Dendritic Cells Injected Via Different Routes Induce Immunity in Cancer Patients[1]

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Dendritic cells (DC) represent potent APCs that are capable of generating tumor-specific immunity. We performed a pilot clinical trial using Ag-pulsed DC as a tumor vaccine. Twenty-one patients with metastatic prostate cancer received two monthly injections of DC enriched and activated from their PBMC. DC were cocultured ex vivo with recombinant mouse prostatic acid phosphatase as the target neoantigen. Following enrichment, DC developed an activated phenotype with up-regulation of CD80, CD86, and CD83 expression. During culture, the DC maintained their levels of various adhesion molecules, including CD44, LFA-1, cutaneous lymphocyte-associated Ag, and CD49d, up-regulated CCR7, but lost CD62 ligand and CCR5. In the absence of CD62 ligand, such cells would not be expected to prime T cells efficiently if administered i.v. due to their inability to access lymphoid tissue via high endothelial venules. To assess this possibility, three patient cohorts were immunized with Ag-pulsed DC by i.v., intradermal (i.d.), or intralymphatic (i.l.) injection. All patients developed Ag-specific T cell immune responses following immunization, regardless of route. Induction of IFN-γ production, however, was seen only with i.d. and i.l. routes of administration, and no IL-4 responses were seen regardless of route, consistent with the induction of Th1-type immunity. Five of nine patients who were immunized by the i.v. route developed Ag-specific Abs compared with one of six for i.d. and two of six for i.l. routes. These results suggest that while activated DC can prime T cell immunity regardless of route, the quality of this response and induction of Ag-specific Abs may be affected by the route of administration. *The Journal of Immunology*, **2001, 166: 4254–4259.**

Dendritic cells (DC)[3] represent the most potent APC of the immune system, uniquely capable of sensitizing naive T cells to novel Ags. The role of DC in initiating or priming immune responses to viral and bacterial Ags in vivo is well established (1–3). Animal studies have demonstrated that DC loaded with an appropriate tumor Ag can prime T cells capable of recognizing and killing tumor cells in an Ag-specific fashion (4–7). Moreover, DC-based immunization can lead to immunologic memory with protection against subsequent tumor challenges (7). These results have generated significant recent interest in the use of Ag-loaded DC as a tumor vaccine in humans (reviewed in Ref. 8). We initially demonstrated that DC can be used to vaccinate B cell lymphoma patients with the induction of Ag-specific T cells and clinical responses (9). Other groups have since used DC to treat malignancies such as melanoma, prostate cancer, and renal cell cancer (10–12).

DC presumably must home to secondary lymphoid organs to prime T cell responses. The extent to which DC cultured and loaded with Ag ex vivo are able to migrate to relevant lymphoid organs in humans is unknown. Labeling studies with radioactive tracers have demonstrated that there are significant differences in the distribution of DC-containing cell products that are administered by different routes (13, 14). Although cells injected i.v. collect in the lung and liver, cells injected s.c. or intradermally (i.d.) can migrate to draining lymph nodes with varying efficiencies, although a significant number of cells remain at the injection site. These experiments, however, were limited by their sensitivity and did not resolve whether sufficient DC are capable of reaching lymphoid organs and priming an immune response. Moreover, the capacity of DC to migrate to secondary lymphoid organs may be dependent on their state of activation. Immature DC are believed to preferentially migrate to peripheral tissues, while activated DC are thought to emigrate from peripheral tissues via lymphatics. Clearly, the optimal route of DC administration must be established for such an immunotherapeutic approach to be maximally immunogenic in humans.

In the study discussed in this report, we loaded DC with a recombinant protein Ag, prostatic acid phosphatase (PAP), in vitro and administered the cells via different routes to patients with prostate cancer. In addition to the i.v. route, we explored the i.d. and intralymphatic (i.l.) routes of administration. In humans, i.d. administration of visual dyes or radioactive tracers can be detected within draining lymph nodes, a technique that is clinically used for sentinel lymph node biopsies (15, 16). Intralymphatic administration involves cannulating lymphatic vessels in the feet as a means of delivering the cells directly into lymph nodes via the afferent lymphatics. This approach is performed clinically for lymphangiography and would presumably represent the most efficient means for delivering DC to secondary lymphoid organs where generation of the immune response is known to occur.

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[3] Abbreviations used in this paper: DC, dendritic cell; i.d., intradermal; PAP, prostatic acid phosphatase; mPAP, murine PAP; i.l., intralymphatic; CLA, cutaneous lymphocyte-associated Ag; VLA-4, very late Ag-4; TT, 5% nonfat dry milk in 0.05% Tween 20; CD62L, CD62 ligand; SI, stimulation index.

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Materials and Methods

Patients

Patients ($n = 21$) enrolled in the study were required to have histologically documented prostate cancer with recurrent or metastatic disease measurable by an abnormal and/or rising serum prostate-specific Ag level as well as detectable serum PAP levels. Patients were hormone refractory or hormone sensitive so long as no hormonal manipulations or other therapies, including immunosuppressive radiation or chemotherapy, were performed during the study. Trial subjects provided signed informed consent that fulfilled institutional review board guidelines before completing the screening process.

Ag production

cDNA encoding mouse PAP was cloned into the pBacPAK8 baculovirus recombination vector (Clontech, Palo Alto, CA) to generate recombinant baculovirus. Recombinant murine PAP (mPAP) was expressed as a His^6 fusion protein. Insect SF21 cells were infected with recombinant baculovirus, and PAP was purified from culture supernatants with a nickel-nitrilotriacetic acid column (Qiagen, Hilden, Germany) to >90% purity by SDS-PAGE.

DC preparation

The patients underwent unmobilized peripheral blood leukapheresis, with two total body blood volumes (8–14 liter of blood) processed with a COBE cell separator. PBMC were obtained by centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and then monocytes were depleted by density centrifugation through Percoll (Pharmacia) as previously described (7). Monocyte-depleted PBMC were incubated with recombinant PAP (2 μg/ml) in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% pooled human AB serum without the addition of exogenous cytokines. After a 24-h culture in a humidified incubator at 37°C with 10% CO_2, DC were further enriched from lymphocytes by centrifugation through a 15% (w/v) metrizamide gradient (Sigma, St. Louis, MO). The enriched DC were then cultured again overnight in medium containing 50 μg/ml recombinant PAP, washed free of Ag, resuspended in normal saline with 5% autologous serum, and infused. The DC dose was determined from the percentage of total cellular dose that expressed HLA-DR and lacked expression of CD3, CD14, CD19, and CD56 by flow cytometry. The average total cell dose was 112×10^6 cells/injection, with an average DC purity of 30%.

Flow cytometric analysis

Four-color flow cytometry was performed using a Becton Dickinson FACSCalibur (Mountain View, CA). APC-conjugated Abs to CD3, CD14, and CD19; PerCP-conjugated Abs to HLA-DR; and isotype-matched control Abs were obtained from Becton Dickinson. APC-conjugated Ab to CD56, PE-conjugated Ab to CD80, and FITC-conjugated Ab to cutaneous lymphocyte-associated Ag (CLA) were obtained from BD PharMingen (San Diego, CA). PE-conjugated Ab to CD83 was obtained from Immunotech (Westbrook, ME). FITC-conjugated Abs to CD44 and CD49d (very late Ag-4 (VLA-4)) were obtained from Serotec (Raleigh, NC). PE-conjugated Ab to CCR5 was obtained from R&D Systems (Minneapolis, MN). Unconjugated Ab to CCR7 was supplied by Dr. Lijun Wu (Leukosite, Cambridge, MA). FITC-conjugated goat anti-mouse Ab was obtained from Caltag (Burlingame, CA). PE-conjugated Ab to CD62 ligand (CD62L) and CD11a (LFA-1) were produced in our laboratory. Cells (1×10^6) were suspended in staining buffer (Dulbecco's PBS with 1% FCS and 0.1% sodium azide) with human IgG at 1 mg/ml (Sigma) for 10 min at 4°C to block the Fc receptors. Samples were then stained with the described Abs at the recommended concentrations for 30 min at 4°C, washed three times with staining buffer, and analyzed. Data were evaluated and presented using FlowJo software (Tree Star, San Carlos, CA).

Allogeneic MLR

PBMC from random donors were used as responders in allogeneic MLR for all of the patients. Stimulators in the assays represent PBMC or enriched DC from the patients. Fifty thousand responders were cocultured with varying numbers of irradiated (3000 rad) stimulators in triplicate in 96-well U-bottom plates (Costar, Cambridge, MA) in RPMI 1640 containing 10% pooled human serum. Proliferation was assessed on the basis of 18 h [^{3}H]thymidine incorporation after 6 days of culture as measured in a Microbeta counter (Wallac, Turku, Finland).

DC vaccination

Twenty-one prostate cancer patients were immunized twice with recombinant PAP-loaded DC, 4 wk apart. Patients were sequentially assigned to three cohorts to receive both DC immunizations via i.v. ($n = 9$), i.d. ($n = 6$), or i.l. ($n = 6$) injections. For i.v. administration, DC were suspended in 100 ml of normal saline with 5% autologous serum and infused by a peripheral i.v. catheter following premedication with acetaminophen and diphenhydramine. For i.d. administration, DC were suspended in 4 ml of normal saline with 5% autologous serum and administered by 16–24 i.d. injections into the medial thighs following application of topical anesthetic. For i.l. administration, DC were also suspended in a volume of 4 ml, but were infused via a catheter cannulating a lymphatic channel in the dorsum of the foot that was identified through a small incision. Patients were evaluated for treatment related toxicity by the National Cancer Institute common toxicity criteria during and following vaccination as well as for the induction of anti-DNA Ab and rheumatoid factor following the vaccination.

T cell functional assays

Blood was obtained from patients before immunization, 1 mo following the DC immunizations, and then every 1–3 mo thereafter until clinical progression. PBMC were obtained by centrifugation over Ficoll-Hypaque (Pharmacia) and were cultured at 100,000 cells/well in triplicate in 96-well U-bottom plates (Costar) in medium containing 10–50 μg/ml of mPAP. Other T cell stimulators used for in vitro assays included influenza protein (Connaught, Swiftwater, PA) and PMA with ionomycin (Sigma) as positive recall controls. T cell proliferation was assessed on the basis of 18-h [^{3}H]thymidine incorporation after 6 days of culture as measured in a Microbeta counter (Wallac). The results are expressed as stimulation indexes representing counts per minute relative to baseline counts without Ag. A stimulation index >2 was defined as a response. Supernatants were also collected from cell cultures, frozen, and assessed for cytokine secretion by ELISA as described below.

Cytokine ELISA

Ninety-six-well Immulon-4 plates (Dynatech, Chantilly, VA) were coated overnight at 4°C with 50 μl of the primary Ab to IL-4, IFN-γ, and TNF-α (BD PharMingen) in 0.1 M carbonate-bicarbonate buffer (pH 9.5). Wells were blocked with Blotto (5% nonfat dry milk in 0.05% Tween 20 (TT)) for 2 h at room temperature. Frozen cell supernatants were added to the wells and incubated at room temperature for 3 h, after which the appropriate biotinylated secondary Ab resuspended in Blotto was added and incubated for 1 h at room temperature. After washing with TT, HRP-conjugated rabbit anti-mouse Ab was added and incubated for 30 min at room temperature. The plates were washed and developed with the substrate tetramethyl benzidine (Zymed, South San Francisco, CA). The reaction was stopped with 1 N HCl, and the OD was read at 450 nm on a microplate reader (Bio-Rad, Hercules, CA). The ELISA sensitivity for the three cytokines assayed was 25 pg/ml.

Anti-PAP ELISA

Sera collected simultaneously with the PBMC were frozen and analyzed in batches. Ninety-six-well Immulon-4 plates were coated overnight at 4°C with mPAP, blocked with 5% dehydrated nonfat milk in 50 mM TBS and TT, and washed with TT. Patient sera were diluted in PBS, added to wells, and incubated for 1 h at room temperature. Plates were then washed and incubated with goat anti-human total Ig Ab labeled with HRP (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for an additional hour at room temperature. The plates were washed and developed with the substrate tetramethyl benzidine (Zymed). The reaction was stopped with 1 N HCl, and the OD was read at 450 nm on a microplate reader (Bio-Rad).

Statistical analyses

T cell proliferative responses, cytokine ELISA, and Ab titers before and after DC vaccination were analyzed with the paired sign test (StatView; SAS Institute, Cary, NC).

Results

To assess the activation state of DC administered to the patients, ex vivo-enriched DC were studied by flow cytometry either from the leukapheresis product before enrichment or after ex vivo enrichment and culture immediately before infusion as

the autologous vaccine. DC were enriched from PBMC with buoyant density-based centrifugation and ex vivo cultured over the course of 2 days as previously described (9). The DC were characterized phenotypically by their expression of HLA-DR and lack of lineage markers CD3, CD14, CD19, and CD56. Pre-enrichment, DC represented ~1% of the PBMC, and a subset expressed CD86 (Fig. 1*A*). Following enrichment, DC possessed an activated phenotype as evidenced by their increased expression of MHC class II, and costimulatory molecules CD80 and CD86, as well as DC activation marker CD83 (Fig. 1*B*). Enriched DC were significantly more potent than PBMC in stimulating an allogeneic MLR (Fig. 1*C*).

We then examined the expression of adhesion molecules, as well as homing and chemokine receptors on DC. When compared with immature DC in the blood (Fig. 2*A*), DC following enrichment maintained their expression of LFA-1, CD44, VLA-4, and CLA (Fig. 2*B*). They, however, lost their expression of CD62L and CCR5, but up-regulated surface expression of CCR7.

During the 2 days of ex vivo culture, enriched DC were coincubated with recombinant mPAP protein to enable the cells to take up and process the Ag. Patients were given two monthly injections of their Ag-loaded cells through one of the three routes examined. The average total cell dose was 112×10^6 cells/injection, with an average DC purity of 30%. The average DC dose for the i.v. route was 10.2×10^6 cells (range, 0.3–32×10^6 cells); for the i.d. route the dose was 12.0×10^6 cells (range, 1.6–32.4×10^6 cells); and for the i.l. route the dose was 12.2×10^6 cells (range, 1.6–40.4×10^6 cells). Administration of DC was well tolerated via all routes, with minor side effects related to the route of administration (Table I). Self-limited transfusion reactions manifesting as National Cancer Institute common toxicity grade 2 fever and rigors were seen in two of 18 i.v. infusions. Self-limited skin erythema was seen following 3 of 12 i.d. injections. Tender adenopathy in a draining inguinal lymph node developed following 1 of 12 i.l. injections. Patients were also assessed for the development of autoimmunity. Induction of anti-DNA Abs was seen in three of nine patients receiving i.v. injections and one of six patients receiving i.l. injections. One of six patients who received his cell via i.l. injection also developed an elevated rheumatoid factor following vaccination. Despite these findings, no patients developed clinical signs of autoimmunity following vaccination.

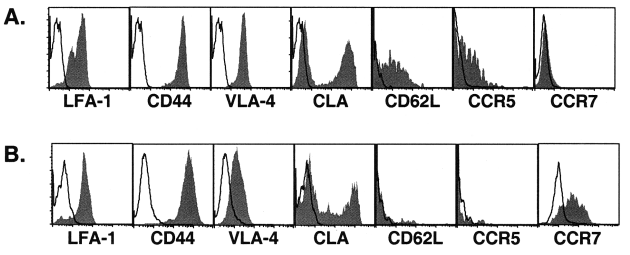

FIGURE 2. DC expression of homing and chemokine receptors. DC contained in the PBMC (*A*) and DC product following culture and enrichment (*B*) were gated by their expression of HLA-DR and lack of lineage markers CD3, CD14, CD19, and CD56. Enriched DC maintain their expression of LFA-1, CD44, VLA-4, and CLA and down-regulate CD62L and CCR5, but up-regulate CCR7 expression. Open histograms represent stain with isotype-matched control Abs. The results shown were obtained from the same patient, but are representative of data from three different patients.

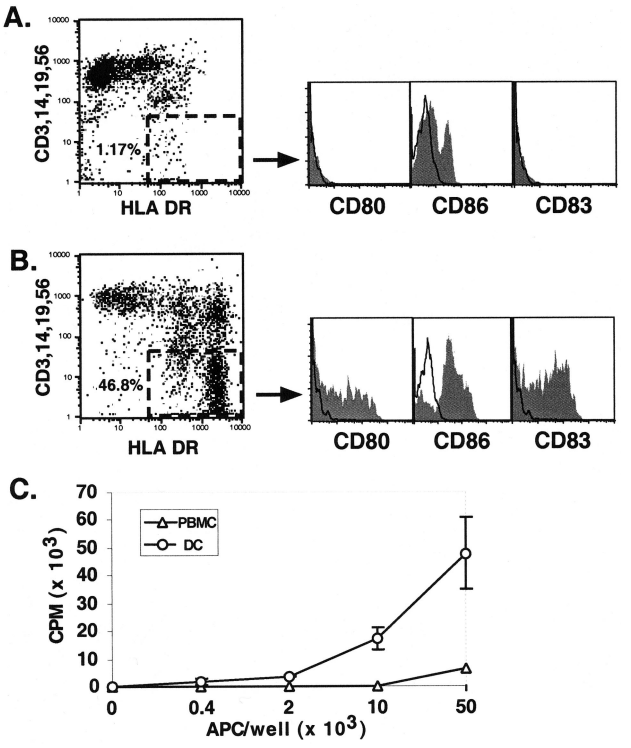

FIGURE 1. Phenotype of activated Ag-pulsed DC. DC contained within the PBMC pre-enrichment (*A*) and DC product following culture and enrichment (*B*) were gated by their expression of HLA-DR and lack of lineage markers CD3, CD14, CD19, and CD56 (dashed rectangle]). Ex vivo-enriched DC possess an activated phenotype, including up-regulation of CD80, CD86, and CD83. Open histograms represent stain with isotype-matched control Abs. Data were obtained from the same patient, but are representative of data from three different patients. *C*, Allostimulatory capacity of DC product (○) and PBMC (△) were compared. Fifty thousand PBMC from random donors were cocultured in triplicate in wells with the DC or PBMC at the indicated cell numbers. Proliferation was assessed after 6 days of culture by [^{3}H]thymidine incorporation over 18 h. The results shown were obtained from the same patient, but are representative of data from three different patients.

PBMC obtained from patients before and after each of the vaccinations were assessed for T cell proliferation. Immunization via i.v. as well as i.d. and i.l. routes induced Ag-specific T cell responses in all of the patients (Fig. 3). Although no patients had an mPAP-specific proliferative response before vaccination, six of nine (66.7%), five of six (83.2%), and four of six (66.7%) patients developed PAP-specific T cell proliferation with stimulation indexes (SI) >2 following a single DC vaccination administered i.v., i.d., and i.l., respectively. Nevertheless, all vaccinated patients developed T cell responses following two DC vaccinations. There was no statistically significant difference in SI following vaccination among the groups.

Table I. *Side effects associated with different routes*

	i.v. 9 Patients/ 18 Injections	i.d. 6 Patients/ 12 Injections	i.l. 6 Patients/ 12 Injections
Transfusion reactions	2/18	0/12	0/12
Skin reaction	0/18	3/12	0/12
Tender adenopathy	0/18	0/12	1/12
Induction of anti-DNA Abs	3/9	1/6	1/6
Induction of rheumatoid factor	0/9	0/6	1/6

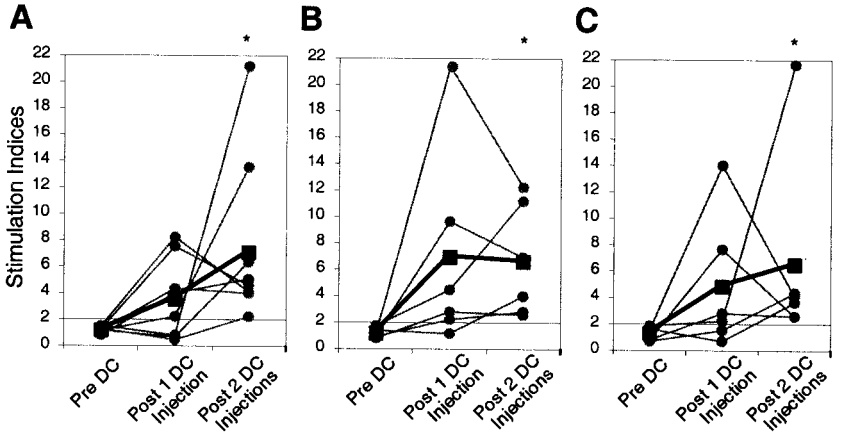

FIGURE 3. Induction of Ag-specific proliferative responses by activated DC administered through different routes. PBMC from each of the 21 patients were assessed for T cell proliferation to mPAP before and 3 wk after the first and second DC vaccinations. T cell proliferation was measured in triplicate wells by [^{3}H]thymidine incorporation over 18 h following 6 days of culture. Serial results for each patient (●) are expressed as SI representing counts per minute relative to baseline counts without Ag for that assay. Patients developed mPAP-specific immunity with i.v. (*A*), i.d. (*B*), and i.l. (*C*) routes of administration. The averages (■) for each time point are also represented. ∗, Induced proliferative responses were statistically significant from preimmunization levels following two vaccinations ($p > 0.05$, paired sign test) regardless of route.

To further characterize the induced immune responses, cytokine profiles were assessed in culture supernatants before or following both immunizations (Fig. 4). One of six patients developed a weak TNF-α response in the i.v. group, while no patients from the other groups developed TNF-α responses. However, when IFN-γ production was assessed, four of six patients in the i.d. and three of six patients in the i.l. groups had induction of responses, and the responses in these groups were statistically significant. Curiously, no patients from the i.v. group had induction of IFN-γ production following immunization. Finally, none of the patients had measurable IL-4 in the culture supernatants at any point.

The induction of PAP-specific Abs was also assessed in serum obtained pre- and postvaccination (Fig. 5). Five of nine patients (55.6%) vaccinated i.v. developed Abs to mPAP, while one of six (16.7%) patients and two of six patients (33.3%) developed Abs following immunization via i.d. and i.l. routes of injection, respectively. Moreover, although the titers of Ab were low in the i.d. and i.l. cohorts (1/40), the titers seen in the i.v. cohort were significantly higher (1/80 to 1/3560).

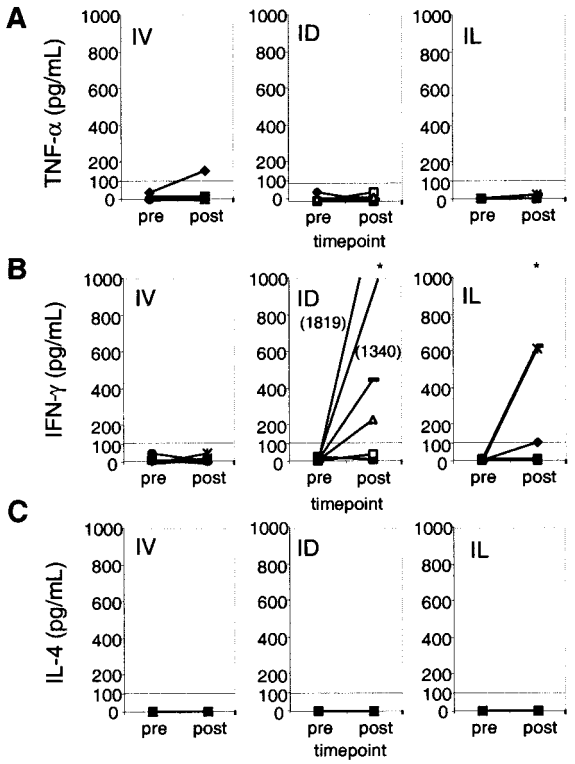

FIGURE 4. Induction of Ag-specific cytokine production. PBMC from 18 patients were assessed for secretion of TNF-α (*A*), IFN-γ (*B*), and IL-4 (*C*) in responses to mPAP before (pre) and 3 wk after (post) the final DC vaccination. Supernatants from PBMC cultures stimulated with mPAP were assessed for the corresponding cytokines by sandwich cytokine ELISA. Serial results for each patient are expressed as picograms per milliliter. Patients immunized via i.v. (*left panels*), i.d. (*middle panels*), and i.l. (*right panels*) were compared. ∗, Induced cytokine production was statistically significant from preimmunization only for IFN-γ following i.d. and i.l. administration of the DC product ($p > 0.05$, paired sign test).

Discussion

We initiated a clinical trial investigating the capacity of DC pulsed with a xenogeneic homologue to prime immunity and break tolerance to self-Ags (manuscript in preparation). In this trial, mouse PAP was used to immunize patients with recurrent and/or metastatic prostate cancer. Mouse PAP is 81% homologous to human PAP at the amino acid level and presumably would be immunogenic in humans. However, because mouse PAP represents a foreign Ag, T cell responses detected following vaccination would represent a primary immune response

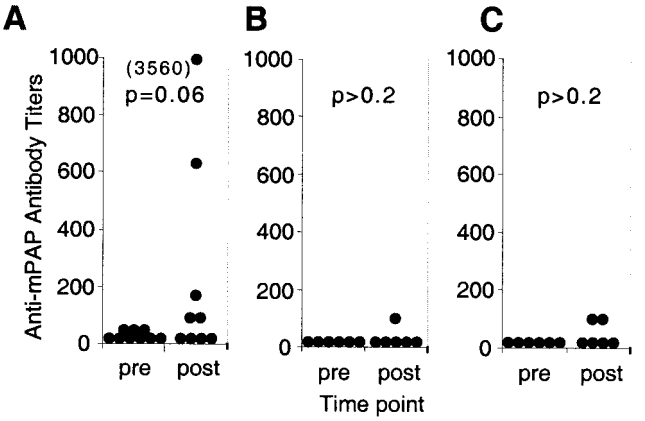

FIGURE 5. Induction of mPAP-specific Ab. Sera from each of the 21 patients were obtained before (pre) and 3 wk after (post) the final DC vaccination and assessed for Abs to mPAP by ELISA. Serial Ab levels for each patient (●) are expressed as end point titers. Patients immunized via i.v. (*A*), i.d. (*B*), and i.l. (*C*) routes were compared. Statistical significance was assessed with the paired sign test.

from naive T cells rather than inducing a recall immune response from memory T cells. Consistent with this, T cell proliferative responses to mPAP could not be elicited in any of the patients before vaccination. Patients in this trial were sequentially accrued to cohorts receiving the cells via differing routes of administration to assess how each route was capable of priming immunity.

During their life cycle, DC vary in their expression of a variety of molecules and receptors to coordinate their migration to particular target tissues (17). DC precursors that circulate in the blood migrate to the various tissues through interactions with their selectins, including CD62L; adhesion molecules including LFA-1, VLA-4, CD44, and CLA; and chemokine receptors including CCR1, CCR5, and CCR6 (18–22). Once DC become activated, a process that would usually occur within the tissues, DC emigrate from tissues via the draining lymphatics and are drawn by chemokines such as macrophage inflammatory protein-3β through CCR7 to lymphoid organs, such as lymph nodes, where they interact with naive T cells (22, 23). DC generated by our enrichment process, which includes 2 days of ex vivo culture in Ag, possessed an activated phenotype. The cells maintained their expression of adhesion molecules important for migrating to tissues, but lost their expression of CD62L. Following i.v. administration, these DC would be unable to home directly to lymphoid organs via high endothelial venules. Enriched DC also down-regulated their expression of CCR5 while up-regulating their expression of CCR7. This pattern would allow the activated DC to migrate to secondary lymphoid organs via afferent lymphatics.

The extent to which immunity was primed was not statistically different among the different routes despite the higher efficiency by which DC are presumably delivered into lymph nodes with i.l. injection. DC injected i.d. and i.v. should be capable of accessing lymphoid organs sufficiently to prime naive T cells such that an expanded pool of memory T cells can be measured in the blood. Because activated DC lack CD62L but express CCR7, DC administered i.v. may access the secondary lymphoid organs via lymphatics within tissues, rather than directly through high endothelial venules.

To our knowledge, this study represents the first to examine the immune-priming capacity of ex vivo activated human DC administered via different routes. Our results indicate that DC can prime CD4 T cell responses when administered by any of the studied routes. These results indicate that relatively small doses of activated DC are capable of priming immunity regardless of the tissue compartment where they are initially located, demonstrating their potency. Although the enriched DC product contained some contaminating T cells, the number of T cells transferred ($<5 \times 10^7$) is less than the sizeable dose required to produce any systemically measurable immune response (24, 25). The DC product also contained some B cells and monocytes, although these cell types have not been demonstrated to prime immunity in vivo in humans. Nevertheless, their potential contribution to the immune response cannot be excluded.

In contrast to the T cell proliferative response, the cytokine profile of the T cells generated by the immunization procedure differed with route of administration. Mouse studies examining the capacity of DC to immunize when given through i.v. and s.c. routes have demonstrated superiority of s.c. injection over i.v. injection in the induction of CTL (26, 27). Our study in humans demonstrates that i.d. and i.l. administrations of DC induce Th1 immunity with greater frequency than i.v. administration. In contrast, i.v. administration was associated with a significantly higher frequency and titer of Ag-specific Abs. Generation of Abs in addition to cellular immunity may be desirable in some clinical situations. Abs specific for certain Ags (e.g., lymphoma Id, CD20, and her2-*neu*) have demonstrated efficacy in treating malignancies expressing these Ags (28). Induction of Abs such as these in vivo would represent an alternative or additive approach as an immunotherapy. On the other hand, the simplicity, lack of transfusion reactions, and frequency of IFN-γ responses seen with i.d. administration are potential advantages with this latter route, especially in the setting where induction of Th1 immunity is desired.

Acknowledgments

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