UC Irvine
UC Irvine Previously Published Works

Title
Soluble suppressor factors elaborated in experimental malignant ascites

Permalink
https://escholarship.org/uc/item/76f870nh

Journal
Cellular Immunology, 86(2)

ISSN
0008-8749

Authors
Wile, AG
Hensen, D
Nahabedian, M
et al.

Publication Date
1984

DOI
10.1016/0008-8749(84)90389-7

License
CC BY 4.0

Peer reviewed

Soluble Suppressor Factors Elaborated in
Experimental Malignant Ascites

ALAN G. WILE,[*,1] DEBORRAH HENSEN,[*] MAURICE NAHABEDIAN,[*]
KENNETH H. IBSEN,[†] AND GALE A. GRANGER[‡]

*Department of Surgery, and †Department of Biological Chemistry, California College of Medicine, and
‡Department of Molecular Biology and Biochemistry, School of Biological Sciences,
University of California, Irvine, California 91717

Soluble suppressor factors in the sera of cancer patients inhibit lectin-stimulated lymphocyte proliferation. These factors, derived from human material, preclude easy corroboration by other investigators. To gain a general understanding of soluble suppressor factors and to avoid the necessary restrictions of human experimentation, an animal model was devised. Sprague–Dawley rats were injected ip with the Walker 256 carcinoma. The resultant ascites proved to be a stable, reproducible source of soluble suppressor factors. Ascites inhibited phytohemagglutinin (PHA)-induced blastogenesis of normal splenocytes by 98%. The possibility of a toxic effect was eliminated by vital staining of splenocytes and by examination in a specific lymphotoxin assay. Suppressor activity persisted after heating at 100°C for 40 min. Extraction by lipid solvents revealed that the bulk of suppressor activity resides in the lipid phase. The active fraction of heat-treated ascites passed through an Amicon PM-10 filter. Thin-layer chromatography revealed the presence of prostaglandins E_2 and $F_{2\alpha}$. Tissue culture supernatants from short-term cultures derived from tumor-bearing animals revealed suppressor activity from thymus, spleen, and liver cultures (97, 91, and 71%, respectively). No suppressor activity was detected in cultures of cancer cells. This study has demonstrated in this animal model that prostaglandins play a major role in suppression of lectin-induced blastogenesis. All suppressor factors appear to be host derived. An understanding of the mechanism of release of these suppressor substances may open new avenues in the immunotherapy of cancer.

INTRODUCTION

Soluble factors which suppress lymphocyte proliferation to lectin stimulation *in vitro* have been demonstrated to be present in the sera of human cancer patients (1–3). However, when these factors have been studied in detail, the physical properties and biologic activity vary among reports. Non-cancer specific, acute phase reactants such as immunoregulatory α-globulin have been found to inhibit lymphocyte proliferation to lectin stimulation *in vitro* and may be responsible for immunosuppression in tumor-bearing patients (4). A substance with a molecular weight of 50,000 has been isolated from the malignant ascites developing in patients with advanced gastric

---

[1] Recipient of ACS Junior Faculty Fellowship and to whom requests for reprints should be addressed: Department of Surgery, University of California Irvine Medical Center, 101 City Drive So., Orange, Calif. 92668.

and ovarian cancers (5, 6). Another factor which suppresses normal human lymphocyte function *in vitro* has been reported to be derived only from tumors (7). These suppressor factors are frequently derived from material obtained from patients in unusual clinical situations which preclude the possibility of easy corroboration by other investigators. Furthermore, it is difficult to separate cancer-related events from the effects of acute and chronic biologic stresses which accompany any human cancer.

To gain a more general understanding of soluble suppressor factors we elected to avoid the necessary restrictions of human experimentation by utilizing an animal model. It was our intent to investigate the elaboration of suppressor factors in a cancer-bearing animal. In this report we describe the initial characterization and partial purification of factors obtained from the ascites of rats injected ip with the Walker 256 carcinoma. These factors inhibit the *in vitro* proliferation of rat spleen cells to phytohemagglutinin (PHA) stimulation.

MATERIALS AND METHODS

Growth and collection of the ascites. Sprague–Dawley rats (Harlan Sprague–Dawley, Madison Wisc.) were injected ip with 1 ml of a fine mince of subcutaneously grown solid Walker 256 carcinoma (Mason Research Institute, Worcester, Mass). This resulted in the production of a malignant ascites. Ten to fourteen days postinnoculation, the abdominal wall became tense due to distention by fluid. The ascites, serum, and tissues were harvested. Ascites was removed by transabdominal needle aspiration from Halothane-anesthetized rats. Blood was obtained by cardiac puncture. The ascites and blood were allowed to stand at 20°C for 1 hr, then centrifuged at 1500 rpm for 10 min, the supernatant serum or ascites was aliquotted and stored at −35°C until assayed for suppressor activity.

Primary tissue culture. Tissue cultures were established using thymus, spleen, liver, and ascites tumor cells obtained from sacrificed tumor-bearing rats. The organs were finely minced with iris scissors in a sterile petri dish containing RPMI 1640 medium (Gibco, Grand Island, N.Y.) and filtered through a sterile fine mesh gauze, to produce a single-cell suspension. The resulting cell suspension was washed three times with PBS. A primary culture was started by adding 10^7 cells/ml to a mixture of RPMI 1640 with 20% FCS. The cell pellet obtained from the centrifugation of crude ascites was similarly resuspended in RPMI 1640 with 20% FCS. This cell pellet consisted of cancer cells with some host derived inflammatory cells. The cultures were incubated at 37°C in a mixed atmosphere of 95% O_2 and 5% CO_2. Tissue culture medium was added as needed. The spent tissue culture medium was collected, centrifuged at 1500 rpm, aliquotted, and stored at −35°C. All cultures were terminated at 96 hr.

Immunosuppressor assay. A proliferation–inhibition assay utilizing normal splenic lymphocytes obtained from healthy Sprague–Dawley rats was employed. The donor animal was anesthetized with Halothane; the spleen was removed and forced through a fine wire screen into a sterile petri dish containing PBS. Red blood cells were lysed by suspending the cells in 1% ammonium chloride. Nonlysed cells were centrifuged at 1500 rpm for 6 min and the supernatant discarded. The cell pellet was resuspended in PBS and washed in similar fashion two times. The cells were counted and the dilution adjusted such that 180 μl of the cell suspension contained 1 × 10^6 cells. The final resuspension was accomplished with RPMI 1640 with 10% fetal calf serum and

containing an appropriate amount of PHA (2.5–5.0 μg/ml; Wellcome Research Labs, Beckenham, England).

The assay was conducted in microtiter test plates (Flow Laboratories, Inc., McLean, Va.), with each sample assayed in sextuplicate. Twenty microliters of the sample to be tested was added to each well. The splenic lymphocyte suspension was added (1×10^6 cells/well) and the plates were incubated at 37°C in an atmosphere 95% O_2–5% CO_2. Maximal response was determined using a control in which the test sample was replaced by 20 μl of pooled normal rat serum. Minimal response was determined using the same control but in the absence of added PHA. Ten microliters of tritiated uridine (50 μCi/ml; Amersham, Arlington Heights, Ill.) was added to the test plates at 24 hr and the cells were harvested 4 hr later. This test monitored early RNA associated events in blastogenesis. In the majority of experiments 10 μl of tritiated thymidine (100 μCi/ml, Amersham) was used to monitor DNA-associated events of blastogenesis. The thymidine was added at 72 hr following initiation of the culture and the cells were harvested 24 hr later.

Isotope uptake was determined by aspirating the cell suspension using an automated multiple-sample harvester (Flow Laboratories, McLean, Va.) which deposited the contents of the wells on a filter paper sheet. The filter paper sheet was dried for 1 hr at 95°C, cut into squares, which were placed in scintillation vials containing 2 ml of scintillation cocktail, and counted on a liquid scintillation counter (Amersham–Searle, DesPlaine, Ill.). The results of the assay for each sample were obtained and a mean and standard deviation were calculated. The percentage of inhibition was calculated by dividing the counts per minute of the experimental sample by counts per minute of the positive control, subtracting the result from one, and multiplying by 100.

Cytotoxicity assay. Cell cultures of lymphotoxin (LT)-sensitive L-929 fibroblasts were established at a density of 100,000 cells/ml in RPMI 1640 with 3% newborn calf serum (NCS, Gibco) and 0.5 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, Mo.). These cultures were incubated overnight at 37°C. Aliquots of test samples were added in triplicate to the cultures. Appropriate controls included RPMI tissue culture medium alone and a tissue culture supernatant known to contain LT. The incubation was continued for an additional 24 hr. The number of viable adherent cells was determined by trypsinizing the monolayer and passing the cell suspension through a Model F Coulter counter (Coulter Electronics, Hialeah, Fla.) by the technique of Spofford *et al.* (8). A significant decrease in the number of viable cells as compared to the control with RPMI alone would indicate the presence of LT.

Heat stability and molecular seiving. Crude ascites was heated to 100°C for 40 min and centrifuged at 30,000 rpm for 30 min. The supernatant was then assayed for suppressor activity. Heat-treated ascites was then subjected to molecular filtration using Amicon PM-30 and PM-10 membranes under a positive pressure of 30 pounds per square inch of nitrogen.

Chloroform:methanol extraction. The heat-treated ascities was extracted twice with 3 vol of a 3:1 (v:v) mixture of chloroform and methanol. The combined extract was dried under streaming nitrogen at room temperature and the slight residual aqueous component was completely dried in a vacuum. The residue was dissolved in a minimal volume of ethylacetate:acetic acid:methanol:2,2,4-trimethylpentane:water (110:30:35:10:100), the solvent AII of Green and Samuelsson (9). The sample was then subjected to silica gel thin-layer chromatography as described by Willis (10). Prostaglandins present were tentatively identified by their R_f values, using known

prostaglandins of the E and F series as standard markers. When utilized in the pro-
liferation inhibition assay the dried extracts were dissolved in a volume of RPMI
equivalent to the original volume of the extracted sample.

RESULTS

The addition of 20 μl of ascites fluid to 180 μl of PHA-stimulated lymphocytes
reduced the rate of uptake of tritiated thymidine to or below the level demonstrated
by nonstimulated cells. Progressive dilution of the ascites with RPMI also caused a
loss of potency, indicating that an additive factor is present. (Table 1). Cell counts
conducted on lymphocytes incubated with ascites fluid under similar conditions showed
no loss in cell number or in cells taking up trypan blue. This demonstrated that the
inhibitory effect was probably not due to cytotoxicity. This conclusion was confirmed
using the lymphotoxin assay summarized in Table 2. The addition of as much as
500 μl of undiluted ascites fluid did not reduce the number of adherent lymphotoxin-
sensitive cells, indicating no loss of viability. A portion of the suppressor activity is
heat stable. Immersion of the ascites fluid in boiling water for 40 min only reduced
its potency slightly (Table 1).

Heat-treated ascites was extracted with chloroform:methanol as described. The
majority of the activity extracts into the nonpolar phase (Table 3). Thin-layer chro-
matography indicated that such extracts contained prostaglandins E_2 and $F_{2\alpha}$ and
other unidentified substances. The active substance in the heat-treated ascites also
passed through Amicon PM-30 or PM-10 ultrafilters (Table 4).

To determine the possible source of the active material, tumor, spleen, thymus,
and liver cells were grown in short-term cultures as described. Aliquots of the media
were then examined in the thymidine assay. The results summarized in Table 5 show
that the conditioned media from the tumor cells actually stimulated thymidine uptake
while the media from the cultures of spleen, thymus, and liver cells suspensions
inhibited blastogenesis.

The data summarized in Table 6 show that the less time-consuming uridine assay
gives results equivalent to those of the thymidine assay.

DISCUSSION

We found that ascites obtained from Sprague–Dawley rats injected ip with a Walker
256 carcinoma exerts a powerful suppressor effect *in vitro* upon proliferation of rat
splenocytes to mitogen stimulation. Serial dilution of the ascites resulted in the loss

TABLE 1

Inhibition of Mitogen-Induced Lymphocyte Blastogenesis by Malignant Ascites

	Crude ascites		Heat-treated ascites	
	cpm	% Inhibition	cpm	% Inhibition
NRS + PHA	28,500 ± 4967	—	—	—
100% Ascites	511 ± 247	98	3,048 ± 1055	89
10% Ascites	19,532 ± 2535	31	23,368 ± 1652	18
1% Ascites	22,164 ± 5774	22	19,299 ±	32
0.1% Ascites	22,624 ± 3059	21	16,521 ± 2897	42
NRS − PHA	1452 ± 332			

TABLE 2

L-929 Cell Viability as an Indicator of Lack of Lymphotoxin Activity

	Sample volume (μl)			
Sample	20	100	200	500
Ascites	—	103, 495 $\pm$ 5158[a]	106, 795 $\pm$ 4021	100, 133 $\pm$ 5061
LT[b]	48, 137 $\pm$ 2882	—	—	—
Control	101, 769 $\pm$ 6130	108, 553 $\pm$ 4316	103, 754 $\pm$ 3888	99, 417 $\pm$ 4960

[a] Cell numbers are expressed cells per milliliter.
[b] Standard LT-containing supernatant is known to contain approximately 50 units/ml.

of the suppressor effect, eliminating the possibility of lack of enhancing factor and demonstrating the presence of suppressor factors. In other words, inhibition of mitogen-induced blastogenesis could be explained by the absence of factors necessary to support blastogenesis or by the elaboration of new factors exerting a biologic suppressor effect. In the first case, lack of essential factors, could be eliminated, since dilution of ascites would be expected to result in sustained inhibition of blastogenesis. The observation that dilution of ascites returns blastogenesis to levels near those of the positive controls confirms the presence of suppressor factors, whose effects were decreased upon dilution. This early work gave impetus to further efforts to characterize these factors and to elucidate their site of elaboration. The observation that the uridine assay yielded results comparable to thymidine assay corroborates the findings of other investigators (11, 12) and supports the use of the uridine assay for the rapid screening of suppressor activity. Both vital staining and lack of LT activity proved that the suppression observed was not the result of a cytotoxic effect upon the responding lymphocytes.

Studies were conducted to establish the molecular characteristics of material(s) with suppressor activity. Activity persisted after heating the ascites to 100°C for 40 min. Indeed, this provided an excellent means of removing protein from the ascites while preserving suppressor activity. We also found that active material passed through both Amicon PM-30 and PM-10 filters without loss of activity, indicating that we were dealing with material of molecular weight less than 10,000.

Next we extracted active preparations with chloroform:methanol. Biologic activity was found to be extractable in nonpolar lipid solvents. This material was chromatographed revealing the presence of prostaglandins E_2 and $F_{2\alpha}$. Prostaglandins E_2 is known to inhibit lymphocyte function (13) and has been shown to be secreted by

TABLE 3

The Effect of Extraction by Lipid Solvents on Lymphocyte Blastogenesis

	cpm	% Inhibition
Aqueous phase	51,886	51
Lipid phase	6,982	94
Crude ascites	4,292	96
NRS with PHA	106,344	
NRS without PHA	4,871	

TABLE 4

The Effect of Molecular Filtration upon the Suppressor Effect of Heat-Treated Ascites

	cpm	% Inhibition
PM-30 retained	571 ± 89	99.4
PM-30 filtered	1220 ± 309	98.8
PM-10 retained	3053 ± 363	97.0
PM-10 filtered	1233 ± 430	98.8
Heat-treated ascites	402 ± 122	99.6
NRS with PHA	122,777 ± 23,250	
NRS without PHA	2,371 ± 535	

suppressor cells during stressful situations (14). The presence of other lipid-soluble suppressor substances, such as slow reacting substances, was not measured.

The cultures prepared from tissue obtained from tumor-bearing animals produced evidence that immunosuppressive factors are produced by the host cells rather than by the tumor. Significant immunosuppressive activity was detected in the thymic, splenic, and hepatic cell cultures, but not in cultures of tumor cells. Other workers have shown that these cell suspensions contain populations of suppressor cells capable of secreting prostaglandins (15). There are a host of protein and lipoprotein substances in serum capable of suppressing mitogen-induced lymphocyte blastogenesis *in vitro* (16). These substances are synthesized in the liver and may account for the presence of suppressor activity in the tissue culture supernatant of the hepatic cell cultures. This lack of suppressor activity arising from tumor cell cultures suggests that all suppressor activity observed in this model is derived from multiple organ host and tissue sites and is not derived from tumor tissue.

These findings then lead to the question of whether or not unique suppressor factors are elaborated in the presence of cancer cells. If unique cancer-related suppressor factors exist, then their utility in the immunodiagnosis and immunotherapy of human cancer would be great. If these factors are not unique to cancer then the problem of clinical immunosuppression as a result of elaboration of suppressor factors could be viewed as a final common pathway in a variety of ills. Indeed, this seems to be the case as suppressor substances have appeared in malignant ascites as well as ascites produced by serious but non-cancer-related states (17). Major trauma (18), surgery (19), and serious medical illnesses such as multiple sclerosis (11) are all associated with suppressor factors.

TABLE 5

Effect of Conditional Tissue Culture Supernatants on Lymphocyte Blastogenesis

Tissue culture supernatant	cpm	% Inhibition
Ascites cells	17,561	−20
Spleen	1,354	91
Thymus	447	97
Liver	4,219	71
RPMI with PHA	14,563	
RPMI without PHA	982	

TABLE 6

Demonstration of Inhibition of Lymphocyte Blastogenesis by the Uridine Assay

Diluted with RPMI	cpm	% Inhibition
NRS + PHA	1996 ± 120	—
100% Ascites	378 ± 101	81
50%	647 ± 174	60
33%	490 ± 245	76
17%	1338 ± 577	33
9%	2243 ± 320	12
NRS − PHA	1361 ± 129	—

The area of soluble suppressor factors appears more complex than initially perceived. Although prostaglandins were detected in this model, there is strong suspicion that protein-like suppressor substances with molecular weights greater than 10,000 are also present. This speculation arises from preliminary work demonstrating loss of a portion of suppressor activity after heat treatment and a persistance of suppressor activity after dialysis which would be expected to remove prostaglandins. Suppressor activity is detected in material adherent in ion-exchange chromatography, again suggesting the presence of protein-like material. Further investigation will be required to elucidate the precise nature of what appears to be multiple suppressor factors elaborated from multiple sites in a tumor-bearing host.

ACKNOWLEDGMENTS

REFERENCES

1. Rangel, D. M., Golub, S. H., and Morton, D. L., Surgery 82, 224, 1977.
2. Guillano, A. E., Rangel, D., Golub, S. H., Holmes, E. C., and Morton, D. L., Cancer 43, 917, 1979.
3. Sinclair, T., Ezdnili E. Z., Boonlayangoor, P., and Wasser, L. P., Cancer 51, 238, 1983.
4. Mannick, J. A., and Schmid, K., Transplantation 5, 1231, 1967.
5. Tamura, K., Shibata, Y., Matsuda, Y., and Ishida, N., Cancer Res. 41, 3244, 1981.
6. Oh, S., and Moolten, F. L., Eur. J. Immunol. 11, 780, 1981.
7. Roth, J. A., Grimm, E. A., Gupta, R. K., and Ames, R. S., J. Immunol. 128, 1955, 1982.
8. Spofford, B., Daynes, R. A., and Granger, G. A., J. Immunol. 112, 2111, 1974.
9. Green, S., and Samuelsson, B., J. Lipid Res. 5, 117, 1964.
10. Willis, A. L., Brit. J. Pharmacol. 400, 583, 1970.
11. Anderson, J. A., Sweet, P., and Armentrout, S. A., J. Clin. Lab. Immunol. 2, 285, 1979.
12. Cooper, H. L., In "Effects of Drugs on the Cell Cycle" (A. Zimmerman, G. Padilla, and I. Cameron, Eds.), p. 137. Academic Press, New York, 1973.
13. Goodwin, J. S., Messner, R. P., and Peake, G. T., J. Clin. Invest. 62, 753, 1978.
14. Novogrodsky, A., Rubin, A. L., and Stenzel, K. H., J. Immunol. 122, 1, 1979.
15. Goodwin, J. S., Messner, R. P., Bankhurst, A. D., Peake, G. T., Saiki, J. H., and Williams, R. C., Jr., New Engl. J. Med.. 297, 963, 1977.
16. Chisari, F. V., J. Immunol. 119, 2129, 1978.
17. Badger, A. M., Beuhler, R. J., and Cooperband, S. R., Cancer Res. 38, 3365, 1978.
18. Constantian, M. D., Menzoian, J. O., Nimberg, R. B., et al., Ann Surg. 186, 73, 1978.
19. McLoughlin G. A., Wu, A. V. O., Saporschetz, I., et al., Ann Surg. 190, 297, 1979.