Carcinoembryonic Antigen-induced Release of a Suppressor Factor from Normal Human Lymphocytes in Vitro

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ABSTRACT

Although it is generally accepted that tumor-bearing patients may be immunosuppressed, the mechanism for this effect is unclear. Therefore, we tested the hypothesis that a tumor-associated macromolecule, carcinoembryonic antigen (CEA), could itself suppress lymphocyte function, as quantitated by uptake of [3H]thymidine by lymphocytes stimulated with the plant lectin, phytohemagglutinin. Normal human peripheral blood mononuclear cells, after exposure to CEA for 48 hr, subsequently released a factor in vitro which markedly inhibited phytohemagglutinin-stimulated lymphocytes. In further experiments, this factor release was confirmed to be initiated by CEA and not by a contaminant, and to be induced over a broad range of CEA concentrations (0.2 to 100 ng/ml). Suppression could not be accounted for by factor-associated cytotoxicity toward indicator cells, nor was it secondary to a mixed-lymphocyte reaction, nor could CEA alone (without factor) modulate proliferation.

In studies to characterize the factor, its molecular weight was >10,000, its activity was partially denatured by heat and proteases, and the isoelectric point was 3.4. Polyacrylamide gel electrophoresis of an "active" fraction revealed protein bands with molecular weights of 52,000, 77,000, and 171,000. Knowledge of immunomodulatory molecules present in cancer patients may suggest new modalities for therapy.

INTRODUCTION

A major challenge for immunobiological research is to define the means by which an apparently immunocompetent host is tolerant of foreign tissue under certain circumstances. For example, despite the presence of paternal major histocompatibility or tumor-specific antigens, respectively, fetal and malignant cells may survive in a host, able to proliferate to form a large amount of antigenically foreign tissue without immunological destruction.

Abrogation of an immune response in these settings may relate to defects in cell-mediated immunity in the pregnant mother and in the tumor-bearing patient, and evidence for this has been reported over the last 15 years (summarized in Refs. 12 and 20). Although the molecular basis for these findings is not clear, many immunosuppressive substances have been implicated. For instance, in pregnancy, numerous components in serum including α₁-macroglobulin (33) and early pregnancy factor (27), as well as soluble products from individual placental components (e.g., trophoblast, decidua, and endometrium) (2, 9, 21), have been described as immunosuppressive in in vitro systems, but their importance in vivo is not known. Likewise, in the sera of tumor-bearing hosts, blocking factors (tumor antigens, immune complexes, or antitumor antibodies) (14), acute-phase α-globulins (5, 7), and lipoproteins of all classes (4, 8, 24), have been associated with depressed lymphocyte function.

Mediating the immunoregulatory effects of these biological molecules may be lymphocytes which are phenotypically and/or functionally suppressor in type, which have recently been detected and partially characterized from lymph nodes draining the gravid uterus (6), from mononuclear cells of the cord blood (28), and from cultured lymphocytes after exposure to tumor antigens (31). In addition, soluble factors with inhibitory properties for immune function have been detected in maternal (16) and malignant sera (10). When studied, these factors have generally been found to be M, 5,000 to 80,000 glycoproteins capable of inhibiting cell proliferation and a wide range of immunological functions (35). Unknown, however, are the mechanisms responsible for release of such factors from regulatory immunocytes. Particularly in the cancer setting, knowledge in this area could provide a means for a selective intervention that would enable a host to react against his own tumor.

Because fetal and malignant tissues share certain oncofetal antigens that could provide a common signal for initiation of host immunosuppression, we examined the hypothesis that one such antigen, CEA, could trigger the release of an immunosuppressive soluble factor from human peripheral blood lymphocytes in vitro, the latter measured functionally by reduction in mitogen-driven lymphocyte DNA synthesis.

MATERIALS AND METHODS

CEA. Initial experiments used commercially available CEA preparations (Roche Diagnostics, Nutley, NJ). Since these contain A-positive plasma at 10⁻⁶ µ/ml of reagent, all subsequent experiments used a CEA preparation devoid of plasma, which was purified from the hepatic metastasis of a colorectal adenocarcinoma and characterized according to the method of Hansen (26). This was a gift from Dr. Darrow Haagensen, Boston City Hospital, Boston, MA. Its purity was confirmed in our laboratory by Laemmli:sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 7.0 and 12.5% gels (18), using a silver stain as described by Merrill et al. (22) and molecular weight standards (Bio-Rad, Richmond, CA).

Preparation of HPBL. Populations of HPBL were isolated from healthy donors by discontinuous density gradient centrifugation performed with 2% Ficoll:Hypaque (3:2) in a 50-ml polypropylene tube (Falcon Plastics, Oxnard, CA). Cells isolated were morphologically 98% mononuclear cells and of greater than 95% viability. Trypan blue dye exclusion did not fall below 67% during the 4-day culture period.

Preparation of Lymphocyte Culture Supernatant. Viable mononuclear cells at 5 x 10⁶/ml were cultured at 37 °C for 48 hr in a total volume of 2 ml, of which 0.5 ml was harvested after the first 24 hr.
of 50 ml RPMI 1640 with 10% FCS (Grand Island Biological Co., Grand Island, NY) and antibiotics. Experimental lymphocyte cultures from pooled or single donors were exposed to CEA in concentrations approximating physiological and pathological levels in vivo at 0.2, 20.0, or 100 ng/ml for an initial 48 hr (Chart 1). The cells were harvested by centrifugation, and the supernatant containing CEA and FCS was discarded. To obtain lymphokine free of CEA and FCS, cells were washed 3 times and recultured at 5 x 10^6 HPBL/ml in RPMI 1640 alone. Supernatants were collected at 48 hr and concentrated 35-fold using ultrafiltration. In control experiments, no CEA was added during the initial stimulatory incubation. Prior to assay, all samples were exhaustively dialyzed against RPMI 1640 and filter sterilized.

Assay for Suppressor Activity. All experiments were performed in triplicate with the initial addition of test supernatant (0.1 ml/well), followed by RPMI 1640 (10% FCS) containing PHA (40 µg/ml) and 5 x 10^7 HPBL/ml in RPMI 1640 alone. Supernatants were incubated for 44 hr at 37°C in 5% CO2:95% air with an initial PHA concentration of 0.2, 20.0, or 100 ng/ml for an initial 48 hr (Chart 1). The cells were harvested by centrifugation, and the supernatant containing CEA and FCS was discarded. To obtain lymphokine free of CEA and FCS, cells were washed 3 times and recultured at 5 x 10^6 HPBL/ml in RPMI 1640 alone. Supernatants were collected at 48 hr and concentrated 35-fold using ultrafiltration. In control experiments, no CEA was added during the initial stimulatory incubation. Prior to assay, all samples were exhaustively dialyzed against RPMI 1640 and filter sterilized.

Unpaired Student's t test was used to compare the statistical significance of experimental versus control values.

RESULTS

Inhibitory Effect of Cell Culture Supernatants. We first examined whether a 48-hr exposure to CEA (0.2 ng/ml) (supplied by Roche Diagnostics, in buffer with 0.01% A-positive plasma) could cause mononuclear cells to release an immune inhibitory substance over the succeeding 48 hr. When mononuclear cells were from pooled healthy donors and the resulting supernatants were tested at 1.0 mg/ml of biuret protein after ultrafiltration and dialysis, a 66 ± 17% (S.D.) inhibition of PHA-driven blastogenesis by normal human peripheral blood lymphocytes was found.

In order to determine that inhibition was due to CEA and not to a plasma component included in the buffer, a highly purified CEA preparation (26) was next used for mononuclear cell conditioning. The purity of the latter was shown by analytical polyacrylamide gel electrophoresis (Fig. 1); a highly sensitive silver stain technique demonstrated that no components entered the gel (7% polyacrylamide). Subsequently, a significant degree of suppression (74 ± 2%) was again found with the culture supernatant after mononuclear cell exposure to highly purified CEA (0.2 ng/ml) (Table 1).
CEA-STIMULATED RELEASE OF SUPPRESSOR FACTOR

Fig. 1. Fifty ng of CEA prepared commercially (Lane 2) and equivalent amounts of highly purified CEA (Lane 3) were subjected to electrophoresis using a Laemmli-sodium dodecyl sulfate system in a 7% slab gel after heating samples to 100° for 2 min. Bands were developed using a highly sensitive silver staining technique.

Table 1
Lymphocyte suppression by CEA-induced supernatant

<table>
<thead>
<tr>
<th>CEA (ng/ml)</th>
<th>% of suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>74</td>
</tr>
<tr>
<td>20</td>
<td>82</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Because the experiments above were carried out with peripheral mononuclear cells pooled from multiple donors, and because the suppressive activity detected may have resulted as a by-product of a mixed lymphocyte reaction, mononuclear cells from a single donor were cultured in the usual manner with highly purified CEA (0.2 ng/ml). After these cells were washed and cultured for an additional 48 hr in medium, the resulting supernatant was highly suppressive. This experiment was repeated twice, and the mean for the suppression achieved was 70 ± 28% (S.D.).

To determine whether pathological levels of highly purified CEA could condition normal peripheral mononuclear cells in a manner similar to that which occurred with more physiological levels (0.2 ng/ml, above), single-donor lymphocytes were exposed to CEA (20 and 100 ng/ml). Suppression of 82 ± 2 and 100%, respectively, was found (Table 1).

Control Incubations. Although the protocol for mononuclear cell exposure to CEA was designed to eliminate CEA contamination of the collected culture supernatant (see "Materials and Methods"), additional experiments were performed in which PHA-stimulated cells were directly incubated with CEA for 48 hr in the assay for suppressive activity (Table 2). CEA in concentrations of 0.2, 20, and 100 ng/ml did not result in suppression of more than 35%.

Trypan blue dye exclusion was performed routinely on cells both in culture and in the microtiter assay in order to determine that suppression was not due simply to cell death. After 4 days in tissue culture, the last 2 of which were in serum-free media, viability of "conditioned" cells remained at 85 ± 4%. After 48 hr in microtiter wells, viability of test cells (PHA-stimulated normal peripheral mononuclear cells) was 62 ± 16%.

Partial Characterization of Macromolecules Responsible for Suppression. Because a number of humoral components of varied molecular weight and composition have been reported as suppressive of mitogen-stimulated lymphocyte DNA synthesis, we undertook characterization studies of this CEA-inducible factor to determine if there were properties held in common with similar components isolated previously. A modest sensitivity to heat was shown by the partial inactivation of suppressive activity with heating of the culture supernatants to 56° and 80° for 30 min (Table 3). This suggested that protein was important for biological activity.

Therefore, susceptibility of active culture supernatants to proteolytic digestion was next tested. Initial control experiments demonstrated that trypsin (50 μg/ml) could hydrolyze 1.0 mg of a test protein substrate (Azocoll) and could subsequently be completely inactivated with Trasylol (128 units/ml) without itself causing inhibition of PHA-driven mitogenesis in the assay for suppressor activity (Table 4). Subsequently, digestion of active-culture supernatants with trypsin utilized in a quantity sufficient to hydrolyze 100% of the available protein resulted in a loss of activity from 99 ± 2 to 59 ± 2%. As before, this suggested that

Table 2
Direct effect of CEA on lymphocyte proliferation

<table>
<thead>
<tr>
<th>CEA* (ng/ml)</th>
<th>PHA</th>
<th>[3H]Thymidine uptake (cpm) % of suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>933 ± 82</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>34,388 ± 1,424</td>
</tr>
<tr>
<td>0.2</td>
<td>+</td>
<td>36,210 ± 3,773</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>35,400 ± 3,945</td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td>22,816 ± 743</td>
</tr>
</tbody>
</table>

* Final concentration of highly purified CEA per 200 μl well assayed in triplicate.

Table 3
Effect of heat inactivation on CEA-induced suppressor activity

Concentrated supernatant with active suppressor factor was collected from HPBL after initial exposure to highly purified CEA (0.2 ng/ml). Aliquots were exposed to varying temperatures for 30 min prior to being tested in triplicate in the assay for suppressor activity.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>% of suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°</td>
<td>100</td>
</tr>
<tr>
<td>58°</td>
<td>64</td>
</tr>
<tr>
<td>80°</td>
<td>71</td>
</tr>
</tbody>
</table>

* Suppression calculated as described under "Materials and Methods."
Effect of trypsin digestion on CEA-induced supernatant suppressor activity

Concentrated supernatant, with active suppressor factor was collected from HPBL after initial exposure to highly purified CEA (0.2 ng/ml). One ml aliquots were treated with 20 µl trypsin (0.25%) at 37° for 4 hr. Trasylol was added to stop proteolysis prior to testing in the assay for suppressor activity.

Table 4

<table>
<thead>
<tr>
<th>Additive tested</th>
<th>PHA</th>
<th>Trypsin</th>
<th>[%]Thymidine uptake (cpm)</th>
<th>% of suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>-</td>
<td>-</td>
<td>256 ± 187²</td>
<td>100</td>
</tr>
<tr>
<td>Medium²</td>
<td>+</td>
<td>+</td>
<td>18,180 ± 2,412</td>
<td>73</td>
</tr>
<tr>
<td>Suppressive</td>
<td>+</td>
<td>-</td>
<td>324 ± 562</td>
<td>99</td>
</tr>
<tr>
<td>Supernatant</td>
<td>-</td>
<td>+</td>
<td>7,633 ± 401</td>
<td>59</td>
</tr>
</tbody>
</table>

²A submaximal dose of PHA (20 µg/ml) was used as described under "Materials and Methods."

⁷RPMI 1640 was incubated with trypsin and treated identically to the test supernatant to control for effects of trypsin and Trasylol in the assay.

Purification of the Suppressive Component by Isoelectric Focusing. Because of its utility in isolating lymphokines in our previous studies (15), isoelectric focusing was used to fractionate active-culture supernatants in the current investigation. Preliminary studies with ampholines having a broad pH range (3 to 10) indicated that suppressive activity was localized to pH 3 to 4, and this range was utilized in all subsequent experiments (Table 5). Arbitrarily assigning 30% as a cutoff, only fractions between pH 3.4 and 3.6 showed suppressive activity (Chart 2). This was true for both CEA preparations, pooled and single donor lymphocytes, and several levels of exposure (0.2 to 100 ng/ml) to the oncofetal antigen. In contrast, electrophoresis of the culture supernatant from a control experiment, in which 0.9% NaCl solution rather than CEA was added to lymphocytes in culture, revealed no suppressive activity greater than 30% in any fraction.

To investigate which components may be responsible for suppression in the electrofocused fraction of pH 3.4 to 3.6, an electrophoretic separation on a 7% polyacrylamide gel under non-denaturing conditions was carried out. Bands developed by a sensitive silver staining technique in the active fractions had apparent molecular weights of 52,000, 77,000, and 171,000 (Fig. 2). It remains possible that a large molecular weight component which did not enter the 7% polyacrylamide carried the biological activity.

DISCUSSION

The molecular basis for immune tolerance of fetal and malignant tissues is not clearly understood. Because CEA is common to both of these tissue types, we wished to determine whether it might suppress, either directly or indirectly, lymphocyte function. A mechanism for interaction between fetal and/or malignant tissues and the immune system was indeed demonstrated in that highly purified CEA was found to inhibit mitogen-driven lymphocyte DNA synthesis, not by a direct suppressing capacity.
of CEA but rather by a novel mechanism, CEA-induced release of a suppressor lymphokine.

This lymphocyte-released factor shares biological and physicochemical properties characteristic of suppressor lymphokines (35). It is antigen nonspecific since it regulates a lymphocyte response (i.e., DNA synthesis) initiated by an antigen-nonspecific stimulus, PHA. In addition, retention of activity above M, 10,000 by membrane filtration, and partial sensitivity to heat and protelysis, is characteristic of lymphokines (1, 35) and suggests a role for a protein component(s) in the active fractions. Furthermore, high biological activity at low protein concentrations, as indicated by low absorption peaks at A260 and minimal reactivity by biuret protein determinations (data not shown), is consistent with high specific activity associated with lymphokines generated in vitro and purified by isoelectric focusing (17).

Separation of components in the crude supernatant by isoelectric focusing with concomitant analysis of each fraction for its suppressive capability revealed that the isoelectric point of the active fraction was at pH 3.4 to 3.6. This is identical to that of human inhibitor of DNA synthesis, an antigen-nonspecific suppressor lymphokine secreted by proliferating T-cells after appropriate in vivo or in vitro stimulation (15). Thus, immunosuppressive activity initiated and/or governed by oncofetal antigens may be mediated by an inhibitor of DNA synthesis-like lymphokine. Another suppressor lymphokine, soluble immune response suppressor, derived from concanavalin A-induced lymphocytes, inhibits immune functions by stimulating macrophages to release a suppressor factor (1). The protocol in our experiments eliminated adherent cells (i.e., macrophages) after the initial 48 hr of culturing and prior to collection of the conditioned medium, reducing the possibility that the CEA-released factor is soluble immune response suppressor. Even less probable is the potential that the CEA-released factor is a lymphotoxin, causing an immunosuppressive effect by cytolysis of lymphocytes (35). No toxin effect was detected in our active fractions by vital dye exclusion.

Explanations for our results other than a CEA-induced lymphokine were considered but thought to be unlikely. To begin with, carryover of CEA from the initial lymphokine-inducing cultures into the assay for suppressor activity might have been responsible for suppression. This possibility was minimized by triple washing of cells after CEA exposure and through the use of CEA-free fresh RPMI 1640 as the medium for collection of elaborated lymphokine. In addition, CEA, with an isoelectric point of 4.8 (11), should not have focused in the pH range (2.5 to 4.0) utilized in our experiments for lymphokine isolation. Possibly, a contaminating moiety in the CEA preparations may have been responsible for inducing lymphocytes to release the suppressor factor. This explanation is qualified by (a) successfully inducing suppressor factor release with 2 different CEA preparations isolated from different laboratories from different sources and (b) failure to visualize any contaminating proteins at levels ≥10 pg/sq mm (22) on polyacrylamide gel electrophoresis of the purified CEA. However, it remains possible that such a contaminant did not enter the gel or else escaped detection by silver stain. Finally, CEA had no direct inhibitory effect on PHA-driven blastogenesis when added to stimulated lymphocytes at concentrations equivalent to those used for lymphokine induction (Table 2) and confirms the findings of others (13, 23, 32) that CEA, at concentrations found in patients, does not directly suppress lymphocytes in vitro.

Also, the possibility that a suppressor lymphokine may have been elaborated as a result of an allogeneic lymphocyte reaction was considered because pooled HPBL were utilized in our initial experiments. However, supernatant from pooled lymphocytes not exposed to CEA and tested by our standard procedure was devoid of suppressor activity (Table 1). Furthermore, suppressor activity was induced in CEA-containing lymphocyte cultures even when single-donor HPBL were used.

It is possible that CEA-induced immunosuppression is representative of widespread immunoregulatory properties invested in oncofetal molecules. In this context, another oncofetal antigen, α-fetoprotein has been extensively studied and found to have both suppressive (19, 25, 29) and enhancing (3, 30) effects, depending on the immunological assay tested. Similar to our studies with CEA, α-fetoprotein suppression of T-cell-dependent immune functions occurs at concentrations of antigen 10-fold more dilute than that found in vivo (29). In contrast, however, is the direct inhibition of mitogen-stimulated lymphocytes by α-fetoprotein (36).

In summary, these studies show that CEA induces the release of a suppressor lymphokine from healthy human lymphocytes in vitro. Further understanding of this interaction and of the precise biological effects of the released lymphocyte factor may lead to new therapies aimed at abrogating pathological states of immune unresponsiveness.

REFERENCES

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