Cancer Immunol Immunother (1997) 44: 137–149

ORIGINAL ARTICLE

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Phase IB trial of picibanil (OK-432) as an immunomodulator in patients with resected high-risk melanoma

Received: 2 October 1996 / Accepted: 28 January 1997

Abstract The microbial immunostimulant OK-432 has been studied intensively in preclinical systems and has shown promise as an anticancer agent in trials that have been conducted over the past 20 years in Japan. To date, no systematic dose response evaluation of this agent has defined its dose-limiting toxicity or immunobiological activity. A phase IA study has been conducted in 25 patients with metastatic cancer at the University of Pittsburgh Cancer Institute Melanoma Center, establishing 30 KE as the maximal tolerable dosage, on the basis of cutaneous reactions. Subsequently, 48 patients with resected high-risk melanoma participated in a phase IB study of OK-432. This study has evaluated the immunomodulatory activity of OK-432 at five dosages ranging from 1 KE to 20 KE, administered ID twice weekly for 3 months. A formal analysis of the treated population in comparison to the randomized control group has been conducted, and profound immunological effects have been defined in the group of patients treated with OK-432. Patients who participated in this trial had a significant depression of OK-432-inducible cytokine production (interleukin-1β, interferon γ, and tumor necrosis factor α) at baseline. Treatment with OK-432 reversed this deficit for interferon γ (IFNγ) production in a dose-dependent manner, and mitigated the inhibition for interleukin-1 (IL-1) across all dosage groups. The impact of OK-432 upon other immunological functions of the treated cohorts is more variable, with durable suppression of mononuclear cell superoxide production, and in vitro cytotoxicity to tumor. Immunological characteristics of the entire cohort demonstrate a strong and significant correlation of elevated blood $CD16^+$ cell counts and natural killer activity with early tumor progression and death due to melanoma. Favorable prognosis is associated with monocyte capacity to produce tumor necrosis factor (TNF), and polymorphonuclear leukocyte formylmethionyl-leucylphenylalanine-inducible superoxide release. This study reveals several new immunological correlates of tumor progression and lethal outcome in resected high-risk melanoma. It demonstrates that the depressed IL-1, TNF, and IFNγ release associated with melanoma may be mitigated by treatment with OK-432. This study has defined treatment and dose response patterns of immunomodulation associated with one of the most complex immunological agents yet evaluated in phase IB trials, in a well-defined population of high-risk patients with resected melanoma.

Key words Picibanil (OK-432) · Cancer · Melanoma · Immunostimulants · Immunomodulator · OK-432

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Introduction

The use of microbial immunostimulants for cancer therapy dates to the studies of mixed bacterial toxins performed by William B. Coley at the turn of the century [4]. Picibanil (OK-432) is a lyophilized product of an avirulent strain of *Streptococcus pyogenes*, inactivated by penicillin G. It has been used for treatment of cancer in Japan since 1975, where clinical evidence from thousands of patients has been acquired. In vitro studies of OK-432 have demonstrated tumoricidal activation of both macrophages and natural killer (NK) cells, the activation of cytotoxic T cell function against tumor-associated antigens, and protection against metastases of ultraviolet-light-induced tumors in mice. Prolongation of survival and inhibition of the development of metastatic disease have been reported with treatment of transplanted melanoma in mice [7, 8, 16], as well as autochthonous ultraviolet-light-induced tumors. A phase I study of OK-432 using escalating doses of this agent, administered intradermally to patients with a range of

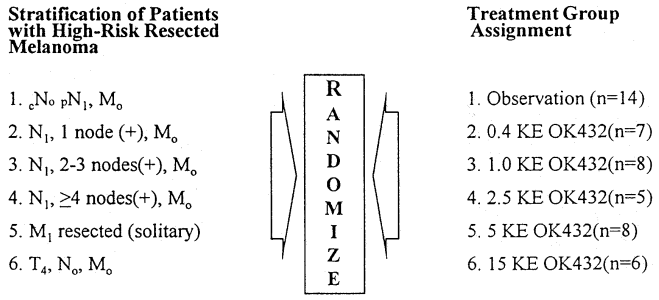


Fig. 1 Scheme of the phase IB trial with OK-432 (picibanil)

cancers, was recently conducted in Pittsburg [10]. The main adverse events noted were local reactions at the site of injection. Redness, induration, pain and itching with ulceration were observed in the most severe cases. These reactions were generally well-tolerated, but limited the administration of OK-432 in single doses to 30 KE (klinische equivalent units = 3 mg). Other events observed with OK-432 therapy included anemia, bone pain, gastrointestinal upset/emesis, abnormal liver and renal functions (elevated blood alkaline phosphatase and creatinine). Among 24 patients treated by the intradermal route, one patient with an angiosarcoma of the liver had a partial response enduring through six courses (months) of therapy. In addition, 1 patient with disseminated metastatic melanoma had objective partial regression of multiple cutaneous metastases. Brain metastasis developed despite the regression of the cutaneous disease after 2 months. Serial evaluations of multiple immunological parameters after single and multiple doses of OK-432 at various dosages during the course of this study suggested that OK-432 doses ranging from 1 KE to 20 KE warranted further study as an immunomodulatory approach to the therapy of cancer.

On the basis of the extensive preclinical and Japanese clinical experience, and the recently completed phase IA trial results, a more detailed evaluation of OK-432 in patients with resected melanoma at high risk of subsequent relapse was initiated at the University of Pittsburgh Cancer Institute Melanoma Center in 1990. The objectives of this study were to further define the toxic, immunological, and potential antitumor effects of OK-432 administered for 3 months, to melanoma patients free of active evidence of disease, but at high postoperative risk of relapse. This trial was also intended to determine which, if any, of the immunological, variables under study might correlate best with relapse-free and overall survival of melanoma. Unexpectedly, we observed a strong, significant correlation between NK cell activity, as well as circulating NK cell numbers, and early tumor progression and death from melanoma. On the other hand, favorable prognosis was associated with higher levels of interferon γ and tumor necrosis factor α (TNFα) and of superoxide produced by monocytes.

Materials and methods

Selection of patients

Patients eligible for this study had completely resected histologically proven cutaneous melanoma of stages IIB-IV as defined by the American Joint Committee on Cancer (AJCC). Patients were required to have normal organ function, as indicated by normal blood chemical, hematological, and urine analyses. No evidence of active metastatic disease was apparent on physical examination or radiological studies including chest X-ray, and computed tomography (CT) scans of head, chest and abdomen. The radiological surveys performed included single-photon-emission computed-tomographic (SPECT) gaillum-67 scans in all patients upon entry into this trial. The radiological survey performed within 1 month of study entry was repeated at the conclusion of the study treatment (3 months), and annually thereafter to confirm the absence of disease recurrence.

A total of 48 patients were accrued to this trial after giving written informed consent. Patients were randomly assigned to one of 6 groups: 0 KE, 0.4 KE, 1 KE, 2.5 KE, 5 KE, and 15 KE (1 KE = 0.1 mg). These dosages spanned the range of those determined to be tolerable in the preceding phase IA trial. Randomization was unbalanced, with 1.5 patients assigned to the control arm for every one assigned to each of the five treatment dosages. The trial originally called for 60 patients to receive treatment (12/dose tier) and 20 to be observed. The study was terminated after accrual of 48 patients by the sponsor, Chugai Pharmaceuticals, for reasons that were economically – and not scientifically – motivated. The scheme for this trial is illustrated in Fig. 1.

Table 1 Numbers of patients entered into dosage groups according to disease stratification (total *n* = 48)

Straturn	Dose group					
	Control	0.4 KE	1 KE	2.5 KE	5 KE	15 KE
1. Microscopic involvement (any TcN0pN1M0)	0	0	1	0	1	0
2. Node positive (any TN1M0)	4	2	3	2	3	2
3. Two or three nodes positive (any TN1M0)	4	2	2	2	2	2
4. Four or more nodes positive (any TN1M0)	1	0	1	0	1	0
5. Distant metastatic site (any T any NM1 resected)	3	2	1	1	1	2
6. Resected primaries (T4N0M0)	2	1	0	0	0	0
Total	14	7	8	5	8	6

Table 2 Dose stratification group versus gender and stage

Dose frequency	Gender		Total	Stage			Total
	F	M		II	III	IV	
0	1	13	14	0	12	2	14
0.4	1	6	7	1	4	2	7
1	5	3	8	0	7	1	8
2.5	2	3	5	0	4	1	5
5	2	6	8	0	7	1	8
15	5	1	6	0	4	2	6
Total	16	32	48	1	38	9	48

Patients who fulfilled the entry criteria for this phase IB trial were stratified by the disease extent prior to resection, to account for the possibility that this might influence the immunological endpoints, as well as to balance the risk of disease burden in predicting disease recurrence. The six stratification categories grouped patients according to the extent of resected disease, as shown in Table 1. Patients were assigned to one of the 6 treatment groups using a modified permuted block randomization [2, 17]. The distribution of patient gender and stage are tabulated in Table 2.

Treatment plan

Patients assigned to each of the six treatment groups received a single intradermal dose at the assigned dose level as an initial course in week 1, and began three 4-week dosing periods in which OK-432 was administered i.d. twice weekly for a total treatment period of 13 weeks. Patients assigned to the observation (0 KE) group were followed for toxicity, immune and disease endpoints at the same intervals as the treated groups, but received no active therapy or placebo.

Disease, toxicity, and immunological evaluation

Disease

The clinical status of melanoma in each patient was established initially by full history, physical examination, and contrast-enhanced CT scans of head, chest, and abdomen, as well as a SPECT gallium-67 scan. Follow-up clinical and radiological studies were repeated at 3 months, 6 months, 1 and 2 years on protocol. Thereafter, studies were obtained clinically, as indicated.

Toxicity

Complete blood counts, prothrombin time (PT), partial thromboplastin time (PTT), urinalysis, electrolytes, chemistry studies of renal and hepatic function, echocardiogram and multiple-gated radionuclide (MUGA) heart scan, throat culture, as well as OK-432 and penicillin skin tests were obtained prior to study entry. Complete blood counts, PT/PTT, urinalysis, and chemistries were repeated on days 8, 15, 22, 29, 36, 64, 92 and 176. The echocardiogram, chest X-ray, throat culture, and skin tests were repeated on days 29 and 92. OK-432 skin tests were repeated on days 92 and 176, while the MUGA scan was repeated on day 92 only. Potential hypercoagulability associated with OK-432 was evaluated by the PT and PT mixing assay, activated PTT and activated partial thromboplastin time mixing assay, protein C activity, thrombin time, plasminogen, antiplasmin, protein S, anti-(thrombin III), factor II (FII), and diluted Russell's viper venom (dRVV) levels on days 29 and 92.

Potential cardiotoxicity was evaluated prior to, during, and following treatment by physical examination, electrocardiogram, echocardiogram, and/or MUGA scans. The ejection fraction was measured to assess cardiac function serially during this trial.

Immunological analyses

Immunological effects of OK-432 were investigated through skin tests and serial blood assays before, during, and after the trial intervention period. The Merieux Multi-test for delayed hypersensitivity to a panel of microbial recall antigens and skin tests for penicillin as well as OK-432 were evaluated before the study and on days 29, 92, and 176.

The blood samples for immunological studies were obtained before treatment and on days 8, 15, 21, and 29 (of month 1), days 64 and 92 (immediately after treatment), as well as on 176 (3 months following conclusion of treatment).

Laboratory methods

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were obtained by centrifugation of heparinized venous blood on Ficoll-Hypaque gradients. The cells were recovered from the gradients, washed in RPMI-1640 medium (Gibco, Grand Island, N.Y.) and counted in the presence of a trypan blue dye. Fresh PBMC were used for cytotoxicity assays, cytokine production, superoxide generation and flow cytometry.

Cytotoxicity assays

Cultured tumor cell targets (NK-sensitive K562 or the NK-resistant melanoma line FEM-X) were labeled with 100-250 µl ^{51}Cr-labeled sodium chromate (specific activity 5 mCi/ml NEN, Boston, Mass.) for 1–2 h at 37 °C. Freshly labeled target cells were washed four times in RPMI medium with 10% fetal calf serum (FCS), resuspended in fresh medium, and divided into aliquots at 5×10^3 targets/well in 96-well U-bottomed plates into which samples of the effector cells had been previously placed at predetermined concentrations. The effector-to-target cell ratios ranged from 50:1 to 6:1. Effector cells were freshly isolated unstimulated mononuclear cells (MNC) or MNC incubated in the presence of OK-432 at concentrations of 1 µg or 10 µg/ml for 18 h. Plates were centrifuged at 65 *g* for 3 min and incubated at 37 °C for 4 h, after which 100 µl supernatant was harvested and transferred to scintillation vials. All determinations were done in triplicate. Radioactivity was counted in a gamma counter and the percentage specific lysis was determined according to the following formula:

$$\text{Cytotoxity \%} = \frac{\text{experimental meanradioactivity(cpm)} - \text{spontaneous release mean radioactivity (cpm)}}{\text{maximal radioactivity mean (cpm)} - \text{spontaneous release mean (cpm)}} \times 100$$

Results are expressed in lytic units (LU), 1 LU being defined as the number of lymphocytes required for 20% lysis of 5×10^3 target cells, and the number of LU (per 10^7) effector cells was calculated as previously described [15].

Cytokine production assays

To measure the ability of patients' MNC to secrete cytokines, these cells (1×10^6/ml) were incubated in medium alone to measure spontaneous release, or in the presence of OK-432 (1µg/ml or 10µg/ml) for

18 h, or in the presence of phytohemagglutinin (20 μg/ml), or lipopolysaccharide (10 μg/ml) for 24 h or 48 h at 37 °C. The supernatants of these cultures were harvested and stored frozen at −80 °C until they were tested by immunoassays. All the supernatants obtained with cells of 1 patient in the course of therapy were always tested in the same assays. The supernatant levels of interleukin-2 (IL-2) were measured by enzyme-linked immunosorbent assay (ELISA) purchased from Collaborative Research, Boston, Mass., those of IL1-β by ELISA (Cistron Biotechnology, Pine Brook, N.J.), those of interferon γ (INFγ) by radioimmunoassay (Centocor, Malvern, Pa.) and those of TNFα by ELISA. The World Health Organization (WHO) cytokine standards were used for calibration of all assays and an internal laboratory standard was used to determine interassay variability (CV = 6%−8% for various ELISA assays, with combined $n = 270$).

Superoxide generation by Monocytes

Freshly separated peripheral blood MNC were counted, resuspended in Krebs Ringer saline (KRS) at a cell concentration of 1×10^6/ml and pipetted into tubes. Cytochrome *c* (type VI, Sigma) and activators, formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe; Sigma), phorbol myristate acetate (Sigma) or opsonized zymosan, were next added to appropriate tubes. No activators were added to the tube containing "resting" MNC. The tubes were incubated for 30 min at 37 °C in a shaking water bath. Immediately after the incubation step, the tubes were centrifuged in the cold to pellet the cells, and the supernatants were transferred to clean cuvettes for measurements of absorbance at 550 nm to determine the amount of cytochrome *c* reduced/10^6 cells during 30 min incubation. Using a differential count, the amount of cytochrome *c* reduced/10^6 monocytes was then calculated for "resting" as well as activated cells.

Superoxide generation by granulocytes

Granulocytes were recovered from Ficol-Hypaque gradients and isolated by sedimentation through 6% dextran (Sigma). After lysis of erythrocytes in NH4Cl lysing buffer, the granulocytes were washed in PBS, counted and resuspended in KRS at a cell concentration of 1106/ml. The amount of cytochrome *c* reacting with 10^6 granulocytes during a 30-min incubation at 37 °C in the presence of various activators was determined as described above.

In vitro stimulation of MNC by OK-432

Peripheral blood MNC were resuspended in RPMI-medium containing 2% (v/v) FCS at a concentration of 1×10^6/ml. OK-432 was added to the cells at two different doses: 1μl/ml or 10μg/ml. Cells with no OK-432 added served as control. The cells were incubated in the presence of the agent for 18 h at 37 °C in an atmosphere of 5% CO_2 in air. They were then washed in RPM medium 2% FCS, counted, checked for viability by trypan blue dye exclusion and tested for cytotoxicity against K562 and FEM-X targets.

Flow cytometry

For two-color flow cytometry, cells were adjusted to a concentration of 0.5×10^6/tube in PBS containing 0.1% sodium azide. Cells were stained with fluorescein- and phycoerythrin-labeled monoclonal antibodies specific for a T-cell-associated antigen: CD3; activation antigens: IL-2 receptor (CD25), CD69 and HLA-DR; NK-cell-associated antigens: CD56, CD11b, and CD16; and a monocyte antigen: CD14. All monoclonal antibodies were purchased as labeled reagents from Becton Dickinson, Mountain View, Calif., and titrated on normal PBMC to determine optimal working dilutions. Staining and two-color flow cytometry were performed with controls including IgG1 and IG2a isotype monoclonal antibodies and PBS. The gate was selected on the basis of forward and side-scatter characteristics and set on lymphocytes or monocytes. A CD14/CD45 monoclonal antibody combination was used to confirm the phenotype of cells in the gate.

Statistical analysis

The analysis of treatment effects related to OK-432 attempted to detect differences indicated on treatment using four different approaches, as follows. The first three analyses were performed only in patients treated with OK-432. The final analysis included all patients from the treatment groups and the control group.

Sustained-effect analysis

Tests for sustained treatment effects computed the medians of all pre-treatment and all post-treatment values for each assay of each patient. The differences between the medians were analyzed to detect effects maintained over the entire post-treatment period. In this analysis and the next, overall tests of treatment effect were carried out using a signed-rank test, and differential dose effects were tested using a Kruskal-Wallis test with dose as the grouping factor.

Acute-effects analysis

Acute treatment effects were examined by taking the differences between the last pre-treatment and first subsequent post-treatment value, and subjecting these to analysis using the same tools as for the sustained-effect analysis.

Analysis of effects of general time interval

General treatment effects to which the above tests may be insensitive were evaluated and grouped according to the interval in which they were drawn: pre-treatment, and for post-treatment days 1−5, 6−18, 19−47, 48−78, 79−134, or 135−200. The Mack-Skillings test (a nonparametric repeated-measures test) was then applied to detect differences among these intervals. This test detects increases, decreases, and patterns of results that neither rise nor fall steadily through time.

Comparative analysis of effects in treated and observed groups

Effects of OK-432 upon immune parameters of patients on treatment were analyzed in comparison to the control group. These effects were sought in analyses of the differences between the pre- and post-treatment medians as follows. In the control group, blood samples drawn prior to the date of randomization were considered "pre-treatment". For the endpoints that showed possible dose effects in analysis under any of the methods listed above, each dose group was compared separately to the control group using a Wilcoxon rank-sum test. A Bonferroni procedure was then applied to control for type I error. This procedure is sensitive to differences that may occur between the control group and one or more dose groups. For endpoints showing no evidence of differences between dose tiers, the treatment group results were pooled and compared to those of the control group using a Wilcoxon rank-sum test.

Disease, outcome, correlation

A Kaplan-Meier product-limit approach was used to estimate survival curves for time to relapse, and death for patients in each group. Relationships between clinical, demographic, and immune variables and time to death or relapse were investigated using Cox proportional-hazards regression. These relationships were investigated in two ways. First, demographic and baseline immunological variables were explored in relation to disease outcome for all patients. Second, immediate and sustained changes in immune parameters from the baseline

Table 3 Summary of all toxicities data pooled over dosage strata for treatment groups. OK-432 dose is associated with toxicity (*P*, two-sided, = 0.0005). Percentages are shown in parentheses

Dose (KE)	No. patients having worst toxicity grade:			Total no. patients
	1	2	3	
0.4	4 (57.14)	3 (42.86)	0	7 (100)
1	3 (37.50)	4 (50.00)	1 (12.50)	8 (100)
2.5	0	4 (80.00)	1 (20.00)	5 (100)
5	1 (12.50)	6 (75.00)	1 (12.50)	8 (100)
15	0	2 (33.33)	4 (66.67)	6 (100)
Total	8 (3.53)	19 (55.88)	7 (20.59)	34 (100)

Table 4 Effect of OK-432 upon cardiac coagulation. *MUGA* multiple-gated radionuclide heart scan, *n* number of patients evaluated, *PT* prothrombin time, *PTT* partial thromboplastin time. Median values are shown

Test	Pre-treatment	Post-treatment	Post-treatment difference	*P*
MUGA scan				
n	45	31	30	
Ejection fraction (%)	68	65	1.5	
Coagulation				
n	48	48	48	
PT (s)	11.3	11.5	0.2	0.004
PTT (s)	27.7	28.5	1.3	0.0051
Protein C activity[a]	2.0	2.03	0.013	0.078
Portein S activity[a]	2.80	1.99	0.27	0.35

[a] Log_{10} values

were investigated using only patients who received a full course of OK-432 treatment, and correlated with disease outcome.

Results

Clinical effects and toxicity

Forty-eight patients with resected melanomas were enrolled into this trial, allocated by risk into the six groups defined earlier. The numbers of patients entered into each stratum according to the OK-432 treatment dose tier ranged from 5 to 8 for treatment groups and 14 in the control (non-treatment group). Reasonable balance in the numbers of patients assigned to each treatment was achieved by the permuted-block method.

Treatment was generally well-tolerated during this trial, and no new or unreported toxicities of OK-432 were identified. General toxicity sustained by patients in this trial is graded using the common toxicity reporting criteria of the U.S. clinical cooperative groups and is summarized in Table 3. Grade III cutaneous effects were encountered in 0–1 patient in all but the highest dosage group (15 KE), as predicted by the preceding phase IA trial results.

No significant manifestation of cardiac toxicity was observed by clinical or MUGA/ electrocardiogram evaluation. The pre- and post-treatment ejection fractions and the pre- to post-treatment differences are shown in Table 4. No clinical suggestion of coagulation disturbance was noted in the trial. The results of coagulation tests that showed changes during treatment are also displayed in Table 4. The changes observed in the PT and PTT were statistically significant, but of small magnitude and dubious clinical importance.

Immunomodulation

A number of immunological parameters were selected for serial monitoring prior to and during therapy with OK-432. The choice of these assays was not arbitrary but governed by pre-clinical observations reported by others [7, 13], as well as a desire to define changes in hypothetical antitumor functions of immune effector cells that might be related to or associated with a favorable prognosis.

Table 5 Sustained effects of OK-432 upon cytokine production, superoxide production, and phenotype of peripheral blood mononuclear cells: influence of treatment and dose. Results of non-parametric tests of differences between pre- and post-treatment median values and differentia effects of dose upon immunological endpoints evaluated serially during the study. *IL-1* interleukin-1, *IFNγ* interferon γ, *TNFα* tumor necrosis factor α, *MNC* mononuclear cells, *PMN* polymorphonuclear leukocytes, significant values are indicated by bold type

Immunological endpoint	Median values		*P*	
	Pre-treatment	Post-treatment	Treatment[a]	Dose evaluated by treatment[b]
Spontaneous IL-1 (pg)	0.075	0.037	0.10	0.75
Stimulated IL-1 (pg)	3.88	3.08	**0.03**	0.77
Spontaneous IFNγ (pg)	0.51	0.51	0.23	0.50
Stimulated IFNγ (pg)	124.9	113.8	**0.02**	0.10
Spontaneous TNFα (pg)	0.01	0.01	0.59	0.22
Stimulated TNFα (pg)	1.37	1.14	**0.04**	0.98
Resting MNC superoxide produced (mmol)	5.75	4.13	0.08	0.06
Resting PMN superoxide produced (mmol)	1.75	1.57	0.71	0.53
Absolute $CD56^+$ $CD69^+$ (%)	0.023	0.023	0.31	**0.05**

[a] Signed rank test
[b] Kruskal-Wallis test

Table 6 Acute effects of OK-432 on cytokine production and phenotype of peripheral blood mononuclear cells: influence of treatment and dose number. Results of non-parametric tests of differences between median $\log_{10}$ values for the last pre- and the first post-treatment samples, and differential effects of dose upon immunological endpoints ($\log_{10}$ values)

Immunological endpoint	Median values		*P*	
	Pre-treatment	Post-treatment	Treatment[a]	Dose evaluated by treatment[b]
Spontaneous IL-1	0.052	0.065	0.08	0.67
Stimulated IL-1	3.99	2.86	0.33	0.58
Spontaneous IFN	0.5	0.5	0.39	0.42
Stimulated IFN	102.0	121.0	0.11	0.29
Spontaneous TNF	0.045	0.065	0.23	0.48
Stimulated TNF	1.69	1.22	0.13	0.72
Absolute $CD3^+$	0.397	0.371	0.48	0.57
Absolute $CD3^+$ $CD25^+$	0.086	0.085	**0.04**	0.25
Absolute $CD3^+$ $CD69^+$	0.009	0.008	0.67	0.11

[a] Signed rank test
[b] Kruskal-Wallis test

Analysis of the immune parameters measured indicated that therapy with OK-432 had either sustained or acute effects. These effects were examined separately in greater detail.

Sustained effects of OK-432

The immunological variables listed in Tables 5, 6 showed sustained treatment effects during the study. Spontaneous production of cytokines was not altered by treatment with OK-432. On the other hand, OK-432-stimulated in vitro production of IL-1, IFNγ, and TNFα by PBMC were all consistently significantly diminished (P = 0.03, 0.02, and 0.04 respectively) in patients receiving OK-432 therapy. No treatment dose effect was identifiable for suppression of OK-432-inducible cytokine production by blood mononuclear cells during therapy.

Superoxide production by resting monocytes directly after harvest showed a marginal dose effect (P=0.06), as shown in Table 5. Production was increased in the 0.4-, 5-, and 15-KE groups, and decreased in the other two groups. Superoxide production by fMet-Leu-Phe-, phorbol-myristate-acetate, and Zymozan-stimulated monocytes showed consistent decreases following OK-432 treatment, which were not significant (data not shown).

No significant sustained OK-432 treatment effects upon cellular cytotoxic functions assessed during this study, nor upon the distribution of phenotypically defined subsets or activation markers expressed by blood lymphoid populations, were documented in this study.

Acute effects of OK-432

To analyze acute treatment effects on immune parameters, we tested for differences between the last pre-treatment and first post-treatment values. The results of this analysis are shown in Table 6. This analysis revealed a marginal (P = 0.08) acute effect of OK-432 upon unstimulated IL-1 production. Decreased IFNγ and TNFα production by blood monocytes after in vitro stimulation with OK-432 was less significant, but consistent with the important results of the global pre-treatment versus post-treatment

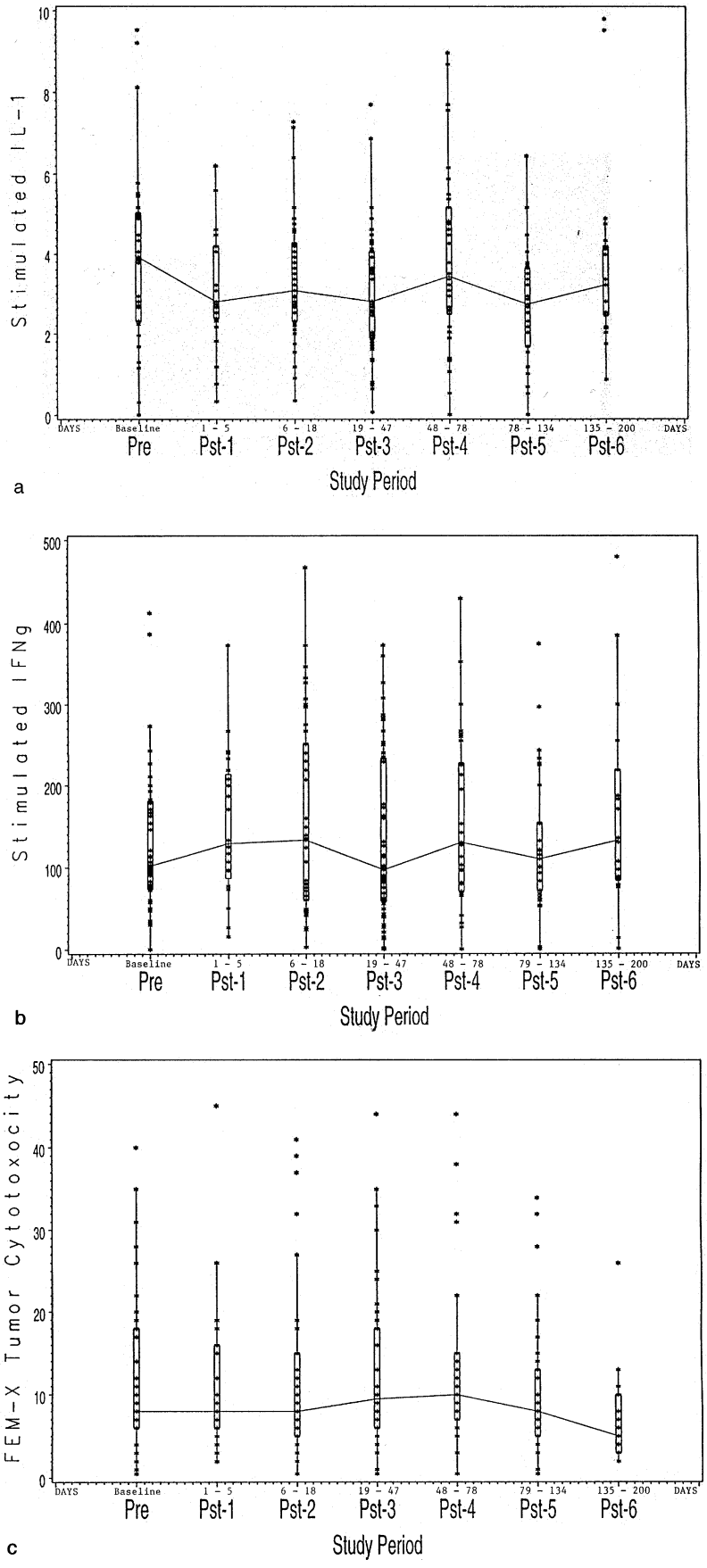
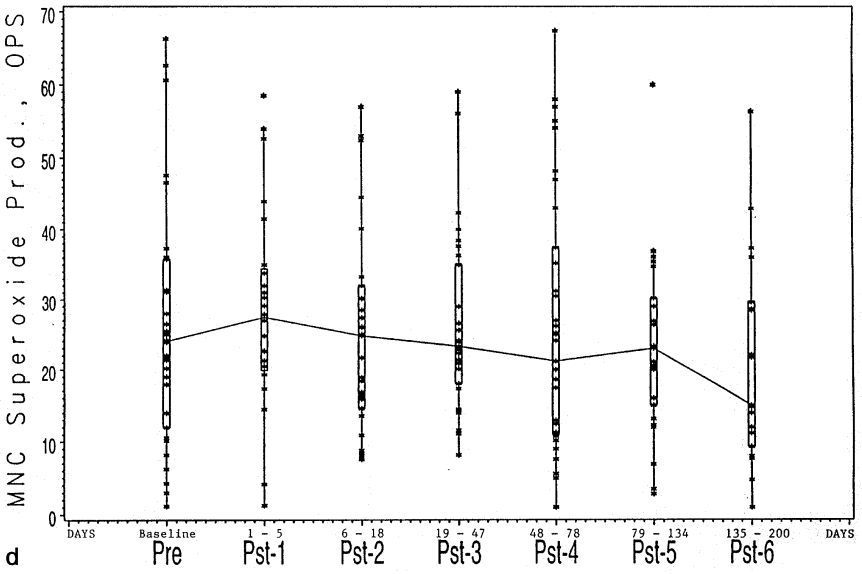


Fig. 2a–g Changes in selected immune parameters observed over time in patients treated with picibanil. **a** Levels of interleukin-1 (*IL-1*) production by mononuclear cells (MNC) incubated in the presence of lipopolysaccharide (10 μg/ml) for 24 h. **b** Levels of interferon (*IFN*γ) production by MNC incubated in the presence of phytohemagglutinin (20 μg/ml) for 24 h. **c** Levels of cytotoxicity against FEM-X melanoma targets, as determined in 4-h ^{51}Cr-release assays. **d** Superoxide production by MNC. **e** Absolute lymphocyte count. **f** Absolute number of CD16^{+} MNC. **g** Absolute number of CD56^{+} MNC. The data are presented as a mid 80% range of measured parameters (*boxes*). The solid horizontal lines show median values


MNC Superoxide Prod., OPS
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DAYS
Baseline
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6 - 18
19 - 47
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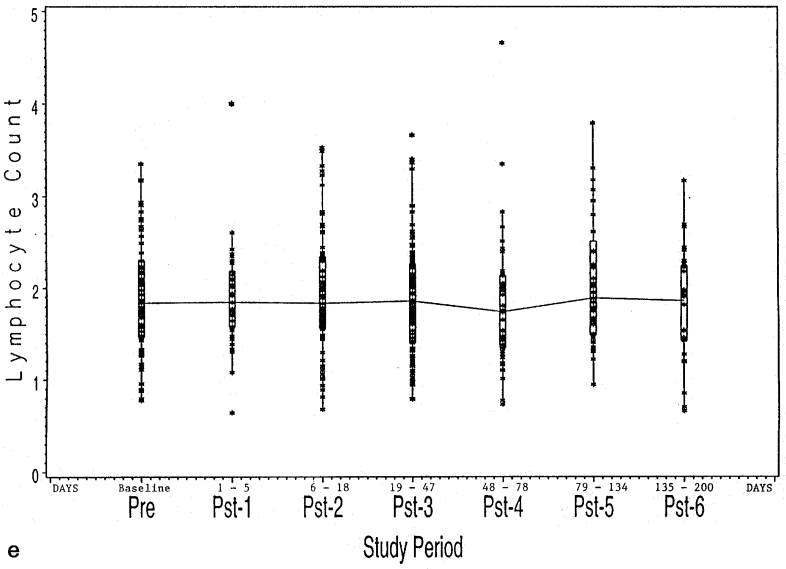
Lymphocyte Count
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4
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1 - 5
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19 - 47
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Pst-3
Pst-4
Pst-5
Pst-6
Study Period

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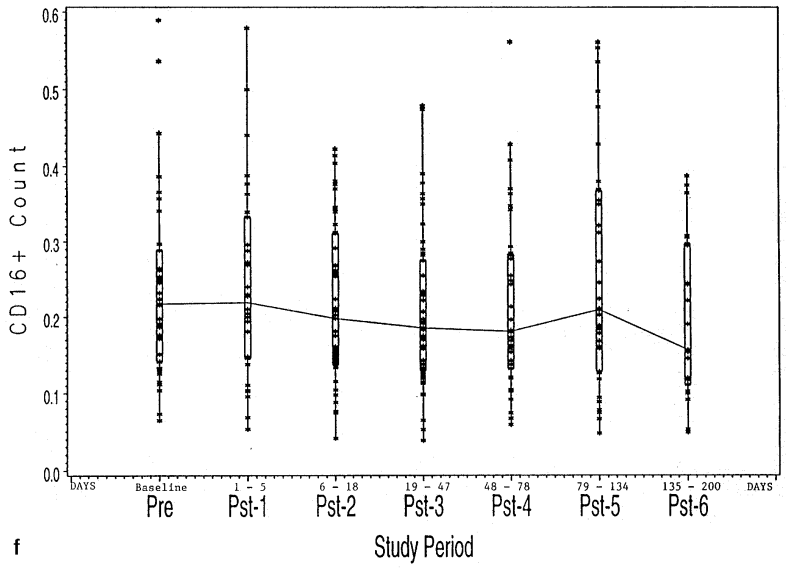
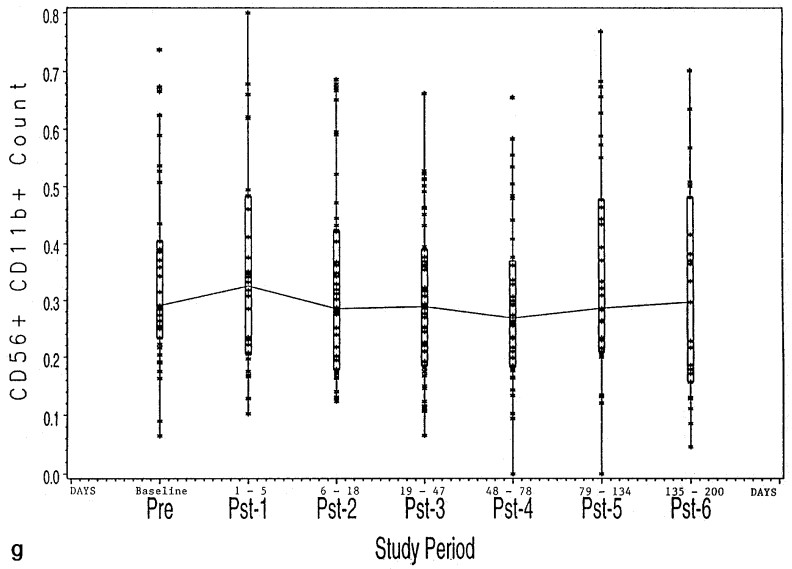

CD16+ Count
0.6
0.5
0.4
0.3
0.2
0.1
0.0
DAYS
Baseline
1 - 5
6 - 18
19 - 47
48 - 78
79 - 134
135 - 200
DAYS
Pre
Pst-1
Pst-2
Pst-3
Pst-4
Pst-5
Pst-6
Study Period

f



g

analysis for sustained effects. Acute treatment effects upon cytotoxic function, or superoxide production (spontaneous or stimulated) were statistically significant.

Acute treatment effects of OK-432 upon circulating lymphoid cell subsets and marker expression are notable for two subpopulations. First, acute augmentation in the $CD3^+$ $CD25^+$ population without a differential effect of dosage was documented by signed-rank test ($P = 0.04$). Secondly, a marginally significant dosage effect upon the $CD56^+$ $CD69^+$ subpopulation was detected in the analysis for sustained effects using all pre- and post-treatment samples ($P = 0.05$), as well as the analysis for acute effects, using the last pre- and first post-treatment samples ($P = 0.07$). These observations suggest a differential impact of treatment dosage without an overall treatment impact when all dosage groups are considered together.

Immunomodulatory treatment effects by time

To better determine effects of treatment over time in the treated population, the Mack-Skillings test was performed. This repeated-measures test of treatment effects uses the six intervals specified earlier as the treatment variable. Stimulated cytokine production (IL-1 and IFNγ) was significantly enhanced (Fig. 2a, b). Similarly, TNFα production rose consistently over the six intervals of assessment, although this increment was not significant. For IL-2, both spontaneous and stimulated production was consistently decreased following treatment with OK-432, although not to statistically significant levels.

Cytotoxicity against the cultured FEM-X melanoma line revealed a progressive long-term decrease following treatment ($P = 0.008$, Fig. 2c), while NK activity was unchanged and monocyte cytotoxicity rose consistently during treatment with a post-treatment drop below baseline, which did not achieve statistical significance (Fig. 2d).

Lymphocyte subsets and activation markers varied significantly for absolute, $CD16^+$, and $CD56^+$ $CD11b^+$ cell counts ($P = 0.009$, 0.028 and $P = 0.041$ respectively (Fig. 2e, f, g). The $CD16^+$ cell counts were consistently significantly depressed, reaching a nadir in the interval between days 19 and 47, while the $CD56^+$ $CD11b^+$ subset increased between days 1 and 18, and decreased from days 19 to 34, normalizing in the final interval of assessment.

Analysis of immunological effects of treatment compared to control populations

The analyses of pre- compared to post-treatment effects for sustained effects, for acute effects, and for general-interval effects were further persued through comparison of treated groups with the untreated control group, as detailed in Materials and methods. This analysis revealed no significant difference between treatment and control immune assay results for the endpoints where possible dose effects had been raised in analysis of the treatment group alone.

A comparative analysis of OK-432-treated and control patients, evaluating the difference between post-treatment median values minus pre-treatment median values, identified two significant differences. Cytokine production stimulated by OK-432 in vitro was diminished less in the treated group by comparison with the control group. This difference was statistically significant in the case of IL-1 ($P = 0.02$ by Wilcoxon rank-sum test), but also notable for TNF and IFNγ. The absolute lymphocyte count showed a significant variation over time in the Mack-Skillings analysis of general treatment effects: the difference between the drop in the control group, and the rise in the treatment group, had marginal significance ($P = 0.07$).

The median interval to relapse, and the median survival for patients in each of the treatment and control groups are

presented in Table 7. The sample size accrued in this phase IB trial was insufficient for compelling conclusions to be drawn with regard to the potential therapeutic impact of OK-432 at the various dosages tested. A Cox proportional-hazards regression was used to evaluate the association of the various immunological features of the treated population and the outcomes of event-free survival and overall survival, where disease relapse or death was scored This analysis was conducted for the entire population entered into this study with intention to treat. Correlations between treatment dose and outcome are presented for the entire intent-to-treat population of patients.

Baseline assay correlations to disease outcome

The initial blood samples from the entire population revealed a predictive (prognostic) correlation between several immune endpoints (cytokine production, phenotype, and function of circulating blood mononuclear cells), and the occurrence of relapse and/or death due to melanoma in this study. These results are summarized in Table 7.

Cvtokine production

Spontaneous IFNγ production was correlated at a marginal level with progression (P = 0.0775) and more so with death (P = 0.0444). Stimulated IL-2 production showed an association with death that was also marginal (P = 0.062).

Circulating mononuclear cell phenotype

The level of circulating peripheral blood $CD16^+$ cells was strongly and directly correlated with the clinical outcomes of progression (P = 0.0148) and death (P = 0.0081), taken together or separately (P = 0.0048); the correlation of either outcome with $CD16^+$ cells was also significant at P = 0.0048. The $CD3/CD69^+$ subpopulation was correlated with death (P = 0.0554).

Effector cell functions

Lytic functions of peripheral blood NK cells against K562 cells showed a correlation with risk of death alone (P = 0.0068).

Treatment effects correlated with disease outcome

Immediate pre- to post-treatment changes

Acute post-treatment rises in OK-432-stimulated IL-2 production were directly associated risk progression (P = 0.0464 and death (P =0.0662). Positive changes in stimulated IL-2 production were significantly associated with progression or death (P = 0.0268).

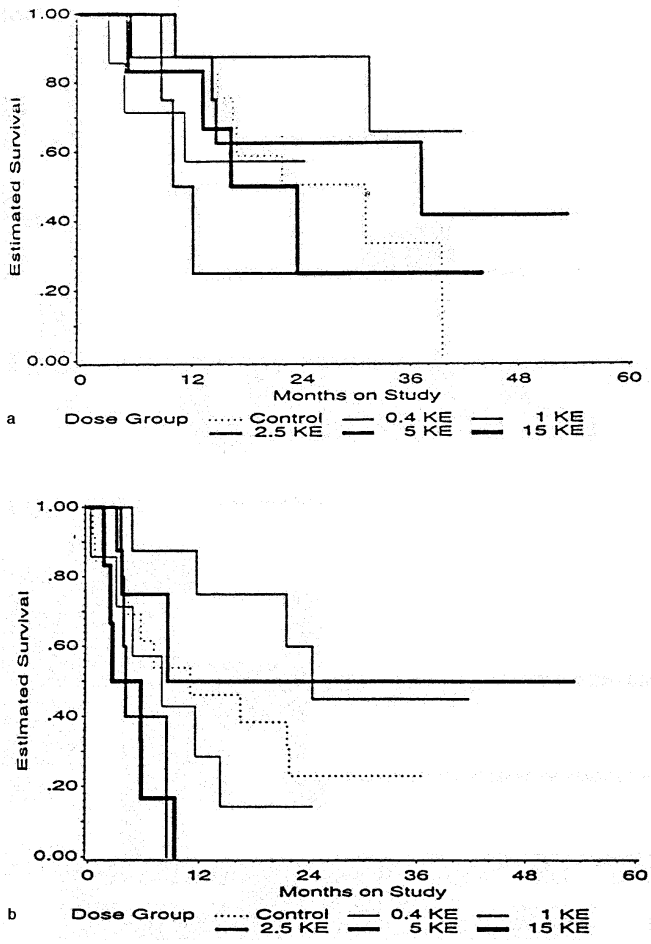


Fig. 3a, b Correlations of picibanil dose with disease outcome: Kaplan Meier plot. **a** Estimated overall survival. **b** Disease-free survival

Sustained pre- to post-treatment changes

Increased spontaneous TNF production was associated with a decreased risk of progression (P = 0.0171) and adverse events altogether, including death or progression (P = 0.0171). The only other laboratory endpoint that demonstrated an association with disease outcome was PMN superoxide induction by fMet-Leu-Phe which was associated marginally with death or progression (P = 0.0517). Tests of the null hypothesis that there is no association between the range of immunological endpoints and the events of death, progression or either death or progression are presented in Table 7.

Correlations of treatment dosage with disease outcome

The intention-to-treat analysis of relapse and death by treatment dose group reveals no clear dose response relationship (see Table 7, and Fig. 3a, b). Doses of 1.0 KE and 5.0 KE are associated with a relatively favorable outcome in terms of disease progression and survival, while the more extreme low and high doses, as well as the intervening dose

Table 7 Analysis of median event-free survival and overall survival according to variables of interest

Group	Estimated median time (months)			
	Death	*P*[c]	Death or progression	*P*[c]
All patients combined	31.1		8.6	
Control	31.1	0.39	11.1	0.019
0.4 KE	[a]		7.9	
1 KE	[a]		24.4	
2.5 KE	11.0		4.0	
5 KE	37.3		[a]	
15 KE	20.6		5.6	
Age <50.5 years	31.1	0.34	11.6	0.56
Age ≥ 50.5 years	37.3		8.5	
Female	37.3	0.18	9.3	0.78
Male	17.0		8.6	
Disease stage II, III	31.1	0.43	8.5	0.60
Disease stage IV	37.3		9.3	
Stratum 1	[a]	0.79	[a]	0.82
Stratum 2	23.5			
Stratum 3	[a]		8.6	
Stratum 4	[a]		11.7	
Stratum 5	37.3		3.6	
Stratum	21.8		11.6	
Low $CD16^+$ count	31.1	0.008	16.6	0.005
High $CD16^+$ count	21.8		4.7	
Low spontaneous IFN produced	17.0	0.21	8.6	0.044
High spontaneous IFN produced	31.1		21.6	
No increase, spont. TNF[b]	20.3	0.60	6.3	0.32
Increase, spontaneous TNF[b]	[a]		[a]	
No increase, stimulated IL-2[b]	[a]	0.054	8.8	0.062
Increase, stimulated IL-2[b]	34.4		6.3	
Low K562 cytotoxicity	37.3	0.007	9.3	0.034
High K562 cytotoxicity	16.6		8.6	
No increase, $CD3^+$ $CD69^+$ count[b]	[a]	0.055	21.6	0.67
Increase, $CD3^+$ $CD69^+$ count	10.2		5.6	

[a] Median time could not be estimated
[b] Treated patients only. Time (months) from final treatment
[c] Test of null hypothesis of no association between group and outcome

of 2.5 KE, are associated with unfavorable disease outcome. Figure 3a, b displays the impact of dose upon survival and relapse interval for the entire population of patients who entered this study.

Discussion

The microbial immunostimulants have had a long and complex history as potential agents for the therapy of cancer, dating to the work of Coley nearly a century ago [4]. The most promising results in the systemic therapy of solid tumors have been observed in melanoma where bacillus Calmette-Guérin [3, 14], *C. parvum* [1] and, more recently, the nonrecombinant and recombinant cytokines including IFNα and IFNγ [9] and IL-2 [11], as well as a series of subsequent cytokines [12], have shown promise. The immunological lesion(s) associated with solid tumors in general, and melanoma in particular, have yet to be defined. As a consequence, single cytokines may be inadequate therapy. The use of multiple cytokines in combination, or the use of complex stimulants, which may trigger the release of multiple cytokines in vivo, are alternatives that may achieve superior results.

OK-432 is the lyophilized pulverized product of the avirulent strain *S. pyogenes* str. OK-432 treated with penicillin G. This product, marketed in Japan since 1975 for treatment of patients with cancer, has been studied extensively in preclinical systems and in phase III trials combined with chemotherapy for a variety of malignant diseases. Despite the demonstration of potent in vitro cytokine induction by OK-432, and activation of macro-

phages, NK cells, and T cells with cytotoxic function against tumor cells, no careful dose response study to define dose-limiting toxicity or immunomodulatory effects has ever been carried out. Based upon a phase IA trial at the University of Pittsburgh Cancer Institute [10], which defined the maximum tolerable intradermal dosage as less than 30 KE/dose, a phase IB trial was designed to evaluate more precisely the immunomodulatory effects of OK-432 treatment in a larger cohort of patients with a single tumor type and minimal tumor burden, appropriate for a more prolonged, focused, evaluation.

Melanoma was chosen for the present evaluation as it is one of the most responsive human tumors to immunologic interventions. Ultimately, it was anticipated that the detailed exploration of this immunological approach to melanoma might allow the examination of the immunological variables associated with disease remission and progression. Patients with resected high-risk disease and no active evidence of tumor remaining were selected for this trial to ensure that the disease burden itself would have the least likelihood of altering immune responses or require interim treatment with alternative modalities. It was initially planned to treat up to 12 patients at each of 5 dosages of OK-432, ranging from the lowest dose level with demonstrable immunomodulatory activity in prior studies (0.4 KE) to the level of tolerance defined in the prior phase IA study (15 KE). This study accrued a total of 58 subjects, with 34 patients entering the five active-treatment dosage groups and with 14 patients in the control group followed for toxicity, immunological changes, and tumor outcome.

Immunological studies have demonstrated a complex influence of OK-432 on cytokine production, both spontaneous and OK-432-induced, through assays performed directly upon circulating blood mononuclear cells and following in vitro incubation of these cells obtained after therapy in vivo. A substantial depression of OK-432-inducible IL-1, TNFα, and IFNγ production was observed in this trial, ranging from 21% to 17% and 9% respectively. The depression of production for all three cytokines was statistically significant in this trial. The analysis of cytokine production over time in the treated and control groups revealed differences in mononuclear cell cytokine production, and especially for OK-432-inducible IFNγ production, which was significantly depressed among controls but less depressed in the OK-432-treated population. Treatment with OK-432 reversed the suppression of OK-432-inducible IFNγ production in a dose-dependent manner. This interesting observation indicates that PBMC obtained from patients with melanoma are poorly inducible by OK-432 to produce IFNγ, and that this lesion can, in part, be reversed by treatment with OK-432. The depression of OK-432-induced IL-1 production by the lymphocytes of patients treated with OK-432 in vivo was significantly mitigated across all dosage groups (P = 0.02). OK-432-induced TNFα production was mitigated, but to a lesser degree (which did not achieve statistical significance). Hence, lymphocytes from patients treated with OK-432 partially regain the ability to produce multiple cytokines when stimulated with OK-432.

The effects of OK-432 therapy upon cytotoxic effector functions showed less consistent effects. In general, depression of cytotoxic functions was observed with treatment. Resting mononuclear cell superoxide production showed a marginal sustained decrement following OK-432, while superoxide production in response to Zymozan-opsonized cells showed an acute treatment-related reduction. Monocyte cytotoxicity to tumor varied significantly when studied over time in a repeated-measure analysis of variance. The treated groups showed depressed cytotoxic functions by comparison to the control group in the final comparative analysis, and cytotoxicity against FEM-X melanoma cells showed the most significant and durable late suppression in the treated group (P = 0.008). These effects of OK-432 upon cytotoxic functions were felt to be unlikely to serve as useful markers for OK-432 antitumor activity.

The impact of OK-432 upon the distribution of circulating mononuclear cell populations reveals a sustained dose-dependent depression of the $CD56^+$ $CD69^+$ activated NK cell population (P = 0.05) A general effect of treatment was observed upon the $CD3^+$ $CD25^+$ subset (P = 0.04). Over time, for the treated groups taken together, there was significant variability in the absolute lymphocyte count (P = 0.009) and the $CD16^+$ and $CD56^+$ $CD11b^+$ counts (P = 0.028, 0.041) respectively. Comparisons of the treated and control groups demonstrate that OK-432 treatment mitigated the drop in lymphocyte counts that otherwise occurred among the control population over 6 months of follow-up during this trial. The FcRγ $RIII^+$ NK $CD16^+$ population showed consistent depression in the treated group while there were significant deviations both above and below baseline during treatment for the NK subset marked by $CD56^+$ $CD11b^+$. It is not possible to implicate an NK-related mechanism of OK-432 activity on the basis of the data obtained in this study.

Acute and sustained treatment effects are observed across all doses of OK-432 when a variety of cytokine induction assays are used. The concordance of findings from different methods of analysis related to OK-432-stimulated IL-1, IFNγ and, to a lesser extent, TNFα production, suggest that these effects are real and causally related to the treatment with OK-432. These observations, in comparison to the observations in the control group where more profound depression of all these cytokines studied was noted (most significant for IL-1 at P = 0.02 for IL-1), offer an important focus for future studies of complex immunomodulators.

This phase IB trial attempted to correlate treatment dosage with outcome, either in terms of death or of tumor progression/relapse and/or death. Two dosages, 1 KE and 5 KE, are associated with an apparent favorable prognosis, but each is bracketed by dosages associated with unfavorable outcomes. The median survival in the 1-KE dosage group was prolonged (indeterminate), and both the median and event-free survivals of the 5-KE group were prolonged (indeterminate and 37.3 months respectively). The relatively poorer results of the 2.5-KE dose group may be questioned, as they interrupt a bell-shaped curve of efficacy suggested by results with the neighboring doses. The small

number of patients studied here does not allow a final conclusion in this regard.

The immunological impact of OK-432 therapy in this trial was assessed in relation to the foregoing disease-specific events, including progression and death. The analysis of immunological endpoints studied during this phase IB trial has revealed an association between the survival patients and the stages into which risk was stratified, as well as age, gender, and immunological characteristics of patients entering the trial. The correlation of survival with age above 50 years, female gender, and low disease stage were not unexpected. It is of note that relapse and death in this study were directly associated with high pre-treatment levels of $CD16^+$ numbers, and high NK cell activity in the circulation. The strong correlation of survival with low numbers of blood lymphocytes bearing CD16 and expressing NK activity against K562 was unexpected, although earlier studies [5, 6] have demonstrated an inverse correlation between primary melanoma lymphoid infiltration by NK cells and the circulating number of NK effector cells. The correlation of relapse and death with higher levels of $CD16^+$ cells and NK activity, as well as IL-2 induction by OK-432 in vitro, is internally consistent in this study. A plausible interpretation of these data is that the effector cells in the tissues, not those in circulation, are responsible for antitumor effects. This would suggest that the decreased number and activity of NK cells in PBMC correspond with an increased extravasation of the relevant effectors into the tissues, particularly since these decreases are accompanied by the highest OK-432-induced ability to produce cytokines. These mediators are likely to promote entry of activated effector cells into tissues and their further disappearance from the peripheral circulation. In support of this hypothesis, Hersey, et al. have previously demonstrated that there is an inverse relationship between tissue and blood level of NK cells in untreated patients with melanoma [5]. Increased spontaneous TNF production was associated with prolonged survival in this study, while IL-2 production was associated with risk of relapse/death concordant with the phenotypic and functional data already noted. Thus, we have identified a set of immunological variables that appear to correlate with survival. These observations will require further evaluation in future trials of adjuvant therapy for melanoma for validation. We have raised fundamental questions regarding the effector mechanisms which may be desirable to induce in the adjuvant therapy of melanoma for prevention of relapse and prolongation of survival. Further study of immunological correlates of disease-free and overall survival will be crucial to the more effective application of immunomodulators such as OK-432 for therapy of melanoma and other solid tumors.

Acknowledgements The authors are grateful to Dr. Franklin Bontempo for assistance with the selection of hematological assays, Dr. William Follansbee for selection of cardiac function studies, and Ms. Marie L. Thompson for secretarial and editorial assistance with this manuscript.

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