

## Original Article

# A Proposal of Effective Immunochemotherapy Using Recombinant Interleukin 2 and Adriamycin, and Its Theoretical Background

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**Abstract:** We performed immunochemotherapy using a continuous infusion of recombinant interleukin 2 (IL2) and intermittent one-shot-injections of Adriamycin (ADR) administered directly to the hepatic artery in 6 cases of advanced hepatocellular carcinoma (HCC). Although the observation periods were short, the tumors reduced in size; most notably, a massive tumor in the right lobe disappeared in one case. After reviewing the 6 patients with HCC, we demonstrated a possible cause of ADR as a kind of immuno-modifier. In an in vitro study, the susceptibility of subpopulations of lymphocytes to ADR was variable with the suppressor T cell being more susceptible to ADR compared to the helper T cell. In HCC patients, the ratio of the suppressor T cells to the helper T cells (suppressor/helper ratio) was significantly higher than that of the healthy controls. Thus, following the administration of the optimal dose of ADR, specific decrease in the suppressor T cell and increase in the helper T cell in response to ADR can be obtained for the recovery of the suppressor/helper ratio. Moreover, ADR-treated lymphocytes of HCC patients exhibited constant killer activity in the presence of IL2. Although the optimal dose of ADR for clinical use may be within a very narrow range, ADR combined with IL2 following tumor mass-reduction treatments may restore the immunity of advanced HCC patients and prolong their survival.

**Key words:** Immunochemotherapy, Recombinant interleukin 2, Adriamycin, Liver cancer, Surface marker

## INTRODUCTION

Recent increases in the incidence of discovery of liver cancer have led to the introduction of various treatments to improve its prognosis. It is certain that, on the one hand, the principle of cancer therapy is the early detection of small tumors and the early curative resection. On the other hand, there are many cases in which it is not possible to perform curative resection because of multiple and diffuse liver metastases, portal invasion and/or distant metastases. The effects of trans-arterial embolization

(TAE) have been noted in the treatment of hypervascular hepatocellular carcinoma (HCC), however, its disadvantages and the limits of its clinical application in advanced cases have recently been reported<sup>1,2)</sup>.

In 1987, we tried adoptive immunotherapy using recombinant interleukin 2 (IL2) both with and without lymphokine activated killer (LAK) cells in the treatment of advanced cases of liver cancer<sup>3-6)</sup>. However no reduction in the size of the tumors was observed, and this treatment was not expected to extend the survival periods. In addition, there were severe side effects such as high fever, ascites, jaundice, thrombocytopenia, etc. After several clinical trials using IL2 as a basic immunother-

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apeutic drug, we have, since 1988, adopted an immunochemotherapy regimen of continuous infusion of a large amount of IL2 and intermittent one-shot-injections of a small amount of Adriamycin (ADR) directly into the hepatic artery. Even over a short period, our clinical experience is more satisfactory than with previous treatments.

The aim of this paper is to elucidate the mechanism of this immunochemotherapy through analysis of the functions of lymphocytes, especially the role of ADR as a kind of immuno-modifier. Although there are a few reports on anti-cancer drugs acting as immunomodifiers<sup>7,8)</sup>, we have found no clinical or experimental reports on such a function of ADR in HCC patients.

## MATERIALS AND METHODS

### I. Clinical study

Table 1 shows the clinical review of the 6 patients with HCC receiving the present immunochemotherapy. All cases were of advanced HCC treated at our department since 1988. In the macroscopic stage, according to "The General Rules for the Clinical and Pathological Study of Primary Liver Cancer"<sup>9)</sup>, 2 cases were Stage III, 3 cases were Stage IV-A, and 1 case was Stage IV-B. Three cases had severe portal invasion. Cirrhosis of the liver was present in all cases. Their respective clinical stages were II in Case 1, III in Case 2 and I in the remaining cases<sup>9)</sup>. All cases, except Case 2, underwent laparotomy, which involved cannulation of the hepatic artery and cholecystectomy. Cannulation was performed in order

Table 1. Clinical review of HCC patients<sup>1)</sup> receiving immunochemotherapy.

Case	ID No. <sup>2)</sup>	Sex	Age	Stage <sup>3)</sup>	Clinical Stage <sup>4)</sup>	Pre-treatments of mass-reduction	IL2 <sup>5)</sup>	ADR <sup>6)</sup>	Duration <sup>7)</sup>	Effect <sup>8)</sup>
1	0401195	M	53	IV-A	II	TAE	1000	10	425	CR <sup>9)</sup>
2	0225429	M	54	III	III	TAE	500	10	276	NC
3	0857451	M	53	IV-B	I	TAE+right lobectomy	500	10	254	NC <sup>10)</sup>
4	0488109	M	44	III	I	TAE+left lobectomy	500	10	199	NC
5	0512181	M	58	IV-A	I	TAE+ lateral segmentectomy posterior segmentectomy	250	10	192	NC
6	0902107	M	52	IV-A	I	TAE+ lateral segmentectomy	500	10	39	NC

1) Liver cirrhosis coexisted with HCC in all cases.

2) Identification number in Yamanashi Medical College Hospital.

3) Macroscopic stages according to the TNM classification<sup>[9]</sup>.

4) Three clinical stages classified according to the clinical and laboratory findings <sup>[9]</sup>.

5) Doses of continuous infusion of IL2 ( $\times 1,000$ U/day).

6) Doses of intermittent injection of ADR ( $\mu$ g).

7) Number of days of the immunochemotherapy up to October 28th, 1989.

8) Judgement of effect <sup>[10]</sup>. There are four categories; Complete Response (CR), no evidence of disease with complete disappearance of all lesions lasting more than 4 weeks; Partial Response (PR), more than 50% decrease of the addition of the diameters of all measurable lesions, with no evidence of new ones; No Change (NC), an objective response inferior to 50% or an increase inferior to 25% in one or more of the existing lesions, and Progressive Disease (PD), increment superior to 25% in one or more of the measurable lesions or the appearance of new ones.

9) Tumors in the liver disappeared on the CT image (See Fig. 1).

10) A metastatic tumor in the medial segment of the remnant liver decreased less than 50% in size, remained in the category of NC, but a coin lesion with a diameter of 2cm in the right lung disappeared on the chest X ray 2 months after the start of this therapy (CR of the lung metastasis).

to administer continuous infusion of IL2, and cholecystectomy was performed so as to prevent necrotic cholecystitis, which often occurs following arterial injection of anti-cancer drugs with lipiodol. The other end of the cannula was connected subcutaneously to the infusion pump (INFUSAID®). In Case 2, the cannulation of the hepatic artery was performed using the modified Seldinger's selective angiography (SAG) technique. To achieve pre-treatment mass-reduction, TAE was performed in all 6 cases and hepatectomy was additionally performed in Case 3 to 6.

The infusion pump set has 2 ports, 1 was for one-shot-injections and the other was connected to the reservoir from which  $0.25-1.0 \times 10^6$  units of IL2 diluted in about 5 ml solution was infused daily to the hepatic artery. In order to refill the reservoir with the IL2 solution and administer the one-shot-injections, patients visited the hospital as out-patients once a week. Ten mg of ADR solution (dissolved in 2 ml of the physiological saline solution) mixed with 0.5 to 1.0 ml of lipiodol was injected once a week. Lipiodol functions to lengthen the retention period of the drugs in the tumor. When liver dysfunction became severe, the weekly interval was extended.

This clinical study was performed according to the guidelines of the Declaration of Helsinki.

## II. Experimental study

**Patients:** Studies were performed on 7 patients. Case 4, 5 and 6 of the clinical study were analyzed in this study as well, but the other 4 patients were not the same. In the macroscopic stage, 3 cases were Stage III and 4 cases were Stage IV-A. Cirrhosis of the liver was present in all cases. Their clinical conditions seemed to be similar to those of the cases in the clinical study. No patients had undergone surgical treatment or chemotherapy before this study.

**Control:** Peripheral blood was taken from 7 volunteers with normal liver function and who were not currently taking any medication.

Peripheral blood mononuclear cells

(PBMC): 20 ml of peripheral blood was taken from the cubital vein. PBMC were isolated from heparinized peripheral blood by the Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient sedimentation technique. The interphase PBMC were suspended in RPMI 1640 (Gibco, New York, USA) culture medium. Blood sampling was performed with the informed consent of both patients and volunteers.

**Medium:** RPMI 1640 powder was dissolved in ultra pure water made by a Milli Q system (Millipore Japan, Tokyo) and sterilized by filtering it through a pore size of  $0.22 \mu\text{m}$ . The medium was completed by the addition of 100U/ml penicillin G (Meiji Pharmaceutical Co., Tokyo), 100  $\mu\text{g}/\text{ml}$  streptomycin (Meiji Pharmaceutical Co., Tokyo) and 2 mM glutamine (Nakarai Chemical, Tokyo) to the RPMI 1640 medium. Cross-matched fresh frozen plasma (FFP) was added after inactivation by heating at  $56^\circ\text{C}$  for 30 minutes.

**Recombinant interleukin 2 (IL2):** S-6820 was supplied by Shionogi Pharmaceutical Co Ltd., Tokyo.

**Adriamycin (ADR):** ADR (Kyowa Hakko Co Ltd., Tokyo) was diluted to a final concentration of 1 mg/ml in ultra pure water made by a Milli Q system and sterilized through a filter with a pore size of  $0.22 \mu\text{m}$ .

**Monoclonal antibodies:** PBMC were treated with the following: anti-IL2 receptor (CD25) labelled with fluorescein isothiocyanate (FITC), anti-Leu2 (CD8) labelled with FITC, anti-Leu15 (CD11) labelled with phycoerythrin (PE), anti-Leu3 (CD4) labelled with PE, anti-Leu8 labelled with FITC, anti-Leu4 (CD3) labelled with FITC and anti-HLA-DR labelled with PE (Becton Dickinson, Mountain View, CA, USA).

**Culture of PBMC:** PBMC were diluted to a final concentration of  $8 \times 10^5/\text{ml}$  in complete media. PBMC were then cultured with 3,000 U/ml of IL2 and various concentrations of ADR for 30 minutes at  $37^\circ\text{C}$  under conditions of 5%  $\text{CO}_2$ , in a  $\text{CO}_2$  incubator (Tabai Espec,

Osaka). The concentrations of ADR were 0  $\mu\text{g/ml}$ , 1  $\mu\text{g/ml}$  and 2  $\mu\text{g/ml}$ . The dose of 1  $\mu\text{g/ml}$  was chosen by approximate calculation of 10 mg of ADR per 60 kg of body weight in Case 1, who clinically showed a Complete Response (CR), with further reference to the hepatic blood flow, the duration of retention of ADR in the liver and the concentration of lymphocytes.

Immunostaining procedures: PBMC were double-stained with monoclonal antibodies, except for CD25. For CD25, the samples of PBMC were single-stained. Briefly,  $1 \times 10^6$  cells were treated with two kinds of antibodies in a concentration of  $1 \times 10^6/7.5 \mu\text{l}$  (cells/antibodies). After 30 minutes of incubation on ice, the cells were washed twice. The combinations of monoclonal antibodies were CD8 & CD11, CD4 & anti-Leu8 and CD3 & anti-HLA-DR. Phosphate-buffered saline (PBS) was used throughout the staining procedures. The viability rate of cells was higher than 95% using the dye exclusion test with 0.1% nigrosin B.

Two-color immunofluorescence analysis: Two-color immunofluorescence experiments were analyzed using a fluorescence activated cell sorter (FACScan, Becton Dickinson, Mountain View, CA, USA). The lymphocyte fraction from PBMC was separated by the scatter gating method. A total of 10,000 cells within the gate were analyzed in each experiment. Double-stained cells were presented as a contour plot seen in two-dimensions of green and red fluorescence. Lymphocytes were gated by auto-fluorescence. The percentage of each fraction was determined by dot plots using a contour-graph.

Induction of killer activity: The percent killer activities of induced lymphocytes in 5 of the seven advanced HCC cases in the experimental study were assayed in 3 groups. In the macroscopic stage, 2 cases were Stage III and 3 cases were Stage IV-A. In Group A, lymphocytes induced only with IL2. In Group B, lymphocytes were treated with IL2 after exposure to ADR. And in Group C, lympho-

cytes induced by exposure to ADR but without IL2 treatment. After confirming the viability rate was over 95% by using the dye exclusion test,  $8 \times 10^5/\text{ml}$  of the samples of PBMC were cultured in complete media at 37°C under 5%  $\text{CO}_2$ , in the  $\text{CO}_2$  incubator for 5 days. Before the culture, in Group B and C, lymphocytes were suspended in a solution of 1  $\mu\text{g/ml}$  ADR in the  $\text{CO}_2$  incubator for 30 minutes and washed with complete media solution 3 times. In Group A and B, lymphocytes were cultured together with 3,000 U/ml IL2.

Target cells: Daudi, a NK resistant human Burkitt's lymphoma cell line was used for the cytotoxicity assay.

Cytotoxicity assay:  $^{51}\text{Cr}$  (ICN, Irvine, CA, USA) was labelled to target cells by incubating 3.7–7.4 MBq of  $^{51}\text{Cr}$  with  $1\text{--}10 \times 10^6$  target cells in 0.2 ml of complete media for 1 hour in the  $\text{CO}_2$  incubator. After washing the cells with complete media solution 3 times, cell viability was checked by using the dye exclusion test, and usually it was over 95%. Into each well of a 96-well U type microtiter plate (Nunc, Roskilde, Denmark),  $1 \times 10^4$  radio-labelled target cells and effector cells were added in the ratios of 40: 1, 20: 1, 10: 1 or 5: 1 to the target cells. Total volume was 220  $\mu\text{l/well}$ . The plate was incubated at 37°C in 5%  $\text{CO}_2$  for 4 hours, and then centrifuged at  $\times 200 \text{ g}$ , at 4°C for 5 minutes. The radio-activity in 100  $\mu\text{l}$  of each supernatant was counted by a  $\gamma$ -counter (Aloka, Tokyo). Spontaneous release was counted in the well, without the addition of effector cells. Maximum release was counted in the well where the same number of target cells were incubated with 0.1% NP-40 for 4 hours. The percentage of killer activity was calculated in triplicate experiments according to the following formula.

% killer activity =  $(\text{Experimental release} - \text{Spontaneous release}) \div (\text{Maximum release} - \text{Spontaneous release}) \times 100$ .

Statistical analysis: Statistical comparisons were made using the Student's t-test for the killer activity, and the paired t-test for the rest

of study.

RESULTS

I. Clinical effect of the immunochemotherapy

Table 1 shows the clinical review of 6 patients with HCC receiving the above-mentioned immunochemotherapy. In Case 1, a massive tumor in the liver had disappeared on computed-tomographic (CT) images (Fig. 1) and remained in remission for 11 months. In Case 3, a metastatic tumor in the medial segment of the remnant liver decreased less than 50% in size and remained in the category of No Change (NC)<sup>10</sup>, but a coin lesion with a

diameter of 2 cm in the right lung had disappeared on the chest X ray taken 2 months after the start of the therapy (CR of the lung metastasis). In Case 2 and Case 5, tumors also decreased less than 50% in size. No patients advanced to the category of Progressive Disease (PD)<sup>10</sup>. Side effects of the immunochemotherapy such as fever, ascites and liver dysfunction were observed only in Case 2, but these symptoms were controllable. No electrocardiographic change was observed despite the use of ADR.

Fig. 1 shows the most effective outcome (Case 1) of the immunochemotherapy. The patient was a 53 year-old male, diagnosed as

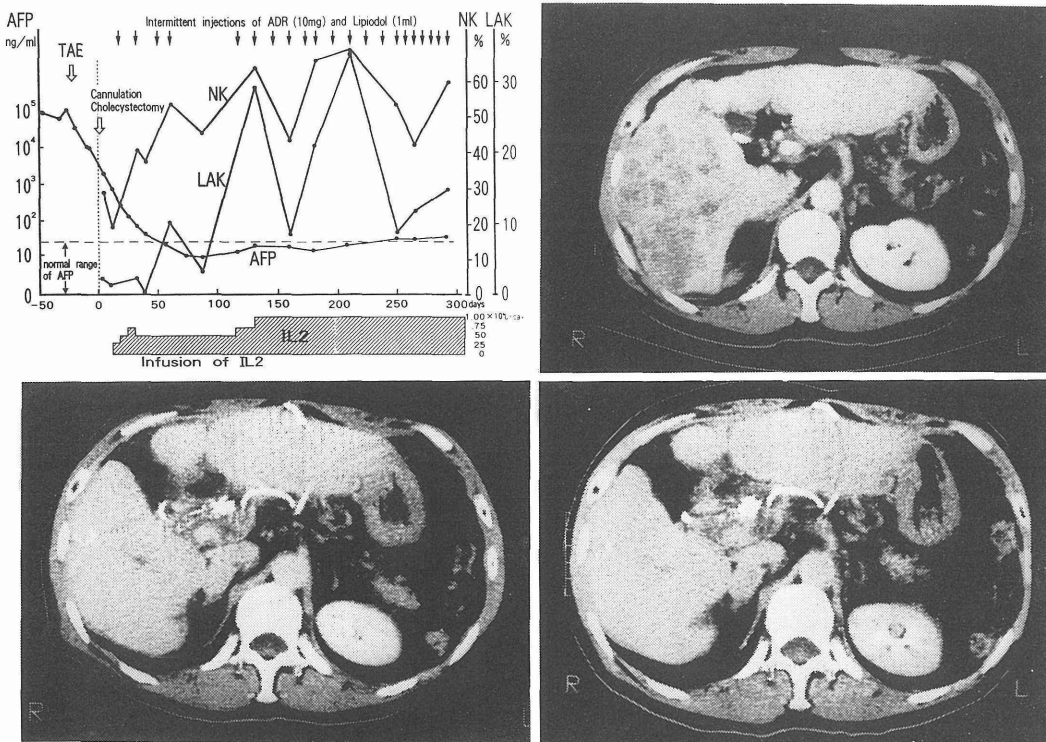
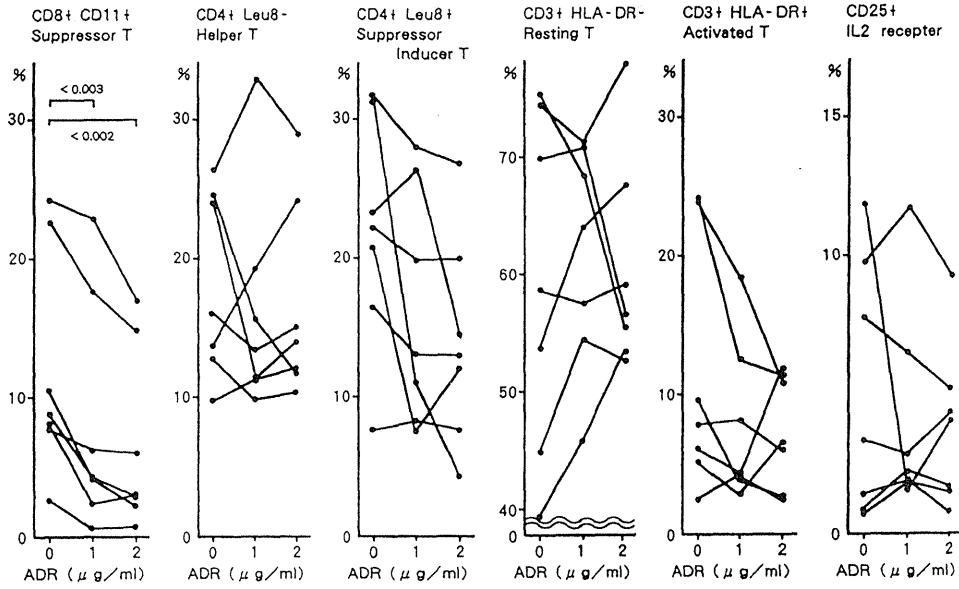


Fig. 1. Time courses of NK and LAK activities and AFP (upper-left). The activities were measured by using <sup>51</sup>Cr releasing assay. The ratio of the effector cells to target cells was 40 : 1. The target cells were Daudi for LAK and K562 for NK. The activities of NK and LAK indicate an upward tendency despite fluctuations. Abdominal CT before the operation (upper-right). Tumors occupy the right lobe of liver (low density area). Another tumor in the medial segment is not seen in this image. Abdominal CT, 5 months after the operation (lower-left). The tumors in the right lobe decreased in size with a compensative enlargement of the lateral segment. Abdominal CT, 11 months after the operation (lower-right). The tumors had disappeared on the CT image of the liver, judged as Complete Response (CR) [10].

Control group

(N = 7)



HCC group

(N = 7)

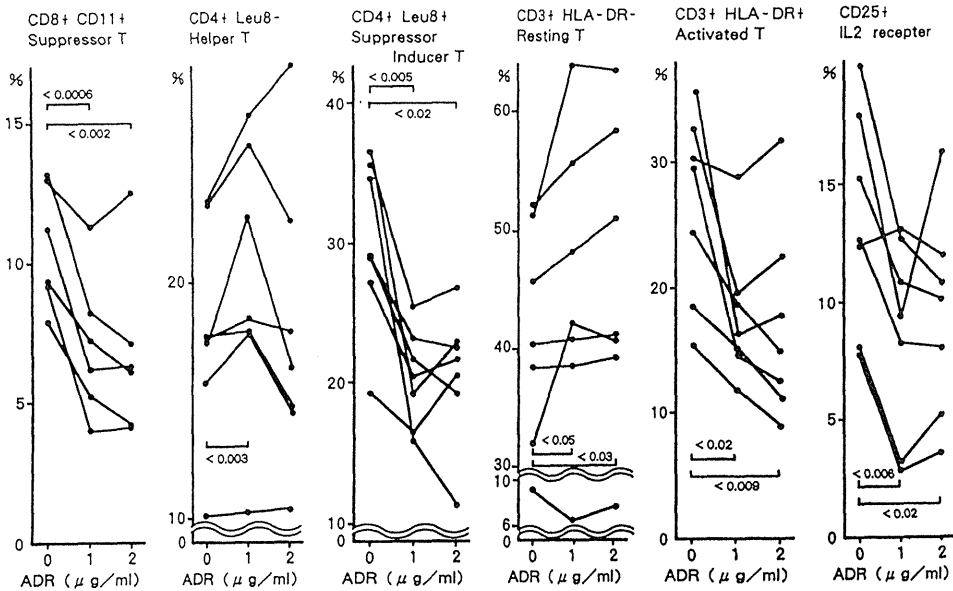


Fig. 2. Changes in subpopulations of lymphocytes after exposure to ADR (0, 1 and 2  $\mu\text{g/ml}$ ). Statistical significances were measured by the paired t-test. The subpopulation of the suppressor T cells decreased with the addition of ADR in both groups.

having HCC and liver cirrhosis by CT, SAG, liver function tests and serum  $\alpha$ -fetoprotein (AFP) assay. Laparotomy was performed 4 weeks after TAE. A massive tumor occupied the right lobe of the liver (Fig. 1, upperright) and there was a metastatic tumor in the medial segment. As the tumors were diagnosed as inoperable, cholecystectomy and cannulation of the hepatic artery were performed. Immunochemotherapy with continuous infusion of IL2 and intermittent injections of ADR began 10 days after the operation. The activities of NK and LAK showed an upward tendency, despite fluctuations (Fig. 1, upper-left). On the abdominal CT, 5 months after the operation, the tumor in the right lobe had decreased in size, with a compensative enlargement of the lateral segment (Fig. 1, lower-left). Finally, 11 months after the operation, the tumors had disappeared on CT scan, and this case was judged as Complete Response (CR)<sup>10</sup> (Fig. 1. lower-right).

II. Experiments in vitro

Susceptibility of the subpopulations of lymphocytes, in the peripheral blood, to ADR was investigated in 7 HCC patients. Their clinical backgrounds were similar to those of the 6 patients in the clinical study. After a 30

minute-exposure of PBMC to ADR, the percentage of each subpopulation were measured by the FACS technique.

The changes in the subpopulations of lymphocytes after exposure to ADR (0, 1 and 2  $\mu$ g/ml) are shown in Fig. 2. In the control group, only the suppressor T cells, of which the surface marker is indicated as positive CD8 and positive CD11 (CD8+CD11+), significantly decreased dose-dependently (Fig. 2, above). On the other hand, in the HCC group, not only the suppressor T cells, but also other subpopulations of mature lymphocytes, such as the suppressor-inducer T cells (CD4+Leu8+), the activated T cells (CD3+HLA-DR+) and the positive IL2 receptor cells (CD25+) decreased after exposure to ADR. However, the resting T cells (CD3+HLA-DR-) increased significantly after exposure. Moreover, with a dose of 1  $\mu$ g/ml ADR, the rate of the helper T cells (CD4+Leu8-) increased significantly (Fig. 2, below)<sup>11</sup>.

Comparisons of the subpopulations and the ratio of the suppressor T cells to the helper T cells (suppressor/helper ratio) following treatment with IL2 but not ADR are summarized in Table 2. In HCC patients, the rate of suppres-

Table 2. Differences in subpopulations of lymphocytes between the control group and the HCC group under a condition with IL2, without ADR.

SUBPOPULATION	SURFACE MARKER	CONTROL mean $\pm$ SD	HCC mean $\pm$ SD	p value	COMPARISON <sup>1)</sup>
Suppressor T cells	CD8+ CD11+	11.96 $\pm$ 8.15	10.25 $\pm$ 2.19	0.602	NS
Helper T cells	CD4+ Leu8-	20.21 $\pm$ 7.41	17.82 $\pm$ 4.52	0.480	NS
Suppressor-Inducer T cells	CD4+ Leu8+	21.94 $\pm$ 7.37	30.10 $\pm$ 6.09	0.049	↑
Resting T cells	CD3+ HLA-DR-	59.80 $\pm$ 14.97	42.07 $\pm$ 7.96	0.017	↓
Activated T cells	CD3+ HLA-DR+	11.36 $\pm$ 8.95	26.60 $\pm$ 7.48	0.005	↑
Positive IL2 receptor cells	CD25+	5.06 $\pm$ 4.58	13.46 $\pm$ 4.68	0.005	↑
Suppressor/Helper ratio		0.313 $\pm$ 0.101	0.516 $\pm$ 0.168	0.0049	↑

1) Statistical significance was examined by Student's t-test.

NS: Not significant

↑: increased in HCC group (p<0.05)

↓: decreased in HCC group (p<0.05)

In the HCC group, the subpopulations of mature lymphocytes increased, and the suppressor/helper ratio increased significantly.

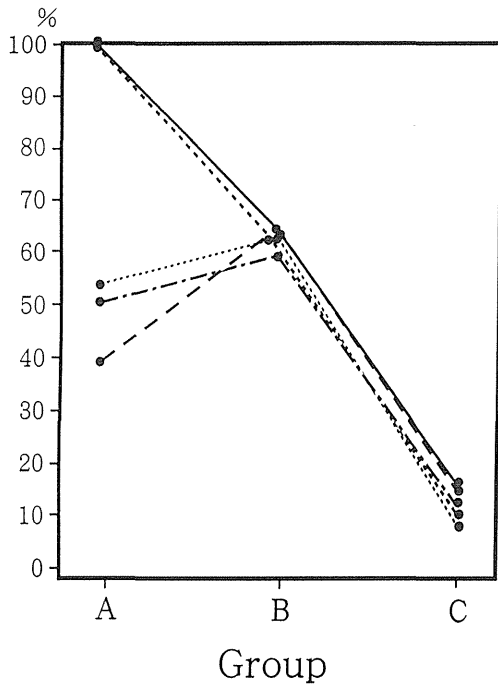


Fig. 3. Percent killer activity of induced lymphocytes in HCC patients. The ratio of the effector cells to target cells was 40 : 1.  
 Group A: lymphocytes incubated with IL2 for 5 days.  
 Group B: lymphocytes incubated with IL2 for 5 days after 30 minute-exposure to ADR.  
 Group C: lymphocytes incubated, without IL2, for 5 days after 30 minute-exposure to ADR.  
 In Group B, the killer activities were higher in 3 cases and lower in 2 cases, compared with those in Group A. The killer activities incubated only with ADR were low in all cases.

sor-inducer T cells, activated T cells and positive IL2 receptor cells were higher, while the rate of resting T cells was lower. Further the suppressor/helper ratio significantly increased in the HCC patients (Table 2).

In order to investigate the killer function of induced lymphocytes after 5 day-incubation with/without ADR and with/without IL2, the killer activities were measured by  $^{51}\text{Cr}$  releasing assay. Fig. 3 shows the percent killer activity of induced lymphocytes of the HCC

patients. The killer activities induced with IL2 (Group A and B) were higher than those without IL2 (Group C). But the killer activities after exposure to ADR (Group B) were higher in 3 cases and lower in 2 cases, compared with those of Group A.

#### DISCUSSION

It has been accepted by clinicians that one side-effect of both antibiotics and anti-cancer drugs is the depression of immunity<sup>1,2</sup>). Recently, some drugs have been reported to improve the immunity of cancer patients<sup>7,8,12,13</sup>). In the present study, after demonstrating the clinical results of immunochemotherapy using ADR and IL2, the possible effects of ADR on the immunity of liver cancer patients were examined in respect to surface markers and killer activities of lymphocytes in vitro.

This in vitro study revealed that HCC patients, with concomitant liver cirrhosis, have a tendency to have mature lymphocytes and are immunologically suppressed, indicating a higher ratio of suppressor T cells/helper T cells. This suppressed condition may be induced by stimulations of the tumor cells and/or immunosuppressive factors released from the tumor cells<sup>14-16</sup>). In recognition of this, reduction of the tumor mass by surgical procedures and/or TAE is required prior to immunotherapy treatment for it to be effective.

With exposure to 1  $\mu\text{g}/\text{ml}$  ADR, all subpopulations changed significantly in the HCC patients, while only that of suppressor T cells changed in the control group (Fig. 2). It seems apparent that, lymphocytes are more susceptible to ADR in HCC patients, but the susceptibility differs between the subpopulations of lymphocytes. The suppressor T cells were more depressed by ADR, while the resting T cells were more resistant to ADR. The helper T cells were also more resistant to ADR than the suppressor-inducer T cells, the activated T cells and the positive IL2 receptor cells. There-



fore, by taking advantage of the different sensitivity of the subpopulations to ADR, it becomes possible to improve suppressed immunity using a suitable dose of ADR that suppresses only the subpopulation of the suppressor T cells and, in turn, lower the ratio. The reason for the different sensitivities to ADR shown by the subpopulations of lymphocytes was not clear. The results of the *in vitro* study, however, indicated a very narrow range for the optimal dose with which to improve the ratio. The dose of 1  $\mu\text{g}/\text{ml}$  was considered optimal to lower the ratio. It may be possible that Case 1 received the dose which coincided with his suppressed immunological condition<sup>17-19</sup>. The killer activity following treatment with IL2 (Group A), that is the functions of the LAK cells, was higher than that of those without IL2 (Group C). The wide variation in the killer activity of Group A implies that, there exists a variety of immunological abilities in HCC patients and also differences in the precursors of the LAK cells in quantity and/or quality. On the other hand, the percent killer activities, induced with IL2 after exposure to ADR (Group B), indicated a relatively constant value, 60% to 70%, in all cases (Fig. 3). This result suggests that the precursors of the LAK cells were affected by ADR. ADR may affect the quantity and/or quality of the precursors. Thus, immunochemotherapy using both IL2 and ADR may be more effective than immunotherapy using the LAK cells in some cases of suppressed immunities.

Clinically, this immunochemotherapy seems to produce less side-effects than chemotherapies using previous anti-cancer drugs for HCC. In advanced HCC patients with suppressed immunological function, this immunochemotherapy, after mass reduction treatments, may possibly improve the prognosis of suppressive conditions and also the quality of life<sup>5,6,20,21</sup>.

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