Native anti-tumor responses elicited by immunization with a low dose of unmodified live tumor cells

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Abstract. The present study demonstrates that immunization with a low dose of unmodified live myeloma tumor cells (FO) elicited tumor-specific immunity. BALB/c mice were vaccinated with 10^4 live dendritic cells (DC)-FO fusion cells or 10^3 live FO cells. 80% of vaccinated mice survived from the later challenge with $1x10^6$ FO cells, whereas all control mice developed tumors. Additionally, vaccination with live FO cells gave no protection against the growth of Lewis lung carcinoma cells in C57BL/6 mice. Cellular immunity was found to be primarily responsible for anti-tumor responses. In an adoptive immune model, the development of myeloma was greatly reduced by transfusion of lymphocytes but not sera from mice immunized with FO. T cells from immunized mice also induced lysis of FO cells in the cytotoxic T lymphocyte (CTL) assay. After co-culture with FO, IFN-γ released from immunized T helper cells increased >10-fold, while IL-4 remained unchanged in comparison with control T cells. These findings provided the first evidence that immunization with a low dose of unmodified live FO cells was safe to mice and capable of eliciting specific protective immunity against tumor growth.

Introduction

Based on the advanced technology of gene transfer (1,2), oncogenes were discovered (3), which disclosed the relationship between tumor cells and their parent cells. Tumorigenesis involves many cascade events, such as generation of limitless replication, inducement of sustained angiogenesis and invasion of tissue (4). Tumor cells usually express tumor-specific antigens or tumor-associated antigens, which consist of targets for the host immune system. Antibodies could attack tumor cells by antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) reactions, while immune cells, such as natural killer cells and cytotoxic T lymphocytes, could lyse tumor cells by intercellular reactions. Additionally, cytokines secreted by T helper cells could regulate the response. A number of mechanisms have been proposed to explain the failure of immune surveillance, such as loss of tumor antigens, decrease of MHC expression, down-regulation of co-stimulatory molecules and over-secretion of inhibitory cytokines (5-8). For over a century, scientists have tried to sew 'loopholes' in the immune systems of cancer patients.

Multiple strategies have been developed to re-evoke the immune system since Coley's toxin was first used to activate systemic immunity in cancer patients (9). Then, lymphokines were introduced and IL-2 demonstrated a prominent effect in cancer treatment despite its dose-related toxicity (10-12). Tumor lysates were also attempted to stimulate anti-tumor immunity with some encouraging results (13; Zehngebot LM, Cancer 53: abs. 17, 1984), comprising whole antigens and inducing polyclonal responses. Nonetheless, the amount of tumor antigens in the lysates was not enough to induce a satisfying effect, even with adjuvants. Vaccination with irradiated tumor cells was another approach, which could provide with a pool of antigens (14-16). However, although they presented antigens on cell membrane and induced cellular immunity, loss of integrity and fluidity caused by irradiation reduced the immunogenicity of tumor cells (17-20). The poor immunogenicity was improved by re-engineering tumor cells with cytokines or co-stimulating molecules (21-24). Methods using tumor-associated or tumor-specific antigens were also explored based on a dramatic progress in searching antigens of melanoma (25-27). A series of tumor antigens, such as MART-1, gp100, and TRP2, were identified. Being the most potent antigen presenting cells, dendritic cells (DCs) have been focused on recently. With MHC and co-stimulating molecules expressed on the surface, DCs were pulsed with tumor antigen peptides, transfected with tumor antigen cDNA or fused with tumor cells to enhance the tumor-specific immune response (28-30). However, preparation of DCs and DC-tumor fusion cells is a difficult and source-limited work.

The immune responses induced by live tumor cells have been discussed in different tumor cell strains (31-33). Here we described immunization with a low dose of unmodified live autologous tumor cells in a myeloma model. To elicit protective anti-tumor immune responses without development of tumors, the safe and effective dose of myeloma cells (FO)

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for injection was determined. Compared with DC-FO fusion vaccines, a similar response was induced by live FO cells. Further results demonstrated that cellular immunity played an important role in the immune response.

Materials and methods

Mice and tumor lines. Female BALB/c and C57BL/6 mice aged 6-8 weeks were purchased from Shanghai Laboratory Animal Center. All procedures in animal experiments were approved by the Animal Study Committee, Institute of Molecular Medicine, Nanjing University. BALB/c myeloma cell line FO and C57BL/6 lung carcinoma cell line LLC were obtained from ATCC. FO cells were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated newborn calf serum (NCS) (Gibco), 2 mM L-glutamine (Hyclone, Logan, UT), 2 mg/ml sodium bicarbonate (Amersco, Cleveland, OH), 25 mM HEPES (Promega, Madison, WI), 100 U/ml penicillin (North China Pharmaceutical Group, Shijiazhuang, P.R. China) and 100 μg/ml streptomycin (Lu-Kang Pharmaceuticals, Jining, P.R. China). LLC cells were grown in DMEM medium (Gibco) supplemented with 10% heat-inactivated NCS, 4 mM L-glutamine, 3.7 mg/ml sodium bicarbonate, 100 U/ml penicillin and 100 μg/ml streptomycin. Freshly isolated T cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco), 50 U/ml recombinant human interleukin (IL)-2 (Four Rings Biopharmaceuticals, Beijing, P.R. China), 5 μg/ml concanavalin A (Promega), 2 mM L-glutamine, 2 mg/ml sodium bicarbonate, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin.

Splenocyte-derived DCs. DCs were obtained from splenocytes as previously described with minor modifications (34). Red blood cells were depleted by 8.3 g/l ammonium chloride in 0.01 M Tris-HCl buffer. The cells were plated in the tissue culture flask in the RPMI-1640 medium supplemented with 2 mM glutamine, 2 mg/ml sodium bicarbonate, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. The non-adherent cells were washed away after 1 h of incubation. The adherent cells were cultured in RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, 2 mg/ml sodium bicarbonate, 25 mM HEPES, 10 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF) (PeproTech, UK) and 10 ng/ml IL-4 (PeproTech), 100 U/ml penicillin and 100 μg/ml streptomycin. After culture for 4 days, adherent and loosely adherent cells were dislodged by gentle pipetting and harvested.

Cell fusion. The fusion of DCs with carcinoma cells was described previously (29). DCs were mixed with myeloma FO cells at a ratio of 1:2.5. The fusion process was carried out with pre-warmed 50% (w/v) polyethylene glycol (Sigma). After washing, fusion cells were maintained in RPMI-1640 supplemented with 10% defined FCS (Hyclone), 2 mM glutamine, 25 mM HEPES, 2 mg/ml sodium bicarbonate, 10 ng/ml GM-CSF, 10 ng/ml IL-4, 5% hybridoma cloning factor (OriGen, Austin, TX), 2% HAT (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin. HAT restricted proliferation of myeloma cells, but not fusion cells (35). Homogeneous fusion cells were obtained after culture for 1 week.

Determination of safe dose of live tumor cells for immunization. FO or DC-FO fusion cells were washed and re-suspended in RPMI-1640 without serum. BALB/c mice were injected subcutaneously in the flank with doses of 10^2, 10^3, 10^4, 10^5 and 10^6 cells per mouse, respectively. The maximal safe dose without tumor growth was determined by daily observation for 2 weeks.

Immunization of mice with live tumor cells

The myeloma model. FO cells, DC-FO fusion cells or DC-FO fusion cells pre-exposed to 20 Gy γ-irradiation were harvested and re-suspended in 1640 medium without serum. Female BALB/c mice were immunized subcutaneously in the flank with 10^3 live FO cells, 10^4 live DC-FO fusion cells or 10^6 irradiated DC-FO fusion cells per mouse, respectively. The injection was given 3 times with 2-week intervals. The mice were challenged with 10^6 live FO myeloma cells subcutaneously 7 days after the final immunization and were monitored for 60 days after all the mice in the control group had died. The survival rate was recorded.

The LLC model. FO cells was collected and re-suspended in 1640 medium without serum. Female C57BL/6 mice were vaccinated subcutaneously in the flank with 10^3 live FO cells per mouse. The mice received live FO cells immunization 3 times biweekly. They were challenged with 10^6 LLC cells subcutaneously 7 days after the final immunization and were monitored until all of them had died.

Flow cytometry. DCs and DC-FO fusion cells were washed with PBS and incubated with Phycoerythrin (PE) conjugated monoclonal antibody (mcAb) against ICAM-1 (BD Pharmingen, CA) and B7-1 (Pharmingen) or fluorescein (FITC)-labeled mcAb against B7-2 (Pharmingen) and MHC-II (Southern Biotechnology, AL) for 1 h at room temperature. All samples were washed with PBS, pH 7.2 and analyzed using a FACScalibur (Becton-Dickinson, CA).

Passive immune transfusion. Donor BALB/c mice were immunized 3 times with 10^3 live FO cells biweekly. Their splenocytes and sera were harvested 7 days after the final immunization. One week after the subcutaneous injection of 10^6 live FO cells, $7x10^7$ splenocytes or 100 μl sera were given intravenously per mouse, respectively. Each recipient mouse was transfused 4 times at 3-day intervals. The size of the tumor was determined by measuring perpendicular dimensions with a vernier caliper daily for 25 days, at which point mice in the control group started dying.

The CTL assay. The T cells harvested from mice spleens by nylon wool were plated into 24-well plates with 50 μg/ml mitomycin C (Union Pharmaceuticals, Beijing, P.R. China) pre-treated FO or DC-FO fusion cells for 72 h at a ratio of 5:1. T cells were then harvested and co-cultured with the target FO cells for 6 h in a 96-well U-bottom plate at different ratios. The supernatant was measured for lactate dehydrogenase (LDH) released from lysed cells using the CytoTox 96 cytotoxicity assay kit (Promega). The percentage of specific release of LDH was determined by the following equation: percent specific release = (experimental release - spontaneous T cell

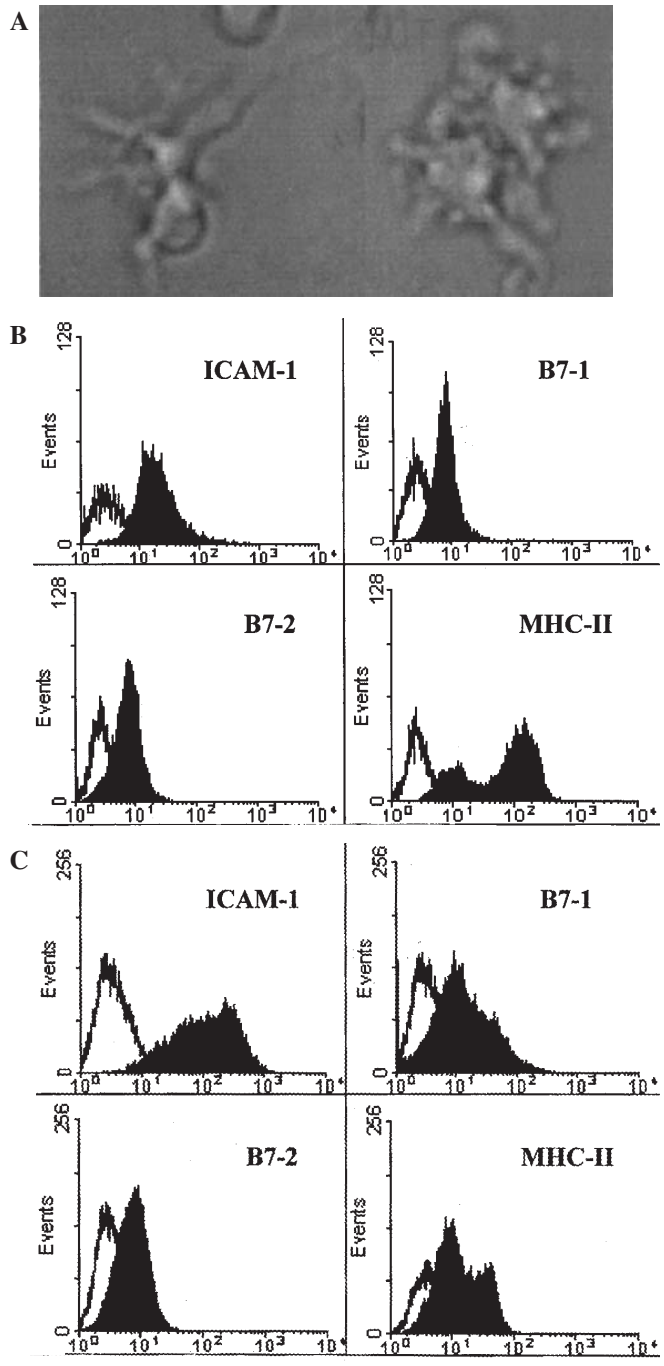

Figure 1. Phenotype of DCs and DC-FO fusion cells. (A) DCs were derived from mice splenocytes in the medium containing GM-CSF and IL-4. Original magnification, x200. (B) DCs were characterized by flow cytometry for indicated antigens. (C) The fusion cells were generated from fusion of FO and DCs. The expression of cell surface antigen was assayed by flow cytometry.

release - spontaneous FO cell release)/(maximal FO cell release - spontaneous FO cell release) x100.

Cytokine assay. Isolated T cells were stimulated by mitomycin C (50 μg/ml), pre-treated FO cells or DC-FO fusion cells for 72 h at a ratio of 5:1. IFN-γ and IL-4 released in the supernatant were measured by using a sandwich ELISA kit (BD Pharmingen).

Statistical methods. Statistical significance was determined using the Student's t-test.

Table I. The maximal safe dose of live vaccines.

	10^5 cells/ mouse	10^4 cells/ mouse	10^3 cells/ mouse
DC-FO fusion cells	3/5[a]	0/5[b]	0/5
FO cells	5/5	3/5	0/5[c]

[a]Tumor incidence was expressed as the number of tumor-bearing mice in the whole group. [b]The maximal safe dose of live DC-FO fusion cells was 10^4 per mouse. [c]The maximal safe dose of live FO cells was 10^3 per mouse.

Results

Phenotypes of DCs and fusion cells. DC-tumor fusion cells have been focused on recently and have demonstrated their prominent anti-tumor effects. In the present study, DC-FO fusion vaccines were prepared for comparative analysis with a low dose of live tumor cells. DCs were derived from mice splenocytes in the medium containing GM-CSF and IL-4, which facilitated the differentiation of monocytes into DCs. They showed a typical morphology of veiled processes and dendrites after being cultured in conditional medium for 4 days (Fig. 1A). At the same time, the expression of co-stimulating molecules, such as ICAM-1, B7-1, B7-2 and MHC-II, were upregulated dramatically (Fig. 1B). The fusion was carried out with 50% PEG. DCs could not replicate limitlessly and the proliferation of FO was restricted by HAT (35). Therefore, only homogenous DC-FO fusion cells were finally obtained in the HAT medium after a 1-week culture. It was found that surface co-stimulating molecules of the DC-FO fusion cells were similarly expressed in comparison with DCs (Fig. 1C).

The maximal safe dose for vaccination with live tumor cells. In the study, development of tumors was found in mice injected with 10^6 fusion cells as well as 10^6 myeloma cells. To determine a safe dose of vaccines, mice were injected subcutaneously with various doses of vaccines. No tumors were found in mice injected with 10^3 live FO or 10^4 live fusion cells, whereas tumors appeared in all groups with a higher dose (Table I). Then 10^3 live FO or 10^4 live fusion cells per mouse were chosen to vaccinate mice biweekly for a total of 3 times.

Prevention of myeloma growth by immunization with a low dose of autologous tumor cells. A tumor vaccination model was used to test the elicited native anti-tumor immunity. Successful immune responses should inhibit the development of tumors and increase the survival rate of mice. BALB/c mice were immunized 3 times biweekly with 10^3 live FO cells, 10^4 live DC-FO fusion cells or 10^6 irradiated DC-FO fusion cells, respectively. After being challenged with 10^6 myeloma cells, all mice in the control group developed tumors and died within 44 days post tumor inoculation, whereas the vaccination groups demonstrated increased survival rates (Fig. 2). More importantly, live FO cells and DC-FO fusion cells were more effective than irradiated DC-FO fusion cells. 80% of mice

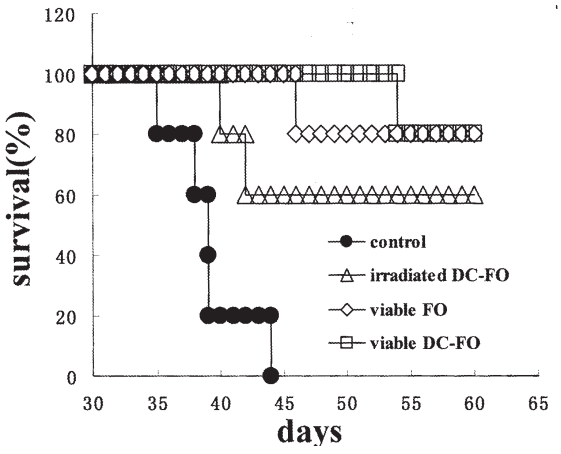

Figure 2. Immunization with live FO cells elicited protective immunity against myeloma. BALB/c mice were immunized subcutaneously 3 times biweekly with RPMI-1640 without serum (●), irradiated DC-FO fusion cells (△), live FO cells (○) or live DC-FO fusion cells (□) respectively, followed by challenge with 10^6 FO cells one week after the last immunization. The survival rate was calculated based on daily observation for 60 days. Each group comprised 5 mice.

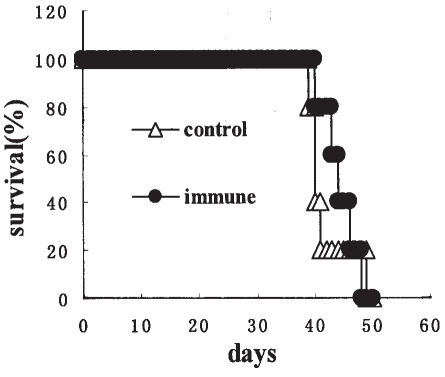

Figure 3. Immunization with live FO cells provides no protection against the LLC challenge. C57BL/6 mice were immunized subcutaneously biweekly 3 times with RPMI-1640 without serum (△) or 10^3 live FO cells (●) and then challenged with 10^6 LLC cells one week after the last immunization. Survival was monitored until all mice had died. Each group comprised 5 mice.

immunized with live vaccines were protected from tumor growth, whereas only 60% of mice vaccinated with irradiated fusion cells were protected. The surviving mice from the vaccination groups remained tumor-free across their lifespan. To assess whether the vaccination with FO protects mice from the challenge of xenogenic tumors, C57BL/6 mice pre-immunized with live FO cells were challenged with LLC. However, no detectable protection effect was found (Fig. 3), suggesting that the immunity was FO myeloma specific.

Transfusion of anti-tumor immunity inhibited tumor growth. To study the mechanism of the anti-tumor immunity, a treatment model of passive immunization was employed. Lymphocytes ($7x10^7$) or 100 μl sera from immunized allogeneic mice were transfused to BALB/c mice, which were pre-inoculated with 10^6 FO cells for 7 days. It was found that the FO myeloma growth was inhibited by the transfusion of lymphocytes but not sera (Fig. 4). Subsequently, the immunized sera showed

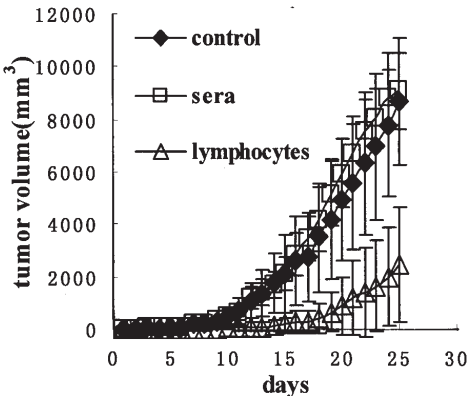

Figure 4. Passive immune transfusion. RPMI-1640 without serum (◆), lymphocytes (△) and sera (□) from BALB/c mice vaccinated with live FO cells were intravenously injected respectively to recipients challenged with myeloma cells. Tumor growth was monitored daily until mice in the control group started dying. The results were expressed as the mean ± SD of 5 replicates.

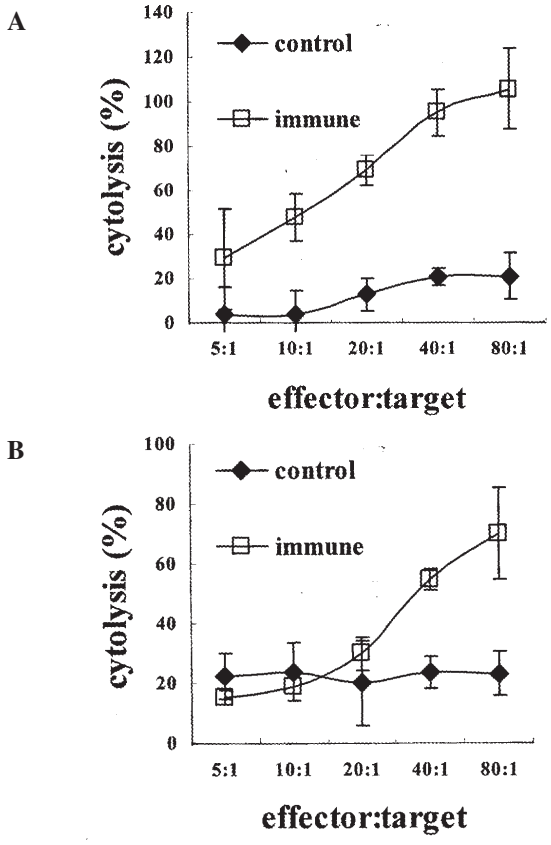

Figure 5. CTL analysis. After activation *in vitro*, CTLs from mice immunized with live FO cells (A) and live DC-FO fusion cells (B) were incubated with target myeloma cells at indicated ratios. LDH released from lysed FO myeloma cells was measured. The data were expressed as the mean ± SD of 3 replicates.

low antibody titer against FO cells, detected by FACS (data not shown). These findings indicated that immunized lymphocytes predominantly contributed to the inhibitory effect of the vaccination in tumor growth.

Elucidation of cellular immunity in immunized mice. T cells were isolated from mice spleens by nylon wool and characterized with anti-CD3 mcAb. The purity of T cells reached

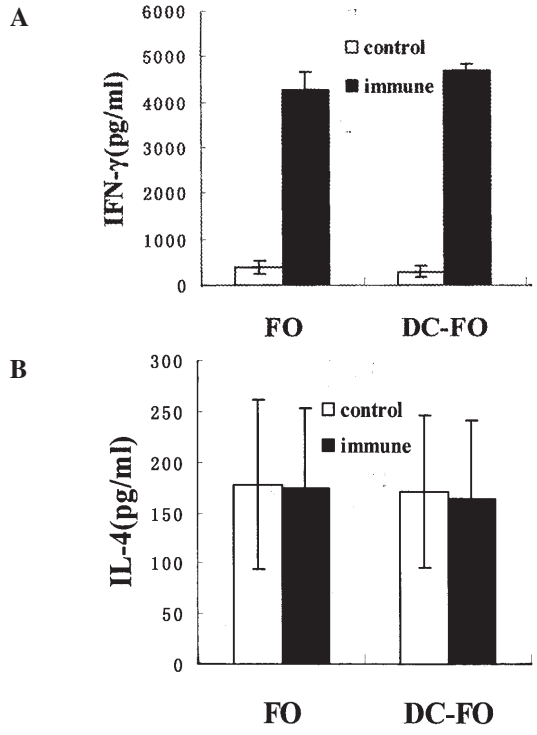

Figure 6. Cytokine release assay. T cells were co-cultured with mitomycin C-treated FO. The supernatants were collected and the concentration of IFN-γ (A) and IL-4 (B) was quantified by sandwich ELISA. The data were expressed as the mean ± SD of 3 replicates.

78.46±8.01%. After incubation with mitomycin C-treated FO cells or DC-FO fusion cells, T cells were co-cultured with the target FO cells. Specific lysis of FO cells was successfully induced by the CTLs either from mice immunized with live FO cells or DC-FO cells but not the control mice (Fig. 5). The extent of specific lysis was correlated with the ratios of effector cells over target cells. The supernatant of immunized T cells incubated with mitomycin C-treated FO cells was collected and assayed for release of cytokines. It was found that the amount of IFN-γ increased more than 10 times, while IL-4 was unchanged in comparison with the supernatant of control T cells (Fig. 6). Since T helper (Th)1 and Th2 cells specifically express either IFN-γ or IL-4, these data suggested that release of lymphokines in Th1 type was increased as a response to cellular immunity, whereas that in Th2 type was unaffected.

Discussion

Tumor vaccines have always been under intensive investigation, evidenced by the many promising approaches that have been explored to elicit protective immunity. In the present study, we provided the first evidence that a low dose of live FO myeloma cells induced native anti-tumor responses. Its protective effect was comparatively studied with live DC-FO fusion cells in a tumor vaccination model. The high survival rate was found in both groups of live FO myeloma cells and DC-FO fusion cells. Since no foreign immunomodulators, such as antigen presenting cells, cytokines and co-stimulating molecules, were introduced into the immunization procedure, this protective immunity should be considered to reflect the natural anti-tumor mechanism. Our data further demonstrated that lymphocytes from the vaccinated donor inhibited the growth of myeloma *in vivo*, while sera from the immunized mice had little inhibition of tumor growth. Since specific lysis of myeloma was induced by the CTLs *in vitro* and a more than 10-fold increase in the secretion of IFN-γ was seen with the immunized T helper cells, we conclude that cellular immunity is primarily responsible for the inhibition of tumor growth. These findings indicated that a low dose of unmodified live myeloma cells elicited a potent cellular immunity that could sufficiently prevent and inhibit myeloma growth.

To avoid tumorigenesis, the safe dose of live tumor or fusion cells was researched. In contrast to a previous finding that DC-tumor fusion cells were not tumorigenic (29), we found that mice injected with 10^6 live DC-FO fusion cells developed tumors. This may be caused by the use of different types of tumor cells. Irradiation was used to prevent the proliferation of DC-tumor fusion cells, while a reduction in protective immunity was seen with irradiated DC-FO fusion cells.

It has been a challenge to reverse immunotolerance and to raise protective tumor-specific immunity since most carcinoma cells are poorly immunogenic. The present study demonstrated that immunization with live FO myeloma cells elicited anti-tumor immunity as potent as that achieved by using live DC-FO fusion cells. This is probably due to the reserved immunogenicity and reduced tumorigenesis of myeloma cells. Firstly, as a special character of myeloma cells, immunoglobulin molecules are secreted on the membrane, whose complementary determination regions are the ideal target for attack by the host (36,37). Secondly, myeloma cells are derived from bone-marrow cells, which express various cell surface antigens, including co-stimulating molecules such as ICAM-1 (38,39). Thirdly, myeloma cells are heterogeneous and only a subtype of them strongly induced tumorigenesis (40). Therefore, the tumor growth could be avoided if the dose of myeloma cells injected is low enough (41). Our data showed that the tumorigenesis of myeloma decreased as the injection dose declined. Collectively, both the reserved immunogenicity and the reduced tumorigenesis make it feasible to induce protective immunity with a low dose of live myeloma cells.

The present approach described a vaccination method without transfection of tumor cells with cytokines or co-stimulating molecules. The recognition and elimination of tumor cells depend on its native immune system. This information could help the study of mechanisms of tumor recognition and elimination by native immunity. It could also be useful for the study of the mechanisms of immune escape of tumor cells. In contrast to the 'sneaking through' of tumor cells (42,43), our tumor model demonstrated that a low dose of tumor cells induced immunity to the development of tumor cells. In conclusion, the present study described a method to induce protective anti-tumor immunity by immunizing with a low dose of unmodified live autologous tumor cells, which could aid the investigation of native anti-tumor immunity.

Acknowledgements

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