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Heterogeneity of Soluble Suppressor Factors in Rat Malignant Ascites

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Abstract. A study was undertaken to enumerate and partially characterize soluble factors generated by tumor-bearing animals capable of suppressing PHA-induced splenocyte proliferation. Sprague-Dawley rats were induced to form malignant ascites by the intraperitoneal injection of the Walker 256 carcinoma. Intact ascites suppressed splenocyte proliferation by 96%. Molecular sieving of the ascites by means of ultrafiltration (10-kilodalton particle cutoff) revealed suppressor activity to reside in both the ultrafiltrate and retentate. Further enumeration of suppressor factors was achieved by preparative polyacrylamide gel electrophoresis of the ascites ultrafiltrate and retentate. Five discrete bands of suppressor activity were resolved in the ultrafiltrate, three of which were heat-labile. Three discrete bands of suppressor activity were resolved in the retentate, none of which were heat-labile. This study underscores the complexity and heterogeneity of soluble factors elaborated in a cancer-bearing animal.

Introduction

The negative consequences of the immunosuppression associated with neoplasia are substantial. In addition to the loss of host defenses against growth and spread of tumor, there are also the problems of opportunistic infection, and intolerance to anticancer therapy. This immunosuppression has been demonstrated to be due, at least in part, to the presence of serum suppressor factor(s). Sera from patients with a variety of malignancies inhibit mitogen-stimulated proliferation in vitro of lymphocytes obtained from healthy donors [1, 2]. Furthermore placing unresponsive lymphocytes from cancer patients into normal serum will restore responsiveness to mitogenic stimulation [3]. Thus, one of the defects in immune function related to cancer may be a reversible inhibition of lymphocyte function mediated by serum suppressor factors.

Despite the importance of the problem of immunosuppression in neoplasia, this area is still not well understood. One of the reasons for this lack of general understanding is the difficulty inherent in human experimentation. Serial sampling of functioning immune tissue from a cancer patient is difficult to justify. In addition, therapeutic manipulation with chemotherapeutic agents, radiation, and surgery may perturb the natural host immune response complicating interpretation of experimental data [4–7]. In order to circumvent these problems we have investigated an animal model capable of generating large quantities of soluble suppressor factors [8].

Sprague-Dawley rats injected intraperitoneally with the Walker 256 carcinoma develop a malignant ascites. We demonstrated the cell-free component of this ascites to be capable of inhibiting mitogen-induced proliferation of normal rat splenocytes. In vitro studies indicated that the source of this material was both lymphoid and nonlymphoid tissues of tumor-bearing animals. Preliminary characterization of suppressor factor(s) in this ascites revealed activity to be heat-stable and of low molecular weight (less than 10,000 daltons). Thin-layer chromatography revealed the presence of prostaglandins E_2 and $F_2\alpha$. The ability

of prostaglandins to inhibit mitogen-induced blastogenesis has been previously documented [9]. The present study incorporates a more detailed examination of the experimental malignant ascites and demonstrates that numerous soluble factors capable of suppressing lymphocyte function are present in the ascites of these tumor-bearing animals.

Materials and Methods

Solid Walker 256 carcinoma (Mason Research Institute, Worchester, Mass.) grown intramuscularly was minced with iris scissors in a sterile Petri dish containing RPMI 1640 tissue culture medium. Adult male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, Wisc.) were injected intraperitoneally with 1 ml of a suspension of finely minced tumor in RPMI. Ten to 14 days after inoculation ascites was harvested by transabdominal needle aspiration from halothane-anesthetized rats. The ascites was allowed to stand at room temperature for 1 h to permit clot formation. The fluid component was separated by centrifugation at 1,500 rpm for 10 min. The supernatant was removed, aliquoted, and stored at −35 °C.

A microplate assay measuring [H³]thymidine incorporation by PHA-stimulated rat splenocytes was employed. The details of this assay have been described previously [8]. Briefly, splenocytes from a normal spleen obtained from a healthy donor were suspended in RPMI 1640 supplemented with 10% fetal calf serum and the cell concentration adjusted to 5×10^6 viable cells/ml. One hundred and eighty microliters of the splenocyte suspension were placed in each well of the microliter test plate. Twenty microliters of the experimental material were added to each well. Proliferation was induced by adding 2.5–5.0 mg/ml of PHA (Wellcome Research Labs, Beckenham, England). Normal rat serum and ascites in various stages of fractionation were also assayed with and without the addition of PHA. Experimental samples were run in sextuplicate, except for assays conducted with material obtained from polyacrylamide gels. Material sufficient only for a single determination was eluted from the gel slices. The plates were incubated for 72 h at 37 °C in an atmosphere of 95% O_2–5% CO_2. Following the 72-hour incubation, 10 µl of [H³]thymidine (100 µCi/ml, Amersham, Arlington Heights, Ill.) were added to each well. Following a second incubation of 18 h, the wells were aspirated and the contents deposited onto filter paper, dried at 95 °C, placed in scintillation vials with 2 ml of scintillation cocktail, and counted in a liquid scintillation counter for 1 min. For samples run in sextuplicate, a mean and standard deviation were calculated. Percent inhibition of DNA synthesis was then calculated by the following formula:

$$1 - \left(\frac{\text{counts/min experimental sample}}{\text{counts/min control}} \right) \times 100.$$

Ascites was first subjected to centrifugation at 30.000 rpm for 1 h at 4 °C to remove fine particulate matter. The supernatant was then passed through a PM-10 Diaflo® ultrafiltration membrane (Amicon Corporation, Lexington, Mass.). The ultrafiltrate was collected. The retentate was further cleansed of low molecular

weight moieties by adding RPMI 1640 tissue culture medium 3 times to the Amicon chamber and subjecting the retentate to repeated ultrafiltration. The ultrafiltrate and PM-10 retentate were aliquoted and stored at −35 °C.

Crude ascites, ultrafiltrate, and the retentate following the ultrafiltration were subjected to heating at 100 °C for 40 min. This resulted in the formation of a precipitate which was removed by centrifugation at 1,500 rpm for 10 min. The supernatant was collected, aliquoted, and frozen at −35 °C.

Polyacrylamide gel electrophoresis (PAGE) was performed by the technique of Davis [10]. A 30-µl sample in 20% sucrose was placed on a 0.5 × 8.0 cm gel column made up of a 1-cm 3% acrylamide stacking gel and a 7-cm 7% acrylamide separating gel in a solution of 50 mM Tris-glycine at pH 9.6. A current of 4 mA/gel was utilized to perform electrophoresis which was run at 4 °C. The gel were cut into 2-mm slices and two adjacent slices were incubated in 200 µl RPMI for 24 h at 37 °C. The eluents were assayed for immunosuppressive activity in the PHA-stimulated splenocyte proliferation assay. The position of the gel slice was identified by its RF value determined by its position in the gel relative to the marker dye, bromophenyl blue.

Results

The suppressor activities of ascites and its components separated by ultrafiltration are summarized in table 1. Mitogen-induced lymphocyte proliferation was consistently inhibited in the presence of crude ascites. Soluble suppressors present in crude ascites were separated into two fractions utilizing ultrafiltration. The low molecular weight moieties present in the ultrafiltrate produced a 71% inhibition when assayed with PHA-stimulated splenocytes. Heat treatment of the ultrafiltrate abolished the suppressor activity and resulted in an enhancing effect. Substances present in the retained material following ultrafiltration were found to suppress PHA-stimulated splenocytes by 95%. Heat treatment of the PM-10 retentate resulted in a loss of suppressor activity. These results were

Table I. Suppression of PHA-stimulated splenocyte proliferation by malignant ascites following ultrafiltration and heat denaturation

| Experimental samples	Percent suppression[b]
Crude ascites	96
Ascites ultrafiltrate[a]	71
Heat-treated ascites ultrafiltrate[a]	−51
Ascites retentate	95
Heat-treated ascites retentate[a]	43

[a] Assayed at 50% concentration.
[b] Negative suppression indicated enhancement of blastogenesis.

observed with a minimum of two experiments and were consistent throughout the study.

Examination of eluted material from polyacrylamide gel slices in the blastogenesis assay proved to be capable of resolving ascites and its components into bands of suppressor activity. Preliminary work revealed that the most consistent results could be obtained when material eluted from the gel slices was further diluted to a 50% concentration with RPMI 1640 tissue culture medium. Hence, all results record-

ed in this study relating to the PAGE work are derived from material assayed at the 50% concentration. A definition of significant suppression was chosen to be greater than 50% inhibition of blastogenesis when the eluted material was assayed at a 50% concentration. This definition of suppression was arrived at since normal rat serum or its components rarely suppressed splenocyte proliferation to this degree.

Ascites eluted from PAGE gels and assayed for suppressor activity was generally suppressive through-
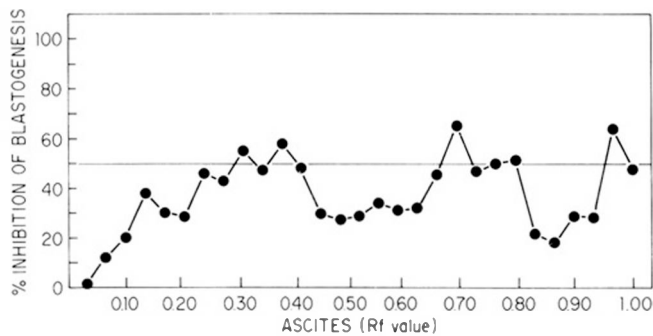

Fig. 1. Examination of suppressor activity following PAGE separation of intact malignant ascites demonstrated significant suppression of splenocyte proliferation by material obtained from the entire gel. Each point represents the activity from material eluted from two adjacent slices. Note that almost all points fall below the 50% inhibition level.
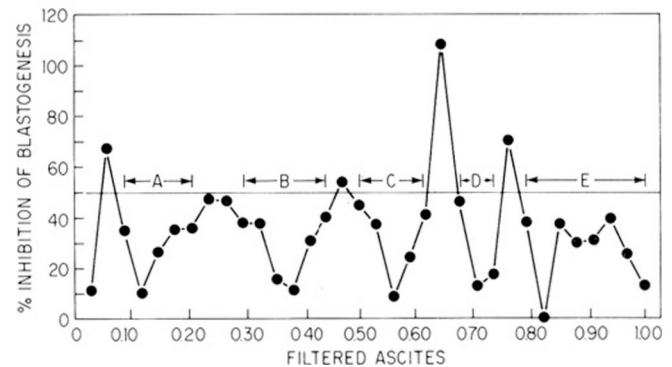

Fig. 3. Suppressor activity of material passing through the ultrafilter after PAGE separation. Five (A–E) relatively discrete bands of suppressor activity could be observed.
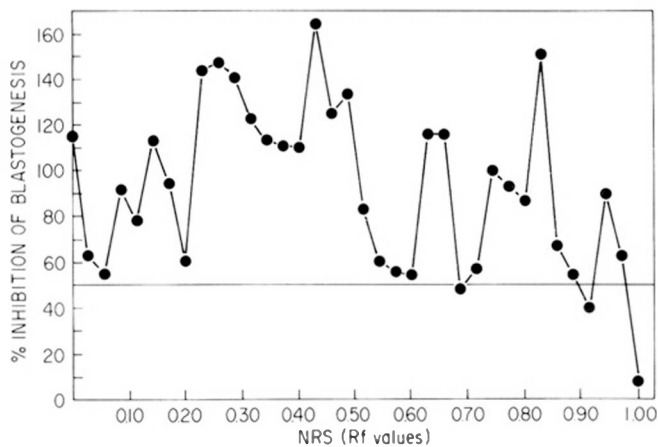
Fig. 2. This figure depicts the pattern of suppressor activity obtained after PAGE separation of normal rat serum (NRS). Note that although there are regions of suppressing and enhancing activity, almost all points lie above the 50% inhibition level.
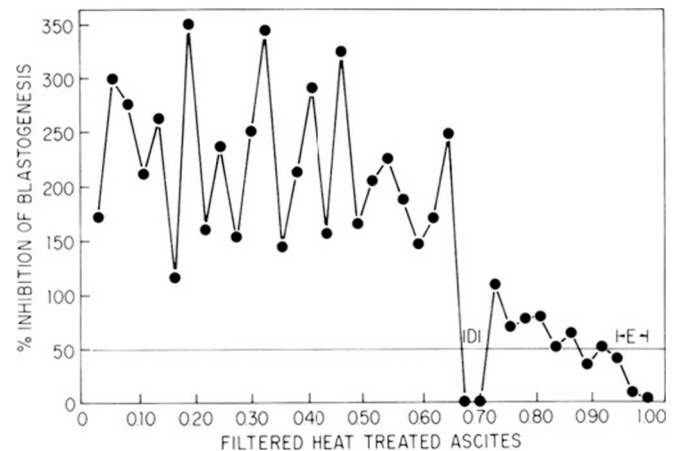
Fig. 4. Suppressor activity of material passing through the ultrafilter, then heated (100 °C × 40 min) and subjected to PAGE separation. Suppressor bands A, B, and C appeared to be heat-labile, whereas regions D and E persisted relatively unchanged.
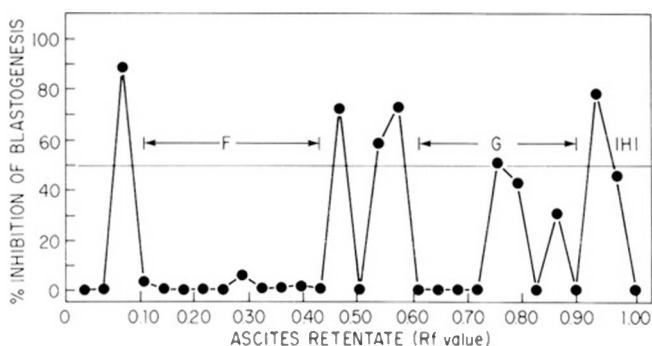
Fig. 5. Suppressor activity of the portion of ascites retained by the ultrafilter. There appeared to be three general bands of suppressor activity: F, G, and H.
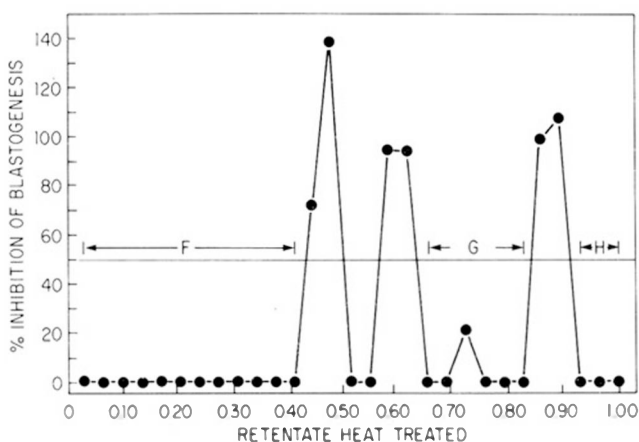
Fig. 6. Suppressor activity of the portion of crude ascites retained by the ultrafilter, then heated (100 °C × 40 min) and subjected to PAGE separation. The three bands of suppressor activity observed previously were noted to be present after this attempt at denaturation. Regions between the suppressor bands became enhancing.

out the gel. This contrasted with the PAGE assay of normal rat serum, which demonstrated rare suppressor activity at the 50% concentration (fig. 1, 2). The ultrafiltrate eluted from PAGE gels and assayed for suppressor activity revealed five discrete areas of suppression (fig. 3). Following heating bands A, B, and C were no longer observed (fig. 4). Band D persisted after heating and band E was diminished. In addition, the areas previously occupied by suppressor bands A, B, and C were found to be strongly enhancing.

The retentate eluted from PAGE gels and assayed for suppressor activity revealed three broad bands of suppression (fig. 5). Heat treatment of the retentate

did not substantially affect the bands of suppression, but did result in the observation of enhancement of blastogenesis in the areas which were previously not enhancing (fig. 6). Although the general trends in suppression were preserved following heating, minor changes were noted in the region of bands G and H.

Discussion

The present studies were undertaken to further examine the characteristics of suppressor factor(s) in the ascites of rats bearing the Walker 256 carcinoma. The initial observation that suppressor factors were multiple occured when substances suppressing splenocyte proliferation appeared in both the retentate and ultrafiltrate following ultrafiltration of malignant ascites. These suppressor factors were further enumerated with PAGE into five bands of suppressor activity present in the ultrafiltrate and three bands of suppressor activity were resolved in that portion of ascites retained by the ultrafilter.

Heat treatment of the ultrafiltered ascites resulted in a decreased suppression of splenocyte proliferation by the PM-10 retentate and enhancement of proliferation by the ultrafiltrate. These studies suggest that some of the suppressor factors are heat-labile. This finding was demonstrated more clearly by examining heat-treated components of ascites following PAGE in the splenocyte proliferation assay. It was observed that the three slowly migrating bands of suppressor activity has been replaced by activity which enhanced splenocyte proliferation. This effect is likely the result of heat denaturation of low molecular weight peptide suppressor substances. Yet, the two rapidly migrating bands of suppressor activity were generally intact. These heat-stable bands may be accounted for by prostaglandins, described by us previously to be present in this model. The large molecular weight moieties which suppressed splenocyte proliferation did not appear to be significantly altered by heating. These large molecular weight substances may consist of complex molecules such as glycolipids or lipopolysaccharides which would not be expected to be altered by heating.

The description of multiple soluble suppressors in this manuscript bears close resemblance to the description of an immunosuppressor peptide isolated from human plasma [11]. The cited reference describes a low molecular weight substance with an estimated

molecular weight of 4–6 kilodaltons (KD) which is noncovalently bound to a large carrier protein. This concept would fit with our observations of both low and high molecular weight suppressor substances. This previous investigation also demonstrated multiple low molecular weight peptides following paper electrophoretic analysis. Our work confirms that numerous low molecular weight substances are present and has demonstrated that each substance has suppressor activity.

A variety of suppressor molecules obtained from malignant ascites have been demonstrated previously. Hess et al. [12] described a substance with a molecular weight between 50 and 100 KD obtained from patients with ovarian neoplasms capable of inhibiting PHA-induced blastogenesis. However, a step in purification was dialysis which would have allowed low molecular weight substances to pass undetected. Sheid and Boyce [13] also examined ascitic fluid from women with ovarian cancer and described a low molecular weight substance which they attributed to macrophage origin. They were able to discern 6 moieties following electrophoresis of lyophilized material. The authors were not certain whether the numerous substances were a result of a complex mixture or were a result of changes in a single molecule brought about by the method of preparation. A third description of an ascites-derived suppressor factor involves a molecule less than 2 KD capable of inhibiting natural killer cell-mediated cell lysis [14]. The authors of this article likewise conclude that this material is of host origin. However, this investigation did not reveal the presence of any large molecular weight suppressor substances.

This study has established that a number of heterogeneous suppressor substances are elaborated in a cancer-bearing animal. This observation would appear to inject a sense of complexity in the area of soluble factors capable of inhibiting cellular immune function. Additional work in our laboratory has restored some order to this area. By examining blood samples from tumor-bearing animals over time we were able to document an initial enhancing effect of serum on in vitro lymphocyte function followed by a suppressor effect [15]. We were able to separate these effects on the basis of molecular weight with the vast majority of suppressor activity residing in the ultrafiltrate.

An issue not addressed in this study is the specificity of these suppressor substances to cancer. Acute-phase reactants present in the sera of animals and humans afflicted with both benign and malignant diseases have the capacity to suppress immune function [16–18]. These acute-phase reactants may account for a number of these suppressor factors demonstrated in this project. This issue could be resolved by applying the methodology utilized in this study in benign and malignant diseases resulting in immune suppression and enumerating the suppressor factors elaborated in each disease condition. In this manner suppressor factors uniquely related to cancer would be sought. The potential clinical utility of specific cancer-associated suppressor factors in the diagnosis and possible treatment of cancer warrants further study in the characterization of suppressor factors related to cancer.

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