Antibody-dependent Cytotoxicity Mediated by Chimeric Monoclonal Antibody Nd2 and Experimental Immunotherapy for Pancreatic Cancer

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In a previous study, mouse monoclonal antibody (MoAb) Nd2 (m-Nd2, mouse IgG1) labeled with ^{131}I exhibited efficacy in *in vivo* radioimmunotherapy against pancreatic cancer. In this study we prepared mouse/human chimeric antibody Nd2 (c-Nd2, human IgG1) for clinical use and examined whether c-Nd2 induced antibody-dependent cell-mediated cytotoxicity (ADCC). Cytotoxicity to pancreatic cancer (PC) cell lines, including Nd2 antigen-positive (SW1990, RWP-1, Capan-1) and Nd2 antigen-negative (Panc-1, MiaPaca-2, Capan-2) lines, was evaluated by mixed human leukocyte and tumor cell culture (MLTC) at an effector cell to target cell (E/T) ratio of 50 with or without Nd2. Cytotoxicities to SW1990 with no antibody, m-Nd2 and c-Nd2 (1 μg/ml) were 26.7%, 38.0% and 55%, respectively; to RWP-1, 28%, 41% and 70%; to Capan-1, 26%, 30% and 52%; to Panc-1, 24%, 28% and 30%; to MiaPaca-2, 18%, 20% and 27% and to Capan-2, 29.7%, 35.0% and 40.6%. Cytotoxic capacity during MLTC with c-Nd2 was significantly higher than during MLTC with m-Nd2 or with no antibody. These findings indicated that cytotoxicity to Nd2-positive PC cells during MLTC is induced by ADCC. Intraperitoneal injection of c-Nd2 inhibited the tumor growth of SW1990 xenografted subcutaneously in nude mice and prolonged the survival of nude mice in which SW1990 tumor was transplanted orthotopically at the tail of the pancreas. These findings suggested that, because of its ability to induce ADCC, c-Nd2 may be clinically useful for the immunotherapeutic treatment of pancreatic cancer.

Key words: Pancreatic cancer — Monoclonal antibody — Chimeric Nd2 — ADCC — Immunotherapy

Pancreatic cancer (PC) continues to have a poor prognosis even with recent progress in diagnostic modalities and therapeutic strategies, due to difficulty in obtaining early diagnosis and to its malignant character. Although various therapeutic strategies have recently been developed to improve the prognosis of patients with pancreatic cancer, tumor-specific monoclonal antibodies (MoAbs) may be particularly useful clinically. We previously examined the usefulness for radioimmunodetection and targeting therapy of murine anti-pancreatic cancer MoAb Nd2 (m-Nd2) directed against purified mucins derived from the human pancreatic cancer cell line SW1990.[1–6] Recently, with the development of genetic engineering, it has become possible to prepare mouse/human chimeric antibodies composed of mouse-derived antigen-binding variable regions and human-derived constant regions.[7, 8] Since clinical application of mouse MoAbs is hampered by the induction of human anti-mouse antibody (HAMA), humanized or chimeric or human antibodies are preferable for clinical trials.[9–11] Chimeric MoAbs are less immunogenic, and HAMA production is reduced. Chimeric MoAbs have been reported to induce antibody-dependent cell-mediated

cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), and are therefore thought to be more useful clinically for targeting therapy than mouse MoAbs.[12–15] We have prepared chimeric Nd2 (c-Nd2) which has high affinity and specificity for pancreatic cancer, as exhibited by m-Nd2,[16, 17] and have reported its usefulness for clinical radioimmunodetection and radioimmunotherapy.[18, 19] In this study, we examined whether c-Nd2 induces *in vitro* ADCC, and studied the *in vivo* effect of c-Nd2 mediated by ADCC in subcutaneously xenografted and orthotopically transplanted pancreatic cancer in nude mouse.

MATERIALS AND METHODS

Preparation of MoAbs   M-Nd2 was isolated from the ascitic fluid of BALB/c mice into which Nd2 hybridoma cells had been injected intraperitoneally, and purified by affinity chromatography on protein A columns using an Affi-Gel Protein A MAPS Kit (Bio-Rad, Richmond, CA). Hybridoma cells which produce c-Nd2 were cultured in E-RDF medium (Kyokuto Pharmaceutical, Tokyo) containing 2 ng/ml recombinant human interleukin-6, but no fetal calf serum. C-Nd2 was purified from the culture supernatant of the cells by affinity chromatography using an Immuno Pure IgG Purification Kit (Pierce, Rockfold, IL).

The antigen-binding affinities of m-Nd2 and c-Nd2 were investigated by competitive radioimmunoassay and Scatchard analyses as described by Hirayama et al.[16]

Pancreatic cancer cell line The human pancreatic cancer cell lines RWP-1 (well differentiated adenocarcinoma), Capan-1 (moderately differentiated), SW1990 (well/ moderately differentiated), Capan-2 (moderately differentiated), Panc-1 (poorly differentiated), and MiaPaca-2 (undifferentiated) were cultured in high-glucose Dulbecco's modified Eagle's minimum essential tissue medium (DMEM; Nikken Biomedical Laboratory, Kyoto) supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY), 100 IU/ml penicillin (ICN Biochemicals, Costa Mesa, CA) 100 IU/ml streptomycin (ICN Biochemicals) and sodium pyruvate (ICN Biochemicals). These cells were used as target cells for cytotoxicity assay.

Flow cytometric analysis Expression of Nd2 antigens on tumor cells was examined by an indirect immunofluorescence method using a FACScan. Cells (1×10^6 cells/ml) were incubated in phosphate-buffered saline (PBS) with m-Nd2, c-Nd2 or no antibody for 2 h at 37°C. The bound immunoglobulin was detected with rabbit anti-mouse immunoglobulin conjugate to fluorescein isothiocyanate (FITC) for m-Nd2 and goat anti-human immunoglobulin conjugate to FITC for c-Nd2. Fluorescence was measured by flow cytometry using a FACScan analyzer.

Effector cells Human peripheral blood was drawn into heparinized tubes. Peripheral blood mononuclear cells (PBMNC) were isolated from healthy donors by Ficoll-Hypaque density gradient centrifugation. After three washes in PBS, the cells were resuspended in DMEM including 10% FCS.

Cell cytotoxicity assay Cell cytotoxicity assay was performed with the Cytotox 96 Non-Radioactive Cytotoxicity Assay kit (Promega Co., Madison, WI), which measures lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis during mixed leukocyte and tumor cell culture (MLTC).[20, 21] Approximately 1×10^3 target cells in 50 μl of medium were placed in each well of 96-well plates and human effector cells were added at various concentrations to obtain the optimal (10–50) effector cell to target cell ratio (E/T ratio). Fifty microliters of MoAb (0.1–5 μg/ml) was added, and the plates were incubated at 37°C in a 5 % CO_2 humidified atmosphere to investigate ADCC activities. Target cells and effector cells without antibody were used as negative controls. After 4, 12 and 24 h incubation, 50 μl aliquots of supernanant of mixed leukocytes and tumor cells (MLTC) were placed in 96-well plates of the Cytotoxicity Assay kit. The plates were incubated for 30 min at room temperature. After the addition of Stop Solution (1 M acetic acid), the absorbance was measured with an MTP-120 Microplate reader at 492 nm. Percentage lysis of cancer cells was calculated as follows: % Cytotoxicity = (experimental − effector spontaneous − target spontaneous) / (target maximum − target spontaneous) × 100.

Anti-tumor effect of Abs in subcutaneous and orthotopic transplantation model mice SW1990 cells were harvested at confluency and resuspended in DMEM. Subsequently, 1×10^7 SW1990 cells in 500 μl of DMEM were inoculated subcutaneously into the left flank of BALB/c nude mice. When the xenografted tumor grew to 0.5−1.0 cm in size (about 14 days after inoculation), PBS (50 μl), m-Nd2 or c-Nd2 (100 μg/50 μl in PBS) was injected into the nude mice (each group, $n=4$) intraperitoneally twice, on the 14th and 15th day after subcutaneous inoculation. Tumor volume was calculated as Tumor volume (mm^3) = 0.4 × (major axis) × (minor axis)^2.

In orthotopic transplantation model mice, the anti-tumor effects of Abs were estimated in terms of survival. The xenografted SW1990 tumors described above were cut into small pieces, 50 mg in weight. The mice were anesthetized with diethyl ether, a left lateral abdominal incision was made, and the tail of the pancreas adjacent to the spleen was exteriorized using forceps. The cut pieces of SW1990 xenograft were fixed at the tail of the pancreas by ligation with a 4-0 Nylon suture. The pancreas and spleen were returned to the abdominal cavity and the incision was closed. When tumor growth was recognized on palpation on the 14th day after ligation, the mice were divided into three groups (PBS-injected group, m-Nd2-injected group and c-Nd2-injected group: each group $n=4$). The groups received 0.5 ml of PBS, 50 μg/0.5 ml in PBS m-Nd2 or c-Nd2 by intraperitoneal injection 4 times at 3-day intervals (approximately 200 μg Ab in total). The survival of these mice was followed.

Binding affinity of MoAb to mouse PBMNC and cytotoxicity with mouse PBMNC To confirm the in vivo anti-tumor effect of c-Nd2 in nude mice, we examined the binding affinity of c-Nd2 and m-Nd2 to mouse monocytes and lymphocytes. Mouse (4-week-female C3H/HeN) whole blood was conjugated to m-Nd2 or c-Nd2 with F(ab)$_2$ fragment of FITC-labeled anti-mouse IgG to m-Nd2 and anti-human IgG to c-Nd2. Fluorescence was measured by flow cytometry using a FACScan analyzer.

Cell cytotoxicity assay was performed with mouse PBMNC using the same method to examine whether c-Nd2 and m-Nd2 have cytotoxicity with mouse PBMNC to pancreatic cancer cell lines (Nd2-positive RWP-1, SW1990, and Nd2-negative Panc-1, Capan-2) with an E/T ratio of 50 and 1 μg/ml concentration of MoAbs. Mouse whole blood (20 ml) was taken directly from the heart (about 1−1.5 ml per body) into heparinized tubes. Mouse PBMNC were isolated by Ficoll-Hypaque density gradient centrifugation.

Statistical analysis Results were expressed as the mean±SD. Student's t test was used for statistical processing and a difference was considered significant if $P<0.05$.

In the orthotopic transplantation model, Kaplan-Meier estimated survival curves were used to compare survival among the three groups.

RESULTS

Cancer cell cytotoxicity with MoAbs (ADCC) during MLTC Of the pancreatic cancer cell lines examined in the present study, SW1990, RWP-1, and Capan-1 exhibited positive expression of Nd2 (89.4%, 60.0% and 50.5%, respectively) on flow cytometric analysis. On the other hand, Capan-2, Panc-1 and MiaPaca-2 were Nd2-negative. No difference was observed in the cytotoxicity induced during MLTC (E/T ratio 50, and without Abs) among the PC cell lines examined. Cytotoxicities by human effector cells to SW1990 with no antibody, m-Nd2 and c-Nd2 (1 μg/ml) were 26.7±2.3%, 38.0±3.4% and 55.0±7.5%, respectively; to RWP-1, 28.3±2.9%, 41.0±6.1% and 70.6±5.1%; to Capan-1, 26.4±3.2%, 30.2±2.8% and 52.3±3.5%; to Panc-1, 24.0±2.4%, 28.3±2.7% and 30.1±3.4%; to MiaPaca-2, 18±2.2%, 20.0±3.3% and 27.6±3.7%, and to Capan-2, 29.7±4.9%, 35.0±3.8% and 40.6±6.2%. With addition of m-Nd2 (1 μg/ml) during MLTC, cytotoxicity towards SW1990 and RWP-1 (positive expression against Nd2) was increased only slightly, but there was no increase in cytotoxicity to other PC cell lines. Cytotoxicity with c-Nd2 (1 μg/ml) (ADCC activity) towards SW1990, RWP-1 and Capan-1 (positive expression against Nd2) was significantly higher than cytotoxicity in PBS and with m-Nd2 (Fig. 1A). On the other hand,
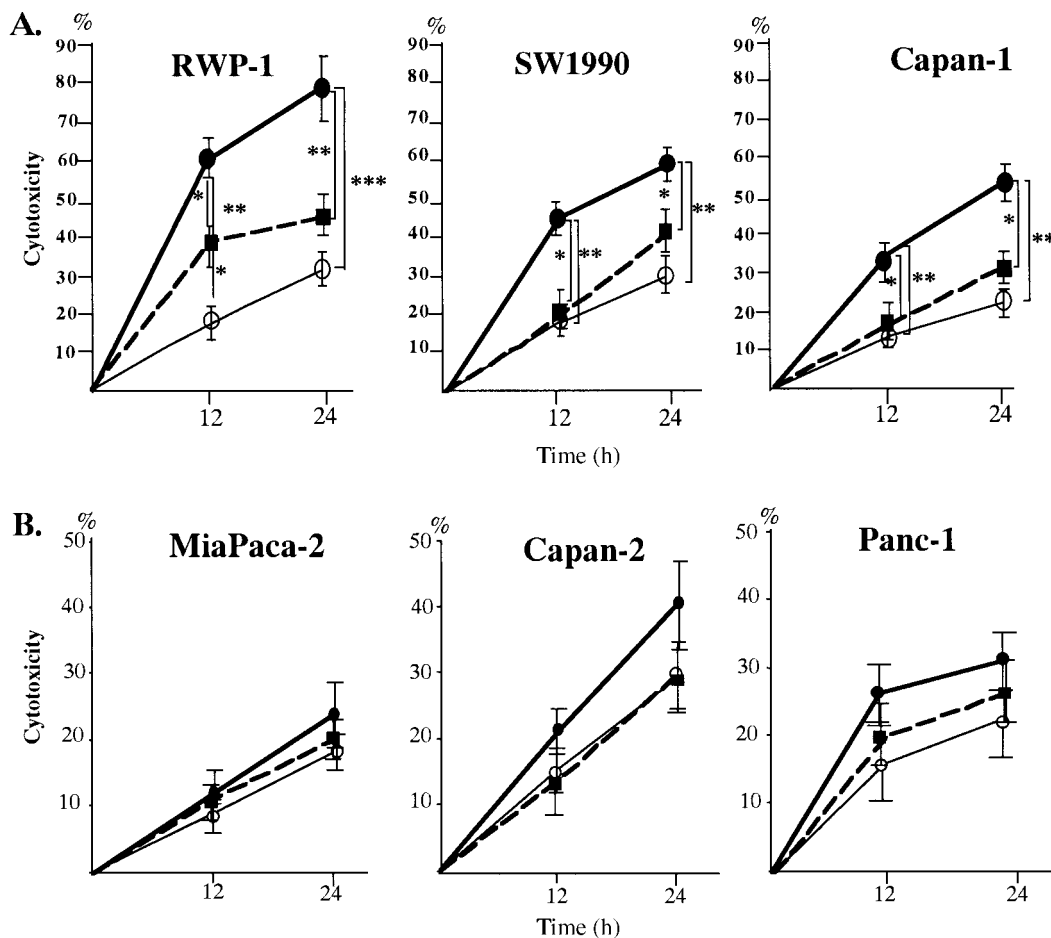

Fig. 1.   Cytotoxicity to pancreatic cancer cells induced during MLTC (E/T ratio: 50) with m-Nd2 and c-Nd2 (1 μg/ml). A. Cytotoxicity to Nd2 antigen-positive cells. C-Nd2 (●) induced significantly higher cytotoxicity compared with m-Nd2 (■) and PBS (○) towards Nd2-positive RWP-1, SW1990 and Capan-1 cells at 12 and 24 h after MLTC. RWP-1 cells were most sensitive to ADCC mediated with c-Nd2. B. Cytotoxicity to Nd2 antigen-negative cells. There is no difference in cytotoxicity with c-Nd2 (●), m-Nd2 (■) and PBS (○). No cytotoxicity (ADCC activity) induced with c-Nd2 was observed for Nd2 antigen-negative MiaPaca-2, Capan-2 and Panc-1 cells. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

no cytotoxicity (ADCC activity) induced with c-Nd2 was observed for PC cell lines which do not express Nd2 (Fig. 1B).

We also examined cytotoxicity during MLTC with various E/T ratios (10, 25 and 50) and various concentrations of MoAbs using RWP-1, for which c-Nd2 induced the highest cytotoxicity. ADCC activity in RWP-1 induced by c-Nd2 was significantly higher than that by m-Nd2, depending on the E/T ratio (Fig. 2A). ADCC activity with c-Nd2 was also concentration-dependent (Fig. 2B).

Anti-tumor effect of c-Nd2 in *in vivo* nude mouse model The growth of subcutaneously injected SW1990 tumor growth in nude mice was significantly inhibited in the c-Nd2 treatment groups compared with not only the PBS group, but also the m-Nd2-treated group by the 7th day after Ab inoculation. By the 14th day after inoculation, the growth of tumors treated with c-Nd2 was sup-

pressed to 48.5% of that of PBS-treated tumors and 67.3% of that of m-Nd2-treated tumors (Fig. 3).

Of orthotopically transplanted nude mice administered PBS and m-Nd2, 50% were dead by 60 days after transplantation, and all were dead by 70 days. In contrast, mice administered c-Nd2 showed 50% survival at 100 days.
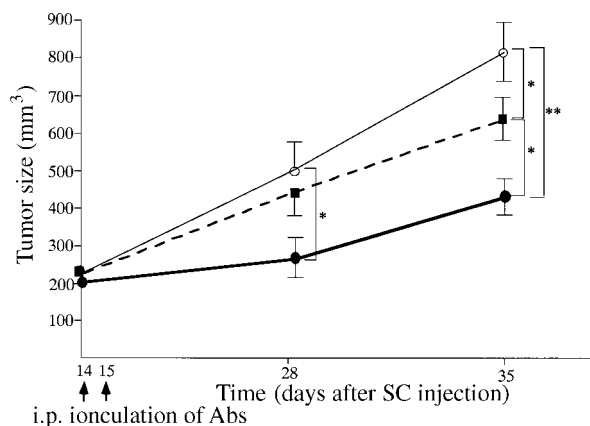
Fig. 3. *In vivo* inhibitory effect of c-Nd2 on subcutaneously-injected SW1990 tumor growth. Tumor growth in nude mice was significantly inhibited by two intraperitoneal injections of c-Nd2 (200 μg per animal in total, ●) compared with m-Nd2 (■) and PBS (○) by the 7th day after inoculation. By the 14th day after inoculation, the growth of SW1990 tumor treated with c-Nd2 was significantly suppressed in 67.3% of m-Nd2 and 48.5% of PBS. ∗ $P<0.01$, ∗∗ $P<0.001$. Tumor volume was calculated as Tumor volume (mm^3) = 0.4 × (major axis) × (minor axis)^2.
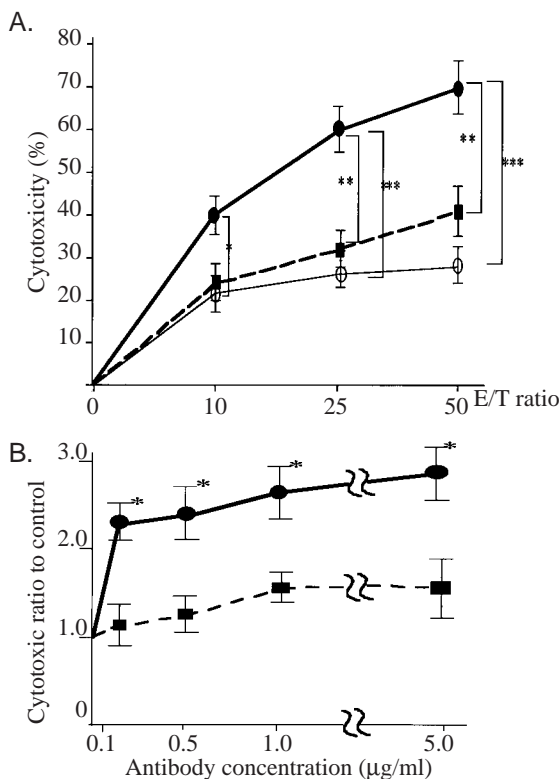

Fig. 2. Cytotoxicity during MLTC for RWP-1 cells at various E/T ratios and concentrations of Abs. A. Titration of effector cells. Cytotoxicity (ADCC activity) induced with c-Nd2 (1 μg/ml, ●) was significantly higher than that with m-Nd2 (1 μg/ml, ■) and PBS (○), depending on the E/T ratio. ∗ $P<0.05$, ∗∗ $P<0.01$, ∗∗∗ $P<0.001$. B. Titration of antibody. Cytotoxicity (ADCC) induced by c-Nd2 (●) at the E/T ratio of 50 increased depending on c-Nd2 concentration. Significantly higher ADCC was observed with c-Nd2 than with m-Nd2 (■). ∗ $P<0.01$.
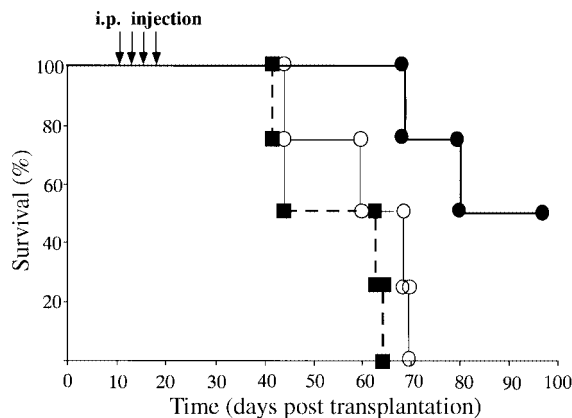

Fig. 4. Anti-tumor effect of c-Nd2 in terms of survival of orthotopically transplanted nude mice. Mice administered c-Nd2 (50 μg/body, total 200 μg, ●) revealed significantly extended survival time, with 50% survival at 100 days after transplantation. The mean survival times of mice undergoing intraperitoneal injection of PBS (○), m-Nd2 (■) and c-Nd2 were 45.8, 39.0 and 73.5 days, respectively.
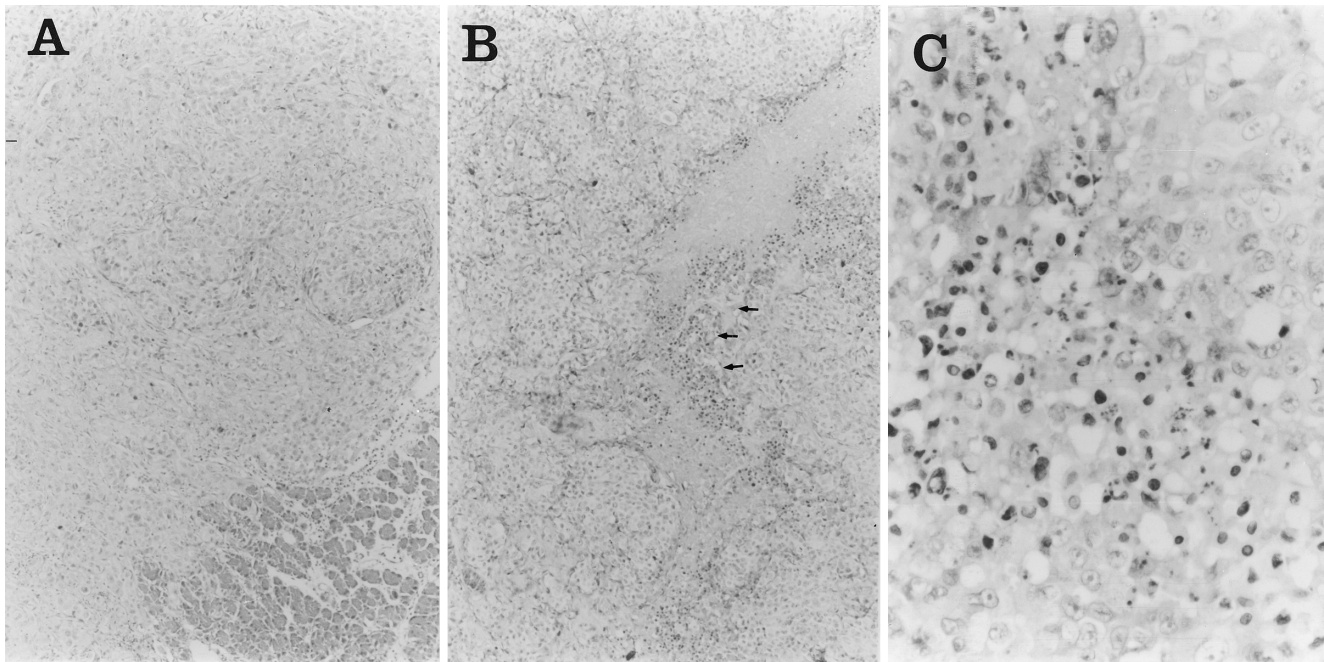
Fig. 5. Histological features of orthotopically transplanted tumor. A: PBS injection, B and C: c-Nd2 treatment (50 μg/body, total 200 μg, intraperitoneal injection). Tumors treated with PBS (A, ×200) were filled with viable cancer cells which invaded to the normal acinar cells. Tumors treated with c-Nd2 (B, ×200) exhibited tumor necrosis with mononuclear lymphocyte infiltration (arrow); most of the infiltrated cells were monocytes (macrophages) and neutrophils as judged from their morphology (C, ×400).

The mean survival times of the mice given intraperitoneal injection of PBS, m-Nd2 and c-Nd2 were 45.8±12.6, 39.0±11.0 and 73.5±15.0 days respectively (Fig. 4). The mice administered PBS exhibited huge transplanted tumors with ascites and more cachexic features than the mice treated with c-Nd2.

We examined orthotopically transplanted tumor treated with PBS and c-Nd2 histologically. Tumors treated with PBS were filled with viable cancer cells without infiltrating lymphocytes. On the other hand, tumors treated with c-Nd2 exhibited tumor necrosis with mononuclear lymphocytes (mostly macrophages as judged from their morphology) and neutrophil infiltration (Fig. 5).

Binding affinity of MoAb to and cell cytotoxicity assay with mouse PBMNC The binding affinities of m-Nd2 to mouse monocytes and lymphocytes were 45.6% and 19.8%, whereas those of c-Nd2 were 62.8% and 20.5%, respectively (Fig. 6).

The cytotoxicities to SW1990 with no antibody, m-Nd2 and c-Nd2 (1 μg/ml) were 17.5±1.7%, 30.3±3.8% and 35.9±3.6%, respectively; to RWP-1, 17.3±1.7%, 30.3± 3.8% and 34.8±3.7%; to Panc-1, 17.7±2.9%, 18.3±1.3% and 19.4±2.2%; to Capan-2, 19.8±3.8%, 21.1±3.7% and 20.3±2.2% (Fig. 7).

Cytotoxicity with c-Nd2 (1 μg/ml) among mouse PBMNC was significantly higher for SW1990 and RWP-1
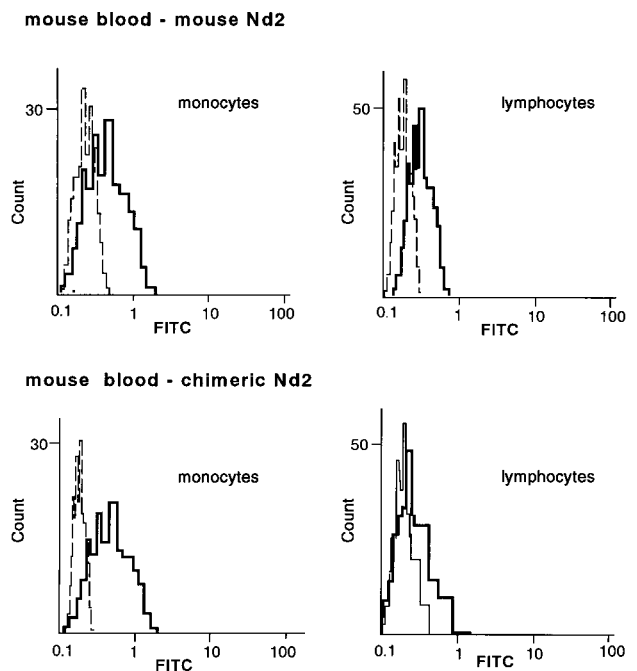

Fig. 6. Binding affinity of mouse monocytes and lymphocytes to c-Nd2 and m-Nd2 by FACScan. The binding affinities of c-Nd2 to monocytes and lymphocytes were 62.8% and 20.5% whereas those of m-Nd2 were 45.6% and 19.8%, respectively.
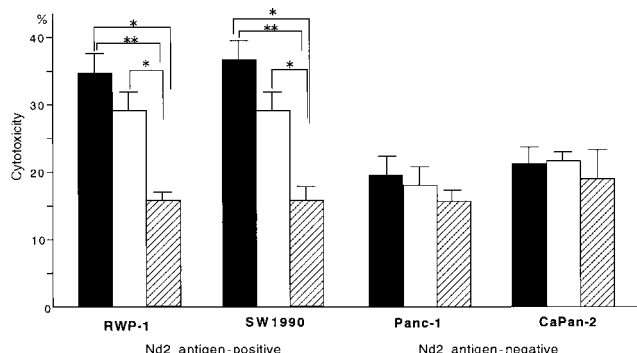
Fig. 7. Cytotoxicity to pancreatic cancer cells in mixed mouse leukocyte cell culture (E/T ratio: 50) with m-Nd2 and c-Nd2 (1 μg/ml). C-Nd2 (■) showed significantly higher cytotoxicity compared with m-Nd2 (□) and PBS (▨) to Nd2-positive RWP-1 and SW1990 at 24 h after MLTC. There is no difference in cytotoxicity with c-Nd2, m-Nd2 and PBS. No cytotoxicity (ADCC activity) induced with c-Nd2 was observed for Nd2-negative Capan-2 and Panc-1 cells. $* P < 0.001$, $** P < 0.05$.

(positive expression against Nd2) than that no antibody or m-Nd2. On the other hand, no cytotoxicity (ADCC activity) induced with c-Nd2 was observed in PC cell lines which lack expression of Nd2.

DISCUSSION

Targeting immunotherapy using MoAbs with high specificity and affinity may be a useful approach to the treatment of pancreatic cancer. Various monoclonal antibodies have been reported to produce tumor cell destruction through ADCC or CDC, but ADCC usually induces stronger cell lysis than CDC.[15, 22] ADCC involves target cell lysis induced by effector cells. Since effector cells with Fc receptors can recognize the Fc portions of immunoglobulins specifically binding to target cells, it is essential to prepare MoAb specific to target cells if ADCC is to be obtained.[23–25] In a previous study, we reported high specificity for and strong accumulation in pancreatic cancer of murine monoclonal antibody Nd2 produced against the human pancreatic cancer cell line SW1990. In order to reduce the side effects resulting from HAMA production in clinical trials, we produced c-Nd2 and found that its specificity and affinity to pancreatic cancer are similar to those of m-Nd2.[16] Since other investigators have suggested that chimeric antibodies are similar or superior to murine antibodies in ability to mediate ADCC, in this study we examined pancreatic cancer cell cytotoxicity during MLTC with Nd2 in order to determine the extent of induction of ADCC by c-Nd2. Cytotoxicity during MLTC for pancreatic cancer cell lines was increased by incubation with c-Nd2 in an E/T ratio- and dose-dependent fash-

ion for Nd2 positive-cells. The findings indicate that c-Nd2 has a strong ability to induce ADCC. Although c-Nd2 exhibited slight cytotoxicity even to Nd2-negative cells, this may be induced by nonspecific cell lysis of effector cells which react to c-Nd2 non-specifically binding to cancer cells.

The efficiency and strength of ADCC are affected by numerous factors, including the population and activity of effector cells, affinity and isotype of sensitizing antibody, quantity and nature of antigens expressed on native target cells, and others. In host defense against cancer, effector cells expressing Fcγ receptor (FcγR) against the Fc portion of IgG have been found to induce ADCC. Sung et al. reported that NK cells are the main effector population mediating ADCC.[26] On the other hand, Hanibuchi et al. and Pullyblank et al. have reported that many ADCC reactions are mediated by activated monocytes in addition to NK cells.[27, 28] It has been reported that rapidly induced ADCC (within 4 h) are mediated mainly by NK/K cells, while slowly induced ADCC (after 16–18 h) are mediated by monocytes/macrophages.[29–31] Johnson et al. have also reported that cancer cell lysis mediated by monocytes required 1 to 2 days for completion.[29] These findings suggest that ADCC by c-Nd2 is mainly induced by monocytes as effector cells, since the ADCC capacity for Nd2-positive cells increased after 12–24 h of incubation during MLTC with c-Nd2. The higher *in vitro* binding affinity of c-Nd2 to mouse monocytes compared with lymphocytes in the present study may also support this hypothesis.

To estimate the *in vivo* effect of ADCC induced by c-Nd2, we examined inhibition of subcutaneous xenograft tumor growth and determined the survival of orthotopically transplanted nude mice following intraperitoneal injection of c-Nd2. Our findings indicated that injection of c-Nd2 significantly inhibited tumor growth and prolonged survival compared with injection of m-Nd2 or no antibody. Preparation and characterization of various chimeric MoAbs have been previously reported. However, few studies of the induction of strong ADCC and *in vivo* inhibition of tumor growth by these MoAbs are available.

Steplewski and Sun reported differences in biological anti-tumor ADCC activity among immunoglobulin subclasses with chimeric IgG1 exhibiting activity superior to those of chimeric IgG2, IgG3 and IgG4.[32] Since the subclass of c-Nd2 used in our study was IgG1, c-Nd2 may have the capacity to induce sufficient ADCC activity and a strong anti-tumor effect *in vivo*. However, the mechanisms by which chimeric MoAbs exert their *in vivo* effect in mouse are not clearly understood. Hogarth et al. reported that mouse IgG antibodies were usually able to be recognized efficiently by FcγII (which is exhibited on monocytes and neutrophils) and FcγRIII (on NK cells) but not by FcγRI (on monocytes/macrophages),[33] while the Fc portion of chimeric or human IgG antibodies could usually

be recognized by not only FcγRII and FcγRIII, but also FcγRI, which has the highest affinity to the Fc portion of immunoglobulin.[34] Furthermore, histological findings of tumors treated with c-Nd2 in the present *in vivo* study demonstrated necrosis accompanied with strong infiltration of monocytes, and these findings are consisitent with the speculation that the main effector cells inducing ADCC with c-Nd2 are monocytes. Another study has also reported that antibody derived from human was recognized by murine FcR-positive effector cells as well as human effector cells, and van der Winkel *et al.* and Ravetch and Kinet reported that human Fc portion exhibits greater multiplicity than mouse Fc portion in ability to be recognized by human or mouse effector cells expressing FcγR.[35, 36] These reports are consistent with our result that c-Nd2 exhibited an *in vivo* anti-tumor effect via ADCC even though the effector cells originated from mouse, and we demonstrated the binding of c-Nd2 to mouse effector cells and ADCC induced by c-Nd2 with mouse PBMNC. These findings together suggest that targeting immunotherapy with c-Nd2 to induce ADCC may be clinically effective.

MoAbs conjugated with anti-tumor drugs or radioisotope have been used for targeting therapy in various clinical situations. While pancreatic cancer has an extremely poor prognosis due to its resistance to all current treatments including surgery, very few studies of targeting therapy using MoAb against pancreatic cancer are available. Targeting therapy using c-Nd2 should be useful in the treatment of pancreatic cancer by virtue of its ability to induce strong ADCC. It will be interesting to determine whether clinical administration of MoAb alone without conjugates can inhibit tumor growth. We are planning a further study aimed at clinical application of c-Nd2.

ACKNOWLEDGMENTS

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