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Acknowledgement—We thank the National Cancer Institute for grant CA 41285.

Eur J Cancer, Vol. 26, No. 9, pp. 965–969, 1990.
Printed in Great Britain
0277–5379/90 $3.00 + 0.00

Intratumoral Injection of OK432 and Lymphokine-activated Killer Activity in Peripheral Blood of Patients with Hepatocellular Carcinoma

Mutsunori Shirai, Seishirou Watanabe and Mikio Nishioka

Lymphokine-activated killer (LAK) activity of peripheral blood mononuclear cells (PBMC) from 33 patients with hepatocellular carcinoma was significantly decreased compared with that of healthy volunteers. There was less LAK activity in PBMC from patients with larger tumours (5 cm or more in diameter) than in patients with smaller tumours (under 5 cm in diameter). In 8 out of 20 patients with larger tumours there was none or little LAK activity. Flow cytometry revealed that the percentage of Leu11b+ cells in PBMC was lower in patients than in normal volunteers, and was lowest in patients with larger tumours. 10 patients with hepatocellular carcinoma were treated with intratumoral injection of OK432. LAK activity was enhanced after treatment in 7 cases, and the percentage of Leu11b+ cells was increased. Enhancement of LAK activity in response to OK432 was more significant in patients with smaller rather than larger tumours. Of the 7 high LAK responders, 4 showed 50–100% tumour regression at 6–9 weeks after injection.
Eur J Cancer, Vol. 26, No. 9, pp. 965–969, 1990.

INTRODUCTION

THE INCUBATION of lymphocytes with interleukin-2 (IL-2) generates cytotoxic cells that can lyse natural killer (NK) resistant tumour cells and a wide variety of other tumour cells without major histocompatibility complex restriction. This cytotoxicity is known as lymphokine-activated killing (LAK) [1–3], and occurs in the absence of any apparent antigenic stimulation. LAK is mediated mainly by IL-2 activated NK cells.

OK432 is a heat and penicillin treated lyophilized powder of the Su strain of *Streptococcus pyogenes* A3 and is a strong immunopotentiator and a useful immunotherapeutic agent for cancer. OK432 induces cytotoxic T lymphocytes against tumour cells [4], activates NK cells [5, 6] and reduces suppressor macrophages against NK cells in cancer patients [7]. Uchida and Micksche [8] demonstrated that autologous tumour killing activity can be induced in peripheral blood mononuclear cells (PBMC) by incubation with OK432. Grimm *et al.* [9] reported that LAK-like cells can be induced by treating PBMC with lower concentrations of OK432 and demonstrated that higher concentrations of OK432 significantly inhibited generation of LAK effectors to the NK resistant Daudi cell line, probably because of penicillin G potassium contained in OK432.

Our aim was to investigate LAK activity in patients with hepatocellular carcinoma and the *in vivo* effect of OK432 on LAK generation and tumour size.

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SUBJECTS AND METHODS

Subjects

For studies of LAK activity in PMBC there were 33 patients with hepatocellular carcinoma (HCC) (27 M/6F, mean age 59.6 years). HCC was confirmed histopathologically and no patient had metastases. 32 of the 33 had liver cirrhosis. 2 were HBsAG and HBeAG positive. The others were not alcoholics and did not have hepatitis B but did have non-A, non-B hepatitis (NANBH). The liver cirrhosis was functionally well compensated. No patient in our study had received previous anticancer therapy or drugs known to cause immunological changes, or any such treatment during this study except for patients 1, 6 and 9 who had had transhepatic arterial embolization 2 months before OK432 injection. Tumour volume and diameter of the ideal tumour sphere were calculated by modelling the tumour portion of computed tomography (CT) scans ("IBAS", Zeiss). 18 patients with liver cirrhosis were also investigated (15M/3F, 58.2 years). These patients were not alcoholics but did have

NANBH. We also studied 20 healthy volunteers (16M/4F, 54.9 years).

For flow cytometry of PBMC 25 of the HCC patients with liver cirrhosis were studied (21M/4F, 59.4 years; not alcoholic but did have NANBH) and 18 of the patients with liver cirrhosis. 25 volunteers were also studied (18M/7F, 53.5 years).

For OK432 studies, 10 of the HCC patients with liver cirrhosis were investigated (9M/1F, 56.0 years; not alcoholic but with NANBH).

Informed consent was obtained from each patient.

OK432 treatment

The 10 patients with HCC received intratumoral injections of OK432 (Chugai Pharmaceutical, Tokyo) 25–65 KE per patient by percutaneous transputic cholangiography (22 A needles) into all detectable hepatoma nodes under ultrasound. (1 KE corresponds to 100 mg dried streptococci [9].)

LAK activity

PBMC were prepared from all subjects by centrifugation on "Ficoll/Hypaque". In the patients treated with OK432, LAK activity was examined in PBMC 1–6 days before and 3–6 days after treatment. After two washes in RPMI 1640, the separated PBMC were resuspended at 5×10^6/ml in complete medium (RPMI 1640 supplemented with 5% human AB serum, antibiotics and glutamine). Recombinant IL-2 (rIL-2) 1500 U/ml was added to the cell suspensions, which were incubated for 72 h at 37°C in 5% CO_2 in air. rIL-2 was from Takeda Chemical (Osaka) [11–13]. The PBMC were washed three times in RPMI 1640 and resuspended in complete medium. The cells were used as effector cells in cytotoxicity assays.

Cytotoxicity assay

The cytotoxic activity of PBMC was evaluated against Daudi cells (maintained in complete medium) in ^{51}Cr release assays over 4 h. 10^6 target cells were resuspended in 0.5 ml RPMI 1640 with 20% human AB serum and labelled by the addition of 3.7 MBq ^{51}Cr sodium chromate (New England Nuclear) for 1 h at 37°C in 5% CO_2 in air. The target cells were washed three times in RPMI 1640 and resuspended in complete medium at 10^5/ml.

In the assay, 10^4 labelled target cells per well were mixed with various numbers of PBMC in round-bottomed 96-well microtitre plates (Costar). After centrifugation at 80 *g* for 5 min, the plates were incubated for 4 h at 37°C in 5% CO_2 in air. The supernatants were harvested and counted in a gamma counter.

Spontaneous and maximum ^{51}Cr release was measured by incubating target cells in control medium or 1 mol/l HCl, respectively. Each assay was in triplicate and cytotoxicity (% lysis) was calculated from: (experimenteal counts per min [cpm] − spontaneous cpm) × 100 ÷ (maximum cpm − spontaneous cpm).

Flow cytometry

PBMC were suspended at 2×10^7/ml in phosphate-buffered saline containing 0.1% azide and stained with fluorescein isothiocyanate-labelled monoclonal antibody to CD16 (leu11b, Becton Dickinson), 40 μl per 10^6 viable cells for 40 min at 40°C. After washing 10^4 cells were analysed in a "FACSIV" [13]. Viability of lymphocytes and target cells was assessed by trypan blue exclusion and was always over 95%.

RESULTS

LAK generation by rIL-2 was observed in PBMC from all the normal volunteers, in all the patients with liver cirrhosis and in most of the HCC patients. However, activity in PBMC from HCC patients was significantly lower than that in liver cirrhosis patients or normal volunteers (Fig. 1). The cytotoxic activity without rIL-2 was low in all samples (less than 8%).

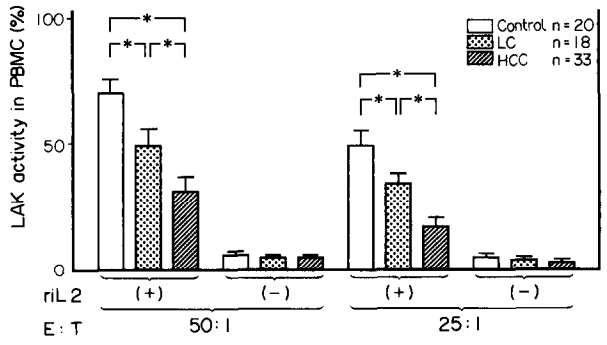

Fig. 1. LAK activity (%) of PBMC from normal volunteers, patients with liver cirrhosis (LC) and patients with hepatocellular carcinoma (HCC), against Daudi cells at 50:1 or 25:1 of effector: target (E:T) ratio. Mean (SE). *$P < 0.01$, *t* test.

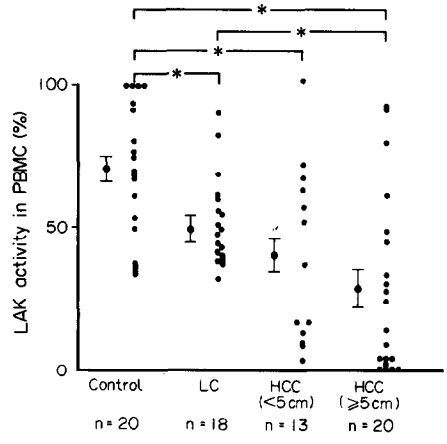

Fig. 2. LAK activity (%) of PBMC in HCC patients with smaller tumours (< 5 cm in diameter) and larger tumours (≥ 5 cm in diameter) compared with that of normal volunteers and that of patients with liver cirrhosis. Effector:target ratio was 50:1; results are same at 25:1. *$P < 0.01$.

HCC patients were divided into two groups on the basis of tumour size (under 5 cm or 5 cm or more in diameter). LAK activity was significantly decreased in PBMC from patients with small and large tumours compared with normal controls (Fig. 2). PBMC showed less LAK activity in the patients with larger tumours than those with smaller tumours (not significant). Of 20 patients with larger tumours, PBMC from 8 generated none or very little LAK activity, including those from 4 patients with tumours more than 10 cm in diameter and who died of tumour growth within 3 months of LAK study.

Flow cytometry revealed that the percentage of Leu11b+ cells was lower in PBMC from HCC patients than in PBMC from normal volunteers (Fig. 3). The percentage in PBMC from HCC patients with larger tumours was significantly decreased compared with that of patients with liver cirrhosis. The percentage of Leu11b+ cell fractions was diminished (under 6%) in PBMC of the patients bearing larger tumours with none or little LAK activity.

We investigated the effects of OK432 injected into the hepatic tumour on LAK activity. After treatment, LAK cytotoxicity was enhanced in cases 5–10 and decreased in cases 1 and 4 (Fig. 4). Patient 1 had low lymphocyte count, reversible shock

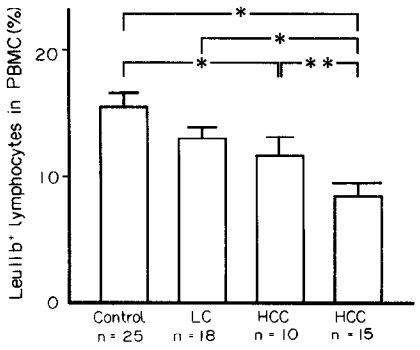

Fig. 3. Percentage of Leu11b+ lymphocytes in PBMC from normal volunteers, patients with liver cirrhosis and HCC patients with smaller (a) and larger (b) tumours. *$P < 0.05$ and †$P < 0.01$.

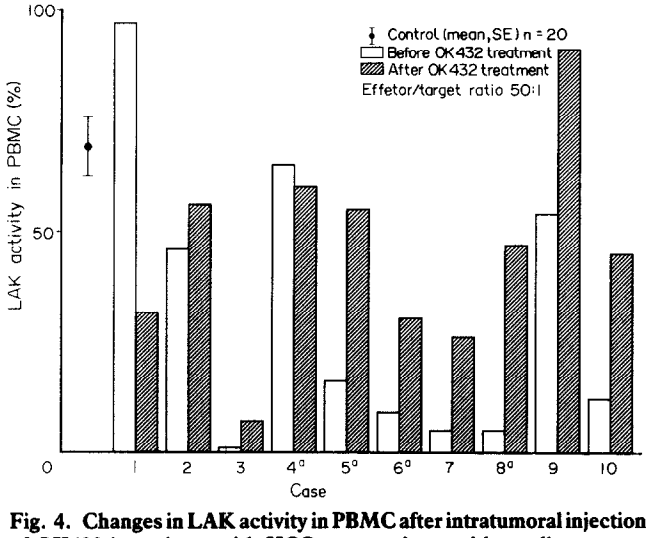

Fig. 4. Changes in LAK activity in PBMC after intratumoral injection of OK432 in patients with HCC. a = patients with smaller tumour (< 5 cm in diameter).

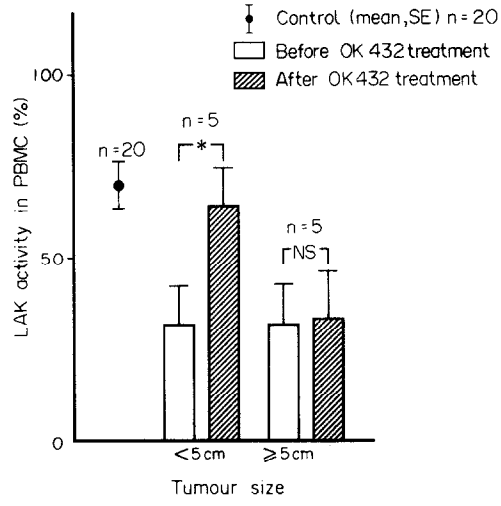

Fig. 5. Changes in LAK activity in PBMC in HCC patients with smaller tumours and larger tumours. Effector:target ratio 50 : 1. *=$P < 0.01$, NS = not significant.

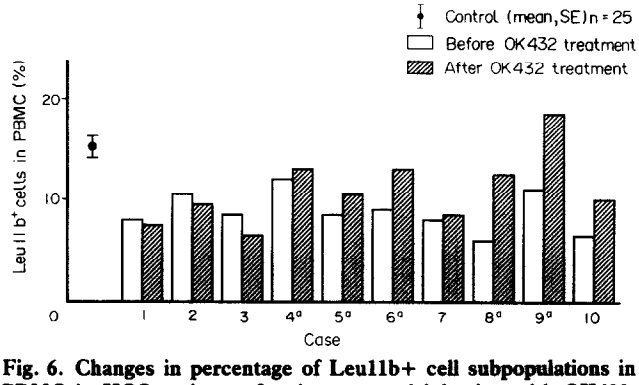

Fig. 6. Changes in percentage of Leu11b+ cell subpopulations in PBMC in HCC patients after intratumoral injection with OK432. a = patients with smaller tumours.

and deterioration of liver function 3 h after treatment with 65 KE OK432. Cases 4–6, 8 and 9 had smaller tumours. Enhancement of LAK activity in response to OK432 was more significant in PBMC from patients with smaller tumours than in patients with larger tumours (Fig. 5). The percentage of Leu11b+ cells was increased in 5 of 7 patients in whom PBMC LAK activity increased in response to OK432 (Fig. 6).

Tumour volume reduction based on CT 6–9 weeks after treatment, indicated no response (< 25%) in cases 1, 2 and 4, minor response (25–50%) in cases 3, 5 and 7, partial response (51–99%) in cases 6, 8 and 10, and complete response (100%) in case 9 (Table 1). CT scans of case 9 before and after treatment with 30KE OK432 are shown in Fig. 7. No tumorous shadow was observed on CT 8 weeks after treatment.

DISCUSSION

Many attempts have been made to discover the mechanisms of immunodeficiency in carcinogenesis. However, the immune mechanism in the progression from liver cirrhosis to HCC has not been well elucidated. There is evidence of association between hepatitis, liver cirrhosis and HCC [14–16]. Diminished NK activity in patients with HCC has been reported [17, 18]. Previous studies on NK cell function in HCC patients [19] have indicated that the NK cell's ability to bind and lyse tumour target cells is depressed. The possibility of cell-mediated suppression (i.e. by monocytes) of NK activity in patients with depressed NK activity has been excluded by Hirofuji *et al.* [18]. These investigators also showed low NK activity of Leu11a+ and Leu7+ cells and a decreased Leu11a+/Leu7+ ratio in patients with low NK activity compared with controls, although percentages of Leu11a+ and Leu7+ cells were similar in controls and HCC patients.

We found deficient LAK activity in PBMC from patients with both small and large tumours. Depletion of LAK precursor cells is thought to be one of the causes for deficient activity. We also found a decreased Leu11a+/Leu7+ ratio in patients with low LAK activity (data not shown). Other experiments have suggested that this deficiency in LAK cytotoxicity is also in part associated with a functional defect closely related to differentiation of LAK precursors to LAK effectors, but without inhibition of IL-2 receptor (p55 chain) expression.

Most of our HCC patients had liver cirrhosis which might be a cause of deficient cellular immunity. However, HCC seems to be more responsible for the deficient immunity than liver cirrhosis, because PBMC from HCC patients had significantly less activity than that from patients with liver cirrhosis, and the activity was decreased as tumour size increased. None of our patients was malnourished.

When other target cells (i.e. Alexander and K562 cells) were used, the depression of cytotoxicity was consistent with our findings (data not shown).

Table 1. *Antitumoral efficacy of intratumoral injection of OK432 in patients with HCC*

Case	OK432 (KE)	Tumour regression (%)	Alpha-fetoprotein (ng/ml)*	
			Before	After
1	65	15	112	163
2	45	19	11	13
3	35	33	35	33
4†	20	0	540	386
5†	30	48	151	56
6†	30	60	36	32
7	20	30	25	29
8†	40	50	7,370	5,770
9†	30	100	23	1
10	35	57	130	45

*Before = 1–2 weeks before OK432 injection; after = 4–8 weeks after OK432 injection.
†Tumour size under 5 cm in diameter.

NK activity is mediated almost exclusively by Leu11-bearing cells [20] and incubation of Leu11+ cells with rIL-2 for over 24 h induces LAK activity [21–24]. However, the mechanism by which IL-2 induces LAK cytotoxicity remains unknown. The cytolytic activity could be a manifestation of several independent mechanisms. Therefore it is difficult to characterise the depression. In our study, PBMC before IL-2 stimulation generated low levels of lysis in patients and controls. HCC patients demonstrated a reduction in number of LAK precursor cells in peripheral blood, but there was often an increase in response to OK432.

In another experiment, we found diminished lymphocyte proliferation in response to IL-2 in HCC patients (data not shown). Interference with the development of LAK is probably in part due to inhibition of the proliferative response of the LAK precursors to IL-2, since lymphocyte proliferation is a prerequisite for the induction of LAK activity [1].

The purpose of immunotherapy with biological response modifiers such as OK432 is to prolong survival. Our approach should prevent recurrences and achieve complete remission when combined with other therapies such as transhepatic arterial embolization or intratumoural injection of ethanol. In 4 of our patients, we demonstrated the efficacy of intratumoural injection therapy with OK432. The antitumour efficacy of local treatment was similar to that obtained previously in patients with gastric cancer treated by endoscopic injection of OK432 [25]. In our data, intratumoral injection of OK432 was more effective for patients with small-size HCC, with parallel augmentation of LAK activity. These results provide evidence of synergism between IL-2 and OK432 on the *in vivo* development of LAK or LAK-like activity.

The major side-effect of OK432 is elevation of body temperature, often to more than 38°C. 1 of our patients, who was in reversible shock, showed an extremely high reaction to the skin test for the streptococcal preparation (injected intradermally before the intratumoral injection of OK432), and it is probable that hypersensitivity to OK432 provoked shock in this case.

Adoptive immunotherapy by LAK in advanced cancer patients has received much attention [26, 27]. This therapy requires the simultaneous and continuous administration of IL-2 to maintain killing activity. OK432 causes IL-2 secretion [9, 28, 29] and is active by itself. Therefore, a possible treatment could be a combination of IL-2, LAK cells and OK432.

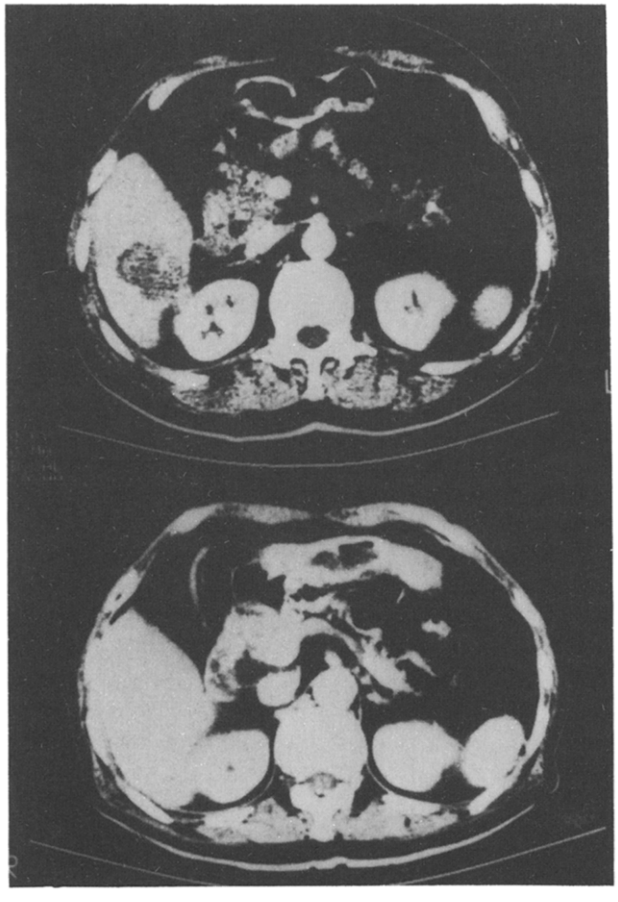

Fig. 7. CT scan of the liver in case 9. Top = demarcated tumour in upper anterior segment of right lobe. Bottom = 8 weeks after intratumoral injection with 30 KE OK432.

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Eur J Cancer, Vol. 26, No. 9, pp. 969–972, 1990.
Printed in Great Britain
0277–5379/90 $3.00 + 0.00

Alpha-fetoprotein–Lectin Binding as a Marker of Tumour Activity or Liver Damage

Jan Marrink, Dirk Th. Sleijfer, Elisabeth G.E. de Vries and Heimen Schraffordt Koops

To establish whether alpha-fetoprotein (AFP) produced in the early post-treatment phase of a patient with a germ cell tumour of the testis or the ovary originates from the tumour or is due to an underlying disturbance in liver function, the binding of AFP to concanavalin A (Con A) was investigated as a discriminative variable. A two-step assay is described that can distinguish the type of AFP produced at levels as low as 10 ng/ml. A Con A-binding ratio of 12–43% was found in the patients with disseminated germ cell tumours and in patients with AFP-positive gastrointestinal carcinomas. AFP from the liver gives ratios below 10%.
Eur J Cancer, Vol. 26, No. 9, pp. 969–972, 1990.

INTRODUCTION

RAISED SERUM levels of alpha-fetoprotein (AFP) are generally associated with tumours of tissues of endodermal origin. Therefore this marker is widely used in the diagnosis and monitoring of patients with hepato-cellular carcinoma and germ cell tumours. To a lesser extent, AFP is found in the serum of patients with gastrointestinal carcinomas. The interpretation of any increase in AFP levels in follow-up is hampered by the fact that the serum concentration also increases in non-malignant diseases in which the liver is involved. Moreover, reversible increases in AFP might be due to the hepatotoxic effect of chemotherapy [1–3]. Thus a method to distinguish AFP produced by maligancies from that due to benign liver activity would be useful, especially in the early phase of a relapse when marker levels are low.

Variations in the carbohydrate moiety of AFP result in different reactivities with lectins [4]. Different patterns of

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