372-fold and the other neuroblastoma patient had an only 48-fold increase in cell number. Thus in four patients, the mitogenic effect of OKT3 + IL2 resulted in an average 500-fold increase in cell number.

Since inhibition of the mitogenic potential of OKT3 by human IgG or serum containing IgG has been previously reported [8], investigation of proliferation with and without IgG-depleted serum was performed (Fig. 2). Normal control peripheral blood mononuclear cells were cultured in medium with IL2, different concentrations of OKT3, and pooled human serum or human serum depleted of IgG. As shown in Fig. 2, if cells were activated in the presence of IgG-depleted serum, less OKT3 antibody was required for cell growth and increased cell yields were obtained at all antibody concentrations tested. Maximum cell number increase was obtained with 3 ng/ml of OKT3. Cell number increase was 2-fold higher in cells cultured in IgG-depleted serum as compred to cells cultured in pooled serum. Thus OKT3 activation of peripheral blood mononu

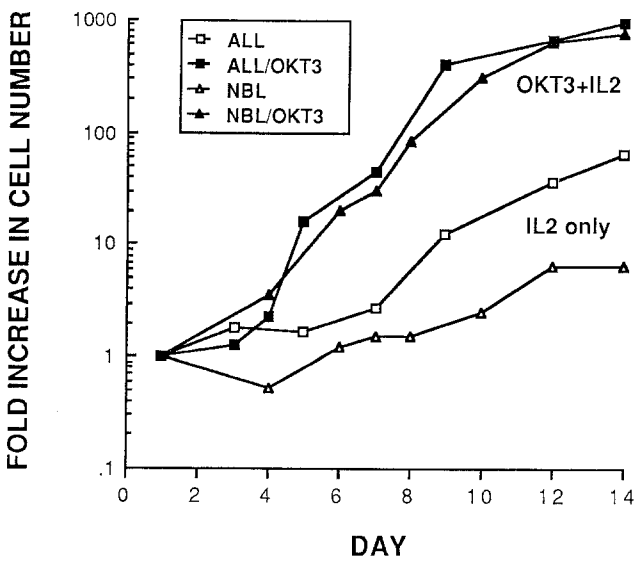

Fig. 1. Cellular increase in peripheral blood mononuclear cell cultured in interleukin-2 (IL2) or OKT3 + IL2. Representative samples from one patient with acute lymphocytic leukemic leukemia (ALL) and another with neuroblastoma (NBL) are shown. A logarithmic scale is used to illustrate the increase in cell number of the rapidly growing OKT3 + IL2-activated cultures

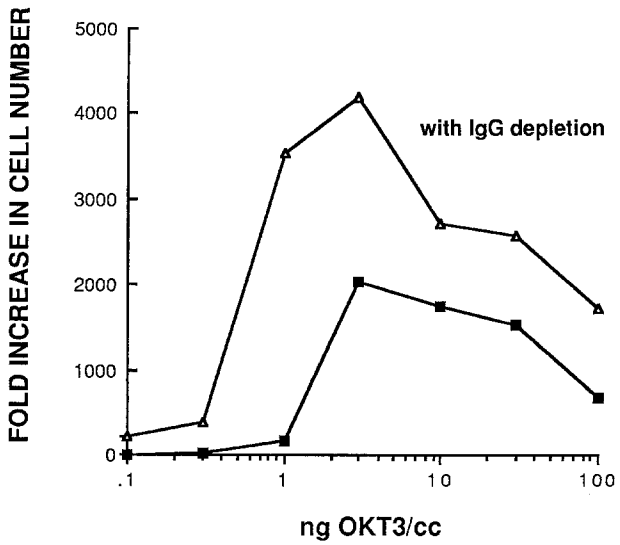

Fig. 2. Proliferation of peripheral blood mononuclear cells after OKT3 + IL2 stimulation. ■ = cultures with IgG (human serum); △ = cultures with human IgG-depleted serum

clear cells is more efficient when done in the presence of IgG-depleted serum.

Cytolytic activity on a per cell basis

The development of cytolytic activity on a per cell basis on days 7 and 14 of culture is summarized in Table 1. On day 7 LAK activity as indicated by lyis of the NK-resistant HL60 target was higher in patient cells activated with IL2 only than cells activated with OKT3+IL2. By day 14 though, the OKT3+IL2 cultures showed significantly increased LAK activity on a per cell basis.

Testing against the NK-sensitive K562 tumor target revealed high cytolytic activity on day 7 in cultures stimulated with IL2 alone; cytolytic activity on a per cell basis on day 14 was also high. Killing of K562 on a per cell basis by OKT3+IL2-activated cultures on days 7 and 14 was inferior to that of cultures stimulated by IL2 alone with the exception of one patient with ALL on day 14.

In summary, LAK activity on a per cell basis had a higher increase between day 7 and day 14 in the cultures stimulated with OKT3 and IL2 (>250-, 13-, 50-, and 3-fold increase) as compared to cells cultured in IL2 alone (>250-, 0.9-, 3.8-, 5-fold increase). In contrast, the development of cytolytic activity against K562 on a per cell basis was more variable. Nevertheless, the high LAK activity of day-14 OKT3+IL2-stimulated cultures was notable, especially when taken in the context of markedly increased cell numbers and the total LAK activity of these OKT3+IL2-activated cultures.

Total LAK activity

Total lytic units were determined by multiplying the number of lytic units/1×10^6 cells by the total number of cells (in millions) obtained in each culture. Presented in Fig. 3 are results of one ALL patient and one neuroblastoma patient using both HL60 and K562 targets. Data from the remaining two patients were similar and therefore not included. On day 14 moderate expansion of total LAK activity (lytic units/culture) was seen in specimens cultured with IL2 only (Fig. 3A); moreover, markedly increased proliferation in OKT3+IL2-stimulated cultures contributed to a significant increase in lytic units/culture. For example, on day 14 the OKT3+IL2-activated culture from the child with neuroblastoma illustrated in Fig. 3A had a

Table 1. Cytotoxicity on a per cell basis of peripheral blood mononuclear cells of patients with ALL and NBL cultured in IL2 or in OKT3+IL2. One lytic unit is defined as the number of effector cells required to induce, (*A*) 20% lysis of the natural killer (NK)-resistant cell line HL60, or (*B*) 40% lysis of the NK-sensitive K562 cell line in a ^{51}Cr release assay. The values represent number of lytic units/10^6 cells for each patient sample, thus representing estimates in the development of the cytolytic efficiency on day 7 and 14 of culture

Activation	Day	Patient[a]			
		1	2	3	4
A. HL60 target					
IL2[b]	7	QNS[d]	550[e]	750	770
IL2	14	5000	500	2850	4000
IL2+OKT3[c]	7	1	75	13	180
IL2+OKT3	14	5000	1000	660	500
B. K562 target					
IL2	7	QNS	2500	3570	4400
IL2	14	500	660	6660	5000
IL2+OKT3	7	280	165	1000	2000
IL2+OKT3	14	14	1800	290	1150

[a] Samples 1 and 2 from children with ALL; samples 3 and 4 from children with NBL
[b] 1000 units/ml
[c] 10 ng/ml on day 0
[d] Quantity not sufficient for analysis
[e] Lytic units/1×10^6 cells

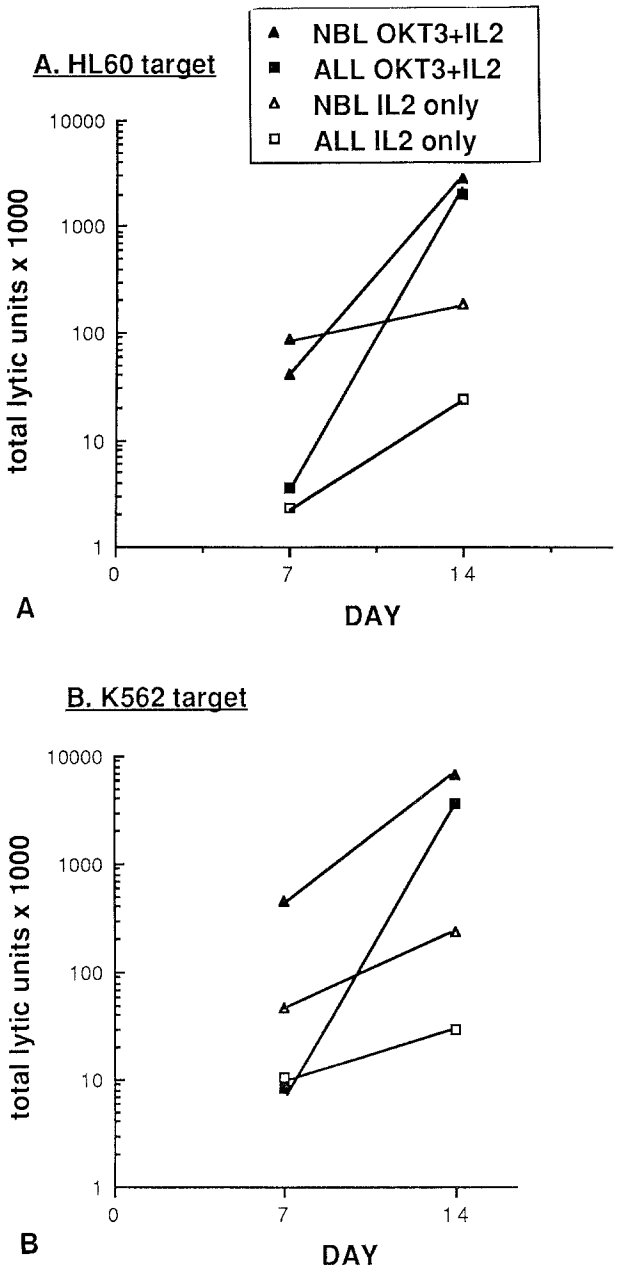

Fig. 3. Cytotoxic potential of day 7 and day 14 cultures of IL2 and OKT3+IL2-stimulated peripheral blood mononuclear cells from a representative patient with ALL and NBL as tested by, (A) theHL60 target, and (B) K562 target. Values on the ordinate represent $10^3 \times$ total lytic units as defined in *Materials and methods.* A logarithmic scale is required to illustrate marked differences in total cytolytic potential between cultures activated with and without OKT3

65-fold increase in total lytic units compared to day 7. In our hands the HL60 tumor target has been more difficult to kill than Daudi, another NK-resistant line, and more reliable than cryopreserved tumor specimens in the assay of LAK activity (data not shown). When tested against the NK-sensitive K562 target, all cultures had increased total cytolytic activity (Fig. 3 B); this was particularly evident in the rapidly growing OKT3 + IL2-stimulated cultures. Thus, OKT3 + IL2 activation resulted in both increased total cytolytic potential against an NK-sensitive target and increased LAK activity per culture as demonstrated by killing of an NK-resistant target.

Phenotypic analysis of long-term OKT3 + IL2-stimulated cultures

Table 2 presents representative immunophenotyping data from patients in this study as well as normal control samples. Cells were phenotyped for CD3+ T cells and for CD16+ NK cells on the first day of culture (day 0), day 7, and day 14 of cultures. By day 7 an increase in the percentage of CD3+ cells was evident for all OKT3 + IL2 cultures (data not shown). By day 14, CD3+ cells had increased to 85%. Approximately 70% of the cells in the OKT3 + IL2-stimulated populations were CD8+; cultures stimulated with IL2 only had variable but consistent increases in CD8+ cells on day 14. In contrast, the percentage of CD4+ cells had decreased in day 14 cultures whether stimulated with IL2 only or OKT3 + IL2. The percentages of CD16+ NK cells were low in initial day 0 cultures; for example 0.9% and 2.2% CD16+ cells were present in the children with neuroblastoma. Analysis of 7 and 14 day OKT3 + IL2 cultures from these same individuals yielded variable low percentages of CD16+ cells (0.4% and 0.1% CD16+ cells on day 7 and 1.6% and 0.2% CD16+ cells on day 14, respectively). However, despite CD16+ NK cells remaining in low percentages in these OKT3 + IL2 cultures, cells with this phenotype had 80- and 74-fold increases in total CD16+ cell number because of the extremely large cell number increase overall. Nevertheless, the majority of cells in these heterogenous long-term cultures were like CD3+, CD8+ cytotoxic/suppressor T cells and unlike CD16+ NK cells.

Table 2. Immunophenotyping of peripheral blood mononuclear cells before and after stimulation with IL2 alone or OKT3 + IL2. Material was processed as described in *Materials and methods* using fluorescein-conjugated monoclonal antibodies recognizing the CD3, CD4, CD8, and CD16 antigens. Values are from one representative (*A*) ALL patient and (*B*) NBL patient. The values of control (*C*) represent the median percentage of fluorescent cells from five normal control samples

	Percent fluorescent cells			
	CD3	CD4	CD8	CD16
Day 0				
ALL[a]	52	26	25	nd[d]
NBL[b]	37	31	6	2.2
Control[c]	67	44	22	8
Day 14 (IL2 only)				
ALL	84	7	70	4.5
NBL	36	4	30	0.9
Control	68	24	33	3.0
Day 14 (OKT3 + IL2)				
ALL	76	3	73	2.8
NBL	85	10	72	0.2
Control	89	16	66	3

[a] Results from one patient with ALL
[b] Results from one patient with NBL
[c] Median percent from five normal individuals
Not determined

Discussion

It has been shown that IL2-activated human peripheral blood mononuclear cells have very broad antitumor target specificity in vitro including many malignancies resistant to radiation and present chemotherapeutic agents [18]. Animal studies and human LAK therapy trials suggest that tumor regression is more significant when the combination of adoptively transferred IL2-activated cells and in vivo IL2 is administered than when IL2 is given alone [5, 18]. For example, only 1 of 21 patients with renal cell carcinoma responded to IL2 alone whereas 12 of 36 patients who received short-term (3–5 day) LAK cells in addition to IL2 had significant responses [5]. One possible reason for variability in the results of human trials may be the difficulty in obtaining, activating, and expanding enough peripheral blood mononuclear cells for the task of eliminating measurable (i.e., bulky) disease. LAK cell doses utilized to date (from short-term LAK cultures) have rarely been greater than 1.5×10^{11} cells. It has been suggested that at least 2×10^{11} cells may be required to provide adults with a cell dosage equivalent to that used in curative murine adoptive immunotherapy models [16]. Thus, it is possible that cell doses employed in many of the patients treated with short-term LAK therapy may be insufficient to adequately judge the merit of this type of immunotherapy of cancer.

One major obstacle that must be overcome before adoptive immunotherapy with IL2 and LAK cells can be utilized in children is the problem of cell dose. Standard LAK protocols in adults have utilized leukopheresis techniques to obtain cells to be cultured. The volume of blood required to prime the cytopheresis apparatus, venous access, and flow rates needed, and, of course, much less cooperation by young children make safe and routine utilization of this type of technology in infants and small children difficult at best. Therefore, this study was directed towards the development of an alternate method of obtaining adequate numbers of activated lymphocytes for pediatric LAK therapy utilizing small quantities of peripheral blood obtained by standard venipuncture as starting material.

Since an average leukopheresis in an adult yields approximately 4×10^9 cells, an over 20-fold expansion of cell number would be required to yield 2×10^{11} cells. This is commonly not achieved in standard LAK short-term cultures where cell yield is often less than 100%. The present study was undertaken to compare yields and in vitro cytotoxic efficiency of peripheral blood mononuclear cells from pediatric patients activated with IL2 or OKT3 + IL2. The latter strategy proved to be much more effective than the former in obtaining large numbers of cells. OKT3 + IL2 stimulation yielded an average 500-fold expansion of cell number versus an only 7-fold expansion for cultures stimulated with IL-2 alone. Mononuclear cell yield of whole blood was about 5×10^6 cells/ml; therefore,

a 500-fold expansion of mononuclear cells obtained from 100 ml of blood could potentially yield 2 to 3×10^{11} cells. Thus the method of OKT3 + IL2 stimulation may circumvent the problem of obtaining enough cells for pediatric LAK immunotherapy protocols.

The mitogenic effect of the OKT3 antibody which recognizes the CD3 determinant has been previously described in detail [8, 21] but only recently in conjunction with IL2 [16, 17]. It appears that efficient OKT3 activation requires binding of the Fc portion of the antibody by accessory cells [21]. Human plasma and IgG have been shown to markedly inhibit OKT3-induced mitogenesis [8]. Our experiments confirmed this inhibitory effect but demonstrated that excellent mitogenesis can nevertheless be achieved in 6% heat-inactivated human serum, a concentration which we had previously found to result in the best growth of IL2-stimulated peripheral blood mononuclear cells. Since significantly increased proliferation induced by OKT3 + IL2 stimulation was observed in serum depleted of IgG using Protein A-Sepharose (Fig. 2), this effect may possible be manipulated in future OKT3 + IL2 activation protocols to result in even further increases in cell yield.

The LAK cultures which were studied were composed of heterogenous populations of activated NK and T cells. Phenotyping results (Table 2) revealed, as expected, a high proportion of cells with T cell markers in OKT3 + IL2-stimulated cultures. Day 14 cultures contained low percentages of CD16+ cells; however, when combined with cell number increases in cultures this represented a significant increase in total numbers of CD16+ cells.

It has been proposed that the majority of the LAK phenomenon in short-term effectors is derived from the activation of NK cells [7]. The NK-associated marker, CD16, remained at low levels of expression in long-term cultures activated with either IL2 only or with OKT3 + IL2. The cytolytic activity of these long-term cultures against K562, an NK-sensitive cell line, appeared to be variable (Table 1). On the other hand, the lytic efficiency of cells in these same cultures against HL60, an NK-resistant cell line, was consistently increased between day 7 and 14 in all OKT3 + IL2-activated cultures. Delineation of the cell populations which contribute to these effects in OKT3 + IL2-stimulated cultures remains to be determined and will probably require cell sorting and double and/or triple color immunofluorescent analysis.

It has been recently demonstrated that about 3% of peripheral blood mononuclear cells are CD3+, CD4−, CD8− [9]. IL2-dependent cell lines established from cells with this phenotype mediate non-MHC restricted cytotoxicity against a variety of NK-sensitive and NK-resistant targets. These CD3+, CD4−, CD8− cells did not express the alpha/beta T cell receptor heterodimer, but rather a T cell receptor which is composed of the gamma/delta heterodimer [12]. It is possible that in addition to the common T cell phenotypes, CD3+, CD4+, CD8− and CD3+, CD4−, CD8+, OKT3 + IL2 stimulation mediates the activation of a CD3+, CD4−, CD8− subpopulation which is cytolytic [17]. Such a cohort of cells with T cell characteristics could contribute to the LAK activity observed in the OKT3 + IL2 cultures.

Despite our success in using OKT3 + IL2 stimulation to augment the proliferative and cytolytic potential of blood mononuclear cells in a small cohort of pediatric patients with relapsed ALL and stage IV neuroblastoma, issues remain to be resolved before the therapeutic potential of OKT3 + IL2 stimulation can be assessed. Although in vivo tumor killing ability of CD8+ cells and NK cells correlates well with in vitro ^{51}Cr release lysis assays [18], this still remains to be shown with activated CD16+ NK cells or CD3+, CD4−, CD8− T cell subpopulations. It is presently unresolved whether facilitation of tumor killing, immunologic help, or both are required for an effectiveimmunotherapeutic approach to cancer eradication in vivo [6]

The availability of a monoclonal antibody which is mitogenic and recognizes the murine equivalent of the CD3 antigen [1, 13] will facilitate the development of murine models to determine in vivo efficacy of anti-CD3 antibody + IL2-activated cells. Since characteristics of OKT3 + IL2 activation include significant proliferation and increase in total LAK activity, a major benefit of this approach would be to provide increased cell numbers for LAK therapy. This would especially facilitate pediatric adoptive immunotherapy protocols.

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