Regional Lymph Node Reactivity in Explanted Bladder Cancer of Mice as Measured by Flow Cytometry

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ABSTRACT

The reactivity of lymphocytes in lymph nodes draining the site of a transplantable experimental bladder tumor (MBT2 in C3H/HeJ mice) has been measured in a multiparameter flow cytometry system. Acridine orange was used as a nucleic acid probe. This dye intercalates in helical DNA, emitting green (530 nm) fluorescence upon exposure to blue light; it stacks to single-stranded RNA, emitting red (640 nm) fluorescence. The relative magnitude of the increase of lymphocyte DNA and RNA has been evaluated simultaneously in tumor-draining nodes, in nondraining nodes of the same animal, and in untreated control animals. Stimulation of the regional node lymphocytes could be observed after 20 days but not after 10 days. It was uniformly high at 35 days. The transcripive response (increased proportion of lymphocytes with high RNA) was more pronounced than the proliferative (increased proportion of lymphocytes with more than diploid DNA). The histological changes in the stimulated nodes resembled closely those described by others in human tumor-draining nodes. The described method has the advantage of being simple, rapid, and able to measure a representative part of the whole-cell population.

INTRODUCTION

Host immune reactions may play a part in the natural history of urinary bladder neoplastic disease. Cellular and humoral immune reactions to bladder tumors have been demonstrated in vitro (for review, see Ref. 10). Peripheral blood lymphocyte cytotoxicity against bladder tumor cell lines is sometimes correlated with the clinical status of the patient (17). That the immune system reacts to tumor is further suggested by lymphocyte infiltration in tumor tissue (18, 20), a phenomenon which may be correlated with peripheral lymphocyte cytotoxicity against the same tumor (13).

The role of the regional lymph nodes in immune recognition and reaction to tumor-related substances has attracted vast interest. Histological alterations have been described in lymph nodes draining tumors of many organ sites (1, 10, 11, 14, 16, 22–24). In several of these studies, certain morphological changes in the lymph nodes were correlated with prognosis. A stimulated pattern with enlarged germinal centers (B-cell proliferation) and expansion of the deep cortex (T-cell proliferation) was linked with improved survival and/or fewer metastases. Depleted lymph nodes generally coexisted with advanced disease, metastases, and poor survival. A standard for evaluating these histological changes was proposed by Cottier et al. (4).

Since the histological examination remains subjective, it would seem desirable to find a simple, objective means of measuring reactivity in lymph nodes draining tumor sites. Recently, Herr (11) was able to demonstrate increased reactivity in lymphocytes from juxtatumoral nodes by measuring [3H]-thymidine incorporation after stimulation with nontoxic mitogens. These measurements were correlated with the typical histological changes described above.

We have previously demonstrated that stimulation of peripheral blood lymphocytes can be quantified in a rapid fashion by multiparameter flow cytometry (3, 7). With this technique, simultaneous measurements of DNA and RNA are carried out on each cell, individually, in a total sample of several thousand. The cell cycle position is estimated from relative DNA content, while transcriptional activity is evidenced by an increase in RNA content (7).

Pilot studies have established the feasibility of this approach for the study of lymph node lymphocytes. We now describe the technique proposed for quantitating lymphocyte stimulation in lymph nodes and report our findings in an experimental bladder tumor of the mouse.

MATERIALS AND METHODS

Tumor Model. The experimental tumor was a s.c. transplantable bladder tumor originally induced by N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide feeding in C3H/HeJ mice. It has been selected for rapid growth and the relative simplicity with which it can be transplanted. The mean survival time after tumor inoculation has been reported to be 41 to 50 days (19). Histologically, it resembles transitional cell carcinoma. While tumor growth after primary syngeneic inoculation is uniform, leading to death in all animals, the secondary response after initial immunization with killed cells or cell extract leads to protection by specific immunity (15).

Transplantation. Tumor tissue was minced and agitated in 0.9% NaCl solution to yield a single-cell suspension. After sieving through multilayered gauze to remove clumps, cells were counted in trypan blue; the suspension was adjusted to a concentration of 5 × 10⁶ viable cells/ml; and 0.33 ml of the suspension was injected s.c. into the left hind leg of adult female C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine). Care was taken to obtain identical inoculation sites in all animals. As it turned out, however, the subsequent tumor...
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PREPARATION OF NORMAL BLADDER CELLS. Bladder mucosa was dissected in 5 healthy syngeneic mice. It was gently teased, agitated, and filtered through multilayer gauze. After trypsin blue staining and counting, the viable cells were adjusted to the same concentration as for tumor cells and used for inoculation in the same amount and at the same site.

LYMPHOCYTE PROCUREMENT. The left and right inguinal and left and right lumbar lymph nodes were chosen for study (Chart 1). At time intervals outlined below, the animals were sacrificed and the lymph nodes were bluntly dissected free. The nodes were disrupted and gently teased in 0.9% NaCl solution to create lymphocyte suspensions. The cell concentration was adjusted to approximately 5 x 10^6/ml, corresponding to a counting rate of 150 to 200 cells/sec in our flow cytometry system.

STAINING AND MEASUREMENTS. Lymphocytes were stained with AO according to the technique described in detail by Traganos et al. (21). In brief, the cells are pretreated with a detergent solution to make them permeable to the dye, stained at equilibrium in AO solution at pH 6.0, and immediately examined by flow cytometry.

The detergent solution consists of 0.1% (v/v) Triton X-100 (Sigma Chemical Company, St. Louis, Mo.), 0.08 n HCl, and 0.15 n NaCl; 0.2 ml of the cell suspension is mixed with 0.4 ml of this solution. Thirty sec later, 1.2 ml of the staining solution are added. The latter contains 6.0 µg of chromatographically purified AO (Polysciences, Warrington, Pa.) per ml in 10^-5 M EDTA/0.15 M NaCl and 0.1 M citrate phosphate buffer (pH 6.0). The final dye concentration in the cell suspension is 1.1 x 10^-5 M, and the final AO/DNA phosphate molar ratio is 2.5. The flow cytometer used by us is an FC 200 cytofluorograph (Ortho Instruments, Westwood, Mass.), interfaced to a Data General minicomputer for data recording and analysis.

AO is a metachromatic dye that intercalates into double-helical DNA in a monomeric form (2). When subsequently exposed to blue light, it emits green fluorescence with a maximum intensity at 530 nm. The dye 'stacks' on single-stranded RNA, fluorescing red with maximum emission at 640 nm when excited. The double-stranded RNA normally present in cells is denatured (converted to single-stranded form) by the treatment with EDTA at high AO concentration and AO/nucleic acid ratio (6).

EVALUATION OF LYMPHOCYTE STIMULATION. Lymphocytes were evaluated with respect to the 2 principal modes of response to stimulation: (a) increase in content of stainable RNA (red fluorescence) per cell, most probably reflecting increased transcriptional activity (5, 8); (b) increased proliferative activity, reflected mainly as an increase in DNA (green fluorescence) due to the increased number of cells in the S and G2 + M phases of the cycle. Although these 2 parameters are thought to be closely correlated, they may vary quantitatively and in time sequence according to the nature of the stimulus and other factors (see "Discussion").

The first parameter, i.e., the increase in cellular red fluorescence, was expressed as the number (percentage) of cells with such fluorescence in excess of an arbitrarily chosen threshold value. This threshold value (usually at Channel 40; see Chart 2) was chosen to discriminate between cells with low and high red fluorescence. Based on this value, 98 to 99% of the control lymphocytes from normal, nonreactive lymph nodes were classified as having low red fluorescence. Thus, the change ("right shift") observed in the lymph nodes draining the transplanted tumors was measured as an increase in percentage of cells with red fluorescence higher than the threshold value, according to the formula:

\[ \frac{\% \text{ cells between Channels 40 and 100 in tumor animals}}{\% \text{ cells between Channels 40 and 100 in control animals}} \times 100 \]

Only cells with diploid DNA content (green fluorescence) were selected for this parameter (Chart 2).

Lymphocyte proliferation, i.e., DNA increase, was also estimated in a green (DNA) versus red (RNA) fluorescence display (Chart 3). The mode of selection of the proliferating proportion of the cells is indicated in Chart 3 and further explained in the legend. The proportion of cells with green (DNA) fluorescence values above the diploid level in tumor-bearing animals was again expressed as percentage of controls.

RESULTS

Ten days after tumor transplantation, when macroscopic tumor was not yet apparent, there was no discernible lymphocytic reaction. At 20 days, when all animals had small but visible and/or palpable tumors, most had marked increase of red (RNA) fluorescence, although with a wide spread. All animals had large tumors at 35 days. At the time the red fluorescence of left lumbar node lymphocytes was uniformly and significantly higher than that in unmanipulated controls (Chart 4). This time (35 days) was subsequently used to compare the different lymph node sites of the tumor-bearing animals.

Eight animals, all with large tumors, were sacrificed at 35 days, and the 4 chosen lymph node sites were examined. Lymphocytes from the nondraining lymph nodes (right lumbar and both inguinal nodes) were normal. Cells from the left lumbar nodes, on the other hand, displayed a significant (p < 0.005) increase of red (RNA) fluorescence (Chart 5).

There was also a slight increase in the number of cells engaged in DNA replication(s) and in the G2 and M phases of the cell cycle (cells with green DNA fluorescence above the
Chart 2. Red (RNA) versus green (DNA) fluorescence scattergrams on the left lumbar (tumor draining) lymph node lymphocytes and right lumbar (control) node lymphocytes both at 35 days after cell inoculation. In these scattergrams, each cell is represented by a dot, its location on the ordinate is a measure of the relative green (DNA) fluorescence of the cell, and its location on the abscissa is the relative red (RNA) fluorescence. Left, lymphocytes from a tumor-bearing animal; right, lymphocytes from a healthy control animal. Top, initial displays in which the diploid cells are selected by a rectangular box; thus, the S, G1, and M cell populations, as well as the dead cells (with low green fluorescence), are rejected from further analysis (7). Bottom, selected diploid cells displayed separately. Taking into account this population only, a further selection for cells to the right of Channel 40 is made; this defines the cells with higher red fluorescence. The proportion of high red (RNA)-fluorescing cells is greater for the juxtatumoral than for the opposite (control) node. The total number of cells measured in each experiment was 5000, although only 2000, chosen at random by the computer, are plotted in this figure. The percentage of cells above the selected threshold (Channel 40) was 0.3 in the control node and 0.7 in the tumor-draining node.

Chart 3. Green (DNA) versus red (RNA) fluorescence displays of lymphocytes from a tumor-draining lymph node in a mouse at 35 days with a large tumor. The mode of selecting the proliferating cells is illustrated. Left, a box is drawn to first exclude low-fluorescing (dead) cells. The selected cells, which are enclosed in the box, are diploid cells with lower red (RNA) values, diploid cells with higher red (RNA) values, and proliferative cells (S, G2 + M) with increased green (DNA) as well as red (RNA) fluorescence. Right, a further selection based on increased green fluorescence intensity defines the proliferating cells (S, G2 + M) from the nonproliferating cells (low green).

Diplot level) in the juxtatumoral left lumbar node. This increase was significant only when compared with the right lumbar node (p < 0.05), but not when compared with the 2 inguinal nodes (Chart 6).

The animals inoculated with normal bladder mucosal cells were also examined at this time. As depicted in Chart 5, they did not differ from the completely untreated controls, except for one animal with an unexplained increase in red (RNA) fluorescence.

Histologically, the draining (left lumbar) lymph node at 35 days displayed a "stimulated" pattern (see above). While the nondraining nodes had a normal appearance (Fig. 1a), the draining nodes were greatly enlarged, with broadened, hyperplastic cortex and prominent germinal centers, with an increased but variable number of plasma cells, and with a marked increase in depth of the deep cortical area (Fig. 1b). There were no tumor metastases in the nodes.

**DISCUSSION**

Immunological events in lymph nodes draining solid tumors of various organs may be important to an understanding of the biological behavior of the tumor, to clinical prognosis, and possibly to the choice of therapy. The difficulty in obtaining objective, reproducible data from histological evaluation alone has promoted measurements of cellular events in regional lymph nodes. Several methods have been described, most of them requiring elaborate preparatory procedures and/or use of radioactive substances.

Herein, reactive changes of lymph node cells have been estimated from measurements of RNase-sensitive red fluorescence (RNA) and DNase-sensitive green fluorescence (DNA) of AO-stained cells. There was a significant increase in the number of cells with high red (RNA) fluorescence in lymph nodes regional to the tumor. There was also some increase in...
the number of S and G2 + M cells, i.e., cells with elevated green fluorescence, but this was minimal. Thus, it appears that the transcriptional response of the lymph node lymphocytes was more pronounced than the proliferative response with this method of measurement. One possible explanation is that many of the stimulated cells remain in the G1 phase and that while in G1, they are involved in antibody production. In this context, it is interesting to review the data discussed by Haskill (9). Regarding the lymphocyte blastogenic response he concludes: "Cell division ... does not appear to be a necessity for the initial production of cells secreting antibody." Although several important differences between the experiments cited by Haskill and our system exist (differences in antigenic strength between, e.g., xenogeneic erythrocytes and our weakly immunogenic, syngeneic tumors; the long time lapse between inoculation and examination in our experiment, allowing for many cell generations and distorting the configuration of the initial response), this type of principally RNA response may well be operative in our system.

Other possible explanations for these reactive changes exist. The assumption that the RNA increase reflects changes only in the original cell population of the node is unlikely. Recruitment of cells almost certainly occurs, and it is the best explanation for nodal enlargement in the absence of more evident proliferation. Histological examination of the regional node showed follicular and cortical hyperplasia and an increased number of plasmoid cells. These may represent reactive and differentiated cells of the original population, or a recruited population, or both. There was no metastatic carcinoma in the lymph node.

The present investigation does demonstrate a simple, quantitative method for evaluation of regional lymph node response to tumor. Lymphocyte stimulation in juxtatumoral nodes can be recorded for a large number of cells in a short time and with a minimum of preparation. The method could easily be applied to clinical situations, e.g., for examination of lymph nodes removed by pelvic lymphadenectomy, presently a common procedure in radical bladder or prostatic cancer surgery.

Do previously reported histological studies of regional lymph node stimulation in patients with solid tumors have a counterpart in the cellular events measured by us? Indeed, the histological changes in the mouse lymph nodes that we have studied closely resemble those described in patients with tumors of the uterine cervix (16), lung (14), bladder (12), and prostate (11), suggesting a close correlation between the mouse model and human disease. We believe that the method described here can support and, in most instances, provide a quantitative value for the hyperplastic nodal reaction observed by histological examination.

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Fig. 1. a, histological section of the unstimulated, right lumbar lymph node at 35 days. The node is small with inconspicuous germinal centers. b, histological section of the stimulated left lumbar lymph node from the same animals as above, also at 35 days. There is considerable enlargement of this node with greatly broadened, hyperplastic cortical and deep cortical zones, and prominent germinal centers. H & E, × 280.
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