Concanavalin A-inducible Suppressor Cells in Regional Lymph Nodes of Cancer Patients

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ABSTRACT

Regional tumor-draining lymph nodes of 11 of 14 patients with urological tumors and one of four controls studied contained suppressor cell precursors that could be activated by concanavalin A (Con A) to suppress the proliferative response of autologous lymphocytes to Con A. In contrast, no suppression of lymphocyte proliferation by lymph node cells that were not activated with Con A was observed in four patients tested. The suppressive effect was not due to decreased viability or increased release of cold thymidine by Con A-activated cells nor to alteration in the time course of the proliferative response of Con A-activated cells. Mitomycin C treatment of lymph node cells 24 hr after activation did not abrogate their suppressive activity. Peak suppression was observed after 72 hr in culture. The amount of suppression measured could be maximized by treatment of suppressor cells with mitomycin C 24 hr after activation and by washing the cells immediately before pulse labeling with tritiated thymidine. The concentration of Con A required to produce peak suppression varied from patient to patient with optimal doses ranging from 5 to 25 \( \mu \text{g/ml} \).

INTRODUCTION

Depression of immune responsiveness associated with progressive tumor growth is a well-documented phenomenon (8). The mechanisms underlying immunosuppression are not clearly understood but have been attributed to various circulating serum factors including blocking antibodies (15), antigen-antibody complexes (2), and nonspecific immunoregulator peptides (12). More recently, suppressor cells have been implicated in the immunosuppression of cancer. These suppressor cell populations include T-lymphocytes (9, 10), B-lymphocytes (13), and macrophages (18).

Suppressor cell function in animal tumor systems is well documented. Fujimoto et al. (10), using a methylcholanthrene-induced sarcoma in mice, found that adoptive transfer of suppressor cells to immune recipients significantly depressed the ability of the recipients to reject a subsequent tumor challenge. Additional experiments showed that the suppressor cells were present in thymus, spleen, and draining lymph node but not in peripheral blood. Other evidence supporting a role for suppressor cells in regulating the growth of methylcholanthrene-induced sarcoma was reported by Greene et al. (14). These investigators demonstrated that daily injections of anti-I-J antisera (I-J antigenic determinants are coded for by the I-J subregion of the H-2 histocompatibility complex in mice and are expressed on suppressor T-cells) produced a significant suppression of the growth of the sarcoma, suggesting that selective removal of suppressor cells augmented the host immune response against the tumor.

Information on suppressor cell function in human tumor systems is limited. Lymphoid cells that suppressed spontaneous cell-mediated cytotoxicity directed against osteogenic sarcoma cell lines have been isolated from the peripheral blood of some osteogenic sarcoma patients (30). Moreover, Bean et al. (3) reported the presence of genetically restricted suppressor T-cells that inhibited the lymphocyte-proliferative response to allografts in a patient with bladder cancer.

Additional information on suppressor cell function in humans has been obtained by nonspecific activation of suppressor cell precursors with mitogens. Mitogen-activated suppressor cells have been shown to belong to a committed suppressor cell population in both animal (17) and human (24) systems. Using this system to detect suppressor cells, several investigators have reported the presence of suppressor cell precursors in the peripheral blood (27), thymus (26), and spleen (25, 26) of humans. In the present investigation, we demonstrate for the first time that regional lymph nodes of many urological cancer patients and similar lymph nodes of some patients with benign conditions possess Con A\(^3\)-inducible suppressor cells that significantly suppress the proliferative responses of autologous lymphocytes. However, spontaneous suppression by LNC that were not activated with Con A was not observed.

MATERIALS AND METHODS

Patients. Lymph nodes and peripheral blood were obtained from 14 patients with genitourinary neoplasms and 4 non-tumor-bearing patients in whom removal of normal lymph nodes was performed to gain access to major vessels for surgical procedures. The clinical data for these patients are shown in Table 1. The availability of LNC from non-tumor-bearing control patients was limited by ethical considerations. LNC from cadaver transplant donors treated with corticosteroids were not used in this study.

Preparation of PBL. Peripheral blood was drawn into a syringe containing preservative-free heparin (10 units/ml) (Fellows Medical, Oak Park, Mich.). PBL were isolated by Ficoll-Hypaque density gradient centrifugation (4). Interface cells were removed, washed 3 times with HBSS, and resuspended...
in Roswell Park Memorial Institute Medium 1640 containing 10% heat-inactivated fetal calf serum and 1% antibiotic-antimyotic solution (penicillin, streptomycin, and fungizone; Grand Island Biological Co., Grand Island, N. Y.), hereafter referred to as complete medium.

Preparation of LNC. Draining lymph nodes were obtained at operation and placed into a flask containing sterile 0.9% NaCl solution. Lymph nodes were trimmed of fat and washed thoroughly to remove peripheral blood, and then LNC were released into complete medium by mincing and passing through a 40-gauge sieve. The cells were drawn through a 25-gauge needle to disperse cell clumps and placed on a Ficoll-Hypaque density gradient to remove dead cells and erythrocytes (4). The interface cells were removed, washed 3 times with HBSS, and resuspended in complete medium.

Con A Activation of LNC. We used a modification of the method described by Breshnihan and Jasin (5). LNC at 2 x 10^6/ml were activated with Con A (6 to 10 μg/ml) and incubated 24 hr at 37° in a 5% CO2 humidified incubator. Parallel control LNC cultures which were not activated with Con A were treated identically except that complete medium was used instead of Con A. The Con A-activated and control cell cultures (hereafter to be called regulator cells) were washed twice with HBSS (previously determined to be sufficient for removal of Con A). In some experiments, the regulator cells were treated with mitomycin C (50 μg/ml) for 30 min at 37° followed by 3 washes in HBSS before resuspension with complete medium. Viability, determined by trypan blue exclusion, was greater than 90% for both Con A-activated and control cells.

Test Tube Assay for Suppressor Cells. One hundred-μl aliquots of the regulator cells at 10^5 cells/ml were added to an equal number of untreated lymphocytes (hereafter to be called responder cells) in a 1-ml volume that had been incubated overnight in complete medium, and Con A (12 μg/ml) was added to each culture. The cultures were incubated for 72 hr at 37° in a humidified 5% CO2 incubator, and [3H]thymidine incorporation was determined as follows. Ten μCi [3H]thymidine (New England Nuclear, Boston, Mass.) were added for the final 4 hr of incubation. At 72 hr, aliquots of 200 μl were pipetted from the assay cultures into triplicate wells of microtiter plates (Cooke Laboratory Products, Alexandria, Va.), and the cells were collected on a Mash II harvester. Filters were dried and counted in a Beckman liquid scintillation counter. Suppressor cell activity was calculated as described above.

Time Course Response of Activated and Nonactivated LNC to Con A. To determine whether the suppressive effect observed was due to differences in the time course responses to Con A stimulation of activated and nonactivated regulator LNC, we set up parallel cultures as described above and harvested at 24, 48, 72, 96, and 120 hr.

Pre-[3H]Thymidine Pulse Label Washing of Cultures. To determine whether the suppressive effect observed was due to increased release of cold thymidine by activated regulator cells, we performed experiments in which cultures were washed or not washed immediately before pulse labeling with [3H]thymidine.

Mitomycin C Treatment of Regulator LNC. To determine whether the suppressive effect observed was due to suppression of proliferative response of the responder cells, we set up cultures in which regulator cells were treated or not treated with mitomycin C (50 μg/ml) (Difco Laboratories, Detroit, Mich.) for 30 min followed by 3 washes prior to adding them to responder LNC. We also set up time course experiments with mitomycin C pretreated cells.

Con A Dose Response. To determine the optimal dose of Con A to induce suppression, we performed parallel experiments using Con A (6, 12, 25, 50, 100 μg/ml) in the cultures.

RESULTS

Time Course Responses of Activated and Nonactivated LNC to Con A. Chart 1 shows that the time course responses to Con A stimulation of activated and nonactivated LNC were similar with peak [3H]thymidine incorporation occurring after 72 hr of culture. A similar time course study (Chart 2) in which [3H]thymidine incorporation by regulator cells was blocked by mitomycin C treatment demonstrated a similar time course and significant suppression.
Pre-[\(^{3}H\)]Thymidine Pulse Label Washing of Cultures. Cultures that were washed to remove cold thymidine immediately before pulse labeling with \([^{3}H]\)thymidine incorporated more label than did cultures that were not washed. However, washing did not abrogate suppression but rather significantly enhanced it (Table 2).

Mitomycin C Treatment of Regulator LNC. Cultures in which \([^{3}H]\)thymidine incorporation of both Con A-activated and nonactivated regulator cells were blocked by mitomycin C treatment exhibited significantly more suppression than was observed with regulator cells not blocked with mitomycin C (Table 3). A time course study of the Con A response in cultures with mitomycin C-blocked regulator cells is shown in Chart 2.

Con A Dose Response. Some variation in the dose of Con A that produced optimal suppression was observed from subject to subject; however, under the conditions of our assay Con A (5 to 25 \(\mu\)g/ml) was found to produce optimal suppression (Table 4).

Con A-inducible Suppression in Regional LNC. The regional lymph nodes of 11 of 14 urological cancer patients studied possessed suppressor cell precursors that could be activated by Con A to significantly suppress the proliferative response of autologous LNC to Con A (Table 5). Only prostatic cancer patients with localized disease lacked suppressor cell activity. LNC from 4 patients with benign conditions were also examined. Significant suppressor cell activity was observed in one of these patients. Although the lack of suppression observed in prostatic cancer patients with localized disease and most non-tumor-bearing patients is interesting, patient numbers are too limited to permit definitive conclusions.

Absence of Spontaneous Suppression by LNC from Cancer Patients. To determine whether spontaneous suppressor cells were present in lymph nodes and, if so, whether they influenced Con A-induced suppressor cell activity, we examined the effect of nonactivated regulator LNC on the proliferative response of normal allogeneic PBL. Previous studies (6, 27) demonstrated no genetic restrictions on suppressor cell function in this system (data not shown). Five experiments were performed using LNC from 4 cancer patients and 1 control. We did not observe spontaneous suppression in any case; however, all patients tested had localized tumors, and their proliferative responses were comparable to those of controls. Data from one experiment (Chart 3) show that the proliferative response of normal responder PBL to which nonactivated LNC were added was unchanged when compared with the response of responder PBL cultured alone or cocultured with nonactivated autologous regulator PBL. Con A-activated LNC regulator cells from the same patient significantly suppressed the response of an identical responder PBL preparation. The other patients not having statistically significant suppression with Con A activation also failed to exhibit spontaneous suppressor cell activity. These results suggest that preactivated nonspecific suppressor cells were not present in the LNC of the patients tested and indicate that the absence of Con A-induced suppression is not due to in vivo activation of suppressor cell precursors.
Suppressor Cells in Lymph Nodes

Table 4
Effect of Con A concentration on the generation of suppressor cells

<table>
<thead>
<tr>
<th>Con A concentration (μg/ml)</th>
<th>Nonactivated regulator cells</th>
<th>% of viability</th>
<th>Con A-activated regulator cells</th>
<th>% of viability</th>
<th>% of suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>47,516 ± 3,901</td>
<td>94</td>
<td>41,466 ± 654</td>
<td>92</td>
<td>12</td>
</tr>
<tr>
<td>12</td>
<td>56,732 ± 819</td>
<td>91</td>
<td>50,506 ± 896</td>
<td>96</td>
<td>11</td>
</tr>
<tr>
<td>25</td>
<td>50,735 ± 1,471</td>
<td>83</td>
<td>36,120 ± 1,002</td>
<td>84</td>
<td>29</td>
</tr>
<tr>
<td>50</td>
<td>13,764 ± 1,054</td>
<td>47</td>
<td>5,571 ± 23</td>
<td>38</td>
<td>60</td>
</tr>
<tr>
<td>100</td>
<td>2,683 ± 24</td>
<td>NR</td>
<td>1,368 ± 40</td>
<td>NR</td>
<td>48</td>
</tr>
</tbody>
</table>

* cpm ± S.E.
** Viability not recorded because of lymphocyte agglutination.

Table 5
Con A-induced suppression of the proliferative response to Con A by LNC obtained from cancer patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Tumor type and stage</th>
<th>Con A-activated regulator cells</th>
<th>Nonactivated regulator cells</th>
<th>% of Suppression</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAH</td>
<td>Renal L</td>
<td>12,686 ± 23</td>
<td>15,054 ± 328</td>
<td>16</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>AS</td>
<td>Renal L</td>
<td>7,657 ± 392</td>
<td>9,108 ± 189</td>
<td>16</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>CB</td>
<td>Renal D</td>
<td>12,328 ± 35</td>
<td>14,456 ± 585</td>
<td>15</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>GR*</td>
<td>Renal D</td>
<td>44,507 ± 2,475</td>
<td>50,443 ± 945</td>
<td>12</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MB*</td>
<td>Renal L</td>
<td>7,987 ± 150</td>
<td>10,000 ± 440</td>
<td>20</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MH*</td>
<td>Bladder L</td>
<td>4,462 ± 130</td>
<td>7,002 ± 209</td>
<td>36</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>HD*</td>
<td>Bladder R</td>
<td>11,948 ± 256</td>
<td>20,404 ± 1,888</td>
<td>41</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>JE*</td>
<td>Bladder L</td>
<td>8,575 ± 208</td>
<td>11,737 ± 251</td>
<td>27</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>SE*</td>
<td>Bladder L</td>
<td>33,516 ± 1,588</td>
<td>37,579 ± 484</td>
<td>11</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>GS*</td>
<td>Prostate L</td>
<td>13,050 ± 927</td>
<td>13,132 ± 1,004</td>
<td>0</td>
<td>&gt;0.4 (NS)</td>
</tr>
<tr>
<td>RS*</td>
<td>Prostate L</td>
<td>4,842 ± 21</td>
<td>7,447 ± 1,736</td>
<td>35</td>
<td>&lt;0.2 (NS)</td>
</tr>
<tr>
<td>JB*</td>
<td>Prostate L</td>
<td>26,842 ± 2,646</td>
<td>32,310 ± 1,849</td>
<td>17</td>
<td>&lt;0.1 (NS)</td>
</tr>
<tr>
<td>CW*</td>
<td>Prostate R</td>
<td>34,684 ± 518</td>
<td>42,758 ± 819</td>
<td>19</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>DN*</td>
<td>Prostate R</td>
<td>31,955 ± 495</td>
<td>36,339 ± 597</td>
<td>12</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

* Con A concentration in all cultures was 10 μg/ml.
# Statistical analysis by Student’s t test.
$ L$, local; $R$, regional lymph node metastases; $D$, distant metastases.
¢ cpm ± S.E.
$ Assays run in microtiter plates. Regulator cells were activated with 12 μg/ml Con A and both activated and nonactivated regulator cells were treated with 50 μg/ml of mitomycin C before addition to responder cells.
$ Regulator cells were activated with 20 μg/ml of Con A, and both activated and nonactivated regulator cells were treated with 50 μg/ml of mitomycin C before addition to responder cells.
9 Tested for spontaneous suppressor cell activity.
h NS, not significant.

DISCUSSION

Mitogen activation of suppressor cells has been regarded as a useful technique for the detection of suppressor cell precursors. Mitogen-activated suppressor cells have been shown to suppress antibody production (22), the mixed lymphocyte reaction (19), proliferative responses to soluble antigens (27), generation of cytotoxic lymphocytes (16, 20), and proliferative responses to mitogens (27). The suppressor cell precursor observed in the mitogen system has been shown to be a terminally differentiated cell that belongs to a specific T-cell subclass (17, 24). Jandinski et al. (17) showed that Con A-activated suppressor cells in mice belong to a T-cell subpopulation that expresses Ly-2, 3 antigenic determinants. Sakane and Green (24) reported that the Con A-activated suppressor cells obtained from the peripheral blood of humans were high-density T-lymphocytes.

The mechanism by which Con A-activated suppressor cells inhibit proliferation of responder cells is unclear. Rich and
Pierce (23) isolated a soluble suppressor substance (SIRS) from Con A-activated mouse spleen and LNC that suppressed antibody production. Tadakuma and Pierce (29) subsequently showed that SIRS exerted its suppressive influence on antibody production on macrophages and not on B lymphocytes. However, SIRS-mediated suppression has been shown to be distinct from suppression mediated by intact Con A-activated cells (21). Redelman et al. (21) showed that suppression by SIRS was abrogated by the presence of 2-mercaptoethanol, while suppression by Con A-activated cells was not affected. Stobo (28), working with human lymphocytes, isolated a soluble factor that suppressed the mitogen-induced proliferative responses of normal allogeneic PBL. The factor was produced by macrophages but required T-cells for its expression. In this regard, attempts to isolate a soluble substance from Con A-activated cells capable of suppressing the proliferative response of autologous or allogeneic lymphocytes have been unsuccessful (1). Previous studies by Catalona et al. (6) and Shou et al. (27) have demonstrated no apparent genetic restrictions on Con A-induced suppressor cell function.

In the present studies, we performed 2 types of experiments to ensure that the suppression observed in our assay system was not spurious. We demonstrated that the time course response to Con A stimulation of cultures containing Con A-activated regulator cells was similar to that of cultures containing nonactivated regulator cells, which indicates that suppression was not due merely to differences in the time course responses of regulator cells. Moreover, we showed that suppression was not abrogated by washing the cultures immediately before pulse labeling with [3H]thymidine, which indicates that suppression was not due to increased release of cold thymidine in cultures containing Con A-activated regulator cells. Furthermore, we demonstrated that cultures containing regulator cells in which DNA synthesis was blocked with mitomycin C also exhibited suppression, indicating that the suppressive influence was exerted on the proliferation of responder cells and that the DNA synthesis was not required for expression of the suppressor cell activity. We also demonstrated that LNC from cancer patients did not spontaneously suppress the proliferation of PBL from a normal donor although none of the cancer patients tested had impaired proliferative responses. Moreover, cell viability studies from our laboratory as well as studies by De Gast et al. (7) showed that the viability of suppressed responder cells was the same as that of non-suppressed responder cells, indicating that suppression was not due to a generation of cells that was cytotoxic to responder cells.

Previous studies in both animal and human tumor systems suggested that the impairment of immune responses in cancer may be caused in part by suppressor cells that have been activated by tumor-associated antigens (11, 14). In this regard, Glaser et al. (11) showed that tumor-bearing animals had splenic suppressor cells that inhibited the proliferative responses to tumor-associated antigens as well as to mitogens. Studies in both animals and humans have also shown that suppressor cells are unevenly distributed in lymphoid organs and peripheral blood (10, 26). Our results demonstrate the presence of suppressor cell precursors in tumor-draining regional lymph nodes of many urological cancer patients and some controls with benign conditions that could be activated by Con A to suppress the proliferative responses of autologous LNC. However, we found no evidence that the suppressor cell precursors had been activated in vivo by tumor antigens to become nonspecifically suppressive (i.e., to suppress a nonspecific response to a mitogen); however, all 4 patients tested had proliferative responses that were comparable to controls. While this appears to suggest that the general impairment of immune responsiveness in cancer patients may not be caused by suppressor cells, it does not rule out the possibility that nonspecific spontaneous suppression occurs in patients with impaired proliferative responses, that antigen-activated suppressor cells may migrate from the regional nodes to other lymphoid organs, or that suppressor cells that are antigen specific may be induced but not detected by our assay.

It might be argued that while Con A induces suppressor cells that act without genetic restrictions, tumor antigens may induce genetically restricted suppressor cells; however, our results in the autologous situation revealed that the addition of non-Con A-activated, mitomycin-treated regulator LNC to responder cells resulted in no suppression of lymphocyte proliferation (Chart 3).

We observed statistically significant suppression in LNC of 11 of 14 (79%) urological cancer patients including 5 patients with renal adenocarcinomas, 4 with bladder carcinomas, and 2 of 5 with prostate carcinomas (the only prostate cancer patients studied having lymph node metastases). Although we observed some suppression in LNC of 2 of 4 controls, it was statistically significant in only one case. While it may appear from our data that Con A-inducible suppressor cell activity is increased in LNC of cancer patients relative to controls, the limited number of controls studied precludes any firm conclusions to be drawn. Moreover, we have conducted a large number of methodological experiments on the reproducibility of our assay system under a variety of conditions. These methodological studies reveal significant variation in the percentage of suppression observed in an individual when tested on different days and indicate that the results of these assays should be regarded in a qualitative sense only. Therefore, we feel that a quantitative comparative assessment of mitogen-activatable suppressor cell function between cancer patients and controls tested on different days would not be meaningful unless the numbers of patients studied were large.

In conclusion, our results demonstrate that regional lymph nodes of many urological cancer patients and some controls contain suppressor cell precursors that can be activated by Con A to suppress proliferative responses of autologous lymphocytes, although we found no evidence that nonspecific suppressor cell precursors had been activated in vivo by tumor antigens. The biological role of these suppressor cells remains to be determined.

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