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ADOPTIVE IMMUNOTHERAPY OF MALIGNANT DISEASES WITH IL-2-ACTIVATED LYMPHOCYTES

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SUMMARY Lymphokine activated killer cells (LAK cells) or interleukin 2 (IL-2)-activated killer cells were induced by recombinant IL-2 (TGP-3) for clinical adoptive immunotherapy of malignant diseases. After incubation of peripheral blood lymphocytes (PBL) with IL-2 and normal human plasma for 1–2 weeks LAK cells were obtained that showed a maximum cytotoxicity against target cells, and did not need a toxic dose of IL-2 to enhance or maintain their cytotoxicity. Both autologous and allogeneic LAK cells were used in five clinical cases without any immune side effects, and were effective in three cases.

INTRODUCTION

Immunotherapy of malignant diseases is now being studied intensively, because theoretically it should be effective for specific treatment of tumors. The discovery of cytokines produced by immune response cells has contributed not only to analysis of the functional relationships of cells but also to the clinical treatment of various malignancies.

Interleukin 2 (IL-2), a representative cytokine, which causes proliferation and activation of T lymphocytes, was first named T cell growth factor (TCGF) (Morgan et al., 1976).

Rosenberg et al. treated patients with malignant diseases, especially cutaneous and pulmonary metastases, with activated lymphocytes expanded by IL-2, which they called LAK cells (lymphokine-activated killer cells) (Rosenberg, 1984; Rosenberg et al., 1985). In their in vivo experiments, they used LAK cells induced from splenocytes in an incubation period of a few days. They found that injection of IL-2 at 8-hour intervals was necessary to maintain the activity of LAK cells, and they obtained effective results when IL-2 and LAK cells were injected simultaneously (Rosenberg, 1985). Based on these experimental results, in clinical studies Rosenberg et al. used high doses of IL-2 and LAK cells induced from patients' peripheral blood lymphocytes (PBL) by their incubation with IL-2 for a few days. This treatment was therapeutically effective, but patients treated with very high doses of IL-2 suffered from toxic side effects, such as the capillary permeability leak syndrome, resulting in marked fluid retention and body weight gain and psycological disorders (Rosenberg et al.,

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1986).

Thus, it is very important to produce LAK cells with higher cytotoxic activity that do not require such high, toxic doses of IL-2.

Another problem in adoptive immunotherapy with LAK cells and IL-2 is that so-called blocking factors (Hellström et al., 1974; Kimoto et al., 1987a, Kimoto et al., 1987b) in the plasma of cancer patients may inhibit the cytocidal effect of LAK cells.

In the absence of human plasma, normal PBL activated by IL-2 show high cytotoxicity as effector cells in vitro. Normal plasma does not block the cytotoxicity of IL-2-activated PBL significantly (Kimoto, 1986), but the plasma of patients' decreases the cytotoxicity of these normal effector cells. When patients' autologous PBL and plasma are used simultaneously in the cytotoxic assay, the cytotoxicity of the IL-2-activated PBL is decreased significantly (Kimoto, 1986), and the continuous presence of IL-2 is required to maintain the cytotoxicity of IL-2-activated PBL in the presence of patients' plasma (Kimoto, 1986).

Therefore, we tried to obtain LAK cells that maintained maximum cytotoxicity even in the presence of patients' plasma. We have reported the change in cytotoxicity of IL-2-activated PBL during incubation with IL-2 and human normal plasma (Kimoto et al., 1987c). Usually LAK cells seemed to be induced after incubation for a few days (Grimm et al., 1982, Doyle et al., 1985). However, the cytotoxicity of the autologous LAK cells induced by our method reached a maximum after incubation for two weeks, and this cytotoxicity was maintained for further two weeks in the presence of IL-2 (Kimoto et al., 1987c). The cytotoxicity of allogeneic LAK cells induced in cultures of mixed PBL obtained from ten donors, reached a maximum after incubation for seven to ten days, and was maintained for another one week even at a low effector to target (E:T) ratio and in the presence of patients' plasma (Kimoto et al., 1987d).

Therefore, considering the possible E:T ratio in the body of patients, the cells transferred for adoptive immunotherapy should be cultured for two weeks in the case of autologous LAK cells and for seven to ten days in the case of mixed cultures of allogeneic LAK cells.

Addition of IL-2 (TGP-3) at a high concentration of 0.5 U/ml could enhance the cytotoxicity of LAK cells in vitro (Kimoto et al., 1987b), suggesting that IL-2 maintained or increased the cytotoxicity of LAK cells in vivo in patients when injected simultaneously with the LAK cells. In in vitro experiments we found that LAK activity reached a maximum at concentrations of 0.1–0.2 U/ml of IL-2, suggesting that this serum level of IL-2 was required to maintain the LAK activity and that a higher concentration was not necessary (Kimoto et al., in preparation). Pharmacokinetic studies (Taguchi, 1986) showed that after intravenous injection of 2000 U of IL-2, the concentration IL-2 reached a maximum of 0.459 U/ml in the patient's serum, the half life of IL-2 was 0.41 h and the AUC (area under curve) was 0.499 U.hr/ml. Thus the serum level of IL-2 decreased rapidly. On the other hand, after intramuscular injection of 1000 U of IL-2 the maximum concentration was 0.060 U/ml, the half life was 1.26 h and the AUC was 0.277 U.hr/ml. Thus the serum level of IL-2 decreased gradually and IL-2 could still be detected 8 h after its intramuscular injection. Consistent with these pharmacokinetic studies, we found in in vitro experiments that for enhancement of LAK activity there was no difference between the effects of intravenous infusion of IL-2 for one hour and its intramuscular injection (Kimoto et al., in preparation).

In this paper we report successful clinical trials of adoptive immunotherapy with LAK cells induced by our method given with nontoxic doses of IL-2. The safety of adoptive transfer of normal allogeneic LAK cells is also discussed.

MATERIALS AND METHODS

1. *Interleukin 2*

Recombinant interleukin 2 (TGP-3) was kindly supplied by Takeda Chemical Industries, Ltd. One unit of TGP-3 was defined as the amount of IL-2 present in 1 ml of supernatant after incubation of 5×10^6/ml of normal PBL in RPMI1640 containing 10% fetal calf serum, 40 μg/ml of Con A and 15 ng/ml of 12-O-tetradecanoylphorbol-13-acetate for 72 hours at 37 C.

2. *Interferons*

In two cases we injected interferons (IFNs) several times simultaneously with adoptive immunotherapy. Recombinant IFN-α, with a specific activity of $2\text{-}4\times10^8$ IU/mg protein and more than 95% purity, and recombinant IFN-γ with a specific activity of more than 1.64×10^6 U/mg protein and a purity of more than 95% were supplied by Takeda Chemical Industries, Ltd. Recombinant IFN-β with a specific activity of 5×10^7 U/mg protein and purity of more than 99% was supplied by Kyowa Hakko Co., Ltd.

3. *Induction of LAK cells*

PBL separated from the blood of patients or normal donors were incubated with recombinant IL-2 (TGP-3) to generate cells capable of killing tumor cells. PBL were isolated from 2000 to 4000 ml of blood by leukapheresis using CS-3000 (Fenwal) and were purified by Ficoll-Paque gradient sedimentation (Böyum, 1968). Then, they were incubated at an initial concentration of 2×10^6 cells/ml in RPMI1640 containing 3 U/ml of IL-2, 10–13% normal human plasma of the same blood type as that of the patient and 5 U/ml of heparin as an anticoagulant. Judging from our previous experiments, IL-2 should be present continuously in the medium and 3 U/ml was sufficient for this incubation. The LAK cells were induced in a cell factory with ten chambers (Nunc) to save time, and medium containing 3 U/ml of IL-2 was added every three or four days. Autologous cells were incubated for two weeks and allogeneic cells were incubated for seven to ten days at 37 C under an atmosphere of 5% CO_2 in air.

4. *Cytotoxic assay in vitro*

Adhesive target cells SW1116, a human colon adenocarcinoma cell line (Leibovitz et al., 1976), were incubated with LAK cells for 2 days in medium with or without IL-2 and plasma of the patient in a flat-bottomed microtest plate. The heparin (5 U/ml) present in the plasma as an anticoagulant did not influence the cytotoxic assays. After incubation, the number of live target cells in the microtest plates was counted by the neutral red dye-uptake method, and the survival rate of target cells was calculated relative to the control, in which the target cells were incubated without IL-2 or LAK cells. The optical density (OD_{550}) was proportional to the number of live target cells. Almost all LAK cells were removed by washing with phosphate-buffered saline before addition of neutral red and they had little affect on the count of target cells. The percentage survival of target cells and the inhibition rate or cytotoxicity were calculated as follows: % survival = (OD_{550} of the experiment / OD_{550} of the control) $\times$ 100; % inhibition or cytotoxicity = 100 – % survival.

5. *Preparation of LAK cells for adoptive transfer*

After incubation in the cell factories, the LAK cells were collected and washed three times with phosphate-buffered saline. Then $1\text{–}10\times10^9$ LAK cells were suspended in 100 ml of saline containing 2000 U of IL-2. The suspension was injected intravenously over a five minute period using a blood infusion set with mesh to remove debris. The cells were injected 1–3 times a week followed by intravenous or intramuscular injection of 1000 U or 2000 U of IL-2 the next day.

RESULTS

1. *Cytotoxicity of LAK cells just before adoptive transfer*

Fig. 1 shows the cytotoxicity of autologous and allogeneic LAK cells prepared for adoptive transfer to patients. Autologous LAK cells showed about 90 to 95% cytotoxicity after two weeks' incubation even at an E:T ratio of 1:1 and in the presence of patient's own plasma, and the cell viability was more than 80%. The LAK cells of blood type B.Rh(+) and O.Rh(+) showed cytotoxicity after seven to ten days' incubation, as shown in Fig. 1. Though some live target cells remained at an E:T ratio 1:1, almost all target cells were kil-

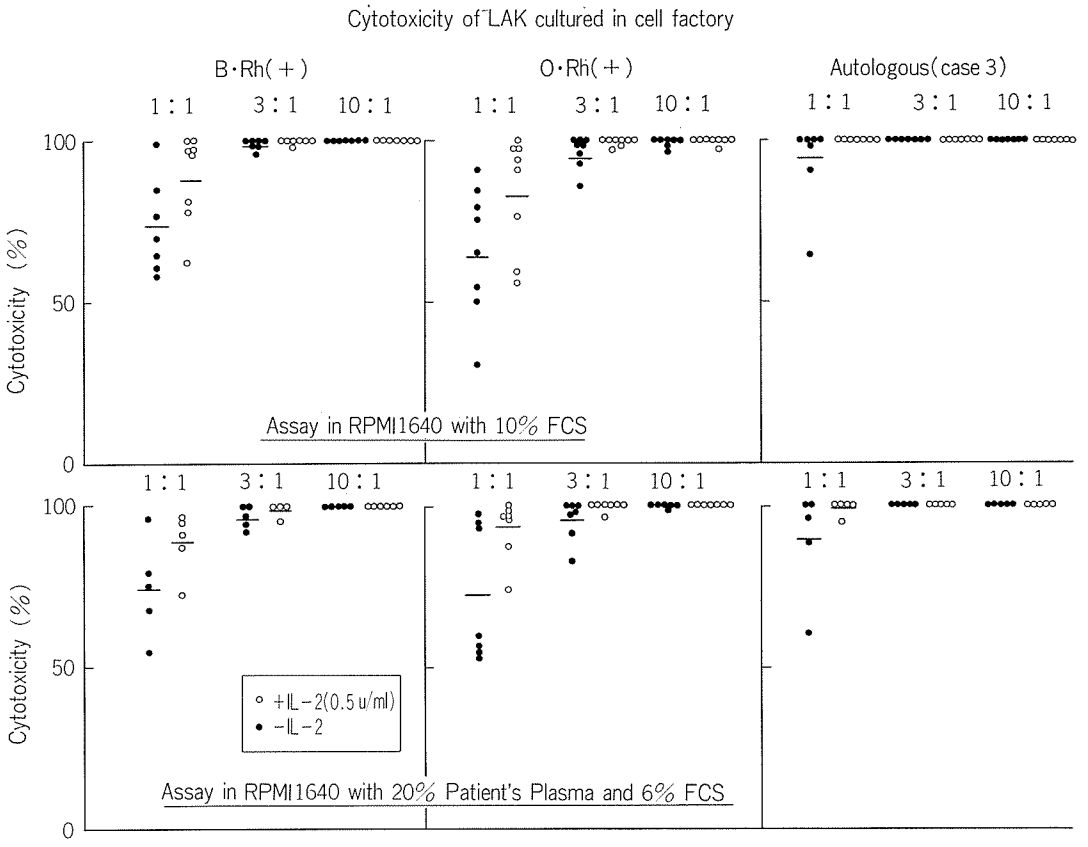

FIGURE 1. Cytotoxicity of autologous and allogeneic LAK cells cultured in a cell factory. The cytotoxic assay against SW1116 was carried out just before adoptive transfer. top: assay in RPMI1640 containing 10% FCS. bottom: assay in RPMI1640 containing 6% FCS and 20% patients' plasma as a blocking factor. The E:T ratios used were 1:1, 3:1 and 10:1. IL-2 enhanced the cytotoxicity of LAK cells (○).

led when the E:T ratio was raised to 3:1 or 10:1. Additional IL-2 at a concentration of 0.5 U/ml enhanced the cytotoxicity.

After incubation, the number of LAK cells increased to one to five times the number of PBL before incubation, but 2- to 3-fold proliferation was usual and the viability of the LAK cells was 80–100%.

2. *Case reports and clinical effects*

Five cases with metastases of the various malignancies shown in Table 1 received adoptive immunotherapy with LAK cells generated by the culture method described above.

In case 1, 8.6×10^7 LAK cells induced from PBL in the patient's pleural effusion were injected into the pleural cavity with IL-2. The LAK cells were left in the pleural cavity for several days, and then continuous drainage was carried out for one week. Tumor cells had disappeared from the pleural effusion before drainage was started. The pleural effusion completely disappeared in five months when the patient died of hepatic metastasis.

Case 2 had five pulmonary metastases of mammary adenocarcinoma and received 1–10×10^9 of autologous LAK cells, 2000 U of

TABLE 1. *Clinical cases*

Case	Diseases		Adoptive immunotherapy				Result
	Primary disease	Metastatic sites	Donor	Dose	Total dose	IL-2 (U)	
1. TH 60 M	Colon Adenoca.	Pleura Pleural effusion Liver	Autologous	8.6×10^7 intrapleural	8.6×10^7	1000	died
2. KM 42 F	Breast Adenoca.	Lung	Autologous Allogeneic	$1\text{–}10\times10^9$ 1–3/week intravenous	$>10\times10^{10}$	2000	1 y+
3. TZA 27 M	Epipharynx Squamousca.	Lung Liver Abdominal wall	Allogeneic	$2\text{–}6\times10^9$ 1–3/week intravenous	4×10^{10}	2000	died
4. MO 43 F	Ileopsoas m. Rhabdomyo-sarcoma	Lung	Allogeneic	$2\text{–}6\times10^9$ 1–3/week intravenous	$>9\times10^{10}$	2000	8 m+
5. FI 61 F	Colon Adenoca.	Liver	Allogeneic	$1\text{–}6\times10^9$ 1/week intraarterial	$>2\times10^{10}$	2000	2 m+

IL-2 and interferon mixture (α, 3×10^6 IU; β, 3×10^6 IU; γ, 2×10^6 IU). One of the metastases of 9 mm diameter completely disappeared and the other two decreased from 10×9 mm to 9×8 mm and from 16×13 mm to 14×13 mm, as shown by chest roentogenograms. After 14 transfers of autologous LAK cells, allogeneic LAK cells of normal donors were transferred with IL-2 once a week because slight pancytopnea developed and sufficient PBL could not be obtained by leukapheresis. These five metastatic lesions, including the one that disappeared, showed slow regrowth in spite of the weekly adoptive transfer. Then combination chemotherapy and adoptive immunotherapy were applied, resulting in effective therapy that is now in progress (Fig. 2).

In case 3, a squamous cell carcinoma of the epipharynx was resected and one year later giant metastatic lesions were found in both lungs, the liver and the abdominal wall. The patient had severe anemia and also antibodies against EB virus. Then allogeneic LAK cells induced from PBL of normal donors were transferred two or three times a week with recombinant IFN-α (3×10^6 IU). Of the multiple pulmonary metastases one of 30 mm diameter and one of 20×10 mm regressed completely, a left hilar metastasis became smaller, one hepatic metastasis disappeared under ultrasonic examination, and a tumor on the abdominal wall became smaller, but no change was observed in the other metastases. The patient died abruptly of massive hemorrhage from a hilar metastasis of the right lung on which this therapy had not apparently been effective (Fig. 3).

Case 4 had one pulmonary metastasis of a rhabdomyosarcoma that grew rapidly in a few months. The patient received allogeneic normal LAK cells systemically two or three times a week. During this therapy for five months growth of the tumor stopped, but the tumor did not become smaller. Then the tumor was resected, and after the operation,

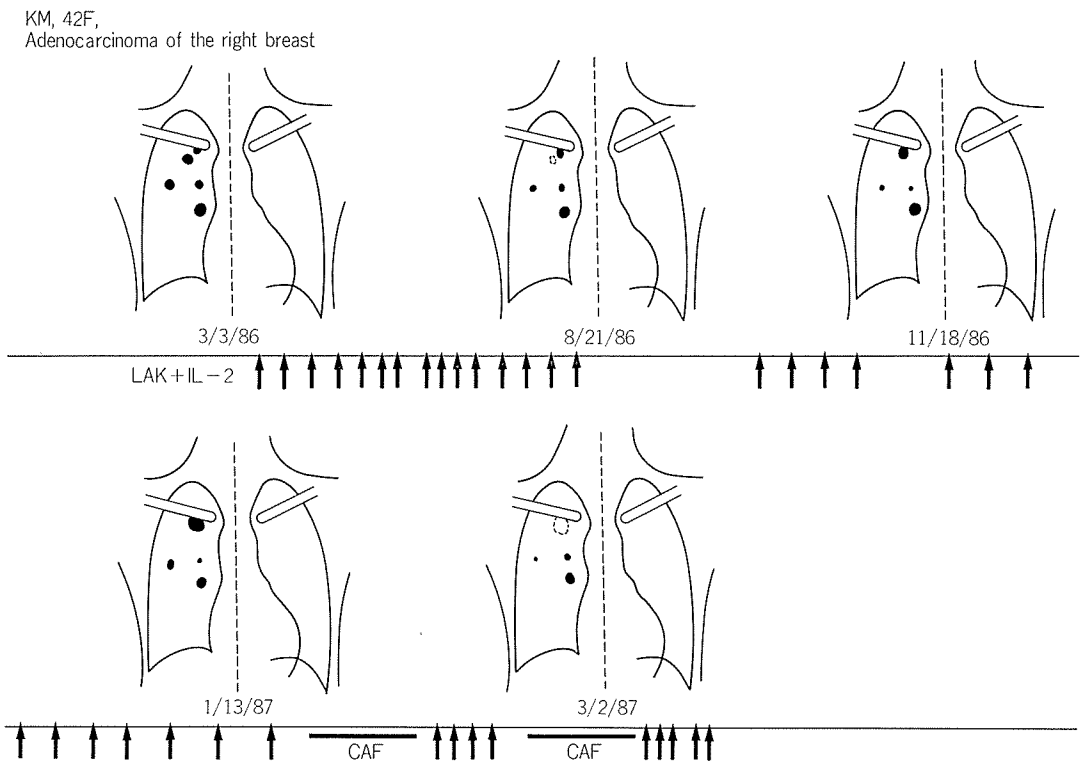

FIGURE 2. Clinical course of a patient (Case 2) who had pulmonary metastatic lesions of a mammary adenocarcinoma. The patient was treated by adoptive immunotherapy alone in 1986, and in combination with chemotherapy in 1987.

adoptive immunotherapy with allogeneic LAK cells and 2000 U of IL-2 has been continued to prevent growth of the remaining micrometastases.

Case 5 has a large metastasis of a colon adenocarcinoma in the liver. LAK cells and IL-2 have been injected into the hepatic artery and therapy is still in progress.

3. *Side effects*

Fever was usually experienced a few hours after each systemic injection of LAK cells and IL-2, which were given concomitantly with indomethacin. Sometimes patients experienced nausea and general fatigue, but without the necessity of use of therapeutic drugs. Slight pancytopnea was seen in case 2 during the period of leukapheresis, and disappeared when normal allogeneic LAK cells were used.

Following rapid injection of LAK cells intravenously, no side effects such as pulmonary embolism or cardiac infarction were experienced. Roentogenograms of the chest taken directly after rapid injection of LAK cells showed no significant differences from those taken before the injection. No infectious diseases or sepsis were experienced during this therapy.

4. *Contamination*

Of more than one hundred cultures, only two were contaminated by *Candida* species, and these were discarded. There were no accidents in patients and no bacteria or my-

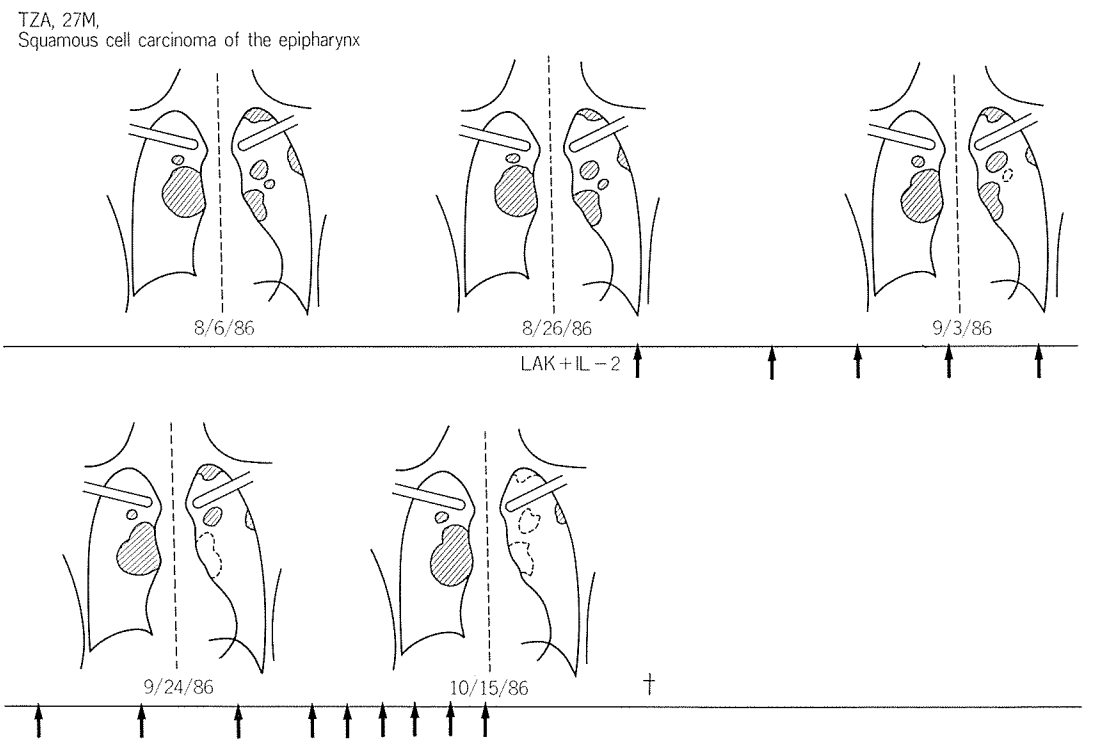

FIGURE 3. Clinical course of a patient (Case 3) with giant metastatic squamous cell carcinoma in both lungs. Some metastases in the left lung diminished, but massive hemorrhage occurred in the right lung.

coplasma were detected in culture media.

5. *Graft versus host reaction*

In cases 2, 3 and 4 allogeneic LAK cells were injected frequently. Only ABO- blood types were matched. No side effect involving an anaphylactic reaction or toxic immune disease was experienced clinically. However, there was a high possibility of occurrence of the graft versus host (GVH) reaction. Laboratory data revealed no significant change in the amount of immunoglobulins. Before each injection of allogeneic LAK cells, a small volume of LAK cells with infusion saline containing IL-2 was tested intracutaneously, but no rapid reactions were detected.

DISCUSSION

Rosenberg et al. in USA were pioneers in adoptive immunotherapy with LAK cells. In their clinical trial, they used autologous LAK cells obtained by frequent leukapheresis followed by a few days' incubation (Rosenberg, 1984; Rosenberg, 1985; Rosenberg et al., 1985). Although no antitumor effect of IL-2 alone was detected (Lotze et al., 1984), they injected an extremely large amount of IL-2 to maintain the LAK activity of the cells injected into patients. This therapy was based on the effectiveness of autologous LAK cells, which might possess autologous tumor-antigen specificity upon autologous tumor cells (Lotze et al., 1981; Grimm et al., 1982; Vanky et al., 1982) and the effectiveness of adoptive transfer of LAK cells into mice

bearing experimentally established pulmonary and hepatic metastases (Mazumder and Rosenberg, 1984; Lafreniere et al., 1985). In some cases they obtained good results, such as complete regression of skin metastases of a malignant melanoma and partial regression of pulmonary metastases of a colorectal cancer, renal-cell carcinoma, melanoma and primary lung cancer (Rosenberg et al., 1985). Moreover, local injection of LAK cells resulted in complete regression of skin metastases of a malignant melanoma and partial regression of a soft part metastatic mammary carcinoma (Adler et al., 1984). However, the side effects of high doses of IL-2 were extremely severe, as described above.

On the other hand, we found that the LAK cells induced after one to two weeks' incubation by our method using IL-2 and normal human plasma possessed maximum cytotoxicity even at a low E:T ratio under the influence of the patients' plasma. The results suggested the importance of the incubation period. Moreover IL-2 injected at the frequency and the doses described in Table 1 for maintenance of the LAK activity in vivo in the patient did not cause any toxic side effects.

Neither autologous nor allogeneic LAK cells caused any side effects, such as infarction or pulmonary embolism. However, when allogeneic LAK cells are used for adoptive transfer, there is a possibility that a toxic immune response against allogeneic antigens involving HLA could occur in these patients after frequent injection of LAK cells from other persons. Theoretically HLA types should be matched, and Kohler et al. used LAK cells obtained from identical or haploidentical donors without any toxic side effects (Kohler et al., 1985). Additional allogeneic LAK cells should be transferred very cautiously, even when there is no significant immune response.

It is sometimes very difficult to obtain sufficient PBL from a patient, and it is very dangerous to culture bacteria- or virus-infected PBL for generating LAK cells. For such patients, allogeneic LAK cells from normal donors could be available. With regard to the safety of transfer of allogeneic cells, it is noteworthy that large amounts of fresh blood can be transferred safely after massive hemorrhage and that no detectable GVH usually occurs.

Moreover, mixed culture of normal allogeneic PBL from more than one donor appears to induce LAK cells capable of lysing other PBL in the culture while reacting with alloantigens of other individuals. The activity of these LAK cells increased earlier than the autologous LAK activity even though the number of LAK cells was smaller due to this interaction.

In three patients who received intravenous adoptive transfer of LAK cells, some pulmonary metastases regressed completely or partially, but other pulmonary metastases, even in the same patient, did not show any marked change. LAK activity is considered to be nonspecific (Rosenberg, 1985; Rayner et al., 1985), but there appear to be differences in sensitivity of malignant cells to LAK cells. Moreover, there are probably some other differences such as in angiogenesis or the distribution of LAK cells.

In two cases we used IFN(s) which could support the activity of LAK cells. IFN-α can kill EB virus and is effective on squamous cell carcinoma of the epipharynx and an IFN-mixture showed a much stronger direct effect on target cells in vitro than one IFN alone (Kimoto et al., 1986), and also enhanced the cytotoxic activity of IL-2-activated killer cells (Kimoto, 1986). However, we usually could not obtain complete regression of pulmonary metastases using IFN(s) alone. The effectiveness in cases 2 and 3 could, therefore, be the effect of a combination of adoptive immunotherapy and IFN(s).

In the future more intensive adoptive immunotherapy will be required, if possible using tumor-specific cytotoxic effector cells. In adjuvant therapy LAK cells should be

transferred to patients who have micrometastatic lesions and a high possibility of recurrence. Probably, combination therapy with biological response modifiers or cytokines such as IFNs would give better results. Further analysis of the blocking factor(s) and its removal also seem important.

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