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Immunological Monitoring of the Peripheral Blood Mononuclear Cell by Two-Color Flow Cytometry after Local Administration of OK-432 in a Patient with Gastric Cancer Displaying Multiple Liver Metastasis

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Summary. **A trial administration of OK-432 by transarterial embolization (TAE) and intra-operative local injection (OLI) was performed to treat the liver metastasis of gastric cancer. The liver metastases decreased in size as assessed by abdominal CT scan. The peripheral level of DR^+ $CD3^-$ monocytes (antigen-presenting cell) were increased on the day following OK-432 administration, and the increase in $CD4^+$ T lymphocytes (helper T cell) was prominent from 3 to 5 days afterward. Furthermore, the $CD8^+$ T lymphocyte (cytotxic T cell) level increased from 5 to 7 days after OK-432 treatment. The increase in the number of these lymphocytes after OLI with OK-432 was more prominent than that after TAE with OK-432. The concentration of DR^+CD4^+ T lymphocytes changed little, however, DR^+CD8^+ T lymphocytes increasing remarkably after TAE and OLI. NK activities decreased significantly on the day following TAE and abdominal operation, but increased afterward to at least pretreatment levels. On two-color flow cytometry using LEU 7 and LEU 11, which recognize NK surface antigens, each subpopulation of NK cells showed similar changes. However, LEU 11-positive cells comprised the majority of the NK cells. The kinetics of the NK subpopulation did not always correspond to that of NK activity, which may be due to the participation of neutrophils induced by the liver biopsy. These results suggest that the local administration of OK-432 by TAE and OLI activates cell mediated immunity by stimulating antigen-presenting cells and helper and cytotoxic T cells, and may therefore be used to treat liver metastasis.**

INTRODUCTION

Gastric cancer with liver metastasis has a poor prognosis. Nakajima[1] reported a 50% survival time of multiple liver metastases with gastrectomy of 5.1 months, and without gastrectomy, 2.7 months. However, in recent years it has been reported that transarterial chemo-embolization (TAE), percutaneus ethanol injection therapy (PEIT) and adoptive immunotherapy (AIT) by OK-432 (Chugai Pharmaceutical Co., Japan) or interleukin-2 (IL-2) are effective for hepatoma and liver metastasis.[2-5]

We performed TAE and operative local injections (OLI) with OK-432 on a patient with gastric cancer showing multiple liver metastasis. We then studied the kinetics, after OK-432 injection, of the peripheral blood mononuclear cell (PBMC) levels by two- and single-color immunofluorescence for CD3, 4, 8, and HLA-DR, LEU 7 and LEU 11, and NK-specific antigens.

PATIENT AND METHODS

Patient and clinical course: A 76-year-old Japanese woman was admitted to our department on December 5th, 1991, with a 4-month history of epigastralgia and 4 kg weight loss. On physical examination, the conjunctiva palpebrae was found anemic and a movable tumor was palpated in the abdomen; however, neither Virchow's nor Schnitzler' metastasis was detected. Although most routine laboratory parame-

ters were within normal limits, the hemoglobin was 6.6 g/dl and the hematocrit was 22.9%. The serum level of carcinoembryonic antigen (CEA) was 2.8 ng/ml and that of CA19-9 was 83.5 U/ml. Upper GI series showed Borrmann type II advanced gastric cancer in the lesser curvature of the pyloric antrum (Fig. 1A). Multiple liver metastasis was detected by abdominal CT scan.

OK-432 (10KE), a preparation from Streptococcus pyogenes A3, was injected into the gastric tumor by endoscopy twice preoperatively. Thereafter, lipiodol TAE treated with mytomicine C(MMC), adriamycine (ADM) and 10KE of OK-432 were given. Subtotal gastrectomy and cannulation into the hepatic artery by Port-A-cath (Fig. 1B) was conducted after the diagnosis of gastric cancer with multiple liver metastais was made. OK-432 (40KE) was injected into each major metastatic liver tumor intraoperatively. Side effects of fever and a slight elevation of transaminases appeared, but only transitorily.

Isolation of PBMC and flow cytometry analysis: PBMC were isolated from heparinized peripheral blood by density gradient centrifugation using Ficoll-Paque (Pharmacia Fine Chemicals, Sweden). Cell populations were analyzed by FACScan (Becton-Dickinson) with FITC- and phycoerythrinconjugated monoclonal antibodies. The following mAbs were used: OKT3 (CD3, all T cells; Ortho USA), OKT4 (CD4, helper/inducer T cells; Ortho USA), OKT8 (CD8, suppressor/cytotoxic T cells; Ortho USA), OKIal (HLA-DR; Ortho USA), Leu7 (CD57, NK cells; Becton-Dickinson USA), and Leu11 (CD16, NK cells; Becton-Dickinson USA). Two-color flow cytometry was done for CD3 and HLA-DR, CD4 and HLA-DR, Leu7 and Leu11.

Cytotoxicity assay: To measure cytolysis by PBMC, a 51Cr-release assay was performed. Target cells (NK-sensitive K562 human cell line) were incubated with 100 uCi of sodium 51 chromate for 1 h at 37℃. After washing twice with HBSS, the target cells were suspended at 5×10^4/ml in 20% fetal bovine serum (Hazleton, USA), 20 mM HEPES, and RPMI-1640. The effector cells were suspended in RPMI-1640 at an appropriate concentration. Targets and effectors were mixed and plated in a 96-well round-bottomed microtiter plate (Nuncs) at an effector/target (E/T) ratio of 20 : 1 and cultured for 4 h in moist air containing 5% CO^2. After culture, the supernatant was harvested using a Titertek Supernatant Harvesting Press (Flow Labs, UK). The radioactivity of the supernatants was measured by an Autowell Aloka gamma counter (Aloka, Japan). Cytotoxic activity was calculated as follows: cytotoxicity (%)

$$=100\times\frac{\text{experimental release (cpm)}-\text{spontaneous release (cpm)}}{\text{total release (cpm)}-\text{spontaneous release (cpm)}}$$

Spontaneous release was measured by counting the radioactivity in the supernatant of target cells incubated without effector cells, and the total radioactivity incorporated was determinated by counting the supernatants of target cells incubated with 2% Triton-X.

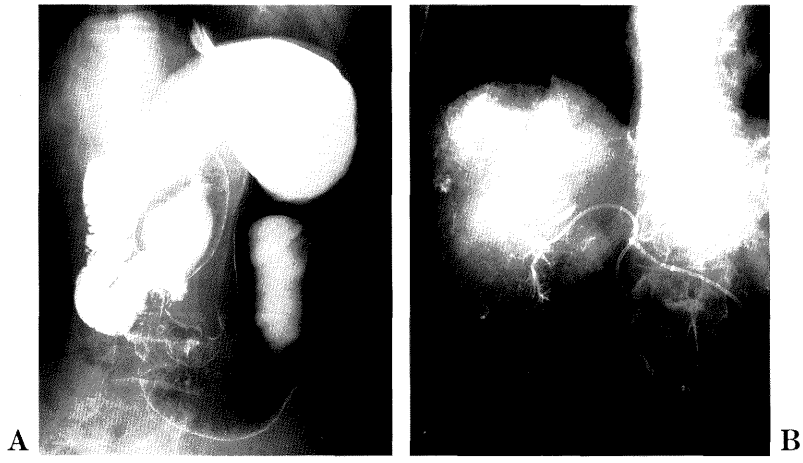

Fig. 1. **A:** An upper gastrointestinal tract examination; Borrmann II type advanced gastric cancer in the lesser curvature of pyloric antrum is revealed. **B:** Plain abdominal roentgenogram after lipiodol TAE by Port-A-Cath. Note the uptake of lipiodol into metastatic liver tumors.

RESULTS

1. Kinetics of CD4⁺ (helper/inducer) T cells and CD8⁺ (suppressor/cytotoxic) T cells

The CD4⁺T lymphocyte count leveled off or decreased on the day following local administration of OK-432. On days 3 to 5, however, it increased from 35.1% to 51.0%. CD8⁺ T lymphocytes, as did the CD4⁺ T lymphocyte count. This, however, increased from 6.9% to 17.6% on days 5 to 7 (Fig. 2).

2. Analysis of HLA-DR expression by PBMC using two-color flow cytometry

DR⁺CD3⁻ cells increased greatly on the day following treatment with OK-432, but decreased thereafter. DR⁺CD4⁺ T lymaphocytes decreased transiently on day 1, but recovered by day 3 or 5, albeit the change was small. The increse in DR⁺CD8⁺ T lymphocytes was most prominent on days 5 to 7 after the local administration of OK-432, and after OLI increased from 2.8% to 9.2% (Fig. 3).

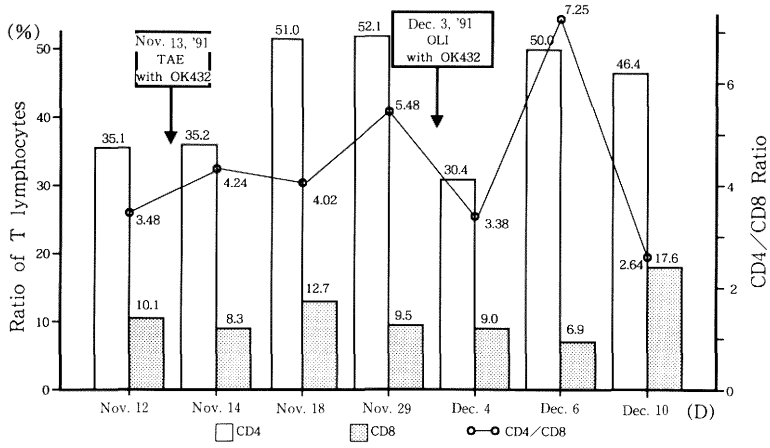

Fig. 2. Kinetics of CD4⁺ and CD8⁺ T cells. CD4⁺ T lymphocytes decreased on the day following local administration of OK-432, although increasing on days 3 or 5. CD8⁺ T lymphocytes changed like CD4⁺ T lymphocytes; however, they increased on days 5 or 7.

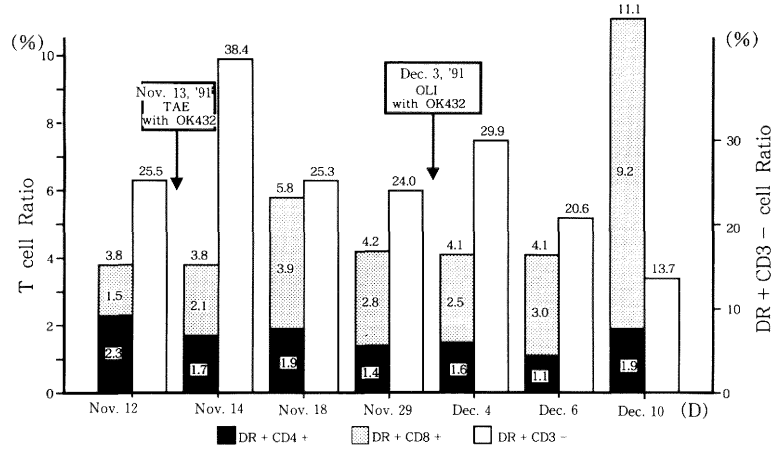

Fig. 3. Kinetics of HLA-DR expression of PBMC by two-color flow cytometry. DR+CD3− cells increased prominently on the day following treatment with OK-432, and decreased thereafter. DR⁺CD4⁺ T lymphocytes decreased transitory on day 1, and recovered on days 3 or 5. DR⁺CD8⁺ T lymphocytes increased most prominently on days 5 to 7 after the local administration of OK-432, especially after OLI increased.

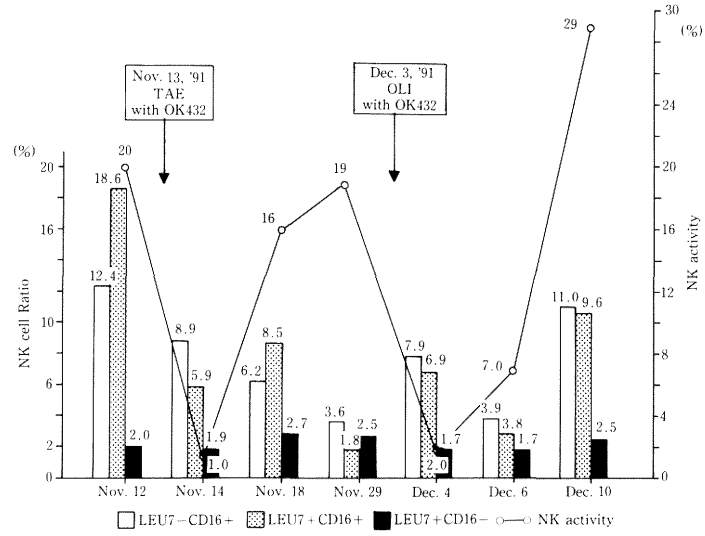

Fig. 4. Kinetics of NK activity and NK cell subpopulations in PBMC. The NK subpopulation changed approximating the NK activity. Each NK subpopulation underwent similar changes. Leu11$^+$ cells comprised the larger share of NK subpopulation, while Leu7 Leu11$^+$ cells were infrequent.

3. NK activity and flow cytometry of the PBMC NK cell subpopulation

NK activity decreased remarkably on the day after TAE or the operation, and recovered by days 5 or 7, respectively. The increase was especially prominent after OLI. The NK subpopulation level varied approximately with NK activity. However, NK activity was sometimes high even when the number of NK cells was small. Each NK subpopulation underwent similar change. Leu11$^+$ cells comprised a large fraction, and Leu7$^+$Leu11$^-$ cells, a small fraction of the NK subpopulation of PBMC (Fig. 4).

4. Clinical evaluation by abdominal CT

Figure shows a computerized tomograph of this patient. Immediately following the TAE with OK-432, lipiodol was taken up by metastatic liver tumors (upper panel). These tumors decreased to about 60% in size 4 months after OK-432 combined TAE and OLI (lower panel) (Fig. 5).

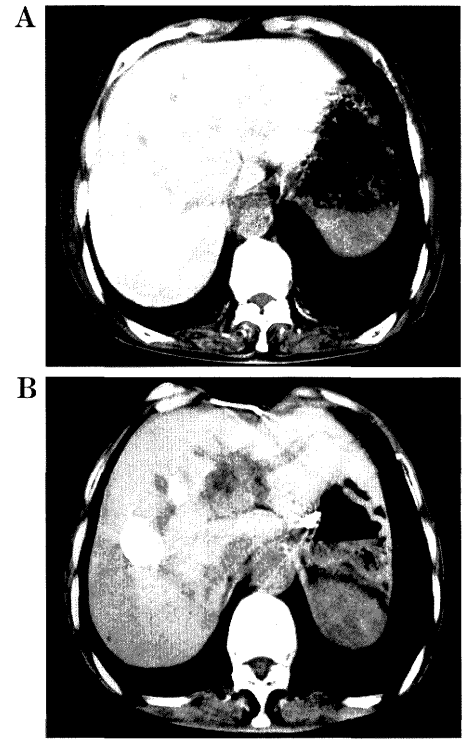

Fig. 5. A: Abdominal CT after TAE with OK-432 preoperatively at November 29, 1991. Lipiodol was taken up into the metastatic tumors. **B:** The tumors decreased remarkably on March 19, 1992.

DISCUSSION

Since Gallo[6] discovered IL-2 in 1976 and Rosenberg[7,8] described lymphokine-activated killer (LAK) and tumor-infiltrating lymphocytes (TIL), AIT has been applied to treating hepatoma and liver metas-

tasis. The effects, however, have not met initial expectations. In an attempt to improve immunotherapy for cancer, we used TAE and OLI with OK-432 to treat multiple liver metastasis in gastric cancer while characterizing PBMC by single- or two-color flow cytometry and measured PBMC NK activity during such immunotherapy.

DR^+CD3^- cells increased remarkably on the day following TAE and OLI with OK-432, and decreased thereafter. $CD4^4$ T lymphocytes decreased on day 1, but then increased greatly by days 3 to 5. $CD8^+$ T lymphocytes increased by days 5 or 7. Two-color flow cytometry of HLA-DR and CD3 or CD4-positive antigens, $HLA\text{-}DR^+CD4^+$ T lymphocytes (activated helper/inducer T cells) decreased transiently on day 1, and recovered by days 3 to 5. $HLA\text{-}DR^+CD8^+$ T lymphocytes (activated suppressor/cytotoxic T cells) increased remarkably by days 5 or 7 after local administration of OK-432, and even further with OLI.

$CD4^+$ T cells are unable to recognize a free antigen. They can recognize antigens only in the context of the major histocompatibility complex (MHC) class II antigen on antigen-presenting cells (APC). Class II MHC antigens are present on B cells, activated T cells. macrophages, Langerhans cells, and dendritic cells.[9] In the present studies, $HLA\text{-}DR^+CD3^-$ cells increased significantly on the day following the local administration of OK-432. While it cannot be denied that they were B cells, it does suggest that APC may be induced by OK-432. With an antigen, the APC products IL-1, and IL-2 stimulate T cells. These events then induce IL-2 and IL-2 receptor expression by T cells. This autocrine mechanism then stimulates the proliferation of T cells.[10] The concentration of $CD4^+$ T cells increased after the concentration of $HLA\text{-}DR^+CD3^-$ T cells (APC) increased. These facts are consistent with T cell activation in cellular immunity. Furthermore, helper T cells which activate cytotoxic $CD8^+$ T cells were found in our studies to increase before $CD8^+$ T cells, with $HLA\text{-}DR^+CD8^+$ T cells (activated suppressor/cytotoxic), especially showing a significant increase. These results suggest that the local administration of OK-432 by TAE or OLI also induces cytotoxic T cells. Kawakami[4] reported that the $CD4^+$ T cell tended to increase, and that the $CD8^+$ T cell $HLA\text{-}DR^+$ cell did not indicate a constant tendency in the adminstration of recombinant IL-2 for primary and metastatic liver tumors. This knowledge might result from a reactivity for immunotherapy. Therefore, this immunological monitoring may appear significant for the evaluation of effects of the immunotherapy.

Natural killer (NK) cells are found in all normal donors and play an important role in the defense against malignant tumor cells. It is reported that NK cells in the peripheral blood vary in their maturity, and that the more mature NK cells are more active. Although the indicators of NK cell maturity are not well established, it has been reported that $Leu7^+$ $Leu11^-$NK cells are relatively immature, and that NK cells become $Leu7^+Leu11^+$ and $Leu7^-Leu11^+$ as they mature.[11,12] Accordingly, we studied the NK subpopulation by two-color flow cytometry with Leu7 and Leu11 after TAE or OLI plus OK-432.

NK activity decreased remarkably on day 1, but returned to at least pretreatment levels by days 5 to 7. Mature $Leu7^-Leu11^+$ or $Leu7^+Leu11^+$ NK cells comprised the majority, while the immature type of $Leu7^+Leu11^-$ NK cell comprised a small fraction of the NK cell population. The proportion of mature NK cells changed with NK activity and decreased on day 1; the proportion of immature NK cells changed minimally after TAE or OLI. NK activity did not always parallel the number of NK cells, perhaps because monocytes, macrophages and neutrophils also have NK activities. IN the operative liver biopsy sample, infiltration of the liver tumor by neutrophils was detected, suggesting that neutrophils may actively work against the tumor.

Mizushima[13] reported that NK activity was lowest on day 7 after TAE, and that four weeks were required to recover to pretreatment levels because liver ischemia by TAE inhibits the proliferation of NK cells (pit cells). In the present case, NK activites recovered within one week after TAE to levels no greater than those before treatment with OK-432 OLI. Indeed, the local administration of OK-432 may enhance NK activity and the anti-cancer effect of TAE.

Kann[5] reported that the anti-cancer effect of adoptive immunotherapy (AIT) only was limited, and proposed the use of immunochemo-lymphocytotherapy (OK-432, cyclophosphamide or mitomycin C, and AIT or IL2). In this study we initially treated with mitomycin C (MMC), and adriamycin (ADM) together with OK-432 TAE, and thereafter used OLI with OK-432. On the abdominal computerized tomogram (CT) in the present case, a main tumor regression of 60% was observed. We conclude that this regression was probably caused by the combined ability of TAE with MMC and ADM, and a combination with OK-432 to activate local immunity, and that further treatment with OLI and OK-432 produced a synergistic effect. Combination therapy using TAE and OLI with OK-432 may be as effective as immuno-chemotherapy using AIT and IL-2. The patient has been alive for 11

months after treatment; hence, this local administration of OK-432 appears effective immunotherapy. The immunological monitoring is essential to evaluate effectiveness of the immunotherapy.

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