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Preventive antitumor activity against hepatocellular carcinoma (HCC) induced by immunization with fusions of dendritic cells and HCC cells in mice

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Background. The prevention of recurrence of hepatocellular carcinoma (HCC) after treatment is very important for improvement of the prognosis of HCC patients. Dendritic cells (DCs) are potent antigen-presenting cells that can prime naive T cells to induce a primary immune response. We attempted to induce preventive antitumor immunity against HCC by immunizing BALB/c mice with fusions of DCs and HCC cells. ***Methods.*** Murine bone marrow-derived DCs and a murine HCC cell line, BNL cells, were fused by treatment with 50% polyethyleneglycol (PEG). Fusion efficacy was assessed by the analysis of fusions of BNL cells stained with red fluorescent dye and DCs stained with green fluorescent dye. Mice injected intravenously with DC/BNL fusions were challenged by BNL cell inoculation. ***Results.*** About 30% of the PEG-treated non-adherent cells with both fluorescences were considered to be fusion cells. The cell fraction of DC/BNL fusions showed phenotypes of DCs, MHC class II, CD80, CD86, and intercellular adhesion molecule (ICAM)-1, which were not expressed on BNL cells. Mice immunized with the fusions were protected against the inoculation of BNL tumor cells, whereas injection with a mixture of DCs and BNL cells not treated with PEG did not provide significant resistance against BNL cell inoculation. Splenocytes from DC/BNL fusion-immunized mice showed lytic activity against BNL cells. ***Conclusions.*** These results demonstrate that immunization with fusions of DCs and HCC cells is capable of inducing preventive antitumor immunity against HCC.

Key words: dendritic cells, hepatocellular carcinoma, cell fusion, immunotherapy

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Epidemiological and prospective clinical studies have demonstrated its strong etiologic association with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infection.[1–3] Although vaccination with hepatitis B surface antigen (HBsAg) and the eradication of HCV by interferon treatment will reduce the incidence of HCC in future, how HCC should be treated in Asia and African countries where HBV and/or HCV are prevalent is an urgent problem. There have been several treatment options available to patients with HCC. Although surgical resection is the most common treatment world wide, the reduction in functional reserve caused by co-existing liver cirrhosis has limited this treatment, especially in HCV-associated HCC. Alternative treatments that have been developed are chemotherapy, transcatheter arterial embolization, transcatheter arterial chemotherapy, percutaneous ethanol injection (PEIT), and percutaneous microwave coagulation therapy (PMCT). However, the recurrence rate after these therapies is high,[4,5] probably because of insufficient therapeutic effect and the multicentric development of HCC in cirrhotic liver.

The increasing knowledge of the mechanisms involved in immune reactions against cancer cells has lead to the development of experimental and clinical immunotherapeutic approaches for the treatment of cancer patients and the prevention of cancer recurrence, with anti-cancer immunotherapy utilizing dendritic cells (DCs) recently attracting much attention. DCs are potent antigen-presenting cells (APCs) which prime naive T cells and initiate a primary immune response.[6,7] The identification of tumor antigens has made it possible to induce tumor-specific cytotoxic T lymphocytes (CTLs) by pulsing DCs with intact protein or synthetic peptides of the tumor antigen[8–12], and by transducing DCs with cDNAs coding the tumor antigen.[13] In most cancers,

however, the tumor antigen has not been identified. Furthermore, immunotherapy against a known shared tumor antigen could be of limited effect, because the tumor cells resistant to immunological attack may downregulate the expression of the antigen that brings about the immunological "escape" phenomenon, or they may express an individually specific tumor antigen that shows quite different antigenecity from that of the known shared tumor antigen.[14,15] Another method for the induction of antitumor immunity is immunization with APC fused with tumor cells. Guo et al.[16] used activated B cells and Gong et al.[17] used bone marrow-derived DCs as fusion partners for tumor cells. This method could induce antitumor immunity against known or unknown tumor antigens that have not been recognized by the immunological system of the tumor-bearing host because whole elements of the tumor cell could be loaded to DCs.

In the present study, we show that the growth of HCC tumors transplanted to mice is suppressed by injection with DCs fused to HCC cells.

Materials and methods

Mice, tumor cell line, cytokines, and antibodies

Female BALB/c mice, 8 to 10 weeks old, were purchased from Nippon SLC (Shizuoka, Japan). All animals received humane care, and the study protocols complied with the institution's guidelines. A murine HCC cell line, BNL[18], was kindly provided by Dr. S. Kuriyama (Nara Medical University, Nara, Japan). Human recombinant interleukin-2 (hrIL-2) was kindly provided by Shionogi Pharmaceutical (Tokyo, Japan). Rat monoclonal antibodies against murine CD4, CD8, CD11c, H-2K^d, I-A^d/I-E^d, and CD54 (intercellular adhesion molecule-1; ICAM-1) were purchased from Pharmingen (San Diego, CA, USA).

Preparation of DCs

DCs were prepared according to the method described by Inaba et al.,[19] with modifications. Briefly, bone marrow cells were obtained from the femora and tibiae of female BALB/c mice (8 to 10 weeks old). Red blood cells were lysed by treatment with 0.83% ammonium chloride solution. The cells were incubated for 1 h at 37°C on a plate coated with human γ-globulin (Cappel, Aurora, OH, USA).[20] Nonadherent cells were harvested and cultured on 24-well plates (10^6 cells/ml per well) in medium containing 10 ng/ml murine recombinant granulocyte/macrophage colony-stimulating factor (GM-CSF) (Becton-Dickinson, Bedford, MA, USA) and 10 ng/ml of recombinant murine IL-4 (Becton-Dickinson). After 5 days of culture, nonadherent or loosely attached cells were collected by gentle pipetting and transferred to a 100-mm petri dish. Floating cells, which included many DCs, were collected after overnight culture. The cells obtained in this manner exhibited dendritic morphological features and the expression of MHC class I and class II and CD80, CD86, and CD54, but not CD4, CD8, and CD45R, on their surfaces (data not shown).

Cell fusion of DCs and BNL cells

The fusion of DCs and BNL cells was performed according to Gong et al.,[17] with modifications. Briefly, BNL cells were irradiated with 35 Gy, mixed with DCs at a ratio of 1:3 (BNL cells:DCs) and then centrifuged. The cell pellets were treated with 50% polyethyleneglycol (PEG 1450; Sigma Chemical, St. Louis, MO, USA) for 1 min at 37°C, after which warm RPMI 1640 medium was added to dilute PEG. The PEG-treated cells were cultured overnight at 37°C in medium containing GM-CSF and IL-4.

Determination of cell fusion efficiency

To determine the efficiency of cell fusion, BNL cells were stained with PKH-26 (red fluorescence)[21] and DCs were stained with PKH-2GL (green fluorescence). The cells stained with the fluorescent dyes were treated with PEG and cultured overnight as described above. The fusions were also stained with phycoerythin (PE)- or fluorescein isothiocyanate (FITC)-conjugated with monoclonal antibodies against I-A^d/I-E^d, CD80, CD86, and CD54 (Pharmingen). Fluorescence profiles were generated with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Histograms and density plots were generated with the Cell Quest software package (Becton Dickinson).

Scanning electron microscopy

Cells were fixed with 1.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Fixed cells were attached to slides previously coated with 0.1% poly-L-lysine, dehydrated in ascending concentrations of ethanol, treated with isoamyl acetate, and critical-point dried with liquid CO_2. Specimens were coated with vacuum-evaporated, iron-spattered gold and observed with a JSM-35 scanning electron microscope (Japan Electric Optical Laboratory, Tokyo, Japan) at an accelerating voltage of 10 kV.

Injection of the fusions to mice

In tumor prevention studies, PEG-treated cells containing DC/BNL fusions were suspended in phosphate-buffered saline (PBS) and injected into the tail vein of

mice (4×10^5 cells/mouse), twice, at an interval of 2 weeks. One week after the second immunization, tumor challenge was performed by subcutaneous injection of 10^6 BNL cells. The mice were monitored each week for 6 weeks for the development of tumor by the measurement of tumor size (≥3 mm scored as positive). The control mice received PBS, irradiated BNL cells (10^5/mouse), DCs (3×10^5/mouse), or a mixture of irradiated BNL cells and DCs (4×10^5/mouse, DC:BNL ratio 3:1) instead of the DC/BNL fusions, and were examined for the development of tumor in the same way as the mice that had received the fusions. Each group consisted of ten mice.

Assay of lytic activity of splenocytes against BNL cells

Splenocytes were obtained by gentle disruption of the spleen on a steel mesh and depletion of red blood cells by hypotonic treatment. Splenocytes from the mice were cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS) in the presence of 50 U/ml of human recombinant IL-2 for 4 days. BNL cells (10^4 cells/well) were labeled with ^{51}Cr and incubated in RPMI-1640 medium supplemented with 10% heat inactivated FCS with splenocytes (effector cells), at various effector/target ratios, in a final volume of 200 μl (in triplicate) in a 96-multiwell plate for 4 h at 37°C. After incubation, 100 μl of supernatant was collected and the percent specific ^{51}Cr release was calculated by the following formula: percent cytotoxicity = 100 × (cpm experimental − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release), where maximum release was that obtained from target cells incubated with 0.33 N HCl and spontaneous release was that obtained from target cells incubated without the effector cells.

Results

Characteristics of fusions of DCs and BNL cells

DCs and BNL cells were combined, treated with PEG, and incubated overnight. Nonadherent and adherent cells obtained from PEG-treated cells exhibited dendritic features and epithelial characteristics, respectively, under a phase contrast microscope (data not shown). Nonadherent cells expressed DC markers, I-A^d (MHC class II) and CD11c by FACS analysis. The finding that the adherent cells were negative for I-A^d and CD11c expression indicated that non-fused BNL cells were in the adherent cell fraction.

Prior to PEG treatment, DCs were treated with an FITC-conjugated antibody against CD11c, and BNL cells were stained with PKH-26. The cells were treated with PEG and observed under a fluorescence microscope. Cells stained with both the FITC-conjugated antibody (green) and PKH-26 (red) were observed among the PEG-treated cells (Fig. 1), indicating the generation of fusions of DCs and BNL cells. FACS analysis was performed for the determination of fusion efficacy. Cells stained with both PKH-2GL and PKH-26, which were considered to be fusions of DCs and BNL cells, are shown in the upper area of the cell scattergram with a high forward scatter and a high side scatter (Fig. 2). The cell fraction of high and moderate forward scatter and low side scatter (Fig. 2) contained non-fused BNL cells, while the cell fraction of low forward scatter and low side scatter (Fig. 2) contained non-fused DCs and non-fused BNL cells. About 30% of the nonadherent cells were fusions, as judged from the width of the area of double-positive cells occupying in the whole scattergram.

The phenotypes of the fusions were analyzed by FACS. The cell fractions positive for both PKH-2GL and PKH-26 were gated on scattergrams and examined for expression of the antigens that characterized DCs. The phenotypic markers of DCs, I-A^d/I-E^d (MHC class II), CD80, CD86, and CD54 molecules, were expressed on the cells in the fusion cell fraction (Fig. 3).

On scanning electron microscopy, BNL cells had short processes on a plain cell surface and DCs had many long dendritic processes. The nonadherent fusion cells were large and ovoid, with short dendritic processes (Fig. 4).

Effect of administration of DC/BNL fusions on prevention of tumor development

The development of BNL tumors was significantly inhibited by injection with DC/BNL fusions prior to the inoculation of BNL cells. By contrast, the injection of DCs or irradiated BNL cells failed to prevent the development of tumors (Fig. 5). The injection of a mixture of DCs and BNL cells, in numbers corresponding to those used to produce the fusions, inhibited tumor growth within 4 weeks, but, finally, tumors grew at rates comparable to those in controls, and tumor incidence was almost same as that in controls 6 weeks after the inoculation of BNL cells (Fig. 5). The tumor incidence at 6 weeks was 20% (two of ten mice) in the fusion-treated mice, significantly lower than that in control mice ($P = 0.0017$ by χ^2 test for independence; computed P values were two-tailed) (Fig. 5).

Lytic activity of splenocytes against BNL cells in mice treated with DC/BNL fusions

Splenocytes derived from mice treated with DC/BNL fusions showed significant cytolytic activity against BNL

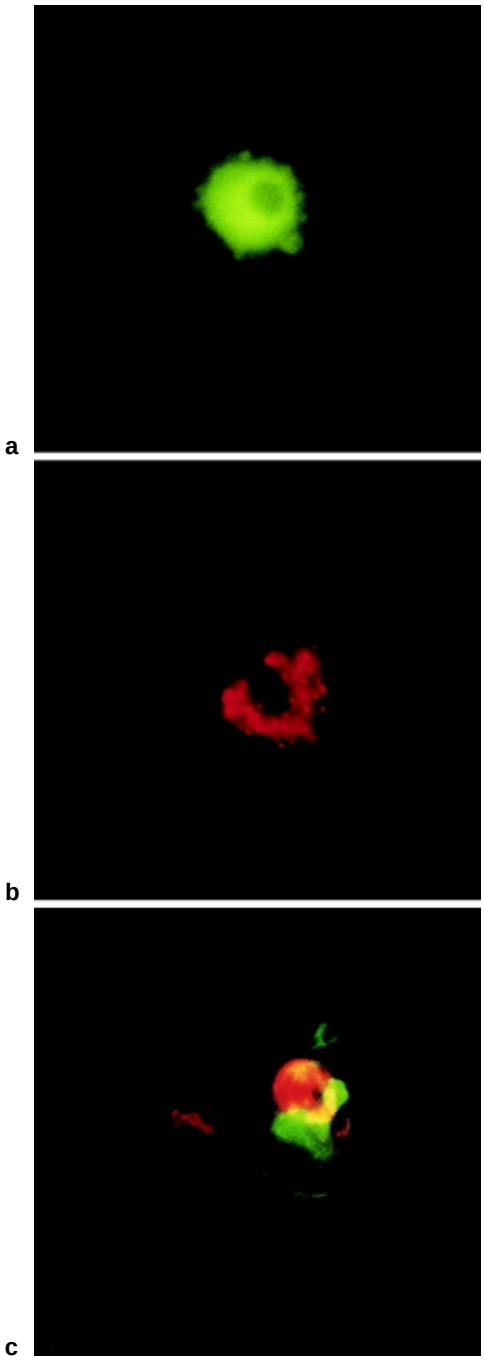

Fig. 1a–c. Fluorescent micrographs of dendritic cell (DC)/BNL (murine hepatocellular carcinoma cell line) fusions. DCs were treated with fluorescein isothiocyanate (FITC)-conjugated antibody against CD11c, and BNL cells were stained with PKH-26. Immediately after the staining of the cells, DCs and BNL cells were mixed and treated with polyethyleneglycol (PEG), as described in "Materials and methods." After overnight incubation, nonadherent cells were collected and observed under a phase contrast microscope. The *green* color is produced by FITC conjugated with an antibody against CD11c, a DC specific marker, and the *red* color is produced by the PKH-26 used for BNL cell labeling. **a** DC; **b** BNL cell; **c** fusion cell

cells, while there was no cytolytic activity of splenocytes derived from untreated mice (Fig. 6).

Discussion

DCs are potent antigen-presenting cells and can present tumor antigens to naive T cells and prime them against tumor antigens.[22,23] A current focus of research is the utilization of DCs as immunotherapeutic agents. Antigenic peptides of tumor antigens combined with MHC class I molecules were expressed and recognized by specific T-cell receptors.[8] However, tumor cells exhibit little expression of costimulatory molecules. Consequently, immunogenic epitopes are not presented to naive T cells for priming.[24] Because DCs can process and present exogenous antigens not only to $CD4^+$ T cells but also to $CD8^+$ T cells, antitumor immunity induced by loading DCs with antigenic peptides of tumor antigens may be a promising antitumor strategy.[10–12,25]

A method for loading DCs with tumor antigens and inducing antitumor immunity is the fusing of APC and tumor cells[16,17]. In this setting, fusion cells can present antigenic epitopes of a tumor antigen to naive T cells and prime them against the antigen, because fusion cells can simultaneously carry antigenic epitopes and retain antigen-presenting capacity.[26] The presence of MHC class I and class II molecules, costimulatory molecules (CD80, CD86), and ICAM-1 on fusion cells suggests that they can present antigens to naive T cells and prime them.[17] By fusing autologous DCs and tumor cells, such obstacles to the induction of antitumor immunity as MHC restriction, unique mutations of tumor antigens,[14,15] and the multiplicity of tumor-specific epitopes may be overcome. Furthermore, problems of peptide-pulsed DCs, such as the low affinity of pulsed antigenic peptides to MHC molecules[7] and the short lifespan of peptide-pulsed MHC class I molecules,[27] do not have to be considered in fusion-based immunization.

It is important to determine the fusion efficacy of DCs and tumor cells by treatment with PEG. After treatment with PEG, we found that the nonadherent cells showed DC markers, $I\text{-}A^d$ molecules and CD11c, whereas the adherent cells did not. This finding indicates that the nonadherent cell fraction included fusion cells and that most adherent cells were non-fused BNL cells. Phase-contrast microscopy and scanning electron microscopy of the nonadherent cell fraction revealed large multidendritic cells, some of which may have been fusion cells. Two-color FACS analysis showed that approximately 30% of the PEG-treated nonadherent cells were positive for both PKH-2GL (fluorescent dye with which DCs were stained) and PKH-26 (fluorescent dye with which BNL cells were stained). Cells stained with both dyes elicited the expression of MHC class I and

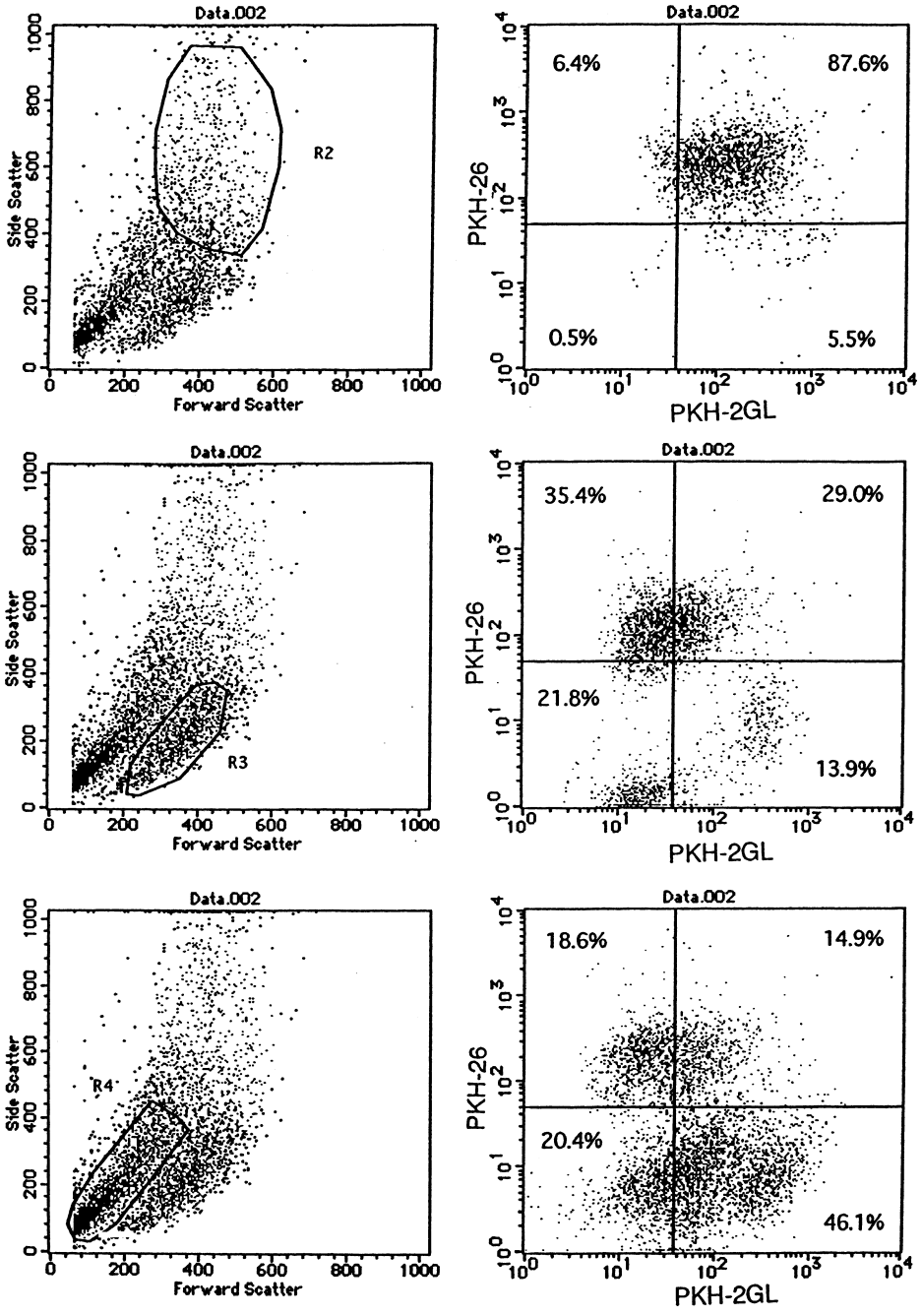

Fig. 2. Two-color FACS analysis of DCs and BNL cells treated with 50% PEG (non-adherent cell fraction). DCs and BNL cells were stained with PKH-2GL and PKH-26, respectively, prior to PEG treatment. Cells gated on the scattergram are shown on the *left* and fluorescence profiles of the gated cells are shown on the *right*. The cells in *the areas enclosed by the lines*, R2, R3, and R4, were gated and examined for their fluorescence profiles. The fluorescence profiles in R2, R3, and R4 are shown in the *upper*, *middle*, and *bottom* figures on the *right side*. In the figures of the fluorescence profiles, the *abscissas* show the fluorescence intensity of PKH-2GL with which DCs were stained, while the *ordinates* show that of PKH-26 with which the BNL cells were stained

class II and CD80, CD86, and CD54 molecules, which are required for antigen presentation. It is conceivable, therefore, that the fusions are able to present BNL tumor antigen(s) to naive T cells by means of DC capability. In the present study, because of the potential difficulty entailed in the preparation of a pure fusion-cell fraction, all of the PEG-treated nonadherent cells were used as immunogens and administered to the mice. However, tumor growth was significantly suppressed in mice that received these cells. Attempts are being made to collect a pure fusion-cell fraction, using a magnetic cell sorting system.

Immunization of BALB/c mice with DC/BNL fusions produced significant resistance against challenge with BNL cells. It is conceivable that DC/BNL fusions injected intravenously reached the spleen, and that T-lymphocytes were primed for the tumor antigen of BNL cells in the spleen. Primed lymphocytes would then spread to the whole body and elicit cytotoxic activity against inoculated BNL cells. Mice immunized with

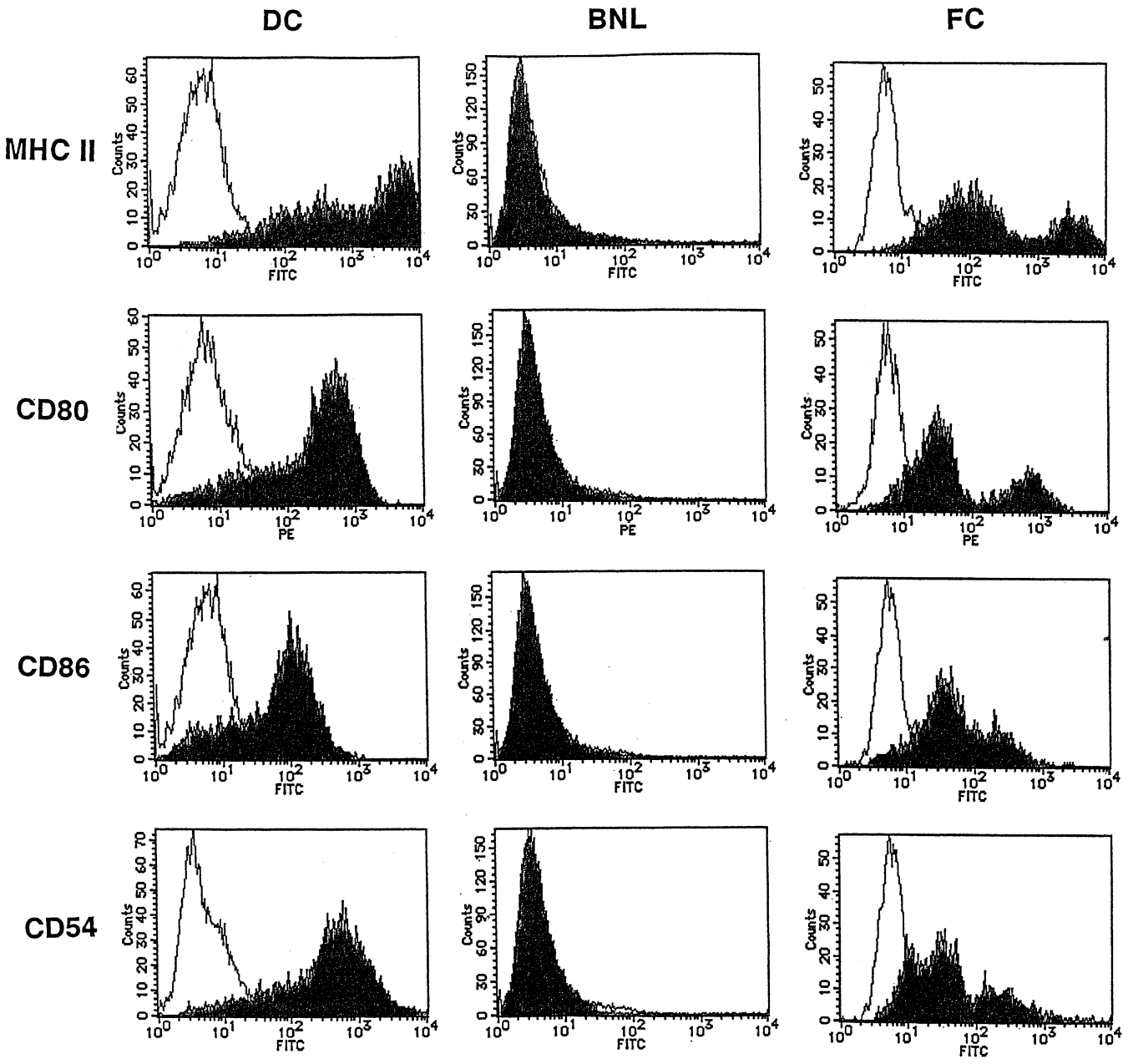

Fig. 3. Phenotypes of DC/BNL fusions. The expression of MHCII (I-A^d/I-E^d), CD80, CD86, and CD54 molecules on DCs, BNL cells, and DC/BNL fusions was examined by FACS analysis. For the analysis of PEG-treated cells, the gate was set on the cell fraction with high side and forward scatter, which contained many fusion cells, as shown in Fig. 2. *PE*, Phycoerythrin

DCs and BNL cells not treated with PEG showed less resistance to BNL cell challenge than did mice immunized with DC/BNL fusion cells. Celluzzi and Falo[28] have reported that immunization with either PEG-treated DC/B16 melanoma cell fusions or with DCs and B16 melanoma cells not treated with PEG produced similar degrees of antitumor immunity. It has recently been shown that some tumor cells secrete tumor antigen in the form of exosomes,[29] and it is possible that DCs could capture tumor antigen from such tumor cells without cell fusion.

Earlier studies have demonstrated that immunization with DCs pulsed with antigenic peptides that are carried on the tumor cell surface produces T-cell-dependent antitumor immunity.[10–12] Because too few peptides might be carried on the cell surface, and because the affinity of the peptide-MHC complex for the T-cell receptor is not high, a large number of tumor cells would

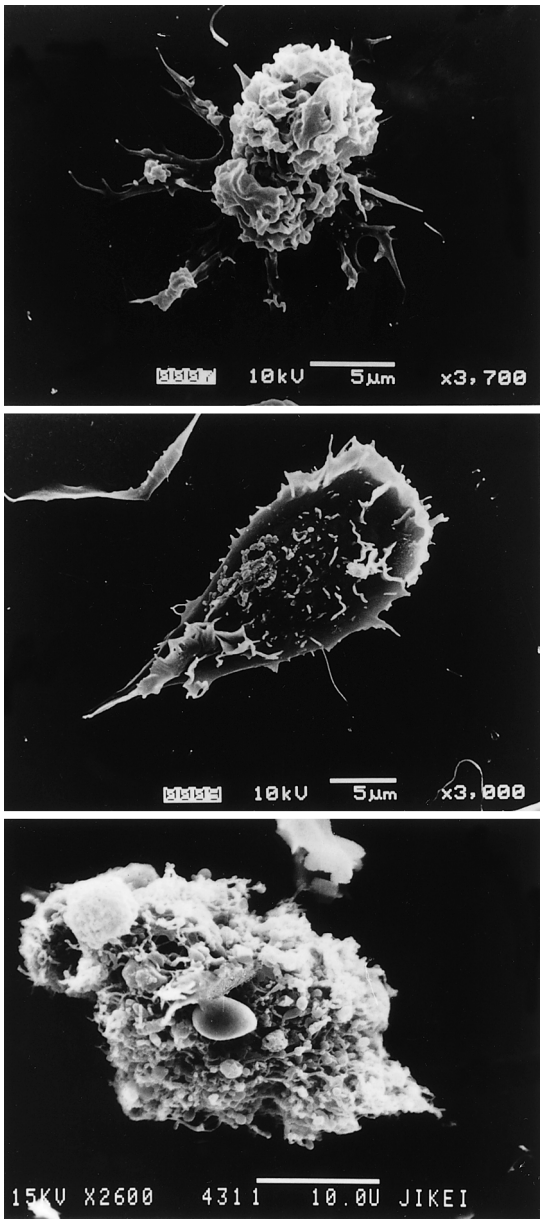

Fig. 4a–c. Scanning electron micrographs of **a** a DC, **b** a BNL cell, and **c** a large nonadherent cell, possibly a DC/BNL fusion cell, after treatment with PEG

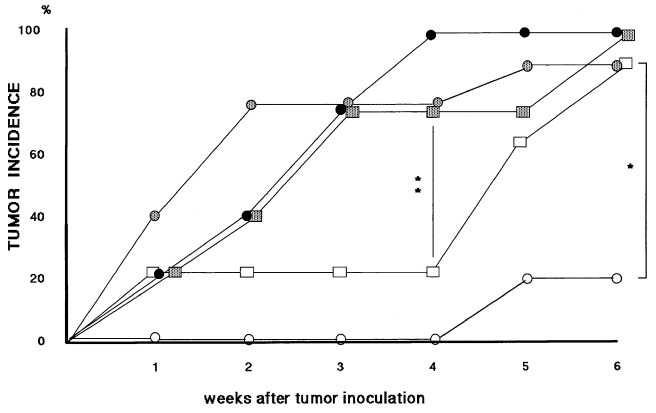

Fig. 5. Preventive effect of inoculation of DC/BNL fusions to mice against challenge of BNL cells. Mice were immunized by the intravenous injection of various cell types twice, with a 2-week interval. BNL cells (10^6/mouse) were inoculated subcutaneously 1 week after the second immunization, after which tumor development was examined; the presence of tumors larger than 3 mm in diameter was regarded as positive. *Round dotted symbols* Phosphate-buffered salive (PBS), as control; *rectangular dotted symbols*, untreated DCs (3×10^5/mouse); *round black symbols* irradiated BNL cells (10^5/mouse); *rectangular white symbols* combined DC and BNL cells without PEG treatment (4×10^5/mouse; DC/BNL cell ratio, 3:1), *round white symbols* DC/BNL fusions after PEG treatment (4×10^5/mouse, DC/BNL ratio, 3:1). $*P = 0.0017$; $**P = 0.0073$ by χ^2 test for independence ($n = 10$). Computed P values were two-tailed

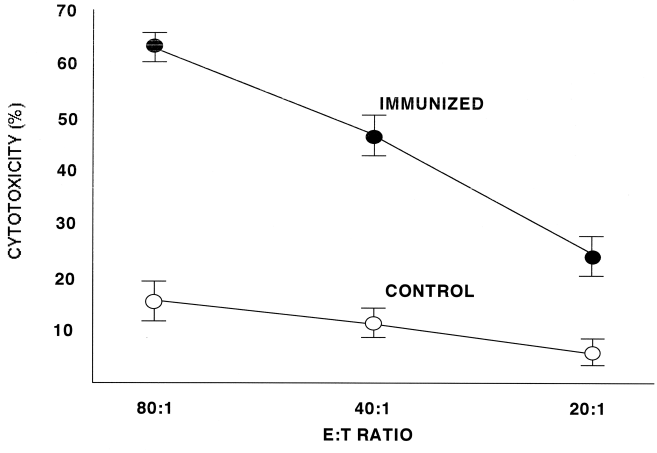

Fig. 6. Cytotoxic activity of splenocytes from mice treated with DC/BNL fusions against BNL cells. Splenocytes were collected from the mice treated with the fusions, as described in the text. Lytic activities of the splenocytes (effector cells; E) against BNL cells (target cells; T) at various E:T ratios were determined and expressed as described in the text. *Solid circles* and *open circles* represent the mean lytic activities of splenocytes ($n = 3$) obtained from mice treated with fusion cells and those that were not treated, respectively. The *vertical bars* attached to the circles represent SDs

be required for peptide elution. The number of BNL cells required for cell fusion is one-half to one-third that of DCs. This smaller number of tumor cells required is a great advantage for the clinical application of fusion-based immunotherapy, as tumor cells that can be obtained at tumor biopsy may suffice as a source of fusion partners for DCs.

The frequent recurrence of HCC after treatment is a serious problem in patients with cirrhosis. Tumor cells obtained at biopsy or resection can be used for fusion with autologous DCs. Recent advances have made it

possible to culture mature DCs from progenitors in the peripheral blood.[30,31] Although the induction of antitumor immunity against HCC in an animal model is not directly applicable to the prevention of human HCC, the induction of cytotoxic lymphocytes against HCC cells is essential for the prevention of HCC by an immunological mechanism in both animal models and humans. In the present study, we demonstrated that the induction of antitumor immunity against HCC was achieved by the immunization of mice with DC/HCC cell fusions. Immunization with fusions of autologous DCs and HCC cells could eventually be a promising method for the prevention of recurrence of HCC.

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