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Protective Antitumor Activity through Dendritic Cell Immunization is Mediated by NK Cell as well as CTL Activation

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Dendritic cells (DCs) are potent professional antigen-presenting cells (APC) capable of inducing the primary T cell response to antigen. Although tumor cells express target antigens, they are incapable of stimulating a tumor-specific immune response due to a defect in the costimulatory signal that is required for optimal activation of T cells. In this work, we describe a new approach using tumor-DC coculture to improve the antigen presenting capacity of tumor cells, which does not require a source of tumor-associated antigen. Immunization of a weakly immunogenic and progressive tumor cocultured with bone marrow-derived DCs generated an effective tumor vaccine. Immunization with the cocultured DCs was able to induce complete protective immunity against tumor challenges and was effective for the induction of tumor-specific CTL (cytotoxic T lymphocyte) activity. Furthermore, high NK cell activity was observed in mice in which tumors were rejected. In addition, immunization with tumor-pulsed DCs induced delayed tumor growth, but not tumor eradication in tumor-bearing mice. Our results demonstrate that coculture of DCs with tumors generated antitumor immunity due to the NK cell activation as well as tumor-specific T cell. This approach would be useful for designing tumor vaccines using DCs when the information about tumor antigens is limited.

Key words : Dendritic cells (DCs), Colon tumor, Antitumor immunity

INTRODUCTION

Over the past several years, dendritic cells (DCs) have been identified as the most effective antigen-presenting cells (APC) (Banchereau and Steinman, 1998). They are believed to be crucial for the induction of a primary T cell response to foreign antigens (Steinman, 1991). They are regarded to be unique in their capacity to capture the Ags and present antigenic peptide fragments, to migrate to lymphoid organs and to induce primary immune responses of both $CD8^+$ and $CD4^+$ T cells. The ability of DCs to act as potent APC for the induction of the T cell response is attributed to the high expression of MHC class I, class II and adhesion and/or costimulatory mole-cules as well as their ability to produce cytokines essential for the acti-vation and proliferation of the T cells (Inaba *et al.*, 1994; Macatonia *et al.*, 1995).

Recently, some strategies of using tumor Ag-charged DCs as vaccines for cancer immunotherapy have been developed. Immunization of DCs pulsed with purified tumor-associated proteins or peptides has been shown to be a powerful method to elicit tumor-specific T cells, host protective and therapeutic antitumor immunity in mice and humans (Celluzzi *et al.*, 1996; Hsu *et al.*, 1996; Porgador *et al.*, 1996; Zitvogel *et al.*, 1996). However, this approach has a limited application in clinics due to a few human tumor rejection antigens so far identified. In addition, the high polymorphism of the human HLA system has also made it difficult to identify tumor-associated antigenic peptides as a vaccine for cancer immunotherapy. To overcome this hurdle, new methods for DC modification such as a treatment with tumor cell lysate, fusion of DCs with tumors, or coculture of them with intact tumor cells have been introduced (Celluzzi and Falo, 1998; Gong *et al.*, 1997; Nestle *et al.*, 1998). Recently, it has been demonstrated that DCs transfected with RNA encoded-antigens could function as potent APCs to induce antigen-specific CTL responses (Nair *et al.*, 1998).

In this study, we examined whether DCs cocultured with intact tumor cells would elicit antitumor immunity

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against a CT-26 colon tumor challenge and immune rejection of established tumor. The cultured DCs derived from mouse bone marrow showed characteristic morphology and phenotype of surface antigen expression. When they were cocultured with weak immunogenic CT-26 colon tumor cells, they were able to induce complete protective immunity against tumor challenges. The tumor protective immunity correlated well with tumor-specific CTL activity. Furthermore, high NK cell activity was observed in mice in which tumors were rejected. Immunization with tumor-pulsed DCs induced a delayed tumor growth, but not tumor eradication in tumor-bearing mice. Our results demonstrate that the cocul- ture of DCs with tumors generated antitumor immunity due to the tumor-specific T cell as well as NK cell activation. This approach would be useful for designing tumor vaccines using DCs when the information about tumor antigens is limited.

MATERIALS AND METHODS

Mice and tumor cell line

Four-to six-week-old male Balb/c mice were provided by the Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea). The mice were main-tained in specific pathogen-free conditions and were used at the age of 6-8 weeks. The studies in mice were performed in accordance with institutional guidelines. Murine Balb/c CT-26 tumor cells, which were initially developed and established as a colon carcinoma cell line in the National Cancer Institute (USA) tumor bank, were obtained from Seoul National Univer-sity Hospital (Seoul, Korea). Renca, a murine (Balb/c) renal adenocarcinoma, was obtained from Samsung Medical Center, Seoul.

Reagents for cell culture, antibodies, and cytokines

All cultures including tumor cell maintenance were performed in RPMI medium (Sigma, St. Louis, USA) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), Hepes (10 mM), 5×10^{-5} M 2-ME (Sigma) and 10% heat-inactivated FBS (Gibco, Grand Island, USA). Growth factors used in the primary cultures of DC precursors were recombinant mouse GM-CSF and IL-4 (Endogen, Woburn, MA, USA). FACS analysis for determining the antigen expression of DCs was performed using a culture supernatant of hybridoma cells against the following surface markers: CD4 (GK1.5), CD8 (3.168), CD11a (FD441), CD11b (M1/70), CD11c (N418), CD32 (2.4G2), CD54 (BE29G1), CD80 (16-10A1), CD86 (GL-1), CD90 (J1j.10), MHC Class I (34-1-2S) and MHC Class II (M5/114). The hybridoma cell lines were purchased from American Type Culture Collection (Rockville, MD, USA). Purified Anti-CD40 mAb was pur-chased from Serotec Inc (Raleigh, NC, USA). Goat anti-rat-PE and anti-syrian hamster-FITC were purchased from Phar-mingen (San Diego, CA, USA) and Jackson Immuno Research Labo-ratory (West Grove, PA, USA), respectively. Mouse or rat IgG_1-FITC/PE isotype control (Pharmingen), FITC-coupled goat F(ab')$_2$ anti-mouse or rat IgG (Biosource International, Camarillo, USA) was used as an isotype control and a secondary reagent, respectively.

Generation of dendritic cells from mouse bone marrow cells

For DC generation a method by Inaba *et al.* (Inaba *et al.*, 1992) was slightly modified. Briefly, bone marrow cells from Balb/c mice were harvested and incubated with an antibody cocktail containing J1j. 10, J11d, 3.168 and GK 1.5 at 4°C for 1 h. They were washed twice with culture medium and treated with rabbit complement (Low-Tox®-M, Cedarlane, Ontario, Canada) according to the manufacturer's instruction. Live cells were isolated by density centrifugation on Histopaque 1077 (Sigma), and washed out twice with RPMI 1640 medium without serum. The lymphocyte-depleted BM cells were then incubated in culture medium (DC medium) supplemented with mouse GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) in 24-well plates (Costar, Cambridge, USA) at $5\text{-}10 \times 10^5$ cells/ml. On day 2 and 4, non-adherent cells were discarded and the culture media were replaced with fresh DC medium. On day 6 or 7, the non-adherent cells were harvested by a gentle swirling, counted and used in the studies. Cell viability was over 95% as determined by flow cytometer after propidium iodide staining.

Flow cytometric analysis for phenotype staining

DCs generated from *in vitro* culture were subjected to flow cytometric analysis using FACScan (Becton Dickinson). Cells were incubated either with various unconjugated mAbs on ice for 30 min, washed twice, then incubated with FITC-conjugated goat anti-mouse IgG for 30 min on ice, or with FITC- or PE-conjugated mAbs, as described previously (Kim and Cohen, 1997). Simultaneous measurement of FITC and PE was performed using 530 nm and 575 nm filters with acquisition in a logarithmic mode.

Determination of mixed lymphocyte reactions

Splenocytes were prepared from C57BL/6 mice and 1×10^5 cells were incubated with various number of irradiated (900 rad) DCs or plastic adherent monocytes from Balb/c mice in U-bottom 96-well microtiter culture plates for 5 days. The proliferation of splenocytes was quantitated by thymidine uptake of cells incubated with 1 μCi of [methyl-^{3}H] thymidine (NEN-DuPont, Boston,

USA) for the last 18 hrs. The cells were harvested onto glass fibre filters (Inotech Biosystems, Zürich, Switzerland) and the radioactivity was measured in a scintillation counter. Results are presented as the mean cpm of triplicate cultures.

Immunization of mice

For measuring protective immunity, Balb/c mice were subcutaneously immunized with either irradiated (20,000 rad) DCs (4×10^5), CT-26 tumor cells (2×10^5) or DCs cocultured with the same number of tumor cells for 40 hrs. Control mice were immunized with phosphate-buffered saline. Seven days after immunization, three mice of each group were injected s.c. with 2×10^5 viable tumor cells. Mice were also injected s.c. with 2×10^5 viable tumor cells for measuring therapeutic effect. Vaccination was done on day 9 once or days 9 and 12 twice by injecting s.c. DCs, tumor cells, or tumor-pulsed DCs after irradiation. The tumor size of each group of mice was measured in two perpendicular dimensions with a vernier caliper.

Cytotoxicity assay

The activity of cytotoxic T lymphocytes (CTLs) and the natural killer (NK) cell activity of splenocytes were determined by measuring the specific cytotoxic activity against ^{51}Cr-labeled CT-26 tumors or YAC-1 target cells in a standard 4-h chromium assay at several effector/target (E/T) ratios. Briefly, 2×10^6 target cells were labeled with 100 μCi (NEN-DuPont) of sodium chromate for 1.5 h at 37°C in CO_2 incubator. A total of 2×10^4 thoroughly washed cells in 100 μl of complete medium were then distributed into wells of round-bottomed microtiter plates in triplicate containing effector cells at various concentrations. After incubation for 4 hrs, 100 μl of supernatant was collected, and ^{51}Cr release was measured in a gamma counter (Wallac Inc., Gaithersburg, MD, USA). The percentage specific lysis was calculated as follows: 100×(cpm of test sample-cpm of medium)/(cpm of maximum control-cpm of medium), where cpm is counts per minute. The spontaneous release of target cells was usually 7-9 %.

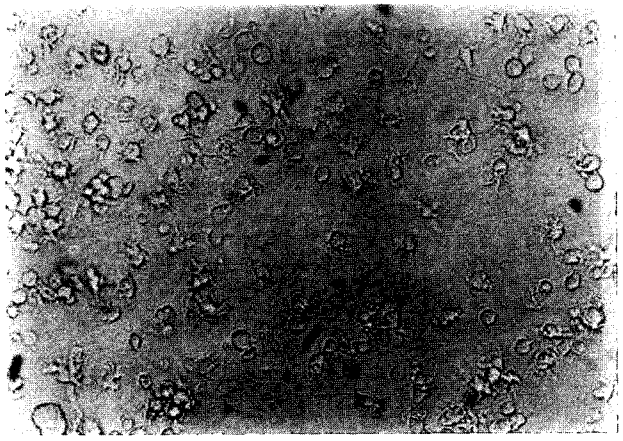

Fig. 1. Microscopic image of mouse DCs derived from bone marrow and cultured for 1 week *in vitro*. The bone marrow cells from Balb/c mice were treated with T and B cell-specific antibodies followed by a treatment with rabbit complement. DCs were generated in the presence of recombinant GM-CSF and IL-4 as described in Materials and Methods. Photograph was obtained by an inverted microscopy with magnification (× 100).

RESULTS

Generation and characterization of dendritic cells from mouse bone marrow cells

Dendritic cells (DCs) were generated from bone marrow cells of Balb/c mice using complete media supplemented with mGM-CSF and mIL-4. On Day 7 of

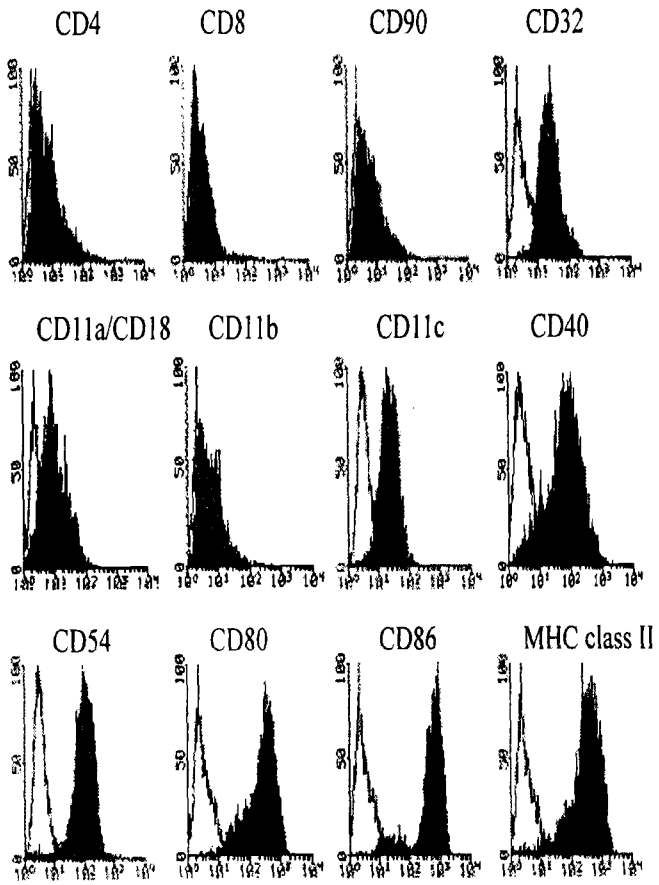

Fig. 2. Expression of surface antigens on DCs generated from bone marrow and cultured for 1 week *in vitro*. DCs were generated as described in Materials and Methods. They were harvested after a culture of 7 days, washed and stained with respective mAbs. The surface antigen expression was measured with a flow cytometer (Becton Dickinson) and analyzed with FACScan® and LYSIS™ software.

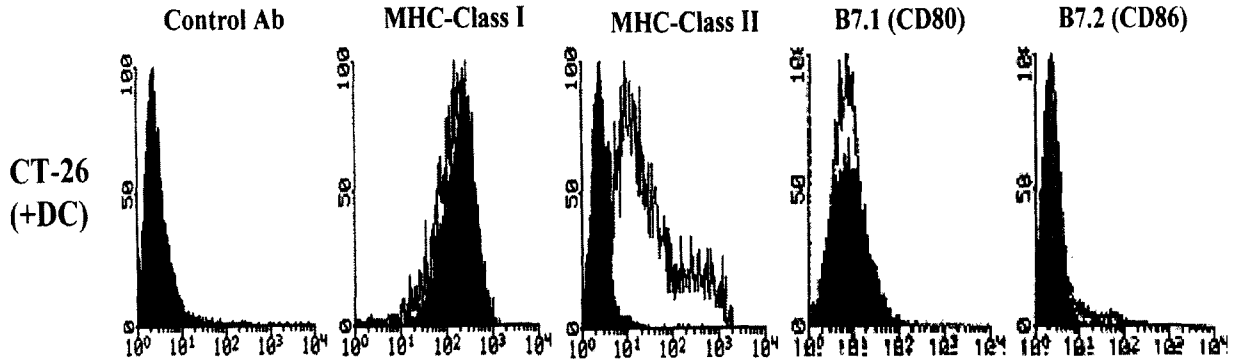

Fig. 3. Changes of surface antigen expression on CT-26 tumor cells after coculture with DCs. Tumor cells were incubated with DCs for 40 hrs in a 12-well culture plate. After staining with respective mAbs, FACScan profiles of the tumor cells were obtained by gating out CD11c-positive DC population.

culturing, numerous clusters of large cells were evident and examination of the cultures using phase-contrast light microscopy revealed that the cells exhibited a characteristic dendritic morphology displaying ruffled membranes (Fig. 1). Analysis of cell surface markers demonstrated that the cultured dendritic cells expressed CD11c, CD32 (FcRII), CD54 (ICAM-1), CD80 (B7.1), CD86 (B7.2) and MHC Class II antigen (Fig. 2). In addition, these cells expressed very low levels of CD4 and CD11a antigens, whereas they did not show expression of CD8, CD11b and CD90 (Thy1.1) antigens. When they were compared with plastic adherent monocytes for their ability to stimulate allogeneic splenocyte from C57BL/6 mice, they were much more potent than monocyte in eliciting allogeneic T cell proliferation (data not shown). These results showed that the cells we generated had the morphology, surface phenotype and potent stimulatory ability of DCs.

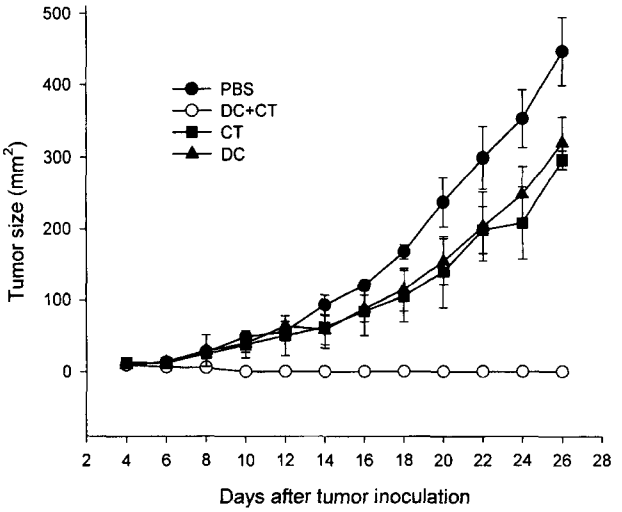

Fig. 4. Protective effect of tumor-pulsed DCs against live CT-26 colon tumor challenge. Mice were subcutaneously immunized with either irradiated (20,000 rad) DCs (4×10^5), CT-26 tumor cells (2×10^5) or DCs cocultured with the same number of tumor cells for 40 hrs. Control mice were immunized with phosphate-buffered saline. Seven days after immunization, three mice of each group were injected s.c. with 2×10^5 viable tumor cells. Tumor size was measured in two perpendicular dimensions with a vernier caliper every other day.

Induction of the host protective effect by tumor-cocultured DCs

On Day 7, the nonadherent DCs were harvested by gentle pipetting and 75-80 % of the cells expressed the N418 Ag (CD11c). They were usually used in further studies, although more than 90 % of the DCs, if purified using 14.5% metrizamide (Sigma) gradient, were N418 Ag positive (data not shown). When the surface antigen expression of CT-26 tumor cells was analyzed, tumor cells expressed MHC class I molecules and low levels of B7.1 molecules, but not B7.2 and MHC class II mole-cules. After coculture with the DCs, the surface antigen expression of tumor cells was not changed for 40 hrs, but a significant increase of class II molecules was observed (Fig. 3). In order to test whether DCs pulsed with tumor cells induce antitumor immunity, 4 groups of mice were preimmunized with PBS, irradiated tumor cells, DCs or DCs cocultured with tumor cells. When the DCs co-cultured with tumor cells were immunized into syngeneic Balb/c mice 1 week before tumor inoculation, they induced a protective effect against tumor challenges (Fig. 4). In the group immunized with DCs or tumors alone, a slight retardation of tumor growth, but no tumor protection, was seen when compared with the tumor growth of the PBS group. Furthermore, we tested whether LPS stimulation of DCs or tumor-pulsed DCs had a better protective effect on tumor challenges, since LPS can induce an activation of DCs such as the upregulation of surface markers mediating T cell stimulation (De Smedt *et al.*, 1996). However, the tumor size in the tumor bearing mice from the LPS-stimulated, tumor-pulsed DC groups

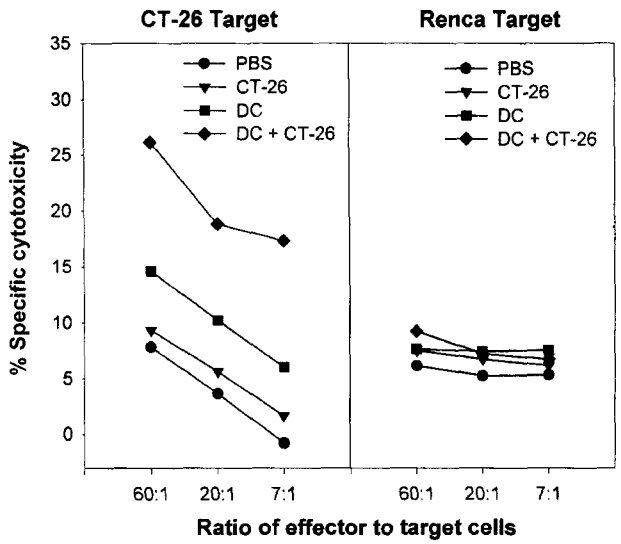

Fig. 5. Enhancement of tumor-specific cytotoxic T lymphocytes (CTLs) activity of splenocytes obtained from a mouse pre-immunized with tumor-pulsed DCs. Mice were preimmunized with PBS, irradiated tumor, DC or DC-tumor coculture and injected with live tumor cells one week later as described in Fig. 4. Mouse splenocytes prepared from each mouse 38 days after live tumor challenge were cultured *in vitro* for five days. CTL activity was determined against CT-26 tumors (left) and Renca (right) target cells by a ^{51}Cr release assay.

was not significantly different from that of the DC groups, suggesting that LPS stimulation *in vitro* of tumor-pulsed DCs did not enhance the tumor-specific immunity (data not shown).

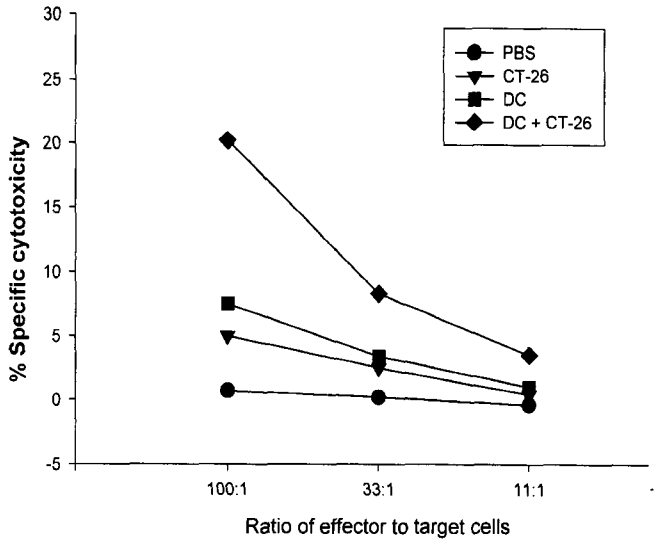

Fig. 6. Vaccination of tumor-pulsed DCs increases natural killer (NK) cell activity. Splenocytes obtained from mice as described in Fig. 5 were tested for their NK cell activity against YAC-1 target cells as described in Materials and Methods.

Immunization of tumor-pulsed DCs augments NK cell activity as well as tumor-reactive CTLs

To determine whether vaccination with DC/CT-26 can elicit tumor-reactive T cells, splenocytes from each mouse of 4 groups were harvested, cultured for 5 days in vitro and tested against CT-26 tumor target cells. Lymphocytes from mice vaccinated with PBS buffer or irradiated tumor cells showed a very weak cytotoxic activity, whereas vaccination with irradiated DCs alone induced an intermediate CTL activity (Fig. 5). In contrast, the cultured lymphocytes from tumor-free mice vaccinated with DC/CT-26 elicited CTLs that specifically lysed CT-26 tumors, but not syngeneic Renca tumor cells, indicating that the antitumor CTL activity was tumor specific. In similar experiments, spleen cells were tested for cytotoxicity against NK target cells, YAC-1. The results showed that splenocytes from mice vaccinated with PBS buffer had no detectable cytotoxicity against YAC-1 target cells (Fig. 6). However, splenocytes from tumor- or DC-vaccinated mice had weak cyto-toxicity against YAC-1, while splenocytes from DC/CT-26-vaccinated mice significantly lysed YAC-1. When NK cells from spleens were stained with antibody (DX5) against pan-NK cell marker antigen, there was no significant difference of NK cell population, suggesting that the increased NK cell activity of splenocytes was not due to the increase of NK cell numbers in spleens. These results demonstrate that the protective immunity against CT-26 colon tumor cells resulting from vac-cination with DC-tumor coculture was mediated by not only tumor-specific CTLs, but also natural killer (NK) cell activation.

Immunization of tumor-cocultured DCs induced growth retardation of established tumors, but not tumor eradication

Recently, it was reported that DCs primed with tumor antigen *in vitro* could mediate effective therapy of established tumors. Thus, we also examined whether tumor-cocultured DCs can induce therapeutic effects on established tumors. CT-26 tumor cells were inoculated on one side of the abdomen. On Day 9, when the tumor with a diameter of 5 to 6 mm was obvious, injection of buffer, irradiated tumors, DCs or tumor-pulsed DCs was performed on the same site. As shown in Fig. 7A, the growth inhibition was pronounced in the groups treated with irradiated tumors or tumor-pulsed DCs. Tumor growth inhibition was not observed in the DC-treated group. The additional immunization 3 days after the first injection also produced a partial response of tumor growth inhibition in the groups of tumors or tumor-pulsed DCs (Fig. 7B). Tumor-pulsed DCs, however, resulted in a better tumor inhibition than tumors without DCs. Complete tumor regression was not noticed in any group. These results indicated

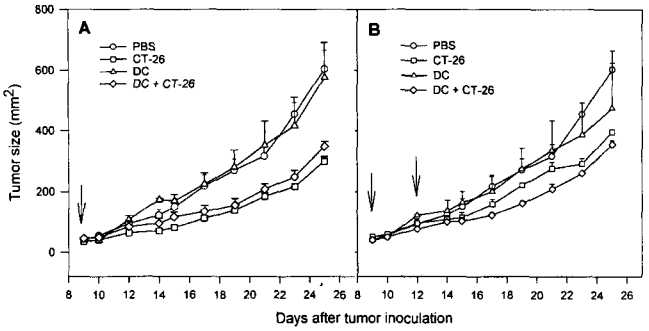

Fig. 7. Induction of tumor growth inhibition by a therapeutic vaccination with tumor-pulsed DCs when injected into tumor-bearing mice. Mice were injected s.c. with 2×10^5 viable tumor cells. Vaccination was done once on Day 9 (A) or twice Days 9 and 12 (B) by injecting s.c. tumor cells (2×10^5), DCs (4×10^5) or tumor-pulsed DCs after irradiation.Tumor size was measured in two perpendicular dimensions with a vernier caliper.

that repeated vaccinations might be needed to induce host antitumor immune responses enough to eradicate the established tumor. This is supported by the observation that tumor growth was inhibited from the beginning in the treatment, but tumors rapidly grew back again (Fig. 7).

DISCUSSION

In this study, mouse DCs were generated from *in vitro* culture of bone marrow cells after a negative selection using T and B lymphocyte-specific antibodies and rabbit complement in the presence of IL-4 and GM-CSF in order to apply them to the induction of antitumor immunity. They showed a characteristic morphology of bone marrow-derived DCs and highly expressed a variety of surface antigens including CD11c, ICAM-1, B7.1, B7.2 and MHC class II molecules as described in detail by other groups (Inaba *et al.*, 1992; Scheicher *et al.*, 1992; Scheicher *et al.*, 1993; Winzler *et al.*, 1997). They were also able to function as potent antigen-presenting cells in allogeneic mixed lymphocyte reaction when compared to plastic adherent monocytes.

To determine the antitumor efficacy of DCs, we selected a CT-26 mouse colon tumor cell line that developed a measurable tumor in syngeneic Balb/c mice when injected subcutaneously. Mice immunized with irradiated cells from DC-CT-26 cocultures were completely protected from challenge with CT-26 tumor cells. Groups of mice injected with PBS, irradiated DCs or tumor cells alone were not protected and developed tumors. The splenic T lymphocytes prepared from tumor-free mice showed a tumor-specific lytic activity, whereas those from tumor-bearing mice had weak or no lytic activities. Furthermore, immunization of DC-tumor elicited significant NK cell activity of splenocytes from tumor-free mice. These results demonstrate that immunization with DC-tumor cell vaccines can induce NK cell activities as well as tumor-specific CTLs. The coincubation of DCs with tumor cells was necessary for immunogenicity, because identical numbers of DCs and tumors that had not been cocultured but were injected together did not induce protection against a tumor challenge (data not shown). The fact that a direct DC-tumor cell contact was necessary was supported by a recent observation in which the separation of two cell types by a porous membrane barrier did not induce lytic activity against tumor cells (Celluzzi and Falo, 1998). However, when the therapeutic potential of this immunization strategy was tested, we observed a retardation of tumor growth, but not a tumor irradiation, in the groups of not only irradiated DC-tumors, but also tumor immunization. These data suggest that tumor regression mediated by host immune response is tumor burden dependent and repeated vaccinations might be needed to induce host antitumor immune responses enough to eradicate the established tumor.

It has been known that insufficient tumor Ag presentation by either tumor cells or host professional APC could be a major cause of the absence of an immune response in tumor-bearing hosts (Schuler and Steinman, 1997). Recent studies showed that vaccination with MC 38 carcinoma and DC fusion cells elicited T cell protective immunity against tumor challenge and induced immune rejection of established tumors (Gong *et al.*, 1997). Vaccination with B16 melanoma and DC fusion cells also induced host protective immunity against B16 tumor challenge (Wang *et al.*, 1998). Although this approach has several advantages compared with other strategies for developing DC-based vaccines such as loading DCs with tumor antigenic proteins or peptides, it remains a technical challenge to efficiently separate the tumor/DC hybrids from parental tumor cells in the fusion preparations, because the unfused tumor cells invariably overgrow the fusion hybrid without separation. In contrast, immunization with a coculture preparation of DC and tumor cells represents a simpler and straightforward strategy to elicit antitumor immunity.

The precise mechanisms by which DC-tumor cell conjugate vaccines induce antitumor immunity remain unclear. It is possible that the association of tumor cells with DCs confers sufficient costimulatory signals to stimulate T cells specific for Ag-MHC complexes expressed by the tumor cell either by coexpression of the costimulatory function or by presenting antigenic ligand in close physical association. It is also possible that intimate association of DCs with tumor cells facilitates the transfer of tumor-associated Ags to appropriate Ag-processing pathways of APCs. Although exogenous proteins do not enter the MHC class I-restricted processing pathway, they can enter the class I pathway of APCs if

it is present in high concentration, is processed or degraded extracellularly, or is associated with cell debris (Jondal *et al.*, 1996; Rock, 1996). Interestingly, vaccination with DC and tumor cells enhanced NK activity in our study. NK cells are known to be potent producers of IFN-γ at an early stage of activation, and may direct the development of a tumor-specific Th1 and CTL response. Therefore, NK and CTL cytotoxic immune responses appear to be cross-regulated and injection of DCs cocultured with tumors may lead to enhancement of both responses (Kos and Engleman, 1996). In this regard, there is evidence that despite their strong class I expression, syngeneic bone marrow-derived DCs and macrophages can be lysed *in vitro* by NK and lymphocyte-activated killer (LAK) cells and this overriding of the inhibitory effect of class I molecules on NK cells has been attri-buted to the strong expression of B7 molecules on DCs (Chambers *et al.*, 1996). Recently, it was also shown that DC vaccines interact with host NK cells as well as with antigen-specific T cells (Cayeux *et al.*, 1999). However, whether the induction of NK activity by DC immunization is crucial for the tumor-specific CTL induction against tumors remains to be defined.

In summary, our data show that the coculture of a weakly immunogenic, progressive tumor with bone marrow-derived DCs may generate an effective tumor vaccine. Immunization with the cocultured DCs was able to induce complete protective immunity against tumor challenges and was effective for the induction of tumor-specific CTL activity. Furthermore, high NK cell activity was observed in mice in which tumors were rejected. In addition, immunization with tumor-pulsed DCs induced a delayed tumor growth, but not tumor eradication in tumor-bearing mice. Our results demonstrate that coculture of DCs with tumor generated antitumor immunity due to NK cell activation as well as the tumor-specific T cell activation. This approach is clinically useful for designing tumor vaccines using DCs when the information about tumor antigens is limited.

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