Increased Vaccination Efficiency with Apoptotic Cells by Silica-induced, Dendritic-like Cells

Delphine Massé, Cécile Voisine, Frédéric Henry, Sandrine Cordel, Isabelle Barbieux, Regis Josien, Khaled Meflah, Marc Grégoire, and Blandine Lieubeau

ABSTRACT

We have demonstrated previously the ability of apoptotic cells to prime a functional immune response using an i.p. vaccination protocol with apoptotic cells and interleukin 2, before injecting a lethal dose of tumor cells into syngeneic rats. This protocol resulted in a survival rate of 33%. To elucidate the nature and the activity of the phagocytes involved in the clearance of apoptotic cells in vivo, we modulated the peritoneal cavity environment by administering either thiglycollate or silica i.p. before injecting the apoptotic cells. Our results showed that thiglycollate abrogated vaccination efficiency, because none of the rats survived under these conditions. In contrast, silica treatment enhanced the vaccination efficiency of apoptotic cells plus interleukin 2 up to 66%. We distinguished a population of dendrite-like cells among the cells derived from the silica-treated peritoneal cavity both by their phenotype (MHC II+/CD80+/CD86+) and by their ability to induce the proliferation of allogeneic T cells in a mixed leukocyte reaction. Our results demonstrate the different roles of macrophages and dendrite-like cells in the physiological clearance of dead tumor cells and their implication in the design of immunomodulating vaccines.

INTRODUCTION

The central role of dendritic cells in controlling immunity makes them ideal tools for cancer immunotherapy (1). In this respect, different strategies have been used to load human dendritic cells with tumor-associated antigen, especially tumor peptides. An alternative way to obtain tumor-associated antigen presentation is based on the ability of dendritic cells to phagocytose nonviable tumor cells (2). The advantage of this approach is that the processing of tumor antigens leads to the presentation of a wide panel of both MHC class I and class II epitopes from tumor cells and consequently to a diversified immune response. The efficiency of such antigen-presenting cells charged with tumor-associated antigen, especially tumor peptides. An alternative way to obtain tumor-associated antigen presentation is based on the ability of dendritic cells to phagocytose nonviable tumor cells (2). The advantage of this approach is that the processing of tumor antigens leads to the presentation of a wide panel of both MHC class I and class II epitopes from tumor cells and consequently to a diversified immune response.

MATERIALS AND METHODS

Materials

Brewer′s thiglycollate medium was purchased from Difco (Detroit, MI), and silica was purchased from Sigma Chemical Co.-Aldrich (St. Quentin Fallavier, France). The following antirat mAbs were obtained from the European Collection of Animal Cell Culture, (Salisbury, United Kingdom): CD1 (OX1, CD45), CD6 (OX6, MHC II), CD17 (OX17, MHC II), CD18 (OX19, CD 5), CD33 (CD45R), CD42 (CD 11b/c), CD41 (Signal Regulatory Protein), and ED3 (sialoadhesin). PE-conjugated anti-CD86/B7.2 (24F) and anti-CD80/B7.1 (3H5) mAbs were purchased from PharMingen (San Diego, CA) and FITC-conjugated anti-CD161a/E 3.2.3 (NKR-P1A) mAb from Serotec (Oxford, United Kingdom).

Animals

BDIX rats, 4–6 weeks of age, were purchased from Iffa Credo (L′Arbresle, France) and housed and bred in our laboratory. Lewis rats were obtained from the Center d′Elevage Janvier (Le Genest-Saint-Ilse, France).

Cell Lines

DHDK12TRb (PROb), a poorly immunogenic rat colon carcinoma cell line, was obtained from the European Collection of Animal Cell Culture. The cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FCS, 2 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technology, Cergy Pontoise, France). Cells were routinely checked for Mycoplasma infection by Hoechst 33258 labeling.

Generation of Apoptotic Cells

Apoptotic cells were prepared and purified as described previously (9). Briefly, PROb cells were treated for 3 days with arginine butyrate (20 mm). Floating dead cells were removed every other day and frozen at ~80°C in complete medium containing 10% DMSO when required. For the analysis of apoptotic tumor cells and IL-2 into the peritoneal cavity of rats before challenging with an injection of viable tumor cells. We sought to indirectly elucidate the role of dendritic cell and macrophage populations in the initiation of the immune response after phagocytosis by injecting apoptotic cells into either a silica- or thiglycollate-treated peritoneal cavity. The peritoneal cavity of rats contains mainly macrophages but also a few dendritic cells (6), and both silica or thiglycollate treatments are known to modulate the peritoneal cavity environment (7, 8).

The results presented in this report show that the immunogenicity of apoptotic cells depends on the nature of antigen-presenting cells and their ability to phagocytose these apoptotic tumor cells. We demonstrate that a prevalence of professional scavenger phagocytes (i.e., inflammatory macrophages after thiglycollate treatment) may favor tumor antigen sequestration leading to a reduction in the immune response. Conversely, the recruitment of dendrite-like cells (i.e., after silica treatment) with the ability to internalize dead tumor cells efficiently confers protection against tumor challenge.
the phagocytic process, apoptotic bodies were first labeled with the general cell linker PKH26GL, according to the manufacturer’s protocol (Sigma Chemical Co.-Aldrich).

**Cell Preparation**

PECs. Sterile Brewer’s thioglycollate medium was prepared 1 week before use. Silica (Sigma Chemical Co.), PBS (Life Technologies, Inc.), or thioglycollate were injected i.p. into rats 24 h (PBS and silica) or 72 h (thioglycollate) before PECs were harvested. Treated peritoneal cavities were aseptically rinsed with 50 ml of PBS. PECs from three to five rats were pooled when necessary to obtain sufficient cells for analysis, and each experiment was performed at least three times. PECs were centrifuged (1800 rpm for 5 min at 4°C), stained with trypan blue, and then counted (>95% viable).

**Purification and Selection of MHC Class II⁺ PECs.** Cells were incubated with a saturating concentration of biotin-conjugated OX17 mAbs at 4°C for 20 min, washed twice, and then mixed with streptavidin-conjugated microbeads, following the manufacturer’s instructions (Miltenyi Biotec, Paris, France). Positive selection was performed on Macs type-positive selection columns (Miltenyi Biotec). Purity was routinely >85%.

**Phagocytosis Analysis in Different Treatment Conditions.** PPKH26GL-labeled apoptotic PROb cells were injected directly into the three differently treated peritoneal cavities of rats. PECs were collected 24 h later and analyzed by fluorescence microscopy and flow cytometry.

**T Cells.** Allogeneic T cells were isolated from Lewis rat lymph nodes by negative selection of MHC II⁺ cells (OX6), NK cells (E 3.2.3), B cells (OX33), and macrophages (ED3), using antimouse IgG-coated magnetic beads (Dynal, Oslo, Norway) as described previously (10). CD3 expression was controlled by flow cytometry. Purity was routinely >98%.

**Cytofluorometric Analyses**

**MHC Class II⁺ Cell Phenotype.** Cells (2 x 10⁵) were incubated with FITC-conjugated mAb (OX1 or OX17) and with PE-conjugated mAb [OX7 or anti-CD86/B7.2 (24F) and anti-CD80/B7.1 (3H5)] for 20 min at 4°C. **Phagocytic Activity of PEC-MHC Class II⁺**, MHC class II⁺ cells (2 x 10⁵) selected as described above were incubated with FITC-conjugated mAb [OX7 or CD86/B7.2 (24F)]. After incubation, the cells were rinsed, resuspended in PBS, and analyzed on a FACScan using CellQuest software (Becton Dickinson, Grenoble, France).

**Mixed Leukocyte Reaction**

Increasing numbers of irradiated MHC class II⁺ PECs were cultured with 5 x 10⁶ purified allogeneic T cells in round-bottomed, 96-well plates in a final volume of 200 μl for 3 days at 37°C, 5% CO₂. 0.5 μCi of [³H]thymidine/well was added for the last 7 h (Amersham, Boston, MA). The cells were then harvested on glass filter fibers, and [³H]thymidine incorporation was measured using standard scintillation procedure.

**Microscopy**

Twenty-four hours after the injection of PKH26GL-labeled apoptotic cells, PECs were harvested and washed. Cells were seeded on coverslips in 6- or 24-well plates and allowed to adhere at 37°C for 3 h.

**Fluorescence Microscopy.** After excitation at 615 nm, active phagocytotic cells were counted by two different experimenters. The percentage of phagocytosis was determined as the number of active phagocytotic cells/total number of cells x 100.

**Confocal Microscopy.** Cells were fixed in 2% formaldehyde in PBS for 30 min at 4°C, blocked in 1% BSA/PBS for 20 min, and washed in PBS, before incubation with mAbs diluted in 0.3% BSA/PBS for 30 min at room temperature. Cells were rinsed three times and then bound with anti-OX17 (class II MHC), anti B7.2 (CD86), or anti-OK42 (CD 11b/c) and were detected using an antismouse FITC-conjugated IgG antibody. Morphology was revealed using reflection interference contrast. Confocal laser scanning microscopy was performed using a confocal laser scanning microscope system attached to a microscope with a krypton/argon laser (Leica). Simultaneous double fluorescence acquisitions were performed using 490 and 615 nm to excite FITC and PKH dyes using a x100 oil immersion objective.

**Immunization Models**

Six rats/experimental condition were coinjected i.p. with apoptotic tumor cells (500 μg) and IL-2 (2 x 10⁴ UI/kg; Chiron, Amsterdam, the Netherlands) at day -35, -28, -21, and -7 before challenging the rats i.p. with 10⁶ viable tumor cells (at day 0). Silica (50 mg/rat), PBS, or thioglycollate (3 ml/rat) were administrated i.p. into six rats, 24 h (PBS and silica) or 72 h (thioglycollate) before each injection of apoptotic tumor cells and IL-2.

**Statistical Analyses**

Each in vitro experiment was done at least twice. Our results were expressed as the mean ± SD. Statistical comparison of two means was done by Student’s t test. Statistical analysis of the in vivo experiment (three independent experiments) was performed using the log-rank test. Comparison of survival curves was considered statistically significant for P < 0.05.

**RESULTS**

**Immunization with Apoptotic Cells and IL-2.** The vaccination protocol used is described in Fig. 1A. Silica and PBS or thioglycollate were administrated 24 h (silica, PBS) or 72 h (thioglycollate) before each injection of apoptotic cells and IL-2. Rats were challenged i.p. with viable PROb tumor cells 7 days after the last apoptotic cell/IL-2 coinjection.

Regardless of the pretreatment, IL-2 alone could not induce any protection against a further challenge with viable tumor cells (control); rats died within 60 days after injection of the tumor cells (Fig. 1, B–D). Coinjection of apoptotic tumor cells plus IL-2 in PBS-treated rats resulted in 33% protection (P < 0.01), as described previously (9). However, treatments with apoptotic cells alone were not sufficient to induce protection or to increase rat survival (difference was not significant).

In silica-treated rats, both apoptotic cells alone and the combination of apoptotic cells plus IL-2 induced rat survival, respectively, by 33 and 66% (P < 0.001; Fig. 1C with control for both). Interestingly, silica treatment before vaccination enhanced the efficiency of apoptotic cells when compared with control (33% versus 0% for PBS-treated rats; P < 0.01; Fig. 1, compare C with B). In contrast, thioglycollate administration totally abrogated the protective effect of apoptotic cells plus IL-2. We only noted a slight increase in the survival time of the injected rats under these conditions (difference was not significant; Fig. 1D).

**PEC Phenotype after Different Treatments.** Thioglycollate treatment induced a significant increase of PEC number, as shown in Fig. 2A. We counted 40 x 10⁶ cells in the thioglycollate-treated peritoneal cavity, compared with 10 x 10⁶ cells in PBS-treated cavities and 20 x 10⁶ in silica-treated cavities. This result is in accordance with previous data showing that thioglycollate induces peritoneal inflammation, causing massive recruitment of activated macrophages (8). To our surprise, the number of cells from the silica-treated peritoneal cavity was also significantly higher when compared with PBS-treated rats (P < 0.001), showing that silica did not damage peritoneal cells as described by others (7). Interestingly, it was evident that the silica had been phagocytosed by the phagocytic cells (Fig. 2B), without leading to disruption of these cells.

We next analyzed the phenotype of the PECs after the different treatments by flow cytometry. In all cases, PECs were strongly positive for CD11b/c (OX42; Fig. 3). These cells were also positive for CD45 (OX1), MHC class I (OX18), and OX41 (signal regulatory protein), showing the myeloid origin of these cells (data not shown).

Also we noted that silica treatment increased the NKR-P1 expression on PECs. However, these cells did not present any cytotoxic activity against the NK-sensitive cell line YAC-1 (data not shown), emphasizing the fact that these cells were not NK cells. A small percentage...
of lymphocytes (CD5+) were also found in the peritoneal cavity, regardless of the treatment (OX19; Fig. 3).

Fifteen % to 20% of the CD45+ cells were also MHC class II+ (OX17), as shown in Fig. 4A, but no significant difference among the PBS, silica, and thioglycollate groups was observed. Nonetheless, only MHC class II+ cells derived from the silica-treated peritoneal cavity expressed a significant amount of CD80 (20%) and/or CD86 (30%) molecules (Fig. 4B).

Allogeneic T-cell Stimulatory Capacity of MHC Class II+ (OX17+) Cell-enriched PECs. To investigate whether the MHC class II+ cells could play a role in T-cell activation, we performed a mixed leukocyte reaction using allogeneic T lymphocytes as responder cells and MACs-sorted MHC class II+ cells as stimulator cells.
As shown in Fig. 5, MHC class II$^+$ cells derived from silica-treated peritoneal cavities were the most efficient in stimulating allogeneic T-cell proliferation. Both PBS- and thioglycollate-treated peritoneal cavity-derived cells induced a basal proliferation of allogeneic T cells independent of the T:MHC class II$^+$ cell ratio. In contrast, when using the silica-treated peritoneal cavity-derived cells as stimulators, the $[^3H]$thymidine incorporation by T cells was four times higher at a 4:1 ratio compared with that observed with PBS- or thioglycollate-treated, peritoneal cavity-derived cells.

**Apoptotic Cell Phagocytosis by PECs.** To determine the nature of cells from the peritoneal cavity that phagocytosed the apoptotic extracts, apoptotic bodies stained with PKH26GL were injected i.p. after PBS, silica, or thioglycollate treatments. PECs were collected 24 h later, and the in vivo phagocytosis was quantified by microscopic analysis (Fig. 6). We observed that 45% of the cells derived from the thioglycollate-treated peritoneal cavity engulfed apoptotic tumor cells. This percentage was quite high because it represented about four times that amount observed in PBS-treated, peritoneal cavity-derived cells. In contrast, silica treatment did not significantly change the ability of PECs to eliminate apoptotic cells (16% versus 10% for PBS).

**Phenotype of PECs after Apoptotic Cell Phagocytosis.** To further assess whether PECs that had phagocytosed apoptotic cells are potential antigen-presenting cells, we next performed flow cytometry on MHC II$^+$ cells selected as described above. This population represents macrophages and immature or mature dendrite-like cells.

Four %, 8%, and 7.5% of the MHC II$^+$ cells derived, respectively, from the PBS-, silica-, and thioglycollate-treated peritoneal cavities were positive for PKH26GL labeling, showing that these cells had phagocytosed apoptotic cells (Fig. 7). Three % (PBS), 5% (silica), and 7% (thioglycollate) of these MHC$^+$ cells were PKH26$^+$/CD86$^-$, indicating that these cells were likely poor antigen-presenting cells, either macrophages or immature dendritic cells.

Of interest, 19% of the MHC class II$^+$ cells derived from the silica-treated peritoneal cavity expressed the B7.2/CD86 costimulatory molecule, including 3% of MHC II$^+$ that also contained apoptotic bodies (Fig. 7). These MHC II$^+$/CD86$^+$ cells can be considered as...
mature dendrite-like cells. Concurrently, we found <1% of these mature MHC II⁺/CD86⁺ in the PBS- and the thioglycollate-treated peritoneal cavities that contained apoptotic bodies. To confirm the flow cytometric analysis, total PECs were seeded on coverslips for immunocytochemical analysis and stained with the anti-CD11b/c (OX42, used as a positive control), anti-MHC class II⁺ (OX17), or anti-B7.2 (CD86) antibodies. Cells were analyzed by confocal microscopy (Fig. 8).

It appeared that whatever the treatment, not all of the phagocytosing cells were MHC class II⁺. It was also evident that in the PBS- and silica-treated peritoneal cavities, a small but significant percentage of the MHC II⁺ cells (~5%) presented long cytoplasmic extensions similar to those observed in dendritic cells (Fig. 8, B and E). CD86⁺ cells containing PKH26-stained apoptotic bodies were only found in silica-treated rats (Fig. 8F).

DISCUSSION

We have shown previously that in vivo treatment of established peritoneal carcinomatosis from poorly immunogenic tumor cells with injection of apoptotic cells plus IL-2 led to tumor regression (9). We have proposed that the beneficial effect of this treatment was the consequence of the in vivo phagocytosis of apoptotic tumor cells by professional antigen-presenting cells (11). In this respect, we have demonstrated that tumor-bearing rats were cured with an 80% success rate by injection of monocyte-derived cells that had phagocytosed apoptotic tumor cells (5).

Here we report the capacity of apoptotic cells to prime a functional immune response in vivo. Coinjection of poorly immunogenic apoptotic cells and IL-2 protected rats against an additional i.p. injection of viable tumor cells (33% survival). Using the B16 melanoma model, Ronchetti et al. (4) obtained similar results to ours, although we never reached the 100% protection obtained in mice vaccinated with immunogenic nonproliferative cells.

The protective effect of apoptotic cells was lost when apoptotic cells were injected in a thioglycollate-treated peritoneal cavity. As described by Van Vugt et al. (6), i.p. administration of thioglycollate induces a chronic inflammatory state characterized by an increase in the total peritoneal cell number. Most of the cells derived from the treated peritoneal cavity were labeled by OX41 and OX42 antibodies, as shown previously by Robinson et al. (12). Both are known to bind...
granulocytes, macrophages, and dendritic cells. By microscopy, we observed that these cells efficiently phagocytosed apoptotic cells in vivo, because 45% of thioglycollate-treated PECs had engulfed fluorescent apoptotic cells after 24 h. However, in these conditions, the percentage of phagocytosis of the purified MHC class II+ cells reached 7.5%, with only 0.5% of the cells being CD86+ (dendritic-like cells). These results strongly suggested that under these inflammatory environmental conditions, macrophages accounted for the massive elimination of apoptotic cells. These inflammatory macrophages (mainly MHC class II+, CD86+), which are poor antigen-presenting cells, may favor antigen sequestration that could inhibit tumor antigen presentation, resulting in the inefficiency of the vaccination with apoptotic cells. Indeed, phagocytosis of apoptotic cells by macrophages induces a suppressive phenotype (13). Whether cross presentation of antigen from internalized apoptotic cells by macrophages results in cross-priming in vivo depends on their degree of activation or their ability to migrate to lymphoid tissues (14). Albert et al. (15) suggested that macrophages scavenge and degrade rather than cross-present the ingested apoptotic cells.

In contrast, silica treatment significantly enhanced the protective effect of apoptotic cells plus IL-2 vaccine. Under these conditions, 66% of treated rats survived (compared with the 33% for control). The nature of peritoneal exudate cells was similar in the different conditions tested in vivo except for NKR-P1 staining, which appeared on PECs derived from the silica-treated cavity. The relevance of this positive staining is unknown because these monocyte-derived cells did not present cytotoxic activity against the NK-sensitive YAC-1 cell line. The expression of NKR-P1 on non-NK cells has been reported previously, in particular on rat spleen dendritic cells (16).

Also, whatever the treatment, 15–20% of MHC II+ cells were present among PECs. However, positive staining with the anti-CD80 and CD86 antibodies was only noted for 20 and 30%, respectively, of the MHC II+ cells derived from the silica-treated peritoneal cavity. Indeed, we showed that these cells were the only cells effectively competent to induce T-cell proliferation in a mixed leukocyte reaction, suggesting their strong allostimulation capacity.

Silica administration at the doses we used did not result in macrophage depletion as described by others (7). On the other hand, in vitro internalization of silica particles has also been described to stimulate peritoneal macrophages to release inflammation-promoting mediators (17). These factors secreted by macrophages were shown to enhance the mixed leukocyte reaction initiated by dendritic cells (18). It was also shown that the accessory activity of PECs was found to increase with the amount of added silica in vitro (19). As reported here, this effect was associated with an increase of the expression of CD80+ / CD86+ at the cell surface, as also reported on mouse dendritic cells (20). Thus, silica may have directly promoted the development of a dendritic-like cell population. In addition, we found a significant number of MHC II+ /CD86+ cells that had phagocytosed apoptotic cells among the silica-induced PECs. By immunocytofluorescence analysis, it also appears that a small percentage of MHC class II+ cells presented long cytoplasmic extensions, typical of dendritic cells. In contrast, this population of mature dendritic-like cells was not found in thioglycollate-treated peritoneal cavities.

All together, our data showed that administration of silica enhanced a mature dendritic-like cell population. It is well described that mature dendritic cells, rather than macrophages, or immature dendritic cells are able to cross-present antigenic material derived from apoptotic cells (15). It has also been shown by using FITC-labeled rat tumor cells that proteins released from these cells were engulfed by inflammatory cells and transported to lymph node T-cell areas, where they can induce a specific immune response (21). Recently, Huang et al. (22) also demonstrated that a subset of rat dendritic cells endocytosed and transported apoptotic epithelial cells to the T-cell areas in lymph nodes.

However, the nature and the degree of maturation of the phagocytic cells strongly affect the immune response from tolerance to immune activation. It is noteworthy that occupational exposure to silica dust has been examined as a possible risk factor with respect to several systemic autoimmune diseases (23). Our results suggest that silica may boost the immune response, probably through regulating dendritic cell activity. Indeed, the protective immune response elicited in vivo by immunization with apoptotic tumor cells has already been shown by Ronchetti et al. (4) to inversely correlate with the presence of professional phagocytes. These authors described a higher vaccination efficiency in apoptotic cell-vaccinated animals injected with carragenan compared with those injected with granulocyte/macrophage-colony stimulating factor. The main difference between our respective models is that in contrast to B16 melanoma cells, PRob cells are not immunogenic. This raises the question of the mechanisms by which apoptotic cells become immunogenic. These mechanisms are currently under investigation.

In conclusion, we provide evidence that the vaccination efficiency with apoptotic tumor extracts not only depends on the phagocytic activity but also on the environment in which phagocytosis occurs. Therefore, future therapies using apoptotic cells should consider the local conditions of inflammation.

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