Demonstration of NonspecificSuppressor Cells in the Peripheral Lymphocytes of Cancer Patients

Phuc Canh Quan and Pierre Burtin
Laboratoire d'Immunochimie, Institut de Recherches Scientifiques sur le Cancer, 94800 Villejuif, France

ABSTRACT

The response to phytohemagglutinin of peripheral blood lymphocytes was studied in 169 cancer patients. There was a significant decrease compared with control groups (normal persons and those with benign disease). By selecting cancer leukocyte samples with reactivity to phytohemagglutinin that was increased by carrageenan, a macrophage-toxic agent, and by mixing them with normal lymphocytes, we have demonstrated that the depressed phytohemagglutinin of six cancer patients' lymphocytes was due to the presence of suppressor cells that possibly were monocytes.

INTRODUCTION

The loss of normal immune surveillance function by the cell-mediated immune system has been postulated as a primary factor in the development of malignant disease (8, 17).

The depression of mitogen responses in vitro was well demonstrated in tumor-bearing animal spleen cells (21, 22, 24, 29) and in cancer patients (4, 6, 7, 12, 16, 18, 29, 31, 32, 36). Many factors were involved in these depressed responses, such as humoral factors (6, 7, 12, 16, 19, 31, 33, 38) and, recently, the presence of suppressor cells (4, 15, 18, 21, 22, 24, 27, 29).

In animal systems, suppressor cells inhibited the mitogen response of normal syngeneic cells; these cells were variously characterized as T-cells (15), probably B-cells (18, 21), or probably macrophages (22, 24, 27, 29). In humans, suppressor cells have been described in noncancerous patients. Waldmann et al. (36) demonstrated that patients suffering from hypogammaglobulinemia have circulating suppressor T-cells that were able to suppress immunoglobulin synthesis of random normal donors.

Sampson et al. (32) have shown that human spleen cell suspensions contain a population of cells that, after being stimulated by plant mitogens, can inhibit mixed-lymphocyte reactions. Recently, Bona2 has demonstrated that specific stimulation of human T-cells by purified protein derivative was inhibited by autologous B-derived lymphocytes.

In cancer patients, very little is known to date about suppressor cells. In this investigation, we tested the in vitro proliferation responses to PHA3 of peripheral lymphocyte cultures of cancer patients. Using carrageenan as a lethal macrophage agent, we have demonstrated that, in some cases, depressed lymphocyte responses to PHA are due to the presence of suppressor cells that are possibly monocytes.

MATERIALS AND METHODS

Patient Groups

Sixty-nine patients (45 males and 24 females), 40 to 91 years old and with colorectal cancer, were examined. All patients were studied after histological confirmation of diagnosis by biopsy. Of these 34 were postoperative. Fifty patients (30 males and 20 females), 37 to 68 years old and with head and neck cancer, were also studied. Twenty of these patients were subsequently included in a continuing trial of active chemotherapy and radiotherapy. Twenty-five patients with primary lung carcinoma, 32 to 65 years old, and 25 patients, 28 to 70 years old with various cancers (ovary, stomach, pancreas), were also investigated.

None of these 169 patients presented a monocytosis (monocytes, 2 to 7.5%).

Controls

The 69 healthy controls were mainly laboratory personnel and blood donors 20 to 55 years old. For further comparison 32 hospital patients, 18 to 75 years old, with various benign diseases (myocardial infarction, duodenal ulcer, hypertension, and respiratory disorder), were included.

Reagents

PHA. Purified PHA (HA 18; Wellcome Research Laboratories, Beckenham, England) was dissolved in phosphate-buffered saline (Dulbecco; Eurobio, Paris, France), divided into aliquots (40 µg/ml), and stored at -20°. PHA was used at a final concentration of 2.5 µg/ml or 0.5 µg/well.

Carrageenan. The macrophage-toxic agent calcium carrageenan (lota; Sigma Chemical Company, St. Louis, Mo.) was dissolved in warm phosphate-buffered saline (10 mg/ml), then sterilized by boiling for 30 min, and kept at 4°. At the time of use, carrageenan was added at a final concentration of 1 mg/ml or 200 µg/well.

1 Supported in part by Grant 76-7-1654 from the Délégation Générale à la Recherche Scientifique et Technique.
3 The abbreviations used are: PHA, phytohemagglutinin; RPMI Medium 1640; E-rosette, rosette formation between human T-lymphocytes and sheep erythrocytes.
**Lymphocyte Separation**

A 25-ml sample of heparinized (100 IU/ml) blood was obtained from each patient. Lymphocytes and monocytes were purified from the blood sample by the Ficoll-Triosil (specific density, 1.078) sedimentation technique (5). After 2 washings, the lymphocytes and monocytes were suspended in RPMI Medium 1640 (Eurobio) supplemented with 2 mM glutamine (Grand Island Biological Co., Grand Island, N. Y.), penicillin (100 units/ml), streptomycin (100 μg/ml), and heparin without preservative (15 units/100 ml; Laboratoire Choay, Paris, France) and adjusted to a concentration of 5 x 10⁶/ml.

**Preparation of Monocyte-free Lymphocyte Suspensions**

For removal of phagocytic cells, 50 ml of heparinized whole blood diluted with 50 ml of RPMI Medium 1640 were incubated with 50 mg of sterile carbonyl iron powder (particles, E; GAF, 63 Louvres, France) in a 250-ml tissue culture flask (Falcon Plastics, Oxnard, Calif.) for 30 min at 37° with mild continuous agitation. The blood was centrifuged over a Ficoll-Triosil gradient as described under "Lymphocyte Separation." Due to their greater specific gravity, iron-loaded monocytes were centrifuged to the pellet of the Ficoll-Triosil layer; thus this procedure yielded lymphocyte preparations depleted of monocytes (less than 0.1% monocytes).

**Preparation of Monocytes**

Monocytes were obtained by incubation of normal lymphocytes for 4 hr on plastic plates at 37° and 4 washings with RPMI Medium 1640.

**Assessment of Monocyte Functional Activities**

Adherent activities and phagocytosis were tested after monocyte monolayers had been incubated for 3 hr at 37° with carrageenan (1 mg/ml) or culture medium. Then the monolayers were washed intensively, and charcoal powder was added for 30 min at 37° and then removed by intensive washing of the monolayers. Adherent cells were counted, as well as the mean number of charcoal grains inside them.

**Lymphocyte Cultures**

Cultures were grown in microtiter plates (3040 microtest II; Falcon) as follows: 0.1 ml of lymphocyte suspension (5 x 10⁶/ml) was added to a tube containing 0.1 ml PHA (2.5 μg/ml), 0.1 ml carrageenan (10 mg/ml), and 0.1 ml decomplemented AB serum, made up to 1 ml with medium; mixed carefully; and distributed to each well at a final volume of 0.2 ml. Control cultures were grown without PHA or carrageenan.

Mixed-lymphocyte cultures were performed with 10⁶ lymphocytes from normal control and 10⁶ lymphocytes from cancer patients.

All cultures were grown in triplicate and incubated at 37° in a humidified atmosphere of 5% carbon dioxide in air for 72 hr. Four hr before harvesting, 1 μCi of [³H]thymidine (TMM48; CEA, Gif-sur-Yvette, France; specific activity, 27 Ci/mmol) in 0.01 ml of medium was added to each well; the cultures were then harvested on glass fiber filters (Reeve Angel and Co., Inc., Clifton, N. J.) with the use of a multiple automated sample harvester (MASH; Microbiological Associates, Inc., Bethesda, Md.). The incorporation of [³H]thymidine was measured with a scintillation counter (Intertechnique, Plaisir, France); stimulation was expressed as the difference in mean cpm between cultures containing PHA and cultures without PHA.

**E-Rosettes**

Rosette formation between human T-lymphocytes and sheep erythrocytes was performed by a modification of the method described by Fournier and Bach (14). We substituted fetal calf serum by sheep RBC-absorbed human AB serum.

**RESULTS**

Depression of [³H]Thymidine Uptake of PHA-stimulated Cancer Patient Lymphocytes. Chart 1 shows the mean and range of incorporation of [³H]thymidine following PHA stimulation of lymphocytes from cancer patients. No signif-
significant difference was observed between healthy control groups and benign disease groups. There is a significant difference in [3H]thymidine incorporation between the cancer groups and the control group (normal and benign disease); \( p < 0.0001 \) for colon and head and neck cancer groups and \( p < 0.01 \) for lung and other cancer groups.

It shows also that no difference was observed between the pre- and postoperative group of colorectal cancer patients and between treated and nontreated groups of head and neck cancer patients.

**Effect of Carrageenan on Normal Monocyte Functional Activities.** After 4 hr of incubation with carrageenan (1 mg/ml), the monocytes lost their adherent activities and there were very few cells still adhering to plastic plates. These monocytes contained very little or no charcoal powder. On the contrary, monocytes incubated with RPMI Medium 1640 always kept their adherent activity and phagocytosis.

**Effect of Carrageenan on Response to PHA of Normal Control Lymphocytes.** Since carrageenan is specifically lethal to macrophages (1) but not to lymphocytes (23, 35) and because of the small volume of blood obtained from patients, we used carrageenan as an agent to destroy monocytes in vitro. Carrageenan was incubated at an optimal concentration of 1 mg/ml or 200 \( \mu \)g/well.

Chart 2 shows the effect of carrageenan on the response of normal control lymphocytes to PHA in vitro; a markedly depressed response (40%) was found with lymphocytes treated with carrageenan on Days 3 and 4 of culture. To verify that the depressed response was due to the absence of macrophages and not due to a decrease of T-cells, we removed the monocytes by treating the whole blood with carbonyl iron powder; Chart 2 demonstrates that a greatly depressed response to PHA was observed in monocyte-free lymphocyte cultures, and no additive effect of carrageenan on this population was found.

The addition of untreated monocytes (3%) to monocyte-free lymphocytes preincubated with carrageenan and washed resulted in an increase in thymidine incorporation after PHA stimulation (Chart 3). Furthermore, the number of T-cells (by E-rosettes) did not change after the lymphocytes were incubated for 6 or 18 hr with carrageenan at 37°C (Table 1).

From the results of the above-mentioned experiments, it seems highly unlikely that carrageenan affects T-cells.

**Effect of Carrageenan on PHA Response of Cancer Patient Lymphocytes.** We tried to destroy macrophages from WBC of cancer patients with carrageenan to see whether the depressed response to PHA of cancer patients might be enhanced, as has been well demonstrated in tumor-bearing animals (22, 24). We examined this possible action in 120 cancer patients. Chart 4 showed that, when cancer patient lymphocytes were cultured with carrageenan, the responses to PHA were generally depressed compared with those not treated \( (p < 0.05) \) as in normal lymphocytes (Chart 2).

**DISCUSSION**

In this study, we have demonstrated the depressed PHA response in a group of 169 cancer patients' lymphocytes as compared to a control group. Their depressed activity was
Human Nonspecific Suppressor Cells

Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control</th>
<th>Incubated with carrageenan (1 mg/ml)(^a)</th>
<th>6 hr</th>
<th>18 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52</td>
<td>56</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>68</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>65</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>63</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>68</td>
<td>65</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± S.D. 61.60 ± 3.43 63.40 ± 2.07 61.80 ± 2.85

\(^a\) After incubation with carrageenan (1 mg/ml), lymphocytes were washed 3 times with phosphate-buffered saline.

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dine uptake between newly diagnosed lung cancer patients and their controls, whether the controls were clinically healthy or hospitalized subjects without malignant disease.

However, patients afflicted with benign diseases had normal reactivity to PHA, although their age was similar to that of cancerous patients. Hence, a depressive effect of the tumors is likely.

No differences were apparent between pre- and postoperative groups of colorectal cancer patients. This may be due to the short postoperative time, which varied from 1 week to 1 month. Watkins (37) observed that the PHA reactivity of peripheral lymphocytes of patients with various types of cancers increased after successful surgery.

No difference was noted between treated and nontreated patients with head and neck cancers. This could be compared to the work of Blomgren et al. (4), who have demonstrated that there was no statistically significant difference between the mitogen responses of irradiated and nonirradiated breast cancer patients' lymphocytes.

However, during our investigation, we found that the lymphocytes of 16 patients had a depressed response to PHA that was increased by carrageenan treatment \((p < 0.001)\). Chart 5 showed the magnitude of this phenomenon in these 16 patients. This observation led us to think that such patients could have immunosuppressive cells in their blood, and we tried to demonstrate the existence of the cells by adding the patients' mononuclear cells to those of normal subjects and by studying the reactivity of the mixture to PHA.

**Demonstration of Suppressive Cells in the Blood of 6 Cancer Patients.** Mononuclear cells isolated from the blood of a normal subject and from a cancer patient were cocultured for 3 days in the presence of PHA and carrageenan. This experiment was made with lymphocytes obtained from the 16 cancer patients described previously. Lymphocytes from 6 of them showed significant inhibition of PHA response in coculture with normal lymphocytes \((p < 0.001)\) (Chart 5).

Depression in coculture was a reproducible phenomenon due, in at least 6 cases, to the presence of suppressor cells.

The control and patient groups were not closely age matched, and the depressed PHA response might reflect the older ages of our cancerous patients. A decrease in the percentage of T-cells and a reduced incorporation of \(^{3}H\)thymidine of PHA-stimulated lymphocytes in old subjects has already been observed \((3, 10, 13, 31)\). Barnes et al. (3) have reported that, when normal controls were age and sex matched, no differences were noted in the thymi-
inasmuch as cells from 2 patients (Cases P and M) caused comparable depression when they were cocultured with cells from 2 other normal cell donors. No depression effect of the lymphocytes from the 10 other patients was observed in coculture with normal lymphocytes (Table 2).

The suppressive effect of these 6 cancer patients’ lymphocytes was at least partially inhibited when carrageenan was added to the cell mixture (Chart 6). The most striking effect was observed in Case P. In the presence of $2 \times 10^5$ Case P cells and $10^8$ normal cells, addition of carrageenan provoked a net cpm increase in response to PHA of 76,647 cpm instead of 4,379 cpm without carrageenan. The PHA response of the mixture of 2 healthy donors’ lymphocytes was always depressed by carrageenan.

We studied in parallel the depression of PHA response and the percentage of E-rosette-forming cells in these 6 patients. As shown in Table 3, there was no correlation between these data. Some patients having a low PHA reactivity had subnormal amounts of E-rosette-forming cells.

The depressed response to PHA of cancer patients’ lymphocytes may be due to a variety of contributing factors, such as decrease of T-lymphocytes, presence of serum factors, and presence of suppressor cells.

A reduction of T-cells in cancer patients has been observed, but this decline was significant only in patients with advanced tumor disease. Furthermore, the depressed PHA response to cancer patients’ lymphocytes could not be based only on a decline in the T-cell population. In fact,


E-rosette-forming cells were studied particularly in the 6 patients with clearly depressed PHA responses. In these cases (Table 3), there was no correlation between the percentage of E-rosette-forming cells and the severity of depression. Furthermore, the 2 patients (Cases M and P) with the most severe depression had normal numbers of E-rosette-forming cells. In addition a depressed PHA response was also observed in a cancer patient’s lymphocytes with a high percentage of T-cells (Case B).

The inhibiting effect of serum factors on PHA-induced lymphocyte responses in cancer patients has been observed (6, 7, 12, 16, 19, 31, 34, 38), but in contrast another...
Table 3
Depression of PHA response and distribution of percentage of sheep RBC-rosette-forming cells of 6 cancer patients' lymphocytes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Rosette sheep RBC (%)</th>
<th>Depression (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>56</td>
<td>M</td>
<td>February 1976: Cancerous stenosis detected by colonic enema. March 31, 1976: Total WBC, 8000 (neutrophiles, 66%; eosinophiles, 4%; basophiles, 1%; lymphocytes, 23%; monocytes, 6%). April 16, 1976: Operation, tumor removal, Lieberkühnian-type adenocarcinoma (T2N4)&lt;sup&gt;a&lt;/sup&gt;.</td>
<td>49 (65)</td>
<td>41</td>
</tr>
<tr>
<td>W</td>
<td>87</td>
<td>M</td>
<td>March 1976: Discovery of rectal adenocarcinoma with lung metastasis. Total WBC, 7200 (neutrophiles, 70%; basophiles, 1%; lymphocytes, 22%; monocytes, 5%). Inoperable due to advanced age.</td>
<td>58 (65)</td>
<td>45</td>
</tr>
<tr>
<td>B</td>
<td>74</td>
<td>M</td>
<td>January 1976: Discovery of colonic polyps. April 12, 1976: Total WBC, 5200 (neutrophiles, 68%; eosinophiles, 2.5%; basophiles, 0.5%; lymphocytes, 22%; monocytes, 7%).</td>
<td>59 (52)</td>
<td>29</td>
</tr>
<tr>
<td>P</td>
<td>52</td>
<td>M</td>
<td>February 5, 1975: Spinocellular carcinoma (floor of the mouth). Submaxillary adenopathy (T2N4M4). Alcoholic habits with severe polyneuritis. December 3, 1975: Radiotherapy (7600 rads). Total WBC: 8800 (neutrophiles, 77%; basophiles, 1%; lymphocytes, 20%; monocytes, 2%).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Depression of PHA response calculated as the percentage decrease in the number of rosette-forming cells compared to preoperative levels.

<sup>b</sup> Tumor size and stage classification as per the American Joint Committee on Cancer.

<sup>c</sup> Values in parentheses indicate the percentage of preoperative levels.

<sup>d</sup> Tumor size and stage classification as per the American Joint Committee on Cancer.
Table 3—Continued

<table>
<thead>
<tr>
<th>Patient Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Rosette sheep RBC (%)</th>
<th>Depression (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperatively.</td>
<td>50 (65)</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoperatively.</td>
<td>56 (64)</td>
<td>87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>February 2, 1976: Removal of 2 invaded cervical lymph nodes. Radiotherapy, 4200 rads.</td>
<td>52 (56)</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk postoperatively.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total WBC: 7400 (neutrophiles, 73%; eosinophiles, 1%; lymphocytes, 21%; monocytes, 6%).</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1 mo. postoperatively.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>February 1977: No recurrence.</td>
<td></td>
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</tbody>
</table>

"% depression (cultured on the same day) = \[(\text{Net cpm control} - \text{Net cpm patient}) / \text{Net cpm control}\] × 100.

T, N, and M, tumor-nodes-metastasis classification system.

Numbers in parentheses, percentage of E-rosette-forming cells of control group.

study showed no significant difference between the PHA responses of lymphocytes incubated in normal serum and those incubated with cancer serum (3, 20, 25). In our preliminary investigations, plasma from 10 cancer patients, mostly with colorectal cancer, did not affect the PHA response of normal lymphocytes.

It is therefore possible that suppressor cells play an important role in the decline of PHA responses of cancer lymphocytes.

In animal systems, the literature has provided ample proof that these suppressor cells exist. Several types of suppressor cells have been described. Thymic-derived suppressor cells (2, 9) have been thought to regulate B-cell activity in a feedback mechanism. Kirchner et al. (22) have demonstrated that a suppressor cell in the spleen of mice bearing primary tumors induced by Moloney sarcoma virus is a cell of the monocyte-macrophage series. In our laboratory, Lespinats and Poupon (24) also showed that suppressor cells in mice carrying methylcholanthrene-induced tumors were probably macrophages.

In the rodent, it seems that much of the suppressor activity of spleen cell suspensions can be abolished by removal of adherent cells with either glass wool or carbonyl iron (22, 24).

Blomgren et al. (4) have shown that lymphocytes from breast cancer patients, which responded poorly to purified protein derivative, exhibited increased reactivity after phagocytic cells were depleted by carbonyl iron. However, the mean PHA responses of lymphocytes from patients and controls, respectively, were not significantly changed after removal of phagocytic cells.

Zembala et al. (40) have recently demonstrated that 1 mechanism of depression of cell-mediated immunity seen in patients with advanced cancer may be the nonspecific suppression of certain T-cell functions by circulating monocytes.

During our investigation, due to the small volume of blood obtained from cancer patients, we did not have the opportunity to remove the monocytes routinely by nylon fiber column or by carbonyl iron; therefore we substituted for these methods the treatment of lymphocytes with carrageenan, a macrophage-toxic agent. Macrophages are known to be necessary accessory cells for the activation of lymphocytes induced by a variety of different stimulating agents in vitro (28, 30). This study indicated that carrageenan is toxic for monocytes. Treatment of lymphocytes with carrageenan gives the same effect as when monocytes were removed by carbonyl iron, i.e., a decrease of PHA response of normal lymphocytes. The addition of 3% untreated monocytes to the cultures of washed monocyte-free lymphocytes preincubated with carrageenan resulted in an increase in thymidine incorporation after PHA stimulation. On the other hand, we have demonstrated by the E-rosette method that carrageenan does not affect T-cells after 6 and 18 hr of incubation with lymphocytes. This is in agreement with other studies on the nontoxic activity of carrageenan on T-cells (23, 35). The response to PHA of the lymphocytes of 120 cancer patients were systematically tested with or without carrageenan. Sixteen of these had an increased response to PHA after treatment with carrageenan, and the lymphocytes of only 6 of these 16 patients have suppressive activity on the response to PHA of normal lymphocytes in mixed cultures. Since the depressed PHA responses in 6 cancer patients' lymphocytes were partially restored by treatment with carrageenan and the suppressive effect of these 6 cancer patients' lymphocytes on the PHA response of normal lymphocytes was partly reversed after the macrophages were destroyed, we suggest that suppressor cells exist in these 6 patients and that these cells are probably monocytes.

The nature of the suppression caused by monocytes from cancer patients is not clear. It may be, as demon-
Stage by Nelson (26), that the inhibitory effect in the
34. Waldmann, T. A., Broder, S., Bleea, R. M., Durm, M., Blackman, M., and
P. C. Quan and P. Burtin


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