INTRODUCTION

Dendritic cells (DCs) are antigen-presenting cells (APC) with a key role in the immune system as initiators and stimulators of naive T-cell responses against microbial pathogens and tumors (1). Since the recognition of DCs in lymphoid organs in 1973 (2), research on these cells has increased because they might be useful for antitumor immunotherapy (1, 3, 4). Furthermore, the central role of DCs in the initiation of immune responses and new methods for the generation of large numbers of pure DCs by culturing progenitor cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α and interleukin (IL)-4, creates possibilities for the development of novel immunotherapeutic strategies against tumors and other diseases (5-8).

DCs are present in small numbers in most tissues, including skin, lung, liver, spleen, blood, lymphoid organs, and bone marrow (9). Morphologically, they are large cells with elongated stellate dendrites. These cells have been shown to internalize and process exogenous or endogenous antigens efficiently, and to present soluble antigens as peptides in conjunction with MHC classes I and II (10, 11). In addition, DCs have the unique ability to cause clustering of naive T-cells. They also respond to antigen by rapid upregulation of the expression of MHC factors and co-stimulatory molecules, the production of cytokines, and migration toward lymphatic organs (12, 13). DCs, helper T-cell-dependent antibody responses, as well as the generation of primary and secondary cytotoxic T-cell responses to tumor-associated antigens, have proven to be effective immunogens when pulsed with tumor-associated antigens (9, 14). However, T-cell defined epitopes that can be presented by DCs have not been identified for most tumors. One study suggested that immunization with undefined tumor antigens might be more effective in eliciting antitumor immunity (15). Although the use of a single antigen-derived epitope has been shown to effect antitumor immunity in murine models, the presentation of multiple antigen-derived epitopes may enhance antitumor immunity. Johnston et al. demonstrated that the increased immunogenicity of tumor cells expressing the B7.1 gene was caused by the expansion of the antigenic repertoire of the tumor (16). Because of the extensive diversity of MHC antigens, designing common peptides for T-cell recognition for different individuals with the same disease may be difficult to prove (17). To circumvent this problem and to pro-

Active Immunization Using Dendritic Cells Mixed With Tumor Cells Inhibits The Growth Of Lymphomas

Dendritic cells (DCs) are potent antigen-presenting cells for the induction and activation of cytotoxic T lymphocytes. We tested whether bone marrow-derived DCs are capable of inducing protective immunity against a murine lymphoma (A20). DCs were grown from tumor-bearing BALB/c mice by culturing bone marrow cells. BALB/c mice were injected (sc) with A20 cells on day 0. Intraperitoneal immunization with DCs mixed with lethally irradiated A20 cells were started when the tumor reached ca. 4-5 mm in diameter (Group A) or on day -7 (Group B). Booster immunizations were given every 3-4 days for four weeks. By 31 days in group A, there was a significant reduction in tumor growth in the mice immunized with DCs mixed with irradiated A20 cells as compared with the control groups (p =0.016). In group B, tumor growth was completely inhibited and there was no tumor growth following extended observations after completion of immunization. Thus, DCs mixed with irradiated tumor cells can induce an antitumor effect. This provides a rationale for the use of DCs mixed with irradiated tumor cells in immunotherapy for minimal residual disease of lymphomas.

Key Words : Lymphoma; Vaccination; Dendritic cells; Neoplasm, Residual
vide DCs with presentable tumor-associated peptides, several methods have been developed to isolate immunogenic peptides from tumor cells. However, there is no defined standard method. Despite the advances in chemotherapy, including high-dose therapy with autologous stem cell transplantation for lymphomas, relapse of the underlying disease remains a significant obstacle (18). Recent advances in cancer vaccine development now make it possible to consider combining active specific immunotherapy as a strategy for the elimination of minimal residual disease.

The aim of this study was to assess the ability of bone marrow-derived DCs to induce therapeutic and protective immunity against a murine lymphoma model. Using immunization with DCs mixed with lethally irradiated target tumor cells, we investigated whether tumor cells, as a source of undefined tumor antigens, could intensify the antitumor immune response of DCs, and evaluated whether the suppression of tumor growth was correlated with T-cell function.

MATERIALS AND METHODS

Mice

Female BALB/c AnN (BALB/c) mice (6-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and housed in specific pathogen-free units of the Animal Resources Center at Asan Institute for Life Science. Mice were maintained and treated according to National Institutes of Health guidelines. All aspects of the studies requiring animal experimentation were approved by the Asan Institute of Life Science Animal Care and Use Committee.

Cell Lines

A20 lymphoma cells were purchased from American Type Culture Collection (ATCC: Rockville, MD, U.S.A.). This is a BALB/c B lymphoma cell line derived from a spontaneous reticulum cell neoplasm found in an old BALB/c mouse (19, 20). Cells were maintained in complete RPMI 1640 medium (GIBCO BRL, Aithersburg, MD, U.S.A.) supplemented with 100 IU/mL penicillin (Sigma, St Louis, MO, U.S.A.), 0.1 mg/mL streptomycin (Sigma), 10^{-5} M \beta -mercaptoethanol (Sigma), and 10% fetal bovine serum (FBS: GIBCO BRL).

Tumor-Bearing Bone Marrow Cell Culture and Isolation of Dendritic Cells

A20 tumor cells (2 \times 10^5) were injected subcutaneously (sc) into the shaved back of BALB/c mice. Animals were sacrificed three weeks after the injection, when the tumor reached 5-10 mm in diameter. Bone marrow cells were obtained from the femurs and tibias of BALB/c mice as described (5). After three washes in RPMI 1640, mononuclear cells were obtained and allowed to adhere to a tissue culture flask for 3 hr at 37°C. The adherent cells were then removed and non-adherent cells were placed in 100 mm diameter tissue culture dishes at a concentration of 1 \times 10^5/mL in the medium supplemented with 20 ng/mL murine recombinant (r) GM-CSF (R & D Systems, Minneapolis, MN, U.S.A.), 10 ng/mL murine IL-4 (R & D) and 2.5 ng/mL murine TNF-\alpha (R & D). Culture dishes were fed once every three days. On day 11, non-adherent cells were harvested and used for assays and immunization.

Evaluation of Cell Yield and FACS Analysis

Cultured cells were washed once and an aliquot volume was mixed 1:1 in Trypan blue solution (Sigma). Trypan blue negative, and large cells were counted as viable under the microscope in a Neubauer chamber and cells were identified by their distinctive morphology. Cells (1 \times 10^6) were incubated with the corresponding antibody for 30 min at 4°C. The cells were then washed and fixed in 1% paraformaldehyde. Flow cytometric analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA, U.S.A.). DCs were phenotyped with antibodies to the following markers: isotype controls for hamster IgG; rat IgG2a; DC markers DEC-205 (NLDC-145) and CD11c; co-stimulatory/adhesion molecules CD80 (B7-1) and CD86 (B7-2); macrophage markers CD14 and F4/80, and granulocyte marker Gr-1 (Pharmingen, Hamburg, Germany).

Preparation of Splenocytes

Spleen cells were obtained from the same mice, and suspended and red cells were lysed with ammonium chloride. To obtain purified T-cells, spleen cells were incubated in tissue culture flasks for 1 hr in medium as above, and non-adherent cells were collected and filtered through a nylon wool column.

Mixed Leukocyte Reaction (MLR)

DCs (5 \times 10^5) from BALB/c AnN mice were irradiated (1,500 rads) to stimulate the proliferation of DCs and were added to T-cells (5 \times 10^5) from BALB/c mice. A20 cells (5 \times 10^5 lethally irradiated at 10,000 rad) were co-cultured in 96-well plates for each immunized group. The cells were co-cultured for three days at 37°C (under 5% CO_2). Cells were divided into three groups: DCs mixed with T-cells, lethally irradiated A20 cells plus T-cells, and T-cells only.

Each group of cells was incubated for 18 hr with 1 \mu ci of [H] thymidine (Amersham, Arlington Heights, IL, U.S.A.) at the end of the three-day culture, and isotope incorporation was determined using a liquid scintillation counter. Results were tested three times in triplicates.
Cytotoxic T Lymphocyte (CTL) Assay

Cytotoxic activity of the stimulated cells was measured using a 6 hr $^{51}$Cr release cytotoxic assay (21). For the immunization group, DCs ($4 \times 10^5$) from BALB/c mice were irradiated (1,500 rads), added to T-cells ($4 \times 10^5$) from BALB/c mice, and $4 \times 10^5$ of lethally irradiated (10,000 rads) A20 cells, and co-cultured in 12-well plates for six days at 37°C (under 5% CO$_2$). Control groups were cultured DCs mixed with T-cells, T-cells mixed with lethally irradiated A20 cells, or T-cells alone. Target cells (A20 cells) were labeled for two hours with $^{51}$Cr at the end of the three day culture, then washed three times and $2 \times 10^5$ of the target cells were mixed with each group of effector cells. After incubation of six hours, supernatants were harvested, and the amount of $^{51}$Cr release was measured using a Packard Parias gamma spectrometer (Packard Instruments, Meridian, CT, U.S.A.). Results were tested three times in triplicate. The maximum and spontaneous release and the percentage of specific release were determined as described (21).

Post-Tumor Induction Immunization

For the evaluation of the effect of immunization on the growth of the established tumor, mice were injected (sc) with $2 \times 10^5$ A20 cells. Intraperitoneal immunizations with $2-4 \times 10^5$ DCs mixed with lethally irradiated (10,000 rads) $2 \times 10^5$ A20 cells were started when the tumors reached 5-10 mm in diameter.

Mice in control groups were given intraperitoneal inoculations of phosphate-buffered saline solution (PBS), or $2-4 \times 10^5$ DCs alone. Each group contained five mice and booster immunizations were performed every three days for four weeks.

Mice were examined daily, and tumor volumes were recorded daily with calipers.

Immunization Before Tumor Implantation

Intraperitoneal immunizations with $2-4 \times 10^5$ DCs mixed with $2 \times 10^5$ lethally irradiated (10,000 rads) A20 cells were started on day -7. The mice in control groups were given intraperitoneal injections of PBS only. Booster immunizations were performed every three days for four weeks. On day 0, the mice were injected sc with $5 \times 10^5$ of A20 cells, and tumor volumes were recorded daily using calipers.

Evaluation of the Effect of Immunization on T-Cells

Splenocytes were obtained from each group of mice after the final post-tumor induction immunization. Viable T-cells were counted using trypan blue vital stain, and FACScan analysis was performed to compare the ratio of CD4+/CD8+ cells using anti-CD4/FITC, anti-clone MT310+/ anti-CD8/PE, and anti-clone DK 205 antibodies (DAKO, Denmark). Estimation of the induction activity of T-cells after DC immunization was performed on $1 \times 10^5$ T-cells in a [H] thymidine uptake test.

For the evaluation of the efficacy of immunization associated with alteration of the Th1/Th2 profile of the T-cells in tumor-bearing mice, $1 \times 10^5$ of splenic T-cells were cultured for three days at 37°C (5% CO$_2$) and the concentrations of IL-2 and IL-4 proteins in supernatants were measured with ELISA kits (R&D).

Apoptosis Assay

For the evaluation of the effect of immunization on tumor tissue, tumor tissues were obtained from each group of mice after the final post-tumor induction immunization.

Demonstration of apoptosis in the tumors was performed by the TdT-mediated biotinylated-dUTP nick end labeling (TUNEL) method using the ApopTag in situ apoptosis detection kit (Oncor, MD, U.S.A.) with slight modifications. Nuclei of tumor sections were stripped from proteins by incubation with 20 µg/mL proteinase K for 15 min at room temperature. The sections were then washed in distilled water and immersed in 5% H$_2$O$_2$ in methanol for 5 min to quench the endogenous peroxidase activity. The tumor sections were immersed in the kit's equilibration buffer for 10 min. Terminal deoxynucleotidyl transferase (TdT) and dUTP-digoxigenin were added to the sections, and the slices were incubated in a humidified chamber at 37°C for 1 hr. After washing in PBS, the sections were incubated with an anti-digoxigenin-peroxidase solution for 30 min. The slices were stained with DAB/H$_2$O$_2$ solution (0.05% diaminobenzidine tetra-chloride and 0.02% H$_2$O$_2$ in 50 mM Tris-HCl buffer), and then counterstained with hematoxylin and eosin (22).

Statistical Methods

Different experimental groups within the study were compared using the non-parametric Kruskal-Wallis, Friedman, or Mann-Whitney tests. A p value <0.05 was considered significant and statistical analysis was done using the SAS program.

RESULTS

Development of BM-DCs

Clusters of round granulocytes developed around the third day, and increasing numbers of macrophages adhered to the plastic bottom of the well. By the fourth to the sixth day, isolated aggregates of matured DCs were visible. Around the seventh to the ninth day, DCs were enlarged and numerous typical long and small dendritic processes were visible.
On day 11, the purity of DCs was >90% with a viability of >90%. Fig. 1 shows a representative flow cytometer result of the cellular phenotype of cultured DCs harvested on that day. Repeated flow cytometry showed cultured cells to be strongly positive for DEC-205 (88%) and CD11 (40.7%), as well as the co-stimulatory molecules CD80 (98%) and CD86 (97%). Only 7% of the cultured cells expressed F4/80, the macrophage-specific marker, and only 2.3% expressed Gr-1, the granulocyte-specific marker.

**Mixed Leukocyte Reaction**

To test whether the DCs generated from bone marrow of tumor-bearing mice stimulate syngeneic T-cells, a \[^{3}H\] thymidine incorporation test was performed. Bone marrow-derived DCs mixed with lethally irradiated A20 tumor cells significantly stimulated syngeneic T-cells, as compared with control groups (Fig. 2). Microscopic examination revealed that DCs mixed with lethally irradiated A20 tumor cells stimulated and induced cluster formation and aggregation of T-cells.

**Cytotoxic T Lymphocyte (CTL) Assay**

Estimation of the induction and functional activity of cytotoxic T lymphocytes after DC immunization was performed by a 6 hr \(^{51}\)Cr release cytotoxic assay. DCs were cultured with T-cells at a ratio of 1:10 for five days and the effector: target ratios were 20:1 in triplicate. DCs generated...
from the bone marrow of tumor-bearing mice mixed with lethally irradiated tumor cells stimulated the CTL as measured by this assay (Fig. 3).

Fig. 3. Dendritic cell (DC) generation from bone marrow (BM) of tumor-bearing mice mixed with lethally irradiated tumor cells stimulate CTL. BM-derived DCs (4 × 10⁵) mixed with 4 × 10⁶ lethally irradiated (10,000 rads) A20 cells showed increased stimulation of CTL (4 × 10⁵).

Effect of Immunization on the Growth of Established Tumors—Immunization After Tumor Induction

To study the effects of immunization on the growth of established tumors, intraperitoneal immunizations with DCs mixed with lethally irradiated tumor cells were started when the tumors reached 5-10 mm in diameter, and continued once every three days for four weeks. A significant reduction in the primary tumor growth of A20 lymphomas was noted.

Fig. 4. The effect of immunization with dendritic cells (DCs) on the growth of established tumors. BALB/c mice were injected (sc) with 2 × 10⁶ A20 lymphoma cells. Intraperitoneal immunization with 2-4 × 10⁵ DCs mixed with 2 × 10⁶ lethally irradiated A20 cells, 2-4 × 10⁵ DCs only or with phosphate-buffered saline solution (PBS) alone, were started when the tumors reached 5-10 mm in diameter. Each group contained five mice. Mean tumor sizes in each group are given. p values for differences between the groups of mice immunized with different methods are shown above the data points.

Table 1. The effect of immunization with dendritic cells on T-cells

| Immunization group | CD4/CD8 (%) | [³H] thymidine uptake test (cpm) | IL2/IL4 (pg/mL) |
|--------------------|-------------|---------------------------------|-----------------|
| A. 2-4 × 10⁵ DC + 2 × 10⁶ A20 cells | 18/47 | 17951 ±1935 | 1536/1150 |
| B. 2-4 × 10⁵ DC | 16/35 | 26324 ±2301 | 2229/1144 |
| C. PBS | 9/27 | 4045 ±796 | 130/81 |
| Normal control | 13/31 | 128 ±53 | 116/70 |

BALB/c mice were injected (sc) with 2 × 10⁶ A20 lymphoma cells. Intraperitoneal immunization with 2-4 × 10⁵ dendritic cells (DCs) mixed with 2 × 10⁶ lethally irradiated (10,000 rads) A20 cells, 2-4 × 10⁵ DCs only, or phosphate-buffered saline solution (PBS) alone, were started when the tumors reached c. 5-10 mm in diameter. For evaluation of the effect of immunization on T-cells, [³H] thymidine uptake test, FACS scan analysis of T-cells and IL-2/IL-4 assays (using ELISA) with T-cells from splenocytes of each group were used.

Effect of Immunization on the Growth of Established Tumors—Immunization After Tumor Induction

To study the effects of immunization on the growth of established tumors, intraperitoneal immunizations with DCs mixed with lethally irradiated tumor cells were started when the tumors reached ca. 5-10 mm in diameter, and continued once every three days for four weeks. A significant reduction in the primary tumor growth of A20 lymphomas was noted.
at day 31, and there were significant differences in the rates of growth of lymphomas between groups (Fig. 4).

Effect of Immunization on the Growth of Established Tumors-Immunization Before Tumor Induction

To test whether immunization with DCs mixed with lethally irradiated tumor cells was capable of inducing protective antitumor immunity, immunization was started before tumor induction. As shown in Fig. 5, immunization before tumor injection could completely prevent tumor growth and there was no growth after the completion of immunization. By contrast, the mice of control groups showed progressive tumor growth.

Effect of Immunization on T-Cells

Spleens were obtained from each group of mice after the
final post-tumor induction immunization. As summarized in Table 1, the ratio of CD4+ to CD8+ cells was highest in the group immunized with DCs mixed with lethally irradiated tumor cells. The mice of both immunized groups and the tumor-bearig control group had higher [3H] thymidine uptakes than normal controls. Mice of the immunized groups showed considerably higher levels of IL-2 and IL-4 than mice of the non-immunized group.

Evaluation of Apoptosis by In Situ End-Labeling

Tumor tissues were obtained from each group of mice after the final post-tumor induction immunization. Microscopically, A20 lymphomas treated with PBS (A, B), DC (C, D) and DC mixed with irradiated A20 cells (E, F), showed some apoptosis with fragmentation of the condensed nucleus in multiple apoptotic bodies (Fig. 6A, C, E), and the apoptotic cells were identified by dark brown nuclear staining using the TUNEL method (Fig. 6B, D, F). The apoptosis indices were 0.5%, 4%, and 6% for B, D and E, respectively.

**DISCUSSION**

In this study, we used bone marrow-derived DCs to induce a therapeutic and protective immune response against a murine lymphoma. DCs mixed with lethally irradiated lymphoma cells as a source of tumor antigens stimulated an effective anti-lymphoma immune response. This was associated with an increase in tumor-specific cytotoxic-T cell responses. Although the exact mechanism of antitumor immunity was not defined, it is possible that the lethal irradiation of the A20 lymphoma cells resulted in rapid cell death with the release of cellular antigens that were then processed by the adjacent DCs (23), which then initiated the stimulation of tumor-specific cytotoxic T-cells.

Bone marrow-derived myeloid lineage DCs provide critical antigen-presenting cell activity for initiating specific T lymphocyte activation and proliferation; thus many studies have used bone marrow-derived DC for immunotherapy (24-26). Several studies applied a combination of cytokines including GM-CSF, TNF-α and IL-4 to harvest a large number of purified DCs from bone marrow cells (6-8). We also obtained DCs with morphologically typical dendrites using GM-CSF, TNF-α and IL-4. In this study, we reduced the dose of GM-CSF and increased that of TNF-α at a later stage of ex vivo culture. This resulted in minimal contamination with granulocytes and macrophages, and we obtained functionally active mature DCs. Our FACS analysis using the DC markers CD11c and DEC-205, and the co-stimulatory molecules CD80 and CD86 showed higher level of DCs at day 11 of culture. However, the macrophage-specific markers F4/80 and CD14, and granulocyte-specific marker Gr-1 were expressed at much lower levels. We also found that DCs mixed with lethally irradiated A20 tumor cells stimulated T-cells and induced cluster formation and aggregation of T-cells.

We performed [3H] thymidine incorporation assays to show that DCs generated from the bone marrow of the tumor-bearing mice significantly stimulated syngeneic T-cells as compared with control groups. The cytotoxic activity of the T lymphocytes measured by 51Cr release was stimulated in the mice immunized with the DCs mixed with lethally irradiated tumor cells. If we were to increase the ratio of the effector cells, this might stimulate the CTL significantly. Immunization before tumor implantation resulted in complete prevention of tumor growth, and there was no tumor growth after completion of the immunization. We observed that these mice were alive without any tumor growth for more than 12 weeks. This result suggests the feasibility of active tumor-specific immunotherapy with autologous dendritic cell transplantation as a strategy for the elimination of minimal residual disease (MRD) of lymphomas. Our results with syngeneic bone marrow-derived DCs for immunotherapy suggest that effective ex vivo expansion and maturation of DC from autologous stem cells could be an important and useful tool for immunotherapy.

In many model systems, the balance between the cytokines induced from Th1 and Th2 effector cells plays an important role in the regulation of immune responses. A Th1-positive cell response is thought involved in cellular and tumor immunity, and Th2-positive cells are associated with the suppression of cytolytic activity (27, 28). In this study, we tried to evaluate the possible role of Th1/Th2 cell balance in the immune response to DCs sensitized with irradiated tumor cells. Tumor progression in control mice was associated with a decreased absolute level of Th1-cell induced cytokine (IL-2) and Th2-cell induced cytokine (IL-4), whereas effective immunization blocks tumor progression, which in turn was closely associated with increased absolute levels of Th1-cells (IL-2). However, we found no significant differences in Th1/Th2 cell ratios between the groups. The induction of antitumor immunity by bone marrow-derived DCs requires the presentation of MHC class-II restricted molecules and activation of CD4+ T-cells (29). Our study demonstrated that the cultured DCs mixed with whole but dead tumor cells as a vaccine and induced antitumor immunity and complete prevention of tumor growth in the co-adjuvant treatment group. Recently, Kim et al. demonstrated that NK cells were required during the priming of cytotoxic T-cell response by DCs-based tumor vaccine and DCs can induce an antitumor immune response by enhancing NK cell-dependent CTL activation (30).

Evaluation of apoptosis by TUNEL demonstrated that the PBS-alone treated control group showed an apoptotic index of 0.5% (Fig. 6B), while in the DC-only treated group it was 4% (Fig. 6D), and in the group treated with DC mixed with irradiated A20 cells treated group it was 6%
(Fig. 6F). Programmed cell death was thus increased over tenfold in the treated group F.

We conclude that DCs mixed with irradiated tumor cells as a source of undefined tumor antigens can induce an effective antitumor immune response and completely prevent tumor growth. It also provides a rationale for the use of DCs mixed with irradiated tumor cells as an immunotherapy for MRD of lymphomas. Further studies on better methods of culturing DCs, optimizing the means of antigen loading on DCs and the most efficacious vaccination schedules and dosages should be investigated. Moreover, the mechanism by which DC vaccine can provide the desired immunity remains to be determined.

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