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Active Macromolecule Uptake by Lymph Node Antigen-Presenting Cells: A Novel Mechanism in Determining Sentinel Lymph Node Status

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Background: Although sentinel lymph node (SLN) biopsy is a powerful staging tool for patients with melanoma and breast cancer, controversy remains regarding specific aspects of technique. We examined particle uptake by antigen-presenting cells (APCs) to determine if this mechanism is responsible for the differential retention of radioactivity in SLNs relative to nonsentinel lymph nodes (NSLNs).

Methods: Mapping was conducted in pigs injected with vital blue dye, fluoroscein isothiocyanate-labeled human serum albumin (FITC-HSA), and one of two ^{99m}technetium-labeled tracers, i.e., human serum albumin, a small macromolecule, or unfiltered sulfur colloid, a mixture of small and large particles. Macromolecule uptake by APCs was studied in vitro by using FITC-HSA and measured by fluorescence-activated cell sorting (FACS). SLNs and NSLNs were analyzed by fluorescence microscopy or FACS, with counterstaining for leukocyte cell surface markers.

Results: Both radiotracers were effective. Cultured APCs rapidly took up FITC-HSA. Microscopy showed FITC-HSA in the subcapsular sinus of SLNs shortly after injection and subsequent distribution to interfollicular areas. FACS revealed increasing amounts of FITC-HSA in SLNs over time. Cells responsible for uptake were APCs, expressing major histocompatibility (locus) class II.

Conclusions: This report establishes active macromolecule uptake as a mechanism that determines SLN status. This mechanism has important implications for performing SLN biopsy.

Key Words: Sentinel lymph node—^{99m}Technetium-human serum albumin—^{99m}Technetium-sulfur colloid—Antigen uptake.

Sentinel lymph node (SLN) biopsy is rapidly becoming the standard for lymphatic staging of melanoma patients and promises to do the same for breast cancer patients, but controversy exists regarding some technical aspects of the procedure.[1–3] Although vital blue dye is considered the best criterion for accuracy in identifying the SLNs, the addition of radioactive tracers has improved sentinel node identification rates.[4–7] The addition of tracers has led to questions regarding the optimal particle size for tracers, the timing of their injection, and the significance of nodes that are radioactive but not stained with blue dye.

Studies of particle kinetics within the lymphatic circulation concluded ideal particle size for lymphoscintigraphy was between 1 and 15 nm.[8] Larger particles were believed to be too large to efficiently enter lymphatic vessels, and smaller particles were absorbed by blood capillaries. Comparisons of the commercially available tracers, human serum albumin (HSA; ~10 nm), sulfur colloid (SC; 50–300+ nm), and albumin colloid (200–1000 nm),[9] in patients and animal models revealed more rapid transit of HSA to higher echelon nodes,[10] similar levels of specific activity in draining nodes,[9] equal efficacy in SLN identification,[11] and a consistent require-

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ment for early imaging in lymphoscintigraphy to capture the first draining node.[12] We have previously confirmed the effectiveness of both HSA and SC, but found that significantly more nodes were removed in patients injected with SC than with HSA.[13] Finally, an animal model demonstrated more radioactive nodes in animals injected with filtered SC than unfiltered SC, when dissections were performed immediately after injection.[14] Most authors who use radiotracers currently recommend using large particles, such as SC, and proceeding rapidly to SLN biopsy after injection of tracer to catch activity in the SLN before tracer passes into higher order nodes.

We believed that clarification of the mechanism by which tracers are differentially taken up or retained by SLNs would help resolve some issues with regard to the type of tracers that can be used, the timing of tracer injection, and the significance of nodes that are radioactive but not blue. We studied macromolecule uptake of APCs in vivo and in vitro to determine if this mechanism contributes to differential radioactivity retention in SLNs.

MATERIALS AND METHODS

Lymphatic Mapping and Identification of SLN

In vivo studies were performed in 2- to 3-month-old female pigs. The animals were cared for in accordance with the guidelines of the animal use committee of the Albert Einstein Medical Center in Philadelphia, where the studies were conducted. After induction of general anesthesia, each limb was injected intradermally with 1 mg of fluoroscein isothiocyanate-labeled human serum albumin (FITC-HSA; Sigma, St. Louis, MO) in 1 ml of phosphate-buffered saline, 0.5 mCi ^{99m}technetium-labeled tracer (^{99m}Tc-SC [unfiltered] or ^{99m}Tc-HSA) in 0.5 ml of saline, and 1 ml of lymphazurin blue dye (US Surgical, Norwalk, CT). Draining lymphatic basins were dissected 10, 40, or 120 minutes after injection of FITC and technetium tracers 10 minutes after injection of blue dye. All nodes from the basin were harvested, separated, and examined for color by visual inspection and radioactivity using a handheld gamma probe (Navigator, US Surgical). Nodes with ex vivo counts more than three times that of adjacent nonlymphatic tissue were considered "hot." After counting, nodal tissue was either snap-frozen in liquid nitrogen for fluorescence microscopy or minced in 10% neutral buffered formalin for fluorescence-activated cell sorting (FACS) analysis.

Uptake of FITC-HSA by APCs In Vitro

Peripheral blood monocytes and lymphocytes were obtained from normal donors by leukapheresis and countercurrent elutriation as previously described.[15] Monocytes were cultured in Macrophage Serum Free Medium (Life Technologies, Gaithersburg, MD) with or without 10 ng/ml granulocyte/macrophage colony stimulating factor 1000 units/ml interleukin-4 (R&D Systems, Minneapolis, MN). These culture conditions induce a dendritic cell phenotype.[16] At various times after initiation of culture, cells were pulsed with 1 mg/ml FITC-HSA and incubated at 37°C for 2 hours (shorter incubations were used for groups harvested before 2 hours). Control groups consisted of similarly cultured B and T lymphocytes and monocytes cultured at 4°C. Antigen uptake was measured by flow cytometry as increase in mean channel fluorescence relative to 4°C controls.

Fluorescence Microscopy of Sentinel and Nonsentinel Nodes

Frozen nodal tissue was embedded in the cryogen OCT, sectioned at 10-μm thickness, and placed on gel-coated glass slides with coverslips. Sections were examined by fluorescence microscopy (IX70 microscope; Olympus, Tokyo, Japan) and photographic exposures made. Exposures were made of tissue from nonsentinel nodes and control nodes from limbs not injected with FITC-HSA. These were matched for magnification and duration of exposure.

Assessment of Lymph Node Macromolecule Uptake

Minced nodal tissue was fixed in formalin and prepared as a single cell suspension by using 70-μm nylon cell strainers (Becton Dickinson, Franklin Lakes, NJ). Cells were washed three times in buffer (phosphate-buffered saline with 1% fetal calf serum and 0.005% NaN_3). Counterstaining was performed by using purified mouse anti-pig CD4, CD8 (T-lymphocyte markers), granulocyte/monocyte marker, and the swine leukocyte antigen DR (SLA-DR; APC marker) (Pharmingen, San Diego, CA) for 20 minutes at 4°C. Nonspecific antibodies of isotypes matched to the experimental stains were used as controls. The secondary antibody was phycoerythrin-conjugated hamster anti-mouse IgGκ. Two additional washes followed each incubation with antibody stains.

RESULTS

SLN Mapping Concordance

SLNs were identified in all limbs. Of 35 SLNs harvested at 120 minutes by using ^{99m}Tc-HSA, 31 (89%) were both hot and blue. Three (9%) were hot only and one (3%) was blue only. Of 36 SLN nodes harvested with ^{99m}Tc-SC, 29 (81%) were hot and blue, and 7 (19%)

were hot only. In animals injected with ^{99m}Tc-HSA, 3.9 SLNs were harvested per limb, whereas 3.6 SLNs per limb were collected in ^{99m}Tc-SC-injected animals. Although counts varied from animal to animal, overall the counts were higher in animals injected with ^{99m}Tc-SC than in those injected with ^{99m}Tc-HSA (Fig. 1A). Mean counts at 120 minutes in SLNs of ^{99m}Tc-HSA-injected limbs was 471 (median, 400; range, 170-1400), and mean for ^{99m}Tc-SC-injected limbs was 1327 (median, 750; range, 50–5000). There was also a trend toward decreasing counts with time in the SLNs from limbs injected with ^{99m}Tc-HSA (Fig. 1B) but not in those injected with ^{99m}Tc-SC (not shown). However, there was no increase in the number of SLNs per basin in ^{99m}Tc-HSA limbs, and the ratio of counts between SLNs and NSLNs remained high in both ^{99m}Tc-HSA- and ^{99m}Tc-SC-injected limbs (Fig. 1C).

Macropinocytosis by APCs In Vitro

Mapping data in this model confirmed that molecules such as HSA are effective tracers in this model, as had been observed in clinical studies.[13] We postulated that this was the result of antigen uptake by APCs in lymph nodes. We used APCs in culture to determine if uptake of such macromolecules was possible. Cultured human monocytes were able to rapidly take up FITC-HSA. This uptake began as early as 15 minutes after pulsing and increased with the duration of incubation with FITC-HSA (Fig. 2 Top). Monocytes were less efficient at macropinocytosis of FITC-HSA compared with dendritic cells. This difference was readily apparent after 24 hours in culture (Fig. 2 Bottom). B cells were much less efficient at uptake of FITC-HSA and T cells failed to demonstrate significant uptake.

Distribution of FITC-HSA in SLN

When FITC-HSA was injected along with radiolabeled tracers into pigs, fluorescence microscopy demonstrated the presence of FITC-HSA in the subcapsular sinus of SLNs as early as 10 minutes after injection. By 40 minutes, fluorescence was distributed to the interfollicular areas of SLNs (Fig. 3). This pattern of distribution, with exclusion of tracer from follicles, persisted through the latest (120 minute) time point evaluated.

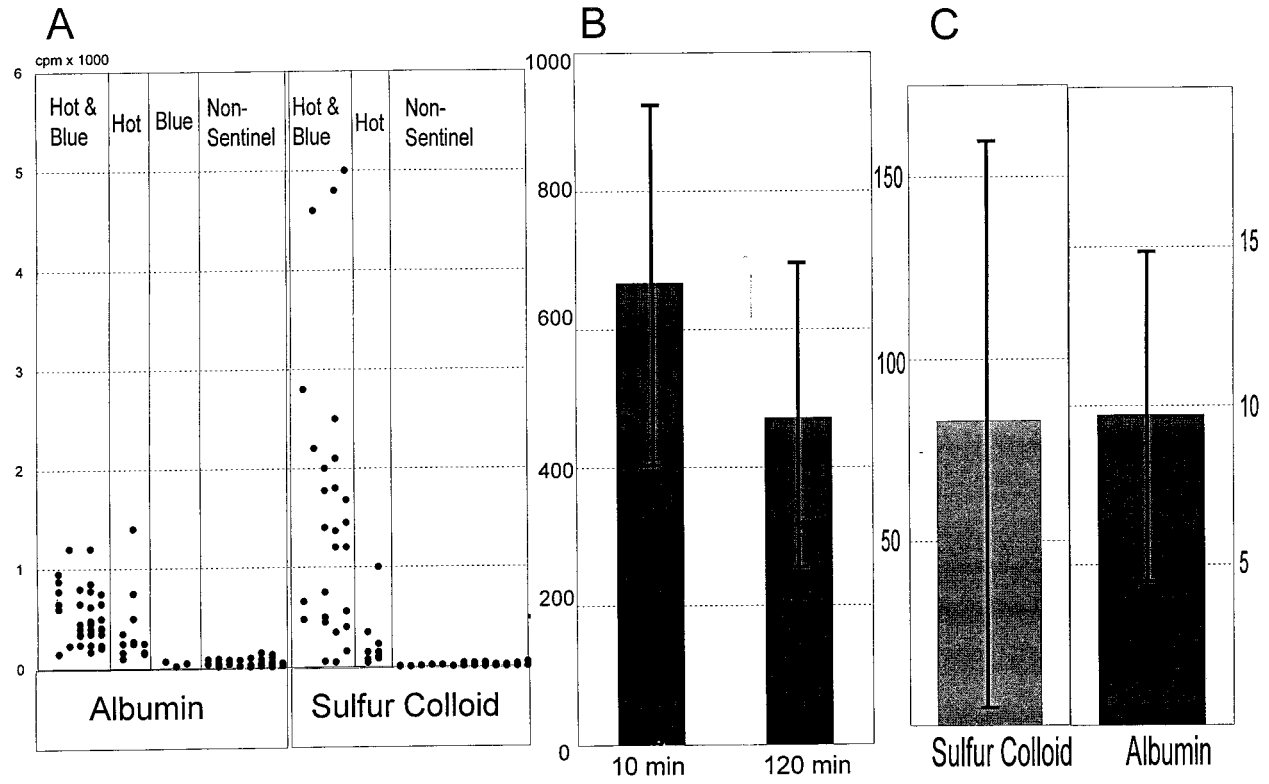

FIG. 1. (**A**) Scatter plot of radioactivity of harvested lymph nodes measured in counts per minute. Counts from sentinel lymph node (SLN) of human serum albumin-injected animals are lower than those of sulfur colloid-injected animals. (**B**) Mean counts per minute of SLN harvested at 10 minutes and 120 minutes after injection of tracer. SDs are marked with error bars. (**C**) Mean ratio of counts per minute between SLN and background. SDs are marked with error bars.

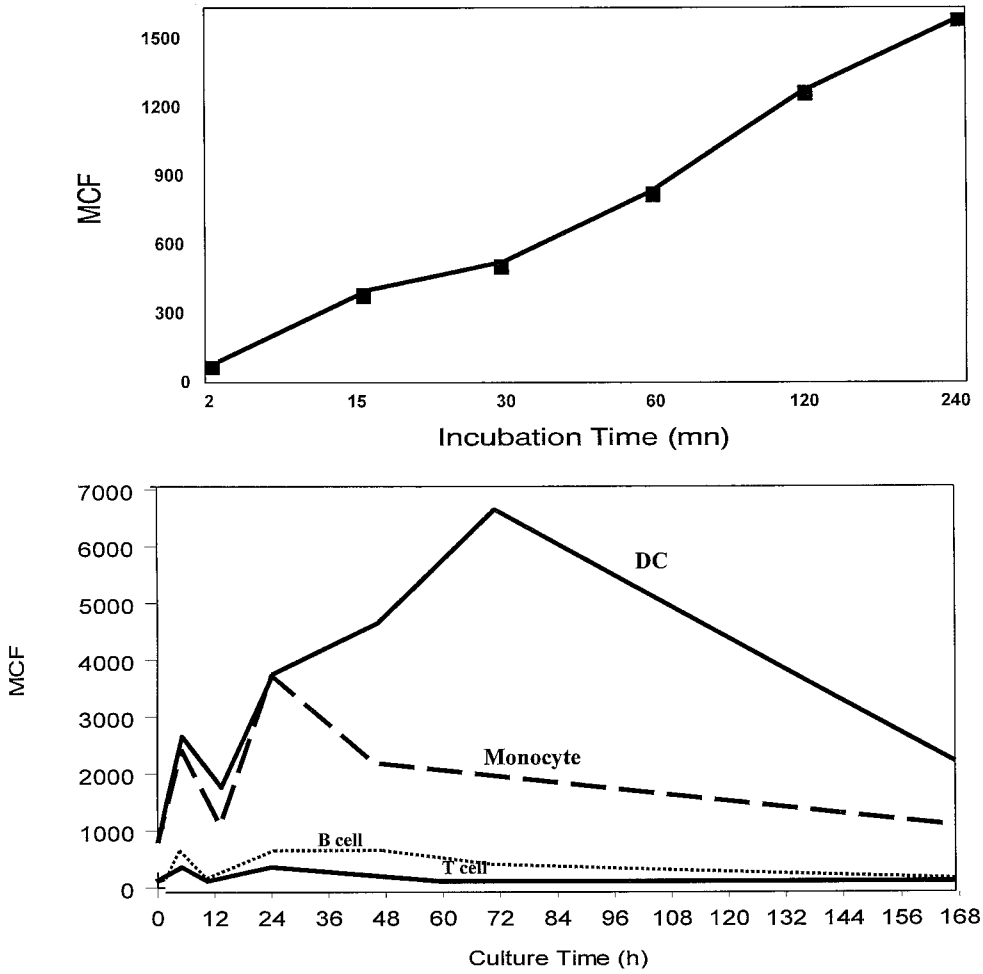

FIG. 2. (Top) Difference in mean channel fluorescence (MCF) between monocytes incubated with 1 mg/ml fluoroscein isothiocyanate-labeled human serum albumin (FITC-HSA) at 37°C and identical cultures incubated at 4°C. (Bottom) Difference in MCF between the indicated cell types after incubation with 1 mg/ml FITC-HSA for 2 hours at 37°C and identical cultures at 4°C. Dendritic cells were generated by culturing monocytes with granulocyte/macrophage-colony forming units and interleukin-4. Untreated monocytes, B cells, and T cells are also shown.

Time- and magnification-matched exposures of both NSLNs and nodes from limbs not injected with FITC-HSA demonstrated minimal background levels of fluorescence.

Uptake of FITC-HSA in Cells of Sentinel Nodes

FACS analysis of cells from SLNs demonstrated fluorescence slightly above control or NSLNs at the 10-minute time point in approximately half of the hot and blue nodes and no increased fluorescence in others (Fig. 4A). By 2 hours, high concentrations of fluorescence were observed in cells from all hot and blue nodes, particularly cells that were high on forward scatter, consistent with APCs. Hot-only nodes from animals injected with ^{99m}Tc-HSA were uniformly positive for FITC-HSA (Fig. 4B), whereas two of five hot-only nodes from animals injected with ^{99m}Tc-SC were negative for FITC-HSA (not shown). NSLNs failed to demonstrate any increased fluorescence above that in nodes from limbs not injected with FITC-HSA.

Uptake of FITC-HSA in APCs

Counterstaining of nodal cells was performed to determine if the large, FITC-positive cells were indeed APCs. Cells that were positive for FITC-HSA were positive for SLA-DR, the swine major histocompatibility locus class II molecule (Fig. 5), and the granulocyte/monocyte marker (not shown.) These cells with FITC-HSA uptake were negative for CD4 and CD8, T-lymphocytes markers. Although only SLA-DR-expressing cells were positive for FITC-HSA, some SLA-DR-expressing cells were not. These negative cells may repre-

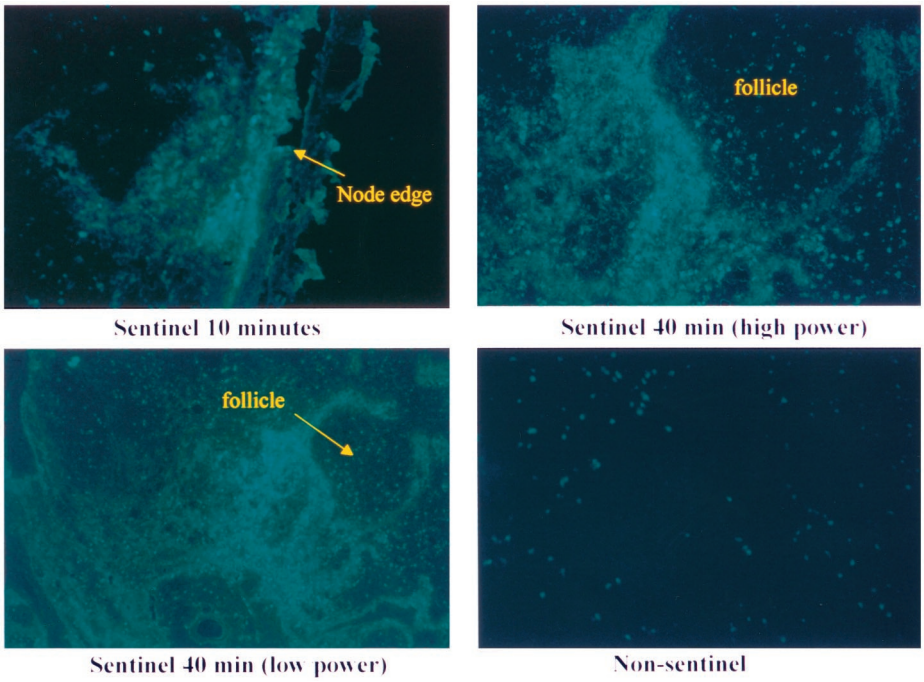

FIG. 3. Fluorescence microscopy of lymph node tissue. *Top left:* Edge of sentinel lymph node (SLN) collected 10 minutes after injection of fluorescein isothiocyanate-labeled human serum albumin (FITC-HSA). *Top right:* SLN collected 40 minutes after injection of FITC-HSA (×400). The distribution of FITC-HSA observed here, and in the *bottom left* panel at a lower magnification (×100), was to interfollicular areas of the node. FITC-HSA remained excluded from follicles at 2 hours, the latest time evaluated. *Bottom right:* Nonsentinel lymph node. Exposure of this image is controlled for duration and magnification, which are identical to the image at top right.

sent B cells, which also express major histocompatibility locus class II molecules or other APCs not exposed to FITC-HSA. FITC staining was not observed in the B cell-dominated follicles of lymph nodes, and B cells took up only minimal FITC-HSA in vitro. This suggests then that dendritic cells and/or macrophages, the cells primarily responsible for antigen presentation in the interfollicular region of lymph nodes, actively macropinocytose small macromolecules such as radioactive HSA and at least the smaller particles in SC.

DISCUSSION

The distribution and kinetics of lymphatic mapping agents have generally been attributed to particle size and physical factors limiting passive transit through the lymphatic circulation. This has led some to recommend the use of tracers with large particle sizes and to proceed to mapping and biopsy as rapidly as possible after injection of the tracer.[11,12] No consensus technique has emerged, however, as a result of these recommendations, in part because very good results have been obtained by using a variety of techniques. These include injection of radiotracer with blue dye in the operating room, and injection of tracer shortly before the procedure, several hours before the procedure, and even the day before the procedure.[13,17–21] Different tracers have also been used with successful results. In this study, we confirm that ^{99m}Tc-HSA can be used effectively for SLN mapping.

We thought that an explanation for the success of these varying techniques may be active antigen retention by APCs within lymph nodes. Normally, antigens travel from the periphery via lymphatics in one of several ways. They may be taken up by APCs in the periphery and travel within these cells. They may also travel extracellularly either free or bound to antibody. Because of the rapidity with which these tracers travel to lymph nodes, extracellular transit is likely in this case. In addition, studies of perfluorocarbon particles demonstrated that smaller particles (60 nm) traveled extracellularly whereas larger particles (340 nm) traveled more slowly and extracellularly.[22]

Once particles reach the node, they must pass via the subcapsular sinus into a system of radial sinuses and into the cortex. The cortex is divided into B cell-dominated follicles and T cell-dominated interfollicular areas. The T-cell areas also contain macrophages and dendritic cells, which are effective in taking up and presenting antigens.[23] After naive T cells have recognized antigen in the context of these APCs, they travel to follicles to form germinal centers, proliferate, and produce an immune response to the antigen.

Several pieces of data from this study and previous work suggest that there are at least two patterns of tracer distribution within the lymphatic circulation after peripheral injection. Initially, there is a period during which particles small enough to freely enter lymphatics travel to nodes. There they are distributed passively as deter-

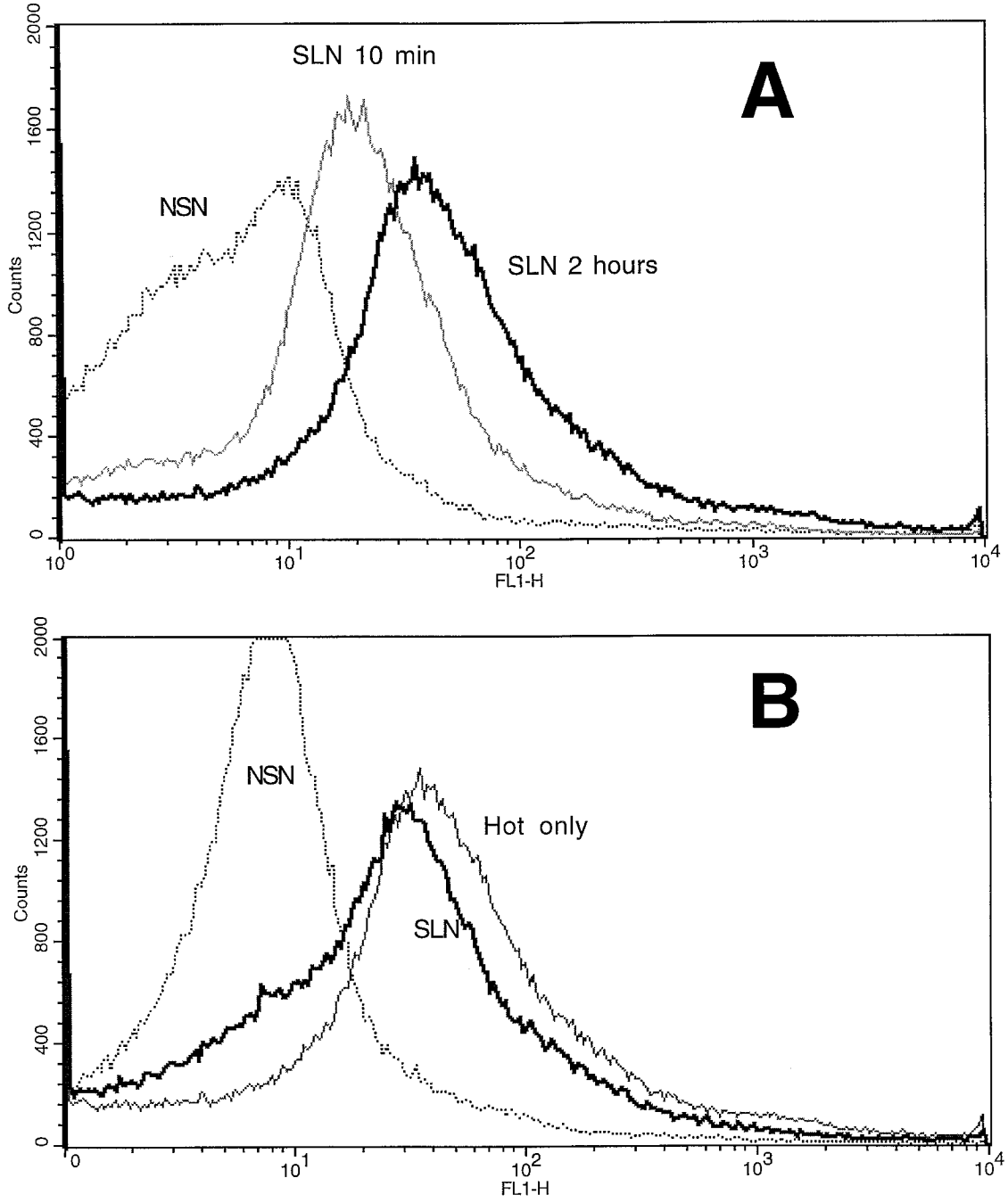

FIG. 4. (**A**) Fluorescence-activated cell sorting analysis of fluoroscein isothiocyanate-labeled human serum albumin (FITC-HSA) uptake by lymph node cells. Several nodes, harvested 10 minutes after injection, demonstrated low levels of uptake of FITC-HSA, as observed here. Others failed to show increased fluorescence. Sentinel lymph node (*SLN*), removed 120 minutes after injection of tracer, uniformly showed high concentrations of fluorescence. (**B**) Fluorescence histograms of nodes harvested at 120 minutes from an animal injected with ^{99m}technetium-labeled human serum albumin and FITC-HSA. *NSN*, nonsentinel lymph node.

mined by lymph flow. At this point, significant accumulation of tracer within cells has not had time to occur. This is supported because nodes harvested early after injection had only low or no concentrations of FITC-HSA, evident by FACS after washing, despite the presence of FITC-HSA as seen by using microscopy.

Antigen uptake then proceeds within nodes that are, by definition, immunologically responsible for screening

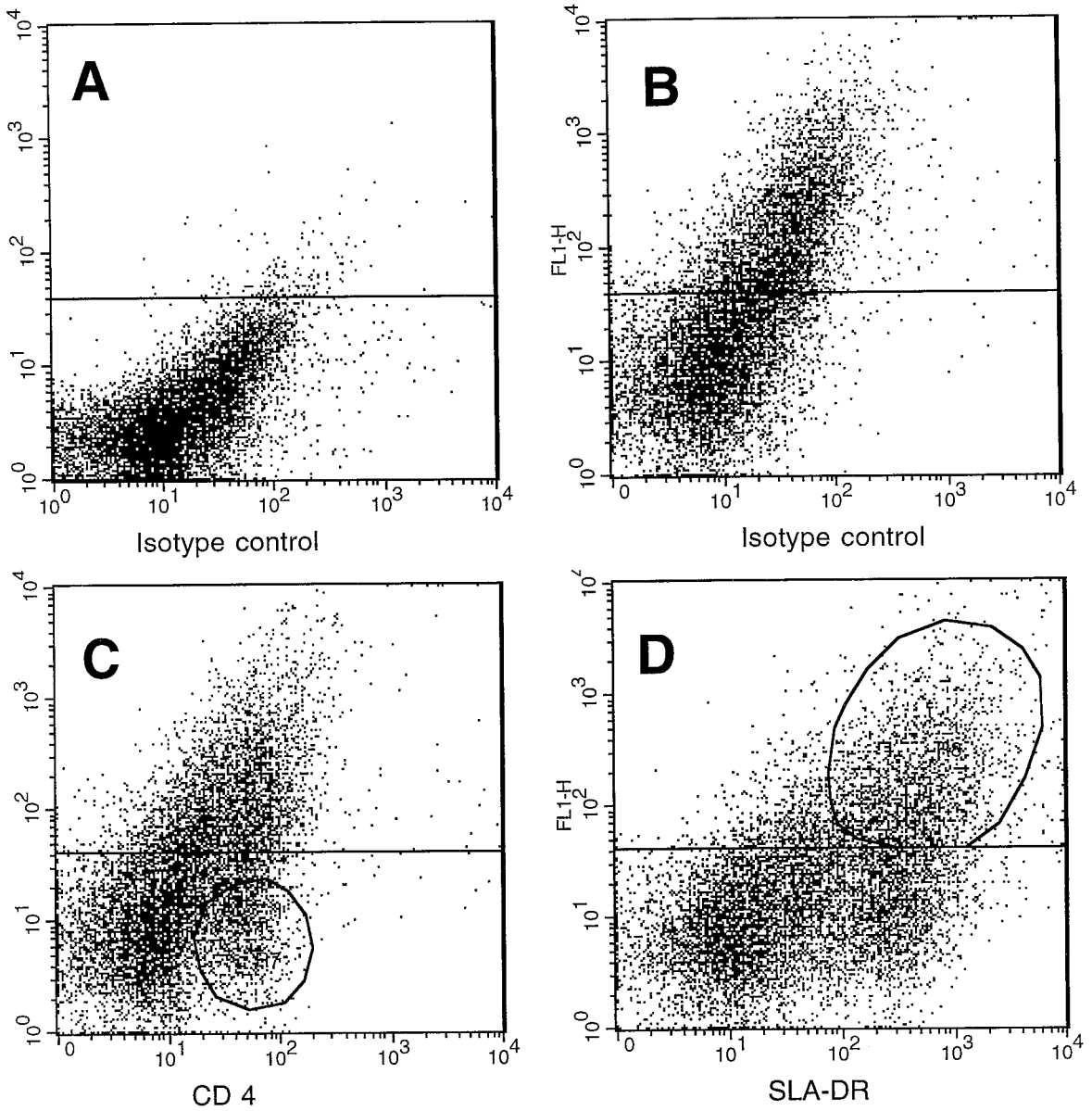

FIG. 5. Dot plots of fluorescence-activated cell sorting-analyzed nodal cells. (**A**) Cells from a nonsentinel lymph node (NSLN) stained with an isotype-matched control antibody. The horizontal boundary marks the maximal concentration of background fluorescence. (**B**) Cells from a sentinel lymph node (SLN) also stained with the isotype control. (**C**) SLN cells counterstained with anti-CD4 antibody. The circled population is positive for CD4 and negative for fluoroscein isothiocyanate-labeled human serum albumin (FITC-HSA). Similar results were observed with anti-CD8 stains. (**D**) SLN cells counterstained with swine leukocyte antigen DR (*SLA-DR*). Note the circled population, which is positive for both SLA-DR and FITC-HSA. Similar results were obtained with the anti-granulocyte/monocyte marker (data not shown.)

lymph from the injection site. This generates the second pattern of distribution, which is consequently immunologically determined. The rapidity with which the second pattern replaces the first may depend on the size of tracer particle used. Larger particles have slower kinetics,[10,12,22] which may lead to a longer persistence of distribution determined by lymph flow. Our previous clinical study found significantly more radioactive nodes and lower concordance with blue dye when SC was used.[13] This study found a trend toward lower concordance with blue dye and several nodes labeled by SC that were not labeled with FITC-HSA. These data suggest that the tracer containing larger particles labels some nodes that are not immunologially determined. With

either tracer, however, a delay between injection and biopsy is not detrimental and may, in fact, be beneficial to the accuracy of the procedure.

Not uncommonly, we have found that lymph nodes that are substantially replaced by tumor do not show uptake of radioactive tracers. This conforms to what would be expected in light of this mechanism of uptake. The loss of APCs caused by an increasing growth of tumor should also lead to a loss of the nodes' ability to take up and retain tracers. Indeed, in a recent large series of SLN biopsies, three of five false-negative cases occurred in patients with nodes considered suspicious during the procedure.[24] Therefore, nodes discovered at the time of biopsy that are clinically suspicious but not radioactive should be removed. In addition, reduction in antigen retention in nodes substantially replaced by tumor may preclude identification of any SLNs, which suggests a complete dissection of draining basins is indicated if no SLN is found.

Finally, there is the question of the clinical relevance of nodes that are radioactive but that fail to stain blue. Active particle uptake was demonstrated for all radioactive, nonblue nodes labeled with ^{99m}Tc-HSA and most labeled with ^{99m}Tc-SC. Authors of a clinical study also documented several cases in which a node that was only labeled by radiotracer revealed disease despite the presence of an early benign node labeled by dye and radiotracer.[24] Based on the immunological function of these nodes seen here and their demonstrated clinical relevance, they should be considered sentinel and be removed as part of a lymphatic staging procedure.

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