

Phase I Evaluation of Humanized OKT3: Toxicity and Immunomodulatory Effects of hOKT3 γ_4

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ABSTRACT

Murine anti-CD3 (OKT3, Muromonab-CD3) is a potent human T-lymphocyte mitogen. A previous clinical Phase I trial examined OKT3 as an immunomodulator for the treatment of cancer. However, the murine monoclonal antibody triggered a potent humoral response that neutralized the antibody activity during subsequent administration. Thus, a “humanized” form of OKT3 (hOKT3 γ_4) was developed to minimize immunogenicity. The genetically engineered human anti-CD3 retained its binding activity and effectively activated T cells *in vitro*. Therefore, we evaluated the safety and activity of hOKT3 γ_4 in a Phase I clinical trial. hOKT3 γ_4 was administered as a 10-min i.v. infusion every 2 weeks for three injections (one course of therapy). Six dose levels ranging from 50 to 1600 μg /injection were evaluated. Headache and fever were common, transient toxicities but were not dose limiting. The dose-limiting toxicities were rigors and dyspnea at the 1600- μg dose level, which defined 800 μg as the maximally tolerated dose in this trial. A dose-dependent *in vivo* T-lymphocyte activation was produced by this treatment, and the most significant T-lymphocyte activation occurred in patients treated at the two highest dose levels (800 and 1600 μg). Persistent CD3 modulation occurred after administration of 1600 μg of hOKT3 γ_4 . Anti-idiotypic antibodies were detected in only 6 of 24 patients after multiple injections and were not associated with attenuation of T-lymphocyte activation. Malignant ascites resolved in three patients, one each with peritoneal mesothelioma, pancreatic adenocarcinoma, and ovarian adenocarcinoma. hOKT3 γ_4 can induce T-lymphocyte activation in patients with cancer, and the immunogenicity of the “humanized” antibody is sufficiently reduced relative to its murine “parent” to permit immunostimulation by repetitive i.v. administration. The therapeutic potential of biweekly i.v. hOKT3 γ_4 at a dose of 800 μg should be further evaluated.

INTRODUCTION

Antibodies reactive with the CD3 ϵ chain of the T-cell receptor complex on T lymphocytes are among the most potent mitogens for T lymphocytes (1, 2). This phenomenon has been described using antibodies directed against both human and murine CD3 ϵ -specific mAbs² (1, 2). Although the current clinical role for anti-CD3 antibody (*e.g.*, OKT3 or Muromonab-CD3) immunotherapy has been the suppression of T-cell responses to prevent rejection of organ allografts (3), we hypothesized that low doses of anti-CD3 might induce T-cell activation in the absence of associated immunosuppression (4). In fact, in the murine setting, administration of low doses of anti-CD3 led to eradication of tumors in the majority of tumor-bearing animals (5). Finally, we have reported previously that low-dose, intermittent administration of OKT3 can be immunostimulatory in cancer patients (6).

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² The abbreviations used are: mAb, monoclonal antibody; hOKT3, humanized OKT3; MCB, Master Cell Bank; IL, interleukin; sIL-2R, soluble IL-2 receptor; TNF, tumor necrosis factor; GM-CSF, granulocyte/macrophage-colony stimulating factor; MFI, mean fluorescence intensity.

However, OKT3 is a murine antibody and is highly immunogenic in humans. Idiotypic and nonidiotypic antibodies develop in treated individuals, even in the presence of intense immunosuppression, such as found after organ allografts (7). In fact, the magnitude of the anti-OKT3 response in immunosuppressed allograft recipients is often sufficient to neutralize the effects of subsequent OKT3 administrations (8). During the previous clinical trial of OKT3, we noted that 100% of the patients developed neutralizing anti-CD3 antibodies, even at the lowest dose (50 μg) of OKT3 (6). The increased immunogenicity is likely due to the immunocompetence of the cancer patients and the dose regimen (every 2 weeks; Ref. 6). Therefore, prolonged immunostimulation of immunocompetent patients after repeated doses of OKT3 depends on an effective way to reduce the immunogenicity of murine antibodies. Genetic engineering of murine antibodies has been shown to be an effective way of reducing immunogenicity while retaining antigen binding activity (9). The variable regions of the heavy and light chains were mutated to change the majority of murine-derived amino acids while retaining the complementarity determining regions and a small number of framework amino acids. These variable regions are engrafted onto a human immunoglobulin Fc to generate a full immunoglobulin for clinical use (9). A “humanized” OKT3 mAb was generated that retained its binding affinity and functional properties. In the present study, a Phase I clinical trial was performed in cancer patients to determine the biological effects and maximum tolerated dose of the humanized anti-CD3 *in vivo*. The data suggest that this mAb activates the immune response in the absence of dose-limiting toxicity or neutralizing immunogenicity.

MATERIALS AND METHODS

Generation of Human Antihuman CD3. Using site-directed mutagenesis, the hypervariable regions of the human DNA coding for an immunoglobulin γ_4 heavy chain and an immunoglobulin λ light chain were altered to produce an antigen-binding site similar to that found on murine OKT3 as described previously (9). Fully assembled heavy and light chain genes were spliced into expression vectors pEe6hCMV.BgIII and pEe12hCMV, respectively. Myeloma cell transfectants were then selected in glutamine-free media taking advantage of the glutamine synthetase gene incorporated into pEe12hCMV (9). High producer clones were selected and expanded for bulk production of hOKT3 γ_4 .

Preparation of hOKT3 γ_4 . Culture flasks containing 200 ml of DMEM F14⁺ were inoculated with 1–1.5 million transfected cells. Cultures were maintained at 37°C in humidified 5% CO₂ until the concentration of hOKT3 γ_4 exceeded 10 $\mu\text{g}/\text{ml}$, when cells and particulate matter were removed by centrifugation. Initial purification and concentration was performed by protein A-affinity chromatography (Protein A Superose HR 16/5 system; Pharmacia LKB, Piscataway, NJ) with a 10-ml bed volume. The column was equilibrated with 100 mM glycine-HCl (pH 8.6), and hOKT3 γ_4 was eluted using a linear gradient of 0–100 mM sodium citrate (pH 2.0). Peak fractions from the Protein A-Sepharose column were pooled and acidified to pH 2.5 with 1 N HCl for 30 min at room temperature to inactivate murine retrovirus. The Tris-HCl (pH 8.5) neutralized product was then extensively dialyzed against 10 mM Tris-HCl (pH 8.5). Dialyzed hOKT3 γ_4 solution was further purified and concentrated using a high-capacity, strong anion exchanger, MonoQ (Pharmacia LKB). hOKT3 γ_4 was eluted using a linear gradient of NaCl from 0 to 500 mM. Pooled

fractions from the ion exchange chromatography purification step were extensively dialyzed against maltose buffer containing 0.55% sodium citrate, 0.025% citric acid, 0.584% NaCl, and 5.265% maltose (pH 6.3). The hOKT3 γ_4 solution was further diluted with maltose buffer to provide an antibody concentration of 0.8 mg/ml, and the final bulk product was aliquoted into individual ampoules containing 0.25 or 1.0 ml. Each ampoule was then flushed with sterile-filtered N₂, and the tops were immediately heat sealed using a fine-point propane flame.

Product Safety Testing. The MCB was tested for murine virus contamination prior to proceeding with production of a Working Cell Bank. All analyses were performed by Microbiological Associates (Rockville, MD). Mouse Antibody Production Testing for K virus, polyoma, Ectromelia, Sendai, reovirus type 3, and Hantaan virus was negative. Mini-MAP for Hantaan virus was negative. Isolation tests for Lymphocytic choriomeningitis, mouse salivary gland virus, mouse thymic virus, EDIM virus, and lactic dehydrogenase virus were negative. Microscopic examination of four replicate sets of plates of S+L cells inoculated with MCB supernatant revealed no foci, indicating the absence of xenotropic virus detectable by the S+L focus assay. Cell lysate from the MCB was inoculated into broth cultures and onto agar plates and incubated anaerobically and aerobically to detect cultivable *Mycoplasma* species. Cell lysate from the MCB was also inoculated into VERO cell cultures to detect noncultivable *Mycoplasma* species by the Hoechst stain procedure. This assay meets the U.S. Regulation CFR 610.30 guidelines. All plates inoculated with the cell lysate from the MCB and subculture plates were examined and no *Mycoplasma* colonies were observed. Therefore, the cell lysate from the MCB was found to be negative for the presence of agar-cultivable and noncultivable *Mycoplasma* in this assay system.

The cell lysate from the MCB did not cause turbidity in fluid thioglycolate broth, peptone yeast glucose broth, or soybean-casein digest broth, all of which detect bacterial contaminants. The MCB cell lysate inoculated into Sabouraud-dextrose agar tubes (which support fungal growth) did not show any growth. Therefore, the MCB cell lysate was found to be negative for bacterial and fungal contamination in this assay system. No ecotropic murine leukemia virus was detected in the XC plaque assay. Transmission electron microscopic examination of the MCB revealed no intracytoplasmic viral particles in 100% of the 200 cells examined. Additionally, no budding or extracellular particles were detected.

Cell lysate from the MCB was cytogenetically characterized by determining chromosome numbers and markers using Giemsa-banded chromosomes, chromosome count, ploidy distribution per 100 metaphases, exact chromosome count per 50 metaphases, and enzyme mobility. The enzyme analysis indicated that the mobilities of the glucose-6-phosphate dehydrogenase, nucleophosphorylase, lactic dehydrogenase, and malate dehydrogenase were comparable with those of the mouse control preparation. The cell line had no chromosomes in excess of those present in the control parental line. An array of marker chromosomes was present that strongly identified it as a hybridoma derived from the P3X63Ag8U1 myeloma cell line.

Final Product Testing. Each vial of final product was clear and colorless and contained 0.25 or 1.0 ml. Each lot of final product had a pH of 6.0–6.5 and was tested for protein concentration by spectrophotometer UV absorbance at 280 nm and found to contain 0.8 mg/ml protein based on the extinction coefficient for OKT3 of 1.375 cm⁻¹. Purity of the final product was assessed by SDS-PAGE using both reducing and nonreducing conditions and by gel exclusion chromatography. The percentage of purity of the final product was >95%.

Functional Activity of hOKT3 γ_4 . A competition assay was performed on the first two lots of hOKT3 γ_4 produced. This assay was configured as a competitive binding colorimetric immunoassay. It was based on a competition between OKT3 antibody and purified product for binding to the CD3 antigen present on the surface of Jurkat T-lymphocytes, a lymphoblastic cell line isolated from the peripheral blood of a patient with acute leukemia. The ability of the antibody to bind antigen was measured by its ability to compete with OKT3 antibody for antigen binding sites. The potency of the purified product was inversely proportional to the amount of OKT3 antibody bound. The reference standard was diluted to ~1.0 mg/ml. In addition, a 70% internal reference standard was diluted to ~0.70 mg/ml to serve as a control. A set of serial dilutions for each lot of final product and reference standard were prepared. A predetermined dilution of OKT3 and a 1.0 × 10⁷ Jurkat cell/ml suspension were prepared. Equal volumes of diluted final product (or diluted

reference standard), OKT3, and a Jurkat cell suspension were incubated in a test tube for 30–45 min in a foil-covered ice bath. The cells were subsequently washed three times by dilution with wash media, centrifugation, and aspiration. The cells were plated in monolayers and were analyzed for the amount of OKT3 bound by an ELISA using anti-murine IgG-conjugated to alkaline phosphatase. The percentage of potency of final product was determined by a comparison of the dilution curve of the final product against a reference standard preparation. Each final product had >90% of the activity of the standard.

General Safety. Each lot of vial final product contained less than 2.5 EU/ml when assayed for the presence of bacterial endotoxin by the Bacterial Endotoxin (LAL) test (Sigma Chemical Co., St. Louis, Mo.). Additionally, each lot was judged sterile by membrane filtration test (21 CFR 610.12f). A general safety test was performed on each lot of final product according to 21 CFR 610.11 using two healthy mice weighing <22 g each. These animals were not used previously for any test purpose. Each mouse was injected i.p. with 0.5 ml of the final product, observed for 7 days, and any responses were recorded. In all instances, animals survived the test period, did not lose weight, and did not exhibit abnormal behavior.

Binding of hOKT3 γ_4 to normal human tissues was assessed using immunoperoxidase labeled anti-murine IgG Fab staining of tissue sections (Dr. Daniel Knowles, Columbia University, New York City, N.Y.). hOKT3 γ_4 was found to bind to T cells in lymph node, thymus, and spleen. No binding of hOKT3 γ_4 was identified in any other human tissues studied.

Immunological Monitoring. Phlebotomy of each patient was performed prior to each antibody administration and 1, 2, 4, and 24 h after each antibody administration to obtain serum and peripheral blood lymphocytes. Phlebotomy was also performed 48 h after the first hOKT3 γ_4 administration. Serum was frozen in 1-ml aliquots at -70°C for subsequent analysis of IL-6, TNF- α , GM-CSF, sIL-2R, and anti-idiotypic antibody. Peripheral blood mononuclear cells were evaluated by flow cytometry within 24 h as described below.

Serum Analyses. Serum samples from hOKT3 γ_4 -treated patients were tested for TNF- α , GM-CSF, IL-6, and sIL-2R using commercial ELISA kits (Endogen, Inc., Cambridge, MA). Briefly, plates came precoated with anti-cytokine antibody. Samples were added in duplicate and incubated for 1 h at 37°C in a humidified incubator. Plates were washed and reincubated for 30 min at 37°C with peroxidase-coupled secondary antibody. After washing, tetramethylbenzidine (TMB) substrate was added and developed for at least 30 min. Absorbance was read at 450 nm. Results were compared to a standard curve generated with recombinant cytokine.

Anti-Idiotypic Antibody. An ELISA was developed to detect antibodies that reacted with the murine OKT3 idiotype. Using patient sera that possessed anti-idiotypic antibodies to murine OKT3, we have previously shown idiotypic cross-reactivity between humanized and murine OKT3 (2). The ELISA was performed by first coating standard 96-well, flat-bottomed ELISA plates (Costar) with OKT3 (1 μ g/100 μ l in bicarbonate buffer) overnight at 4°C. Plates were washed and blocked with 1% BSA, and serial dilutions of control sera (*i.e.*, sera containing idiotypic anti-murine OKT3 antibodies) were added. Also tested were 1:100 dilutions of sera from hOKT3 γ_4 -treated patients. Plates were incubated for 1 h and washed, and peroxidase-coupled, anti-human IgG polyclonal antibody (Kirkegaard and Perry, Gaithersburg, MD) was added. Plates were incubated and washed, and substrate was added; absorbance was measured at 405 nm.

Flow Cytometry. Circulating peripheral blood T cells were monitored by flow cytometric analysis.

Peripheral blood was collected in EDTA and kept at room temperature until processed. Lymphocytes were isolated using Isoprep, a Ficoll density gradient (Robbins Scientific Corporation, Sunnydale, CA) and resuspended in RPMI 1640 containing 5% FCS. Cells were aliquoted at 2 × 10⁵ cells per tube and stained with appropriate antibodies. The cells were incubated for 15 min, and excess antibody was washed out with PBS wash buffer containing 0.5% BSA and 0.1% sodium azide (PBA). The samples were fixed in 1% paraformaldehyde prior to analysis on a FACScan (Becton Dickinson, San Jose, CA) flow cytometer. Data were collected on at least 5000 lymphocytes for each sample.

Analysis was performed using the following antibodies: CD4-FITC, CD4-PE, CD8-PE, CD25-FITC, CD20-FITC, mIgG-FITC (all purchased from Becton Dickinson, San Jose, CA), and CD45-TriColor (Caltag Laboratories, So. San Francisco, CA). OKT3 and OKT3d were provided by R. W. Johnson PRI (Raritan, NJ) and FITC conjugated in our lab. NHS-FLUOS (Boehringer

Table 1 Patient characteristics

Age	Median, 47 years	Range, 29–73
Karnofsky Performance Status	Median, 100	Range, 80–100
Gender (n)	Female (11)	Male (13)
Malignancy (n)	Melanoma (9) Ovarian (3) Sarcoma (2) Hepatoma (1) Mesothelioma (1) Colon (4) Lung (2) Renal (1) Pancreas (1)	

Mannheim Biochemicals, Indianapolis, IN) was dissolved in DMSO (Sigma Chemical Co., St. Louis, MO), resulting in a 1 mg/ml solution. Seventy-five μ l of this solution were added to purified mAb (2 mg/ml, pH8.0) and incubated at room temperature for 45 min. Unreacted NHS-FLUOS was removed using a PD-10 Sephadex G-25 M column (Amersham Pharmacia Biotech, Piscataway, NJ). Conjugated antibody was stored in the presence of 0.1% sodium azide at 4°C. All data analysis was performed using Lysis II software (Becton Dickinson, San Jose, CA). The percentage of cells staining positive for CD4 and CD8 was used to calculate the ratio (CD4:CD8) of these T-cell subsets.

The percentage of CD25-positive cells and MFI was also determined. An activated population was defined by at least a 2-fold increase in CD25-positive cells over pretreatment results. Modulation of CD3 was determined by OKT3d-FITC fluorescence (2). A decrease in staining intensity of <25% was considered negative. A decrease of 25–50% was considered moderate modulation, whereas >50% decrease was considered strong modulation.

Patient Characteristics. Eligibility for this trial was based on biopsy-proven malignancy for which there was no standard therapy available or which had progressed following standard therapy. In addition, patients were required to have a Karnofsky Performance Status of 80 or higher and to be able to give informed consent for this treatment, which was performed under an Institutional Review Board-approved protocol. Table 1 lists key patient characteristics.

Treatment Schema. Patients received hOKT3 γ_4 i.v. over 10 min and were observed for 48 h following the first administration. Treatments were given every 2 weeks, and subsequent treatments were followed by a 24-h observation period. A treatment cycle consisted of three injections of hOKT3 γ_4 and was followed by a reevaluation of measurable or evaluable disease. Patients without evidence of tumor progression or grade 3 or 4 toxicity were eligible for subsequent cycles. Six dose levels of hOKT3 γ_4 (50, 100, 200, 400, 800, and 1600 μ g) were defined for this trial. Three patients were entered at each dose level, and additional patients were entered if grade 3 toxicity was observed in one patient. Dose-limiting toxicity was defined as two grade 3 or one grade 4 toxicity in a dose level. Standard NCI Toxicity Criteria were followed.

Tumor Evaluation. All patients underwent complete physical examination before enrollment in this trial and before each administration of hOKT3 γ_4 . Tumor measurements or evaluations were performed by physical examination before the first treatment and 1 week after completion of each cycle (three treatments) of therapy. When required, computerized tomography scans and serum tumor markers were used to measure or evaluate changes in tumor burden before and after each cycle of therapy. An increase of >25% in the sum of cross-sectional diameters of indicator lesions or appearance of new lesions constituted evidence of disease progression. A 50% increase in disease-appropriate serum tumor marker was considered evidence of tumor progression.

RESULTS

Dose-Limiting Toxicity. Twenty-four Patients were treated in this Phase I study. Six patients were treated at the first dose level (50 μ g), and of these patients, four completed 1 cycle of treatment, one completed 3 cycles of treatment, and one completed 6 cycles of treatment. An additional three patients were enrolled at the first dose level due to the appearance of persistent headaches, one of which was a grade 2 (Table 2). Headache was always limited to the first 48 h after treatment, and no other neurotoxicities were identified. Because of the transient nature of this toxicity and its lack of serious sequelae,

headache was not deemed a dose-limiting toxicity in this or subsequent dose levels. Grade 1 fever, hypotension, and nausea were observed in two of the six patients at this dose level and were transient. Three patients were entered in the second dose level (100 μ g), and two received one cycle and one received two cycles of treatment. Except for mild fever and headache in one patient, no treatment-related toxicities were observed. Only grade 1 toxicities were observed at the third and fourth dose levels (200 and 400 μ g of hOKT3 γ_4 , respectively), and these toxicities were limited to fever, headache, nausea, and rigors (Table 2). At the third dose level, patients received one, three, and five cycles of hOKT3 γ_4 , and at the fourth dose level, two patients received one cycle of hOKT3 γ_4 and one patient received two cycles. Four patients were entered at the fifth dose level (800 μ g). All patients experienced fever and nausea of various severities (Table 2), and three of the four patients experienced grade 1 nausea and rigors. One patient experienced transient dyspnea, which was treated with supplemental oxygen. Except for fever, no grade 3 toxicities were observed at this dose level.

Five patients were entered at the sixth dose level (1600 μ g), but only three of the five patients completed 1 cycle of hOKT3 γ_4 treatment at this dose level. Two grade 3 episodes of dyspnea, the last following severe rigors and associated with grade 3 headache, defined dyspnea as the dose-limiting toxicity. The maximally tolerated dose given by the schedule in this trial was defined as 800 μ g.

Immunomodulatory Effects. Temperature elevation was consistently observed after administration of hOKT3 γ_4 (Fig. 1). The maximum temperature was recorded at a median of 4 h after administration of hOKT3 γ_4 . Similar fever responses within individual patients were observed with repetitive treatments, suggesting that tolerance to treatment did not develop. The greatest fever response was observed at the two highest dose levels tested (800 and 1600 μ g), suggesting an increase in the amount of induced endogenous pyrogens, most likely TNF- α (10).

A transient depletion in peripheral blood lymphocytes was observed after the administration of hOKT3 γ_4 . As illustrated in Fig. 2, the magnitude of the lymphocyte depletion increased with the dose of hOKT3 γ_4 administered, but absolute lymphocyte counts returned to pretreatment values by 48 h after each treatment. The magnitude of the lymphocyte depletion and the time course of the depletion and recovery were reproduced with repeated hOKT3 γ_4 administrations, which suggested that tolerance and hOKT3 γ_4 neutralization were not occurring with repetitive treatments.

Expression of the M_r 75,000 component of the IL-2R (CD25) was used as an indicator of T-cell activation. After the administration of 50, 100, or 200 μ g of hOKT3 γ_4 , neither the percentage of peripheral blood lymphocytes expressing CD25 nor the surface density of CD25 expression was altered. However, at the 400- μ g dose level, two patients responded with small increases in the percentage of CD25-positive lymphocytes over 48 h following hOKT3 γ_4 administration (Fig. 3). Two of three patients treated at the 800- μ g dose level and three of five patients treated at the 1600- μ g dose level responded to

Table 2 Summary of clinical toxicities

Dose	Patients (n)	Fever n (grade)	Headache n (grade)	Nausea n (grade)	Hypotension n (grade)	Dyspnea n (grade)	Rigors n (grade)
50 μ g	6	2 (1)	3 (1)	2 (1)	2 (1)	0	0
100 μ g	3	1 (1)	1 (1)	0	0	0	0
200 μ g	3	2 (1)	2 (1)	1 (1)	0	0	2 (1)
400 μ g	3	1 (1)	1 (1)	2 (1)	0	0	2 (1)
800 μ g	4	1 (1)	1 (1)	3 (1)	0	1 (2)	3 (1)
			2 (2)	3 (2)			
			1 (3)				
1600 μ g	5	1 (1)	4 (1)	3 (2)	0	2 (3)	3 (1)
		3 (2)	1 (3)			1 (3)	

Fever Response After hOKT3 γ ₄ Administration (+/- SEM)

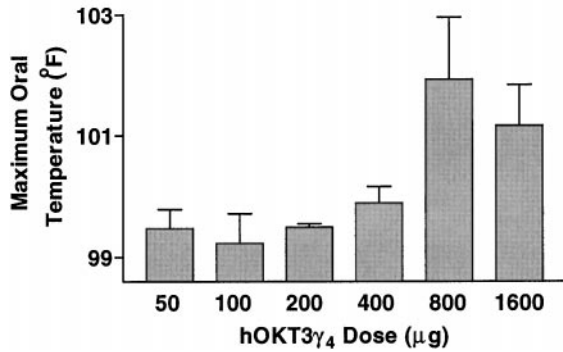


Fig. 1. The mean peak oral temperature within 48 h of initial hOKT3 γ ₄ administration is displayed for each dose level evaluated. Bars, SE.

Absolute Lymphocyte Count Following hOKT3 γ ₄ Administration

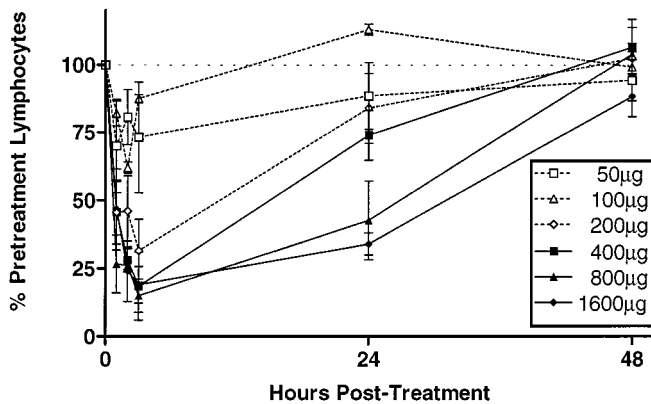


Fig. 2. The mean percentage of pretreatment lymphocytes in the peripheral blood at 1, 2, 4, 24, and 48 h after initial hOKT3 γ ₄ administration is plotted for each dose level. Bars, SE.

hOKT3 γ ₄ administration with more substantial increases in the percentage of CD25-positive lymphocytes. The interpatient variability in CD25-positive lymphocytes and small numbers of patients in each dose level did not permit a statistical evaluation of the significance of these changes. However, increases in CD25 expression at the two highest dose levels tested (Fig. 4) indicated a dose-dependent enhancement of CD25 expression. The percentage of lymphocytes expressing CD4 or CD8 and the CD4:CD8 ratio was unaffected by hOKT3 γ ₄ administration.

In the majority of patients (67%), the surface expression of CD3 was not altered by the administration of hOKT3 γ ₄. Modulation of CD3 was detectable in the presence of hOKT3 γ ₄ using murine OKT3d, which binds to a stearily independent site on the CD3 complex. Early CD3 modulation (within 2 h of hOKT3 γ ₄ administration) was observed in one patient each at the 50- and 200- μ g dose level, two patients each at the 400-, 800-, and 1600- μ g dose levels, and none of the patients at the 100- μ g dose level. CD3 modulation was transient in most cases, and only one of the patients (200- μ g dose level) treated in the first five dose levels had evidence of CD3 modulation at 24 h. Therefore, at doses through 800 μ g, hOKT3 γ ₄ did not induce persistent CD3 modulation. However, after administration of 1600 μ g of hOKT3 γ ₄, three of three patients had evidence of persistent CD3 modulation at 24 h.

Serum samples obtained prior to and 1, 2, 4, 24, and 48 h following

administration of hOKT3 γ ₄ were assayed for IL-6, TNF- α , GM-CSF, and sIL-2R. GM-CSF and TNF- α were not detectable in any of the serum samples. Increases in serum IL-6 were detected at 2 h after hOKT3 γ ₄ administration in one patient at the 400- μ g dose level, two patients at the 800- μ g dose level, and three patients at the 1600- μ g dose level. Fig. 5 illustrates the mean increase (\pm SE) in serum IL-6 2 h after hOKT3 γ ₄ administration at each dose level tested. These results suggest a dose-response relationship, which does not reach statistical significance.

Increases in serum sIL-2R were greatest 24 h after the administration of hOKT3 γ ₄. As seen in Fig. 6, the mean increase in sIL-2R level 24 h after hOKT3 γ ₄ administration appeared to be related to dose.

Anti-Idiotypic Antibody. Serum obtained from patients before each treatment was tested for the presence of anti-idiotypic antibody as described in "Materials and Methods." In all but six patients, no anti-idiotypic response was detected. Six patients developed anti-idiotypic responses (Fig. 7). This response was not related to the dose of hOKT3 γ ₄ administered because anti-idiotypic antibody was found in 2 patients at the 50- μ g dose level, none at the 100- μ g dose level, and in one patient at each of the remaining dose levels. Only 6 of the 24 patients in this trial received multiple cycles of therapy, and 4 of these patients developed anti-idiotypic antibody responses. These results suggest that repeated treatment can lead to the development of

Effect of hOKT3 γ ₄ on CD25 Expression

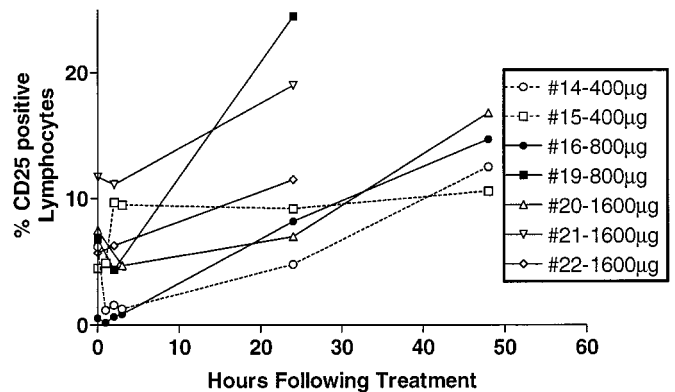


Fig. 3. The percentage of peripheral blood lymphocytes that were CD25-positive after hOKT3 γ ₄ administration are illustrated for the seven patients that developed increased numbers of CD25-positive lymphocytes.

Increase in Surface CD25 Expression

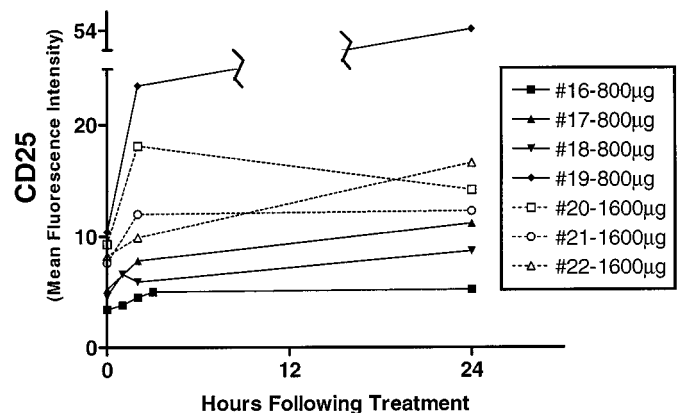


Fig. 4. MFI of CD25 within 24 h of hOKT3 γ ₄ administration is illustrated for the seven patients who demonstrated increased CD25 MFI.

Increase in Serum Soluble IL-2 Receptor 24 Hours Following hOKT3 γ ₄ Administration

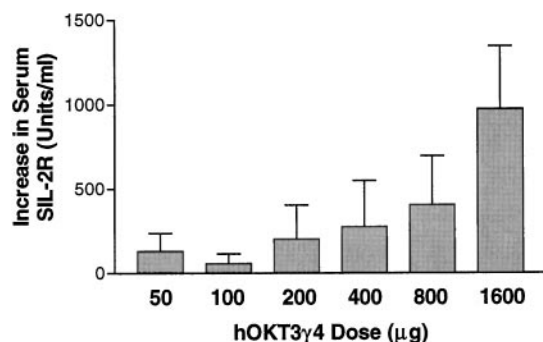


Fig. 5. The mean increase in serum sIL-2R at 24 h after initial hOKT3 γ ₄ administration is plotted for each dose level. Bars, SE.

Increase in Serum IL-6 Concentration 2 Hours Following hOKT3 γ ₄ Administration

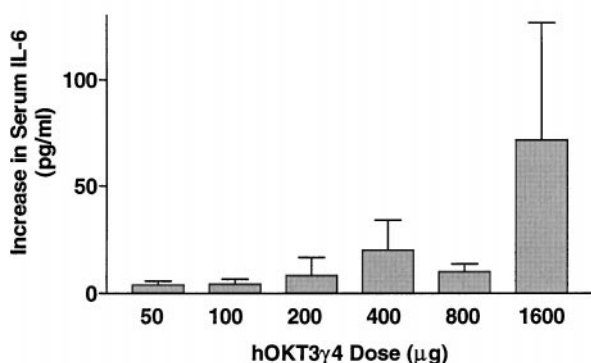


Fig. 6. The mean increase in serum IL-6 at 2 h after initial hOKT3 γ ₄ administration is plotted for each dose level. Bars, SE.

anti-idiotypic antibody response. However, these antibody responses were not associated with a decrease in the indicators of T-lymphocyte activation. Specifically, the magnitudes of hOKT3 γ ₄-induced fever response, lymphocyte depletion, sIL-2R, and IL-6 were not attenuated by the anti-idiotypic responses.

Clinical Response. Three tumor responses were documented in this Phase I trial. A patient with chemotherapy refractory ovarian cancer treated at the 50- μ g dose level had a >50% reduction in CA-125 serum tumor marker and resolution of ascites and back pain for 14 months. A patient with chemotherapy refractory pancreatic cancer and tense ascites treated at the 50- μ g dose level had complete resolution of ascites for 5 months before biliary and small bowel obstruction occurred, and a patient with peritoneal mesothelioma and tense ascites treated at the 200- μ g dose level had complete resolution of ascites for 6 months on treatment; response was maintained without additional therapy for an additional 6 months.

DISCUSSION

Humanized anti-CD3 (hOKT3 γ ₄) was administered to 24 patients in a standard Phase I study designed to assess toxicity and immunomodulatory effects. hOKT3 γ ₄ was administered i.v. over 10 min every 2 weeks for three doses, and this cycle was repeated in the absence of tumor progression. Coincident mild headache and nausea

were the most common clinical toxicities (71%). As a result of two episodes of severe dyspnea and cyanosis at the highest dose level tested (1600 μ g), we conclude that 800 μ g is the maximally tolerated dose of hOKT3 γ ₄ given by this route and schedule.

Lymphocyte depletion from circulation was maximal 2 h after hOKT3 γ ₄ administration, but all peripheral blood counts returned to baseline values at 48 h. This phenomenon alone indicates that T-cell activation is occurring early after treatment with hOKT3 γ ₄. Buysmann *et al.* (11) have demonstrated up-regulation of CD11a and CD18 on the surface of T lymphocytes after *in vitro* treatment with OKT3. These investigators demonstrated that adhesion of OKT3-activated human T lymphocytes to endothelial cell monolayers could be completely blocked by the addition of anti-CD18 or anti-CD11a. These early activation events are likely the critical events leading to lymphocyte depletion after OKT3-induced T-cell activation by facilitating adhesion of activated T cells to endothelial cells bearing the intercellular adhesion molecules ICAM-1 and ICAM-2. The magnitude of *in vivo* lymphocyte depletion observed in this clinical trial is, therefore, an indirect measure of *in vivo* T-cell activation by hOKT3 γ ₄. As illustrated in Fig. 2, peripheral blood lymphocyte depletion was more pronounced after administration of larger doses of hOKT3 γ ₄. However, the depletion and recovery of lymphocytes after the administration of 800 or 1600 μ g of hOKT3 γ ₄ are indistinguishable. This finding suggests that comparable T-cell activation is achieved at the two highest dose levels tested in this trial. However, the increased toxicity supports a subtle but clearly increased level of T-cell activation and most probably cytokine production at the highest dose level.

An increase in CD25 expression on peripheral blood lymphocytes was variably observed 24 h after hOKT3 γ ₄ administration at the 800- and 1600- μ g dose levels (Figs. 3 and 4). Because up-regulation of CD25 is a late event in T-cell activation and re trafficking of activated T cells precedes their CD25 up-regulation, evaluation of CD25 on circulating lymphocytes could be a falsely negative marker of T-cell activation. However, the finding of increased CD25 expression on lymphocytes after administration of the highest doses of hOKT3 γ ₄ suggests that T-cell activation does occur after hOKT3 γ ₄ treatment. Soluble factors released by T lymphocytes in response to activation were less likely to be affected by lymphocyte adhesion and could be a more reliable measure of T-cell activation by hOKT3 γ ₄. Therefore, to more accurately assess CD25 expression, the presence of the soluble form of IL-2R (sIL-2R) was examined (12). An increase in

Development of Anti-OKT3 Response

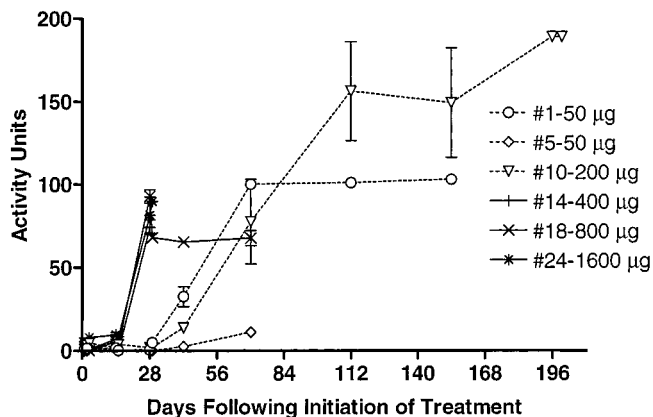


Fig. 7. Development of anti-idiotype antibody was assessed as described in "Materials and Methods." Anti-idiotypic responses were detectable in six patients. The level of this response, while receiving treatment, is depicted relative to the days from initial treatment. Bars, SE.

sIL2R was observed 24 h after hOKT3 γ_4 administration, and Fig. 6 strongly suggests that T-cell activation, as represented by sIL-2R production, is directly related to the dose of hOKT3 γ_4 administered.

An increase in serum IL-6 was observed 2 h after the administration of hOKT3 γ_4 , and although this response was highly variable between patients, the data suggest that this response was dependent upon the dose of hOKT3 γ_4 administered (Fig. 6).

Together the evidence strongly suggests that *in vivo* T-cell activation occurs after the administration of hOKT3 γ_4 . Fever response, peripheral blood lymphocyte depletion, and increased CD25 expression suggest that the two highest dose levels tested (800 and 1600 μg) produce the greatest T-cell activation. Soluble products of T-cell activation (sIL-2R and IL-6) suggest that an hOKT3 γ_4 dose of 1600 μg produces the greatest T-cell activation, but this dose exceeds the maximally tolerated dose identified in this trial and cannot be recommended for further evaluation. Rather, the 800- μg dose of hOKT3 γ_4 can be safely administered on the schedule tested while providing measurable T-cell activation, which is comparable to the 1600- μg dose.

None of the patients treated at the 800- μg dose level and only one of the patients treated at all of the lower dose levels had evidence of persistent CD3 modulation. This finding suggests that doses of hOKT3 γ_4 at or below 800 μg are unlikely to be immunosuppressive. However, the finding of persistent CD3 modulation after the administration of 1600 μg of hOKT3 γ_4 suggests that infusion administration of doses at 1600 μg and higher may be immunosuppressive. This finding suggests that the optimal biological dose for immunostimulation is <1600 μg .

Perhaps the greatest impediment to the use of OKT3 for *in vivo* immune activation has been the anti-idiotypic antibody response, which can neutralize OKT3 and prevent T-cell activation in repetitive treatments (8). This phenomenon was not clinically apparent in this trial. The magnitude of lymphocyte depletion 2 h after hOKT3 γ_4 administration (*i.e.*, the most reliable indicator of T-cell activation) did not diminish with repetitive treatments. Additionally, febrile response and sIL2R responses did not diminish with repetitive treatments. Anti-idiotypic antibody was detectable in four of six patients who received more than six doses of hOKT3 γ_4 (Fig. 7). However, no attenuation in the indicators of T-cell activation was observed in these patients following repetitive treatments. These findings demonstrate that neither tolerance nor significant antibody neutralization was induced in these patients. The data also emphasize the relatively nonimmunogenicity of the humanized Ab. The fact that only 25% of the patients produced an idiotype response in the face of a dose regimen (every 2 weeks) maximized for its T-cell activating effects is quite encouraging. This is especially true as compared with the previous clinical experience with the murine OKT3 that led to neutralizing anti-idiotypic antibodies in 100% of patients (6).

Therapeutic efficacy was not the primary goal of this clinical trial. However, clinically significant responses were observed in three patients with histologically different diseases. A 42-year-old woman with chemotherapy-refractory, premenopausal ovarian adenocarcinoma complicated with moderate ascites had resolution of ascites and >50% decline in CA-125 and survived 19 months from the time she entered the trial. A 45-year-old woman with chemotherapy-refractory

adenocarcinoma of the pancreas had resolution of ascites and survived 9 months from protocol entry. Finally, a 35-year-old man with peritoneal mesothelioma had resolution of tense ascites and remains asymptomatic 44 months from protocol entry. These were the only patients with known ascites treated on this trial, and each had a significant clinical response. However, it should be noted that there was no obvious correlation between the treatment dose and clinical effects observed. Whether hOKT3 γ_4 has a particular role in the treatment of ascites or *i.p.* disease of any histology should be explored in future trials.

In conclusion, hOKT3 γ_4 produced and formulated as described can be safely administered to patients. The maximally tolerated dose of hOKT3 γ_4 is 800 μg when administered as a 10-min *i.v.* infusion every 2 weeks. When administered by the route and schedule described, maximal *in vivo* activation of human T lymphocytes by hOKT3 γ_4 occurred at the 800- or 1600- μg dose levels. The potentially immunosuppressive persistent CD3 modulation induced by 1600 μg of hOKT3 γ_4 suggests that immunosuppressive effects may result from higher doses and further suggests that 800 μg is the optimal biological dose, as well as the maximally tolerated dose of hOKT3 γ_4 . The three antitumor responses observed in patients with *i.p.* malignancies (pancreatic, ovarian, and mesothelioma) suggest that Phase II clinical evaluation of the 800- μg dose is warranted.

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