The potential of the CD3 monoclonal antibody (MoAb) OKT3 to selectively target lymphokine-activated killer (LAK) cells and T-cell clones in vitro against autologous tumor cells was studied using material from patients with acute leukemias (19 acute myeloid leukemias [AML], and 3 acute lymphoblastic leukemias [ALL]). Cytotoxicity mediated by patient LAK cells against AML blasts, but not against all cells and autologous Epstein-Barr virus--transformed B cells, was enhanced 1.5-fold to 5.3-fold by OKT3 in all AML patients studied. The following findings suggest that the major target molecule on AML cells for OKT3-coated LAK cells is the high-affinity Fc receptor for IgG (FcγRI; CD64): (1) susceptibility to killing by OKT3-coated effector LAK cells segregated with target cell expression of CD64; (2) preincubation of AML blasts with monomeric OKT3 (murine IgG2a), the Fc portion of which is known to have preferential binding affinity to CD64, resulted in lysis by autologous T cells that were not spontaneously cytotoxic; (3) OKT3-dependent increase in lysis of primary and relapsed AML cells by autologous T-cell clones correlated with the amount of target cell expression of CD64; (4) antileukemic cytotoxicity of OKT3-coated T cells could partially be inhibited by monomeric human Ig, the natural ligand of CD64; and (5) expression of CD64 (FcγRI) on fresh AML cells could be increased by interferon-γ (IFN-γ) and IFN-α transducing into further enhancement of lysis by autologous OKT3-coated LAK cells. Nonmalignant CD34+ cells sorted from peripheral blood were found to lack expression of CD64 and hence were not affected by OKT3-triggered T-cell targeting, as detected by colony formation assays. In conclusion, the in vitro data presented provide a rationale for the combined clinical use of recombinant interleukin-2, IFN-γ, and low doses of CD3 MoAb to eliminate AML cells while sparing nonmalignant hematopoietic progenitor cells, for example, in the setting of purging procedures for autologous bone marrow transplantation. © 1993 by The American Society of Hematology.

The relapse-free survival advantage in patients with acute myeloid leukemia (AML) or chronic myeloid leukemia eligible for allogeneic bone marrow transplantation is partially lost after the depletion of T lymphocytes demonstrating potential antileukemic activity of T cells that is associated with clinical benefit. This graft-versus-leukemia reaction suggests that immune cell-mediated leukemia cell elimination may become a therapeutic option also in the nontransplant setting of remission therapy or in autologous marrow transplantation purging procedures. Immunointervention with a predicted antileukemic efficacy requires a profound knowledge of the interaction between immune cells and both malignant and nonmalignant autologous cells on a cellular and molecular level. At present, there is no evidence that cytotoxic T cells of patients with myeloid leukemia recognize tumor-specific antigens encoded by a unique family of genes in the context of HLA molecules on autologous tumor cells, as it is the case in malignant melanoma. However, HLA-restricted tumor cell recognition can be circumvented by bispecific antibodies that, in addition to the T-cell receptor (TCR)/CD3 complex, bind to a target cell antigen, as has been shown for solid tumors and common acute lymphoblastic leukemia (ALL). CDw32 and CD16 both have low affinity to monomeric IgG and bind to the membrane through a phosphatidylinositol glycan motif of CDw32 exists, giving rise to functionally and biochemically distinct forms. CD16 occurs bound to the membrane through a phosphatidylinositol glycan linkage on neutrophils, and as transmembrane form on monocytes and natural killer (NK) cells. CDw32 is found on myeloid cells, B cells, some T cells, and some endothelial cells. Allelic polymorphism of CDw32 exists, while sparing nonmalignant hematopoietic progenitor cells, for example, in the setting of purging procedures for autologous bone marrow transplantation. © 1993 by The American Society of Hematology.

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eloidal human leukemic target cells are responsible for triggering T cells carrying surface-bound CD3 antibodies.

The purpose of this study was (1) to investigate the efficiency and specificity of the immunostimulatory potential of the CD3 antibody OKT3 in vitro in patients with acute leukemia, (2) to identify the FeR subtype on leukemic cells involved in the interaction with CD3 MoAb-coated T lymphocytes, and (3) to determine the toxicity of CD3 MoAb-mediated T-cell targeting for nonmalignant hematopoietic progenitor cells.

MATERIALS AND METHODS

Patients and autologous test systems. For functional immunologic studies, fresh leukemic cells were cryopreserved using a rate-controlled biologic freezer (TRA-12; Crysogen, Schoellkrippen, Germany) following standard Ficoll-Hypaque (Pharmacia, Freiburg, Germany) isolation from peripheral blood obtained at diagnosis and in 5 cases at relapse from consecutive nonselected patients with AML (n = 19) and ALL (n = 3). Only patients presenting with elevated initial white blood counts (>20,000/μL) were studied. The immunophenotype of leukemic cells was extensively characterized, as described.15,16 The percentage of leukemic cells was determined by immunophenotyping with MoAbs directed against a myeloid antigen in the case of AMLs or against CD10 or CD24 in the case of common-ALL and B-ALL, respectively. The leukemic cell content ranged between 85% and 99%. If the leukemic cell fraction was less than 90% in the case of relapsed AML, contaminating normal T cells were depleted using OKT3 MoAb (Ortho, Raritan, NJ) and goat-antimouse-coated magnetic beads (Dynal, Hamburg, Germany) according to the manufacturer’s instructions, resulting in greater than 95% pure leukemia cell preparations.

Permanent EBV-B cell lines were established from the leukemia patients studied as described17 and were used as autologous nonmalignant target counterparts in cytotoxicity testing. In brief, 4 x 10⁶ peripheral blood mononuclear cells (PBMC) per well were cultured in RPMI1640 (GIBCO, Grand Island, NY) with 20% fetal calf serum (FCS; Seromed, Berlin, Germany) supplemented with L-glutamine (2 mmol/L; Seromed), sodium pyruvate (1 mmol/L; Seromed), and cyclosporin A (60 ng/mL; Sandoz, Basel, Switzerland) (EBV medium) in 24-well multidishes (Nunc, Roskilde, Denmark) in a final volume of 2 mL per well. At the beginning of culture, 50% EBV-containing B95/8 supernatant was added. EBV-producing B95/8 cells were generously provided by Dr J. Walter (German Cancer Research Center, Heidelberg, Germany), and supernatant was harvested when viability of B95/8 cells in culture had decreased to 60%. Cultures were fed weekly with EBV medium and continuously growing EBV-B cell lines were obtained between 4 and 6 weeks of culture. In cases in which no EBV-B cell lines were obtained, phytohaemagglutinin (PHA)-stimulated blast cells were prepared by culturing 5 x 10⁶/mL PBMC in B medium with PHA (5 μg/mL; Wellcome, Dartford, UK). On day 3, lymphoblasts were pelleted and expanded in recombinant human IL-2 (rIL-2; 25 U/mL)–containing B medium (RPMI 1640 [GIBCO] supplemented with 10% FCS, L-glutamine [2 mmol/L], and sodium pyruvate [1 mmol/L]) for 3 to 4 weeks. rIL-2 was a generous gift from Biogen (Cambridge, MA).

Informed consent was obtained from patients. The volume of blood samples obtained never exceeded 60 mL and did not interfere with patient management.

Maintenance of cell lines. The K562 and U937 cell lines, (all obtained from the German Cancer Research Center) and patient EBV-B cell lines were maintained in suspension culture in RPMI1640 (GIBCO) supplemented with 10% heat-inactivated FCS B medium. Only cells in logarithmic growth phase were used for experiments. Every 2 weeks, cell lines were tested for the presence of mycoplasma by the DNA staining procedure using bisbenzimide (Seromed). Contaminated cultures were discarded.

Generation of CD3ε effector cells by long-term culture with rIL-2. Patient PBMC were isolated by Ficoll-Hypaque gradient centrifugation in complete clinical remission when PB counts had recovered from chemotherapy. PB lymphocytes (PBL; 10⁷/mL) were cultured in RPMI1640 with 10% heat-inactivated human serum pooled from 7 to 9 healthy male donors, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL) (T medium) in the presence of rIL-2 (1,000 U/mL) in upright standing tissue culture flasks (final volume, 10 mL for Falcon 3013 or 20 mL for Falcon 3024; Lincoln Park, NJ) for 10 to 35 days. Cell concentration was adjusted every 3 to 4 days by renewing T medium, rIL-2, and culture flasks. This culture system resulted in expansion of CD3ε cells. The content of T cells was greater than 85% by day 10 of culture.

Production of T-cell clones. Autologous mixed lymphocyte tumor culture (AMLTC) was performed as described previously18 with the modification that mitomycin C–treated autologous tumor cells were used as stimulator cells. Mitomycin C inactivation consisted of 30 minutes of incubation at 37°C and 5.5% CO₂ of 2 to 10 x 10⁶ cells/mL B medium with 100 μg/mL mitomycin C, followed by three washing steps with phosphate-buffered saline (PBS) to remove soluble mitomycin C. Responding T cells were cloned by limiting dilution in 96-well round-bottomed plates (Nunc; 163320) in T medium containing 25 U/mL rIL-2 at 0.3 lymphocytes/well using 2 x 10⁶/well mitomycin C–treated allogeneic EBV-B cells as feeder cells and 3 x 10⁶/well mitomycin C–treated autologous tumor cells as stimulator cells. After 7 and 14 days, 100 μL of supernatant was replaced by fresh medium with the same concentration of feeder and stimulator cells. On day 21, restimulation was repeated by adding 200 μL fresh stimulator and feeder cell suspension with rIL-2 (25 U/mL) to responder cells that had been transferred to 96-well flat-bottomed plates (Nunc; 167008). Seven days later, wells were screened for T-cell expansion and positive microcultures were expanded in 24-well plates by adding 2 x 10⁶/well allogeneic EBV-B and 5 x 10⁶/well autologous tumor cells (mitomycin C–treated). Clones were screened for cytotoxicity against autologous tumor and EBV-B cells and cell lines, respectively. Phenotyping of interesting clones was performed by indirect immunofluorescence analysis. Southern blot analysis of the T-cell clones described using a T-cell receptor β (TCRβ) probe was performed as described.19 The clones presented showed clonal TCRβ gene rearrangements.

Activation with CD3 MoAbs. The CD3/Ti MoAbs OKT3 (Ortho), Leu4, and WT31 (Becton Dickinson, Heidelberg, Germany) were added to effector cells 45 minutes before cytotoxicity testing and were present throughout the assay at a final concentration of 80 ng/mL in most experiments. In some experiments, OKT3 MoAb was bound to either effector or target cells by incubating indicated amounts of OKT3 MoAb with given numbers of effector or target cells in 200 μL B medium for 30 minutes at 4°C, followed by three washing steps with PBS to remove unbound MoAb. The OKT3-coated effector or target cells were then used in cytotoxicity assays. In blocking experiments, OKT3-induced T-cell–mediated cytotoxicity was measured in the presence of monoclonal or complexed (incubated at 56°C for 30 minutes) human IgG (hiG; German Red Cross, Hagen, Germany).

Cell-mediated cytotoxicity assay. Cryopreserved leukemic cell samples were thawed 1 day before assay and cultured overnight at 10⁶ cells/mL in B medium. If the viability of leukemic cells as determined by Trypan blue (Seromed) exclusion was less than 70%, dead cells were removed by Ficoll-Hypaque gradient centrifugation. Au-
tologenous EBV-B cells or cell lines were used when they showed logarithmic growth in culture. Target cells (2 × 10^4) in 100 µL FCS were labeled with 300 µCi (leukemic cells) or 100 µCi (cell lines) Na^2CrO_4 (Behring, Marburg, Germany) for 2 hours at 37°C and 5.5% CO_2. Cells were washed once with PBS followed by a second incubation at 37°C and 5.5% CO_2 for 30 minutes in 5 mL B medium. Labeled cells were washed twice with PBS and B medium, respectively. Target cells (5 × 10^5) in 100 µL B medium were added to 100 µL B medium containing effector cells in various dilutions. Determinations were made in duplicate or triplicate in 96-conical well plates (Costar 3898; Cambridge, MA). Plates were centrifuged for 5 minutes at 150g and incubated for 4 hours at 37°C and 5.5% CO_2. Supernatants were then harvested using a Skatron system (Skatton, Lier, Norway) and counted in a gamma counter (LKB, Wallac, Bromma, Sweden). The percentage of specific 51Cr release was calculated using the following formula: percentage of specific release = (ER - SR) × 100/ER - SR, where ER was experimental 51Cr release, SR the spontaneous 51Cr release as measured by incubating 5 × 10^5 target cells in 200 µL B medium alone, and MR the maximum release after the addition of 100 µL 1% Triton X100 (Sigma, St. Louis, MO). Standard deviations were less than 10%, and only results of experiments with SRs not exceeding 25% were included in the final analysis.

Flow cytometry analysis of effector T cells and of fresh leukemic cells and target cell lines. Effector T cells were analyzed by incubating 10^5 cells with the following antibodies: Leu 4 (CD3), Leu3a (CD4), Leu19 (CD56), WT31 (all from Becton Dickinson), OKT8 (Ortho), and TCRd1 (T-Cell Sciences, Boston, MA). Fresh leukemic cells and established target cell lines were investigated for expression of Fcy Rs by using the following MoAbs: Leu11a (CD16; Becton Dickinson), IV.3 (3Dw32; Medarex, Lebanon, NH), 2E1 (CDw32; DiaNoN, Hamburg, Germany), 32.2 (CD64; Medarex), and Tuel (CD23; Biotech, Dreieich, Germany). After 30 minutes of incubation at 4°C, cells were washed once, resuspended in 50 µL goat-antimouse FITC Fab' fragments (Immunotech, Marseille, France), and incubated on ice for 30 minutes. Stained cells were washed and analyzed on a FACScan Cytometer using the Lysis II software package (Becton Dickinson). Human IgG (German Red Cross) was present throughout all incubation steps at a final concentration of 4 mg/mL to block nonspecific binding via FcRs. Human IgG did not interfere with specific binding of CD64 MoAb (32.2), because its epitope is distinct from the IgG binding site.

Induction of Fcy Rs on leukemic cells. Thawed fresh leukemic cells showing greater than 90% viability were cultured at 10^5 cells/mL for 1 to 4 days in B medium containing 100 U/mL IFN-γ or IFN-α. Cells were then tested for expression of Fcy Rs as described above. IFN-pretreated cells were also simultaneously used as targets in cell-mediated cytotoxicity assays using autologous lymphokine-activated killer (LAK) effector cells.

Colony formation assays. PBMC (5 × 10^5) of a healthy donor and 200 or 2,000 cells of the monocytic leukemic cell line U937 were seeded in semisolid culture media (HCC-11.3a; Terry Fox Laboratory, Vancouver, Canada) in 3.5-cm petri culture dishes (Nunc) and were incubated at 37°C in a high-humidity 5% CO_2/95% air incubator. After 14 days, the total number of colony-forming units (CFU), clusters (<40 cells), CFU-granulocyte-macrophage (CFU-GM), CFU-erythroid (CFU-E), burst-forming unit-erythroid (BFU-E), and CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) were counted. The effects of LAK on cells or T-cell clones of nonleukemic malignant progenitor cells and on the leukemic cell line U937 were studied by preincubating autologous PBMC for 4 hours with autologous LAK cells or with an autologous CD4+ T-cell clone that was coated with OKT3. LAK cells or the T-cell clone did not form colonies.

Sorting of CD34+ nonmalignant hematopoietic progenitor cells. PBMC (5 × 10^5) were isolated by Ficoll-Hypaque centrifugation of whole blood of a patient with Hodgkin’s disease with minimal residual disease who underwent leukapheresis after peripheral stem cell mobilization with chemotherapy and granulocyte colony-stimulating factor (G-CSF). 2.3% of PBMC were CD34+ at the time of PBMC collection. PBMC were incubated with human IgG (4 mg/mL final concentration) and canine serum (1:10 final dilution; Sigma, Munich, Germany) to block Fcy R binding sites before adding HPCA-1 MoAb (Becton Dickinson; 1:50 final dilution) for 30 minutes at 4°C. After washing with PBS/0.5% bovine serum albumin (BSA), cells were incubated with rat antimouse IgG1 MoAb coupled with paramagnetic microbeads (Miltenyi, Cologne, Germany) for 15 minutes at 4°C and, after washing, were passed through an A2 steel wool column positioned in a MACS sorting device (Miltenyi, Cologne, Germany) following the manufacturer’s instructions. Subsequently, cells were stained with goat-antimouse phycoerythrin (GAM-PE; Medac, Hamburg, Germany) and with fluorescin isothiocyanate (FITC)-labeled 32.2 (CD64) or IV.3 (CDw32; both from Medarex, Lebanon, NH). One round of sorting resulted in cell populations that contained 60% to 65% CD34+ progenitor cells, 15% to 25% monocytes, and 10% to 15% lymphocytes.

Statistics. Linear regression analysis and Wilcoxon test for paired rank sums were used as appropriate.

RESULTS

Cytotoxic activity of LAK cells against autologous AML and autologous nonmalignant EBV-B cells. Table 1 shows screening results of a series of 22 nonselected patients (19 with AML and 3 with ALL). LAK cells were generated from remission blood lymphocytes of acute leukemia patients by culture in high-dose rIL-2 (1,000 U/mL), resulting in greater than 85% CD3+ effector cell populations. LAK cells were tested for cytotoxic activity against autologous leukemic and EBV-B cells. This test system showed three patterns of cytotoxicity. In cases no. 2, 4, 6, 9, 12, 14, 17, and 19, LAK cells were selectively cytotoxic for leukemic cells. LAK cells of patients no. 3, 10, 11, 13, 15, 16, and 18 were similarly cytotoxic for both tumor and EBV-B cells. In ALL patients and in AML patients no. 1, 5, 7, and 8, an overall small or absent cytotoxic response was detected.

In contrast to these heterogeneous specificity patterns of LAK cells, a rather uniform picture emerged when the same screening assay was performed in the presence of the CD3 MoAb OKT3. In all AML patients studied, the addition of OKT3 MoAb enhanced antileukemic cytotoxicity 1.5- to 9.3-fold. Cytotoxicity against autologous EBV-B cells was not affected or even reduced. The low cytotoxic activity against the 3 lymphoid leukemic cell samples studied was unaltered. In summary, addition of OKT3 MoAb increased efficiency and specificity of effector cells against autologous myeloid leukemic cells in this test system.

Mechanism and potency of anti-CD3–mediated enhancement of cell-mediated cytotoxicity against myeloid leukemic cells. To dissect the cytotoxic antileukemic response observed, PBMC of patients no. 2 (AML-M2) and 12 (AML-M4eo) were activated by AMLTC. Responding T cells were cloned by limiting dilution. The specificity analysis of 4 representative clones is shown in Fig 1. The two CD3+ CD8+ clones L.37 and G27 expressing T-cell receptors of the
recognition. subsequently tested for their cytotoxicity against SG AML OKT3, followed by removal of unbound MoAb. They were AML-M4eo were incubated with varying amounts of

icity by OKT3 MoAb, LAK cells of patient no. 12 with

range of specific cytotoxicity of 20% to 60% at effector target

4-hour "chromium release assays and maximum responses are shown. Screening was performed in the absence or presence of OKT3 (BO ng/mL).

antileukemic cytotoxicity was reached at 180 pg (I .2 fmol)/

spontaneous cytotoxicity against autologous leukemic cells

NK-like activity against K562 and

killing of autologous fresh blast cells was clearly dissociated,

selective cytotoxicity against autologous tumor cells. It seems noteworthy that, on a clonal level, antileukemic selec-
tivity of OKT3-induced enhancement of cytotoxicity was

NK-sensitive target K562. Figure 1 B demonstrates that

Mean

that are not killed by autologous cytotoxic T cells renders

them susceptible to lysis, as shown in Fig 2B for patient no. 8 with AML-M3v. This finding indicates that binding sites for OKT3 on AML cells exist. Assuming that selective target-
ging of activated T cells involves binding of the F(ab')2 part

As evident from Table 2, increased lysis of AML cells by autologous LAK cells in the presence of OKT3 was associated with target cell expression of the high- and low-affinity receptor for IgG, CD64 (Fcγ RI) and CDw32 (Fcγ RI), but not CD16 (Fcγ RII). The high expression of CD23 (Fcε RII) on EBV-B cells with no susceptibility to lysis by OKT3-coated LAK cells excludes a role of this Fc R for OKT3-mediated selective T-cell targeting. The ALL cells studied, which were not lysed by OKT3-coated LAK cells, were CD64− and CD16− and had only low-level expression of CDw32.

The natural ligands for CD64, ie, monomeric and complexed human IgG, were subsequently used in blocking ex-

periments. LAK cells were preincubated with saturating

plexed human IgG, were subsequently used in blocking ex-

sponses and specific cytotoxicity of 20% to 60% at effector target

300,000 cells. Similarly, OKT3 pretreatment of AML target cells

and γδ type, respectively (Fig 1A), had selective cytotoxi-
city against autologous leukemia cells. OKT3 present dur-
ing the effector phase potentiated antileukemic activity

without affecting killing of autologous EBV-B cells or of the

NK-sensitive target K562. Figure 1B demonstrates that both CD4+ (L41) and CD8+ (L42) T-cell clones without

spontaneous cytotoxicity against autologous leukemia cells could be induced to selectively kill autologous tumor cells. The efficiency of this enhancement was documented by the range of specific cytotoxicity of 20% to 60% at effector target (ET) ratios of 1:1 or even below 1:1 (clone L 37 and L41). It seems noteworthy that, on a clonal level, antileukemic selec-
tivity of OKT3-induced enhancement of cytotoxicity was

maintained, and that NK-like activity against K562 and killing of autologous fresh blast cells was clearly dissociated, indicating different mechanisms of target cell binding and recognition.

To assess the efficiency of triggering antileukemic cytotoxic-

ity by OKT3 MoAb, LAK cells of patient no. 12 with AML-M4eo were incubated with varying amounts of OKT3, followed by removal of unbound MoAb. They were subsequently tested for their cytotoxicity against SG AML and SG EBV-B cells. Figure 2A shows that half-maximum antileukemic cytotoxicity was reached at 180 pg (1.2 fmol)/

10^8 cells. Similarly, OKT3 pretreatment of AML target cells

that are not killed by autologous cytotoxic T cells renders

them susceptible to lysis, as shown in Fig 2B for patient no. 8 with AML-M3v. This finding indicates that binding sites for OKT3 on AML cells exist. Assuming that selective target-
ging of activated T cells involves binding of the F(ab')2 part

of OKT3 to the CD3 molecule of the effector cell, and of the

Fc portion to Fc receptors of the target cell, Fc receptor

expression on leukemic and on EBV-B cells of the studied

patients with acute leukemia was analyzed. As evident from

Table 2, increased lysis of AML cells by autologous LAK

cells in the presence of OKT3 was associated with target cell expression of the high- and low-affinity receptor for IgG, CD64 (Fcγ RI) and CDw32 (Fcγ RII), but not CD16 (Fcγ RII). The high expression of CD23 (Fcε RI) on EBV-B cells with no susceptibility to lysis by OKT3-coated LAK cells excludes a role of this Fc R for OKT3-mediated selective T-cell targeting. The ALL cells studied, which were not lysed by OKT3-coated LAK cells, were CD64− and CD16− and had only low-level expression of CDw32.

The natural ligands for CD64, ie, monomeric and complexed human IgG, were subsequently used in blocking ex-

periments. LAK cells were preincubated with saturating concentrations of OKT3 and unbound MoAb was removed. Cytotoxicity of OKT3-coated LAK cells against autologous AML-M3v leukemia cells of patient no. 8 was

Table 1. Selective Targeting of Autologous LAK Cells Against AML Blasts by OKT3

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Patient Initials</th>
<th>Diagnosis</th>
<th>Leukemia Cells - OKT3</th>
<th>EBV-B or T Cells - OKT3</th>
<th>Leukemia Cells + OKT3</th>
<th>EBV-B or T Cells + OKT3</th>
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<tr>
<td>1</td>
<td>AM</td>
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<td>4.6*</td>
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<td>8.5*</td>
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<td>IE</td>
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<td>24.1</td>
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<td>5</td>
<td>IBu</td>
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<td>9.4 ± 8</td>
<td>52.5 ± 14.4</td>
<td>13.5 ± 10.6</td>
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</table>

LAK cells were generated by long-term culture (7 to 35 days) of PBMC with 1,000 U/mL rIL-2. Cultures were tested in weekly intervals by standard 4-hour ^1^chromium release assays and maximum responses are shown. Screening was performed in the absence or presence of OKT3 (80 ng/mL). Diagnoses including FAB subtypes are indicated.

* Cases in which EBV-B cells could not be established and PHA blasts were used as nonmalignant reference cells instead.
LAK CELL TARGETING AGAINST CD64 OF AML CELLS

T cell clone | % Specific Lysis from Target: AML | EBV-B | K562
--- | --- | --- | ---
L 37 | 40 | [Graph] | [Graph] | [Graph]
CD3+ CD8+ CD4+ TCR αβ+
20 6 2 0.7
G 27 | 80 | [Graph] | [Graph] | [Graph]
CD3+ CD8+ CD4- TCR γδ+
30 10 3 1
L 41 | 40 | [Graph] | [Graph] | [Graph]
CD3+ CD8- CD4+ TCR αβ+
20 6 2 0.7
L 42 | 80 | [Graph] | [Graph] | [Graph]
CD3+ CD8+ CD4- TCR αβ+
30 10 3 1
Fig 1. Cytotoxicity of T-cell clones for autologous AML tumor cells, EBV-B, and K562. T-cell clones derived from patient AL (AML-M2; L 37, L 41, L 42) and patient SG (AML-M4eo; G 27) were tested in the presence (—) and absence (----) of OKT3 (80 ng/mL). Phenotypes of the clones are indicated on the left.

measured in the presence of increasing concentrations of monomeric or complexed human IgG. At supraphysiologic levels of 20 mg/mL, monomeric and complexed IgG caused 67% and 86% reduction, respectively (Fig 3). An increase in lysis of AML cells by control LAK cells preincubated with an irrelevant IgG2a MoAb up to 14% was observed at IgG concentrations ≥10 mg/mL. Antibody-dependent cytotoxicity by LAK cells may be responsible for the enhancement of lysis when complexed human IgG was added.20 We have no direct data to explain why addition of monomeric human IgG increased cytotoxicity mediated by control LAK cells. Ramos et al21 have shown that complement attached to CD11b/CD18 of target cells enhances lytic activity of NK cells. We found that all myeloid leukemic blast cell samples expressed CD11a-b/CD18 and, to a variable extent, CD11c/CD18 adhesion molecules and complement receptors, respectively (data not shown). Accordingly, one explanation for the increased target susceptibility of leukemic blast cells observed in Fig 2A may be that contaminating complement present in the monomeric human IgG preparation that could not be inactivated by heat pretreatment has led to increased killing by LAK cells.

In 5 patients, AML cells were obtained at diagnosis and in first relapse. Relapsed blasts showed decreased expression of CD64 (56.1% ± 13.9% vs 12.9% ± 5.4%; P < .05) but not of CDw32 (59.7% ± 21% vs 50.2% ± 36%). In 3 patients, 5 cytotoxic T-cell clones that had no NK-like activity against K562 were analyzed for their cytotoxic potential against autologous AML blasts in the presence and absence of OKT3. There was a significant correlation between the OKT3-induced increase in killing and expression of CD64 (R = .85; Fig 4), but not of CDw32 (R = .39). No such correlation was found if clones with NK activity or LAK cells were used. Furthermore, this finding points towards a major role of the high-affinity Fcγ RI in OKT3-dependent targeting of T cells against AML blasts.

Expression of CD64 on fresh AML blasts can be increased by IFN, resulting in increased killing by autologous OKT3-coated LAK cells. Having identified CD64 as likely candidate target cell ligand for OKT3-coated cytotoxic T cells, in the next set of experiments we addressed the question of whether IFN-mediated increased expression of CD64 on fresh AML blasts translates into further enhancement of OKT3-mediated T-cell targeting. In vitro viability of 8 AML samples was sufficient to perform 4-day incubation experiments with medium, IFN-γ, or IFN-α. As evident from Table 3, IFN-γ profoundly increased expression of CD64 in all cases. However, IFN-α upregulated CD64 in 3 of 5 tested samples to a lesser degree when compared with IFN-γ. In contrast, CDw32 was upregulated by both IFN-γ and IFN-α only in patient no. 12 with AML-M4eo. Both cytokines had no or only marginal effects on CDw32 ex-
measured simultaneously with susceptibility for killing by autologous LAK cells in the presence of OKT3. Twenty-four-hour exposure of AML blasts of patient no. 1 (AML-M1) with IFN-γ resulted in increased expression of CD64 (3.97 ± 6.28 mean fluorescence intensity arbitrary units) and decreased expression of CDw32 (9.6 ± 3.61 mean fluorescence intensity arbitrary units). IFN-γ–pretreated AML blasts were two-fold more susceptible to autologous LAK cell lysis as compared with control cells cultured in medium. This effect could be inhibited by monomeric human IgG, suggesting that indeed CD64 is the target cell ligand responsible for the increased lysis observed.

**Inability of OKT3 to target LAK or T cells against nonmalignant CD34+ progenitor cells.** We performed coculture experiments of OKT3-coated LAK and T cells with autologous freshly isolated PBMNC depleted of monocytes by adherence. After a 4-hour incubation period, the mixture of cells was plated in semisolid medium and colony-forming units were counted on day 14. As a control, the same coculture experiments were performed with the leukemic cell line U937. Figure 6 demonstrates that LAK cells reduced and a CD4+ T-cell clone completely inhibited U937 CFUs when effector cells had been precultivated with OKT3, but not with an irrelevant control antibody. In contrast, they did not affect the total number of CFUs derived from nonmalignant autologous PB hematopoietic progenitor cells. In the case of coculturing the T-cell clone with autologous PBMNC, there was a nonsignificant trend towards increased numbers of CFU-E and BFU-E, and cloned T cells appeared to be more effective in reducing U937 colony formation. We hypothesized that the resistance of nonmalignant hematopoietic progenitor cells to OKT3-mediated T-cell targeting could be explained by the absence of CD64 on early progenitor cells, thereby preventing triggering of OKT3-coated T cells. We therefore used the PB of a Hodgkin's disease patient that had undergone peripheral stem cell mobilization using chemotherapy and G-CSF as a source to positively enrich CD34+ cells..

**DISCUSSION.**

Our test system to screen cytotoxicity of patient LAK cells against autologous leukemic blast cells and nonmalign-

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**Table 2. Fc-Receptor Expression on Cells of Acute Leukemia Patients and Sensitivity to Lysis by Autologous LAK Cells in the Presence of OKT3**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>CD64 (32.2)</th>
<th>CDw32 (V.3)</th>
<th>CD16 (Leu11b)</th>
<th>CD23 (Toe 1)</th>
<th>% N</th>
<th>Change in % Specific Lysis* (mean ± SD) by Autologous LAK Cells in the Presence of OKT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>15/19</td>
<td>16/19</td>
<td>13/16</td>
<td>0/19</td>
<td>4/16</td>
<td>19</td>
</tr>
<tr>
<td>ALL</td>
<td>0/2</td>
<td>0/2</td>
<td>1/2</td>
<td>0/2</td>
<td>0/2</td>
<td>3</td>
</tr>
<tr>
<td>EBV-B</td>
<td>0/13</td>
<td>0/13</td>
<td>4/13</td>
<td>0/16</td>
<td>12/13</td>
<td>13</td>
</tr>
</tbody>
</table>

* FcR expression on target cells was determined by immunophenotyping proximate to the time of cytotoxicity assays. MoAbs used are indicated in parentheses. Autologous LAK cells were generated as described in legend to Fig 1. Results are expressed as means ± SD.

* LAK cell lysis in the presence of OKT3 (80 ng/mL) minus LAK cell lysis in the absence of OKT3 at ET ratio 30:1.
Fig 3. Effect of (A) monomeric and (B) complexed human IgG on OKT3-induced T-cell targeting. LAK cells at day 31 of culture of patient GW with AML-M3v were preincubated with saturating concentrations (200 ng/10⁶ cells) of OKT3 (m) or irrelevant mouse IgG2a MoAb (□) and tested against autologous AML blasts in the presence of monomeric or complexed human IgG. The x-axis represents IgG concentration.

Fig 4. Correlation between CD64 expression on primary and relapsed AML blasts and susceptibility for OKT3-enhanced killing by autologous T-cell clones. AML blasts obtained at diagnosis (○) and in relapse (■) of patients AL (AML-M2), SG (AML-M4eo), and EP (AML-M5b) were analyzed for expression of CD64 and were used as targets for autologous T-cell clones. If the proportion of contaminating T cells exceeded 10%, immunomagnetic depletion of T cells was performed, which resulted in greater than 95% purity of AML blast preparations. Clones are indicated by numbers and were of the following phenotype: 1 and 2 (patient AL): CD3⁺CD4⁻CD8⁺TCRαβ⁺; 3 (patient SG): CD3⁺CD4⁺CD8⁻TCRαβ⁺; 4 and 5 (patient EP): CD3⁺CD4⁺CD8⁻TCRαβ⁺. The OKT3-induced increase in the percentage of specific lysis was calculated as described in Table 2.

The efficiency of antileukemic OKT3-mediated targeting of LAK cells is reflected by the fact that 180 pg bound to 10⁶ effector cells is sufficient to cause half-maximum enhancement of killing. Assuming a molecular weight of 150 Kd for the OKT3 IgG2a molecule and neglecting OKT3-CD3 dissociation, this corresponds to approximately 720 CD3 molecules per single effector LAK cell that need to be occupied at ED₅₀. This number is within the order of magnitude that has been determined by Harding and Unanue for the number of peptide/MHC class II complexes (210 to 340 per antigen-presenting cell) required for the stimulation of T cells. Thus, antileukemic T-cell targeting by CD3 MoAb in quantitative terms appears to mimic MHC-restricted T-cell recognition of tumor cells.

On the target cell level, the following findings suggest that the high-affinity Fc receptor for IgG (FcγRI, CD64) is the molecule predominantly responsible for cross-linking OKT3 MoAb molecules bound to T cells. (1) Killing by OKT3-coated LAK cells segregated with target cell expression of CD64 (Table 2). (2) Preincubation of CD64⁺ AML cells with monomeric OKT3 resulted in susceptibility to LAK cell lysis (Fig 2B), suggesting the existence of binding sites. CD64 is a candidate target cell ligand in this system because it is known to preferentially bind murine IgG2a.
Table 3. Modulation of CD64 on Fresh AML Cells

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Medium</th>
<th>IFN-γ</th>
<th>IFN-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>Fl</td>
<td>%</td>
</tr>
<tr>
<td>6 KK</td>
<td>AML-M3 relapse</td>
<td>10.5</td>
<td>474</td>
<td>31.8</td>
</tr>
<tr>
<td>8 GW</td>
<td>AML-M3v diagnosis</td>
<td>91.4</td>
<td>456</td>
<td>96.7</td>
</tr>
<tr>
<td></td>
<td>AML-M3v relapse</td>
<td>77.5</td>
<td>426</td>
<td>98.3</td>
</tr>
<tr>
<td>12 SG</td>
<td>AML-M4eo relapse</td>
<td>28.9</td>
<td>443</td>
<td>62.8</td>
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<tr>
<td>10 HG</td>
<td>AML-M4eo diagnosis</td>
<td>48.6</td>
<td>575</td>
<td>64.4</td>
</tr>
<tr>
<td>17 EP</td>
<td>AML-M5b diagnosis</td>
<td>9.6</td>
<td>387</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>AML-M5b relapse</td>
<td>4.2</td>
<td>392</td>
<td>15.7</td>
</tr>
<tr>
<td>15 MR</td>
<td>AML-M5 diagnosis</td>
<td>16.3</td>
<td>397</td>
<td>81.1</td>
</tr>
</tbody>
</table>

Fresh AML blasts were incubated with medium, IFN-γ (100 U/mL), or IFN-α (100 U/mL) for 4 days followed by flow cytometry analysis. CD64 and CDw32 was detected by 32.2 and IV.3, respectively. Percentages (%) of positive cells including mean fluorescence intensity (FI) in arbitrary units are indicated.

Abbreviation: ND, not done.

However, pretreatment of AML blasts with murine IgG2a (mIgG2a) in this experiment did not block OKT3-redi-rected cytotoxicity. We presume this was due to higher localized concentrations of T-cell-bound OKT3 compared with target-bound mIgG2a. On a per cell basis, 20,000 to 100,000 CD3 molecules per T cell27 binding F(ab')2 portions of OKT3 face 3,400 to 34,000 Fcγ R1 molecules per myeloid tumor target cell28 binding the Fc portion of mIgG2a molecules. Hence, we think that the density of T-cell-bound OKT3 exceeds the density of target-cell-bound mIgG2a at the ET membrane interface, resulting in displacement of mIgG2a with subsequent triggering of effector cell function.
Fig 6. Inability of OKT3 to target LAK and T cells against nonmalignant hematopoietic progenitor cells. LAK cells (A) or a CD3+CD4+ T-cell clone (B) of a healthy donor were used as effector cells. Monocyte-depleted autologous PBMNC (5 × 10^5) and 200 (A) or 2,000 (B) U937 cells were plated in semisolid culture media alone (1) or after 4 hours of preincubation with effector cells coated with an irrelevant mouse IgG2a MoAb (200 ng/10^5 cells) (2), or with OKT3 (200 ng/10^5 cells) (3) at an ET ratio of 1:1. Nonmalignant hematopoietic progenitor colonies and leukemic U937 colonies were counted on day 14. Mean values of 4 and 7 replicate determinations, respectively, are shown. Standard deviation was < 15%. LAK or T cells alone were not clonogenic. (m) Total CFU; (□) cluster; (■) CFU-GM; (△) CFU-E; (■) BFU-E; (■) CFU-GEMM.

Fig 7. Lack of expression of Fcy RII (CD64) and Fcy RI (CDw32) by CD34+ nonmalignant progenitor cells. CD34+ progenitor cells were positively enriched by 1 round of immunomagnetic cell sorting of PBMNC of a patient with Hodgkin's disease using HPCA-1 MoAb. Cells gated to include monocytes for internal control were subsequently stained with GAM-PE and with FITC-labeled 32.2 (CD64) or FITC-labeled IV.3 (CDw32) (A). Fcy R expression of U937 cells is shown in (B).
the amount of target cell expression of CD13 or CD10 on leukemic blast cells and the targeting potential of autologous LAK cells activated by 1,000 U/mL IL-2 and targeted by bispecific CD13 × CD3 or CD10 × CD3 MoAbs, respectively. The investigators have no explanation for this discrepancy and speculate on unknown factors inherently present in target tumor cells. The lack of correlation between expression of the target cell ligand and redirected cytotoxicity of LAK cells may be the result of confounding CD3-dependent and NK-like recognition mechanisms used by this effector population. We assume that by deletion of NK-like killing (lysis of K562) after cloning of effector T cells, the correlation between CD64 expression and OKT3-dependent cytotoxicity became apparent. (4) Antileukemic cytotoxicity of OKT3-coated T cells could partially be inhibited by monomeric human IgG, the natural ligand of CD64. It is noteworthy that even supraphysiologic concentrations of human monomeric IgG caused only incomplete reduction of OKT3-dependent T-cell targeting. We explain this phenomenon by high localized concentrations of T-cell–bound OKT3 that cannot be overcome by soluble IgG. In fact, opsonization would not be possible under physiologic conditions if Fcγ Rs were blocked by serum IgG.

The finding that the murine MoAbs Leu4 and WT31 of the IgG1 subclass preferentially binding to human CD3210 were similarly potent in targeting LAK cells against AML blasts (data not shown) suggests a possible role of CDw32 in the toxicity of OKT3-coated T cells. Our attempts to analyze the quantitative contribution of CD64 and CDw32 for targeting by using blocking MoAbs were unsuccessful. It seems necessary to completely block or downmodulate all molecules during the entire assay period, which may not be possible with MoAbs alone. Transfection experiments may be a more appropriate approach to clarify this issue and to definitely establish the role of CD64 as major target cell ligand in this system. These studies are in progress. (5) Upregulation of CD64 on AML cells induced by IFN-γ was associated with increased lysis by autologous OKT3-coated LAK cells (Fig 5). We found that, in all cases of primary and relapsed AML cells amenable to in vitro incubation studies, IFN-γ increased expression of CD64, indicating that the IFN-responsive element is preserved in the organization of the CD64 gene of fresh AML cells.30 In contrast, upregulation of CDw32 after IFN-γ exposure was seen only in 3 of 8 cases, suggesting that heterogeneity of CDw32 exists on myelomonocytic cells of the malignant phenotype. IFN-α had only marginal effects on expression of CD64 or CDw32 or was less potent compared with IFN-γ.

Interestingly, Munn et al32 reported the low-affinity Fcγ RI1 and Fcγ RII1 to be the primary effector molecules involved in antibody-dependent tumor cell phagocytosis mediated by human macrophages. The role of Fcγ RII was less clear in their system and required further study. Cemeresic et al33 have shown that IFN-γ treatment of neutrophils and PBMC resulted in increased lysis of chronic lymphocytic leukemia cells coated with MoAbs, which was due to induction of FeRα on effector cells. On malignant AML blasts, Fcγ RII (CD64) remains responsive to IFN-γ stimulation and can, in reverse, be used as a target molecule for OKT3-coated LAK and T cells. Compatible with the concept of CD64 cross-linking OKT3 bound to T and LAK cells and thereby triggering cytotoxic effector function is a recent study that showed that T-cell proliferation mediated by murine IgG2a CD3 MoAb depends on CD64 expressed by accessory cells.34 IFN-induced upregulation of other adhesion molecules such as ICAM-1 (CD54) or LFA 3 (CD58) may also have contributed to enhanced killing of AML blasts, as it has previously been shown for neuroblastoma35 and fresh T leukemia cells.36 However, the complete inhibition by human IgG when limited concentrations of OKT3 were used suggests that CD64 is essential and sufficient in triggering cytotoxicity.

One important consideration in using antileukemic targeting strategies for therapy is their toxicity for nonmalignant hematopoietic progenitor cells. Oblakowski et al37 have proposed that one mechanism of selective killing of myeloid CD34+ leukemic blast cells by CD2+ LAK cells is increased expression of CD54 on CD34+ cells of the malignant phenotype compared with normal CD34+ bone marrow cells. We found that the formation of hematopoietic colonies in a healthy donor was not affected by OKT3-mediated targeting of autologous LAK and cloned T cells and that this lack of toxicity can be explained on the basis of absent expression of CD64 and CDw32 on early CD34+ progenitor cells. Considering Fc receptors as functional adhesion structures for MoAb-coated effector T cells, we find here another example of how differential adhesion receptor expression on malignant versus nonmalignant target cells can be used for highly selective elimination of leukemic cells.

The data presented provide a rationale for the combined use of IL-2, IFN-γ, and CD3 MoAb for the selective elimination of myeloid leukemic blast cells sparing nonmalignant stem cells. In syngeneic animal tumor systems, it has been shown that in vivo administration of CD3 MoAbs can have antitumor activity.38,40 Human AML represents a malignant disease entity in which, due to the presence of the IFN-inducible CD64 on AML blasts, local activation of T cells carrying CD3 MoAbs can be expected to occur. However, several caveats exist to caution premature use of murine CD3 MoAb for in vivo therapy in humans. (1) Although the cellular distribution of CD64 is mostly restricted to the monocytic lineage, its local expression on tissue macrophages of the skin, liver, lungs, brain, and other organs containing cells with antigen-presenting capacity may cause considerable toxicity when CD3 MoAb-carrying T cells circulate through these organs. Supporting this assumption, significant neurotoxicity has followed administration of CD3 MoAb to patients with refractory solid tumors in a clinical phase I trial.41 (2) Human IgG interferes with the binding of OKT3-coated T cells to CD64 of the target cells. This may be bypassed by the construction of heteroconjugates consisting of CD3 MoAb and CD64 MoAb, such as 32.2. Shen et al39 have shown that such constructs target monocytes in the presence of human IgG, because 32.2 binds to an epitope of the CD64 molecule that is distinct from the IgG binding site. (3) The combined use of murine CD3 MoAb with immunopotentiating cytokines causes...
rapid formation of human antimouse antibodies (J.A. Bluestone, personal communication, July 1992). Hence, humanization of murine MoAbs by genetic engineering is preferential, and a humanized version of OKT3 MoAb has recently been described.\(^4\)

(4) IFN-\(\gamma\) induces expression of CD64 on neutrophils not present under constitutive conditions. Although the degree of induction is 100-fold less pronounced compared with the induction on monocytes,\(^4\)

these nonmalignant cells would represent a competitive target in vivo. However, this still could lead to AML blast elimination in vivo provided leukemic cells were susceptible to bystander killing. (5) Because CD64 is a trigger molecule for cytotoxicity of monocytes and macrophages, in vivo administration of OKT3 may result in depletion of T cells. In a 4-hour in vitro assay, we detected no killing of LAK cells coated with OKT3 by autologous macrophages activated by high-dose IL-2 for 7 days, whereas LAK cells were significantly induced by OKT3 to kill autologous macrophages (data not shown). The in vivo studies in a murine tumor system performed by Ellenhorn et al.\(^6\) indicate that the dose of CD3 MoAb administered in vivo is critical, with lower doses causing persistent T-cell activation with antitumor effects and lasting tumor immunity and higher doses causing T-cell depletion and immune dysfunction.

Due to its lack of toxicity for nonmalignant early hematopoietic cells in vitro, a potentially interesting clinical application of the combined use of IL-2, CD3 MoAb, and IFN-\(\gamma\) lies in the field of purging in autologous bone marrow transplantation. Purging based on targeting of T cells would offer the dual advantage of selective elimination of leukemic blasts and at the same time of providing effector T cells with potential cytotoxic activity against residual leukemic host cells in vivo.

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Selective targeting of human lymphokine-activated killer cells by CD3 monoclonal antibody against the interferon-inducible high-affinity Fc gamma RI receptor (CD64) on autologous acute myeloid leukemic blast cells

M Notter, WD Ludwig, S Bremer and E Thiel