Soluble Suppressor Factor from the Spleens of Tumor-bearing Mice

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ABSTRACT

Spleen cells from tumor-bearing mice when cultured for 3 to 5 days released a soluble factor into the media that suppressed the stimulation of lymph node and spleen cells by tumor antigen or mitogens. Spleens from mice bearing MC43 tumors for 14 days were capable of producing suppressor factor in vitro, while those from mice bearing the tumor for 10 days or less failed to do so. Lymph node cells from the same animals did not produce suppressor factor in vitro. The suppressor factor was produced by a nonadherent cell population, was heat stable, was lost on dialysis, and did not appear to be tumor antigen or thymidine.

INTRODUCTION

In tumor-bearing individuals several immunological functions have been shown to be impaired. It is not clear whether the tumor grows progressively because of blocking factors, blocking antigen, antibodies, or antigen-antibody complexes (2); because of a defect in the host's immune response, either cellular or humoral (11, 15); or because of suppressor elements, the source of which could be the lymphoid cells (7, 8), macrophages (6, 10), or possibly the tumor itself, that actively antagonize the response of the host to tumors.

Previous work from this laboratory has shown that in vitro lymphocyte stimulation by mitomycin C-blocked tumor cells can be used to measure tumor-specific immune responses (3–5). This work describes a soluble suppressor factor released from the spleens of mice bearing methylcholanthrene-induced tumors. The factor that was released in vitro into the media of cultured spleen cells suppressed the DNA-synthetic response of tumor-immune cells when they were stimulated with mitomycin C-blocked syngeneic tumor cells or nonspecific mitogens. Lymph node cells from the same animals failed to produce the suppressor factor. Production of suppressor factor by the spleen was directly related to the duration of tumor growth.

MATERIALS AND METHODS

Mice. C3H/HeJ females between 8 and 16 weeks old were used for all experiments (The Jackson Laboratory, Bar Harbor, Maine). These were kept in air-conditioned animal rooms, fed Purina laboratory chow, and given water ad libitum.

Tumors. The MC43 fibrosarcoma was originally induced in 1970 in a female C3H/HeJ mouse by the injection of 0.5 mg 3-methylcholanthrene dissolved in 0.1 ml trietanol. The tumor is passed by s.c. injection or banked in liquid nitrogen. Specific tumor immunogenicity was demonstrated in syngeneic mice in this laboratory. Mice given s.c. injections of 5 × 10⁶ tumor cells develop microscopic metastasis in regional lymph nodes, spleen, and lungs in 14 days. A 100% mortality caused by tumor growth occurs 4 to 6 weeks after the tumor injection, depending on the number of cells injected.

We prepared tumor cell suspensions by aseptically removing a s.c. tumor, mincing it, and treating it with 0.25% trypsin at room temperature for 60 min while the mixture was stirred continuously.

DNA synthesis in these tumor cells was blocked by treatment with mitomycin C (Sigma Chemical Co., St. Louis, Mo.). Tumor cells at a concentration of 10² per ml were incubated with 25 μg mitomycin C per ml of tumor cell suspensions at 37° for 60 min. Following this the cells were washed 4 times with medium, and viability was determined by trypan blue exclusion.

Immunization. Tumor cells (5 × 10⁹) were injected into a hind limb of the mice, and the tumor-bearing limb was amputated 6 to 7 days later. The mice were used 2 to 3 weeks after the amputation.

Lymph Node and Spleen Cell Preparations. The mice were sacrificed with ether anesthesia. The superficial and deep cervical, axillary, brachial, inguinal, lumbar, caudal, and mesenteric lymph nodes were removed aseptically and pooled together in RPMI Medium 1640 (Microbiological Associates, Inc., Bethesda, Md.) containing 10 mm N-hydroxyethylpiperazine-N'-ethanesulfonic acid buffer (Microbiological Associates) with penicillin and streptomycin (100 mg of each per ml; Microbiological Associates) at 4°. The spleens were treated in the same manner. We prepared single-cell suspensions by gently pressing minced lymph nodes and spleens through a 60 mesh stainless steel screen. The cell suspensions were placed in centrifuge tubes and centrifuged at 180 × g for 10 min, and the supernatant was discarded. RBC were lysed with Tris-buffered ammonium chloride. The lymphoid cells were then centrifuged and washed twice before being resuspended in RPMI Medium 1640 containing 10 mm N-hydroxyethylpiperazine-N'-ethanesulfonic acid buffer, penicillin, streptomycin, and 5% pooled human serum. The cells were counted in a hemocytometer chamber, and viability was determined by trypan blue exclusion.

Materials from Lymphocyte Cultures. Lymph node and
spleen cell suspensions were prepared as described previously. Ten × 10⁶ cells/ml were cultured in 100- × 15-mm Lux No. 5211-5276 plastic dishes (Lux Scientific Corp., Newburg Park, Calif.) and incubated for 3 to 5 days (unless otherwise specified) in a humidified 5% CO₂-air atmosphere at 37°. For harvesting of the media, the culture fluids were spun at 1200 rpm for 10 min to settle out all the cells, and supernatants were removed and stored at -70°.

In Vitro Lymphocyte Stimulation Assay. Cultures were set up in quadruplicate in Falcon Microtest II No. 3040 microtiter plates (Falcon Plastics, Oxnard, Calif.). The appropriate cell suspensions and media from lymphocyte cultures were dispensed with a repeating Hamilton syringe. One million responder lymph node cells or 5 × 10⁶ spleen cells were incubated with 100 µl (0.1 ml) of media from lymphocyte cultures and 10⁶ mitomycin-blocked stimulator tumor cells per well (0.2 ml medium) in a humidified 5% CO₂-air atmosphere at 37°. Cultures were incubated from 1 to 6 days. PHA-M (Difco Laboratories, Detroit, Mich.), Con A (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), pokeweed mitogen (Grand Island Biological Co., Grand Island, N. Y.), or RPMI medium alone were added in maximally stimulatory doses to parallel sets of lymphocyte cultures which were then incubated for 48 to 72 hr.

Twenty-four hr prior to harvesting, the cultures were labeled with 2 µCi of tritiated thymidine (specific activity, 1.9 Ci/mmol; Schwarz/Mann, Orangeburg, N. Y.) in 0.05 ml of serum-free RPMI Medium 1640. The cultures were harvested on glass fiber filters with a semiautomated multiple sampling apparatus. The samples were dried, and the incorporation of tritiated thymidine was determined by liquid scintillation counting. The results in this study are expressed as percentage of stimulation.

% stimulation = 100 ×

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\frac{\text{cpm (responders + media from tumor-bearing lymphocyte cultures)}}{\text{cpm (responders + media from normal lymphocyte cultures)}}
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RESULTS

Release of an Immunosuppressor Factor In the Media by Different Lymphocyte Populations Growing In Vitro. Lymph nodes and spleen cells from normal and tumor-bearing mice (mice that had tumors for at least 19 days) were cultured in vitro for 3 to 5 days. The viability of lymphoid cells when the media were harvested was uniformly high, ranging from 85 to 88% by trypan blue exclusion. The harvested media were tested for their activity on tumor-immune lymph node and spleen cells. Media from “tumor-bearing” spleen cells were highly suppressive when compared to media from normal spleen cells, showing a 58% suppression of the response of immune spleen responder cells stimulated by mitomycin C-blocked tumor cells (Chart 1). Tumor-immune lymph node responder cells were not as well suppressed as were spleen responder cells. Media from tumor-bearing lymph nodes did not suppress the stimulation of immune spleen or lymph node cells.

Activity of Media from Cultured Tumor Cells. For determination of whether a suppressor factor was being produced by the tumor cells themselves and carried by the spleen cells, MC43 tumor cells were cultured alone for 5 days in vitro. The activity of the supernatant was tested on tumor-immune lymph node and spleen cells that were stimulated with mitomycin C-blocked tumor cells. The thymidine uptake was higher in lymphoid cells stimulated by tumor cells in the presence of tumor cell media, indicating the presence of a stimulating factor in the media, possibly antigen shed from the cells during culture (Chart 2). Clearly, there was no suppression by media from tumor cells grown in culture.

Suppression of Response to Mitogens. In addition to stimulation by syngeneic tumor cells, responsive lymphocytes were also stimulated by the mitogens PHA, Con A, and pokeweed mitogen. Stimulation of tumor-immune spleen cells by each of these mitogens was inhibited when they were cultured in the presence of media from cultures of “tumor-bearing” spleen cells (Chart 3). Media from cultured lymph node cells did not elicit as much suppression when tested on parallel sets of responding cells.

Chart 1. The effect of media from cultures of lymph node cells (LC) or spleen cells (SC) of tumor-bearing mice on lymphocyte stimulation by tumor cells. Responder cells were immune lymph node or spleen cells. Results are expressed as percentage of stimulation of responder cells in control media from cultures of normal spleen cells. Uptakes of [³H]thymidine by immune lymph node responder cells were 7,980 ± 437 and 7,310 ± 492, and by immune spleen cells they were 13,057 ± 687 and 12,819 ± 184 when stimulated with tumor cells in the control media.

Chart 2. The effect of media from cultures of tumor cells on stimulation of immune spleen cells (SC) by tumor. Controls are media from spleen cells of normal and tumor-bearing mice.
The Effect of Media from Cultures of Tumor-bearing Spleen Cells on the Stimulation of Normal Lymphoid Cells. Suppression of immune responder cells to nonspecific mitogens and specific tumor antigen was demonstrated in Charts 1 and 3. In this experiment spleen cells from normal mice were stimulated with mitogens and tumor cells in the presence of media from cultures of tumor-bearing spleen cells and normal spleen cells. A 40 to 65% suppression of normal spleen cells to stimulation by mitogens and tumor cells suggested that prior sensitization of responder cells is not required (Chart 4).

Kinetics of Release of Suppressor Factor. Spleen cells from tumor-bearing and normal mice were cultured for different periods of time, from 0 to 7 days, and the media were harvested and then stored in the frozen state. Complete sets of samples were tested at the same time for their capacity to inhibit stimulation of tumor-immune spleen cells by tumor cells and mitogens. Suppressor factor appeared in the media on the third day of culture but did not increase in concentration after that time (Chart 5).

The Effect of Tumor Growth on the Production of Suppressor Factor. For determination of the stage at which the tumor induces the production of suppressor factor, spleens were removed periodically from 1 day to 4 weeks after tumor injection. Eight to 14 days after tumor injection, spleen cells began to release suppressor factor and continued to do so through the fourth week. During this time the spleens grew larger, and there was a general increase in cell number paralleling the tumor growth (Chart 6).

Media from Cultured Regional, Mesenteric, and Distal Lymph Nodes of Tumor-bearing Mice. Previous work from this laboratory showed that the total cell content of regional nodes and spleen increased following tumor injection, with the relative percentage of macrophages increasing more than other cells. Regional, distal, and mesenteric nodes and spleen cells from tumor-bearing mice were cultured for 4 days. The media from regional and mesenteric nodes increased the DNA-synthetic capacity of responding tumor-immune spleen cells upon stimulation with PHA or Con A, compared to media from distal lymph nodes, which had no effect. Only media from spleen cells were suppressive (Chart 7).

Media from Adherent and Nonadherent Subpopulations of Spleen Cells. Normal and tumor-bearing spleen cells were incubated in plastic Petri dishes for 2 hr at 37° in serum-free media. The nonadherent cells were removed at the end of incubation and were further separated with carbonyl iron (Technicon Instruments Co., Tarrytown, N. Y.) to ensure removal of phagocytic macrophages. Nonphagocytic cells were then recovered, washed, plated in serum-containing media at 10^7 cells/ml, and cultured for 4 days. Samples from nonadherent cells tested by polystyrene bead ingestion and Wright's staining showed a decline of macrophages of from 15 to 20% to 0 to 0.5%. The adherent cells were similarly cultured for 4 days in serum-containing media. The harvested media were tested for activity on stimulation of tumor-immune spleen cells. Media from adherent and nonadherent normal spleen cells were used as
controls. Only the nonadherent cells from tumor-bearing spleens released the suppressor factor (Chart 8). The tumor-immune spleen cells incubated in media from adherent cells showed increased incorporation of tritiated thymidine upon stimulation with PHA or tumor cells. The suppressor factor in our system appears to be released by nonadherent cells without the addition of macrophages.

The Effect of Mitomycin C on Production of Suppressor Factor. Tumor-bearing spleen cells at a concentration of 10^7/ml were incubated with 25 μg mitomycin C per ml of cell suspension at 37° for 30 min. At the end of this incubation, the cells were washed 4 times with media and cultured for harvesting of media. The media that were harvested after 4 days were tested for their suppressive activity on tumor-immune spleen cells. Untreated normal spleen and tumor-bearing spleen cell media were used as controls (Chart 9). Media from mitomycin C treated spleen cells did not suppress the DNA-synthetic response of tumor-immune spleen cells, showing that DNA synthesis and subsequent transcription steps were probably necessary for the release of suppressor factor.

Effects of Dialysis on the Suppressor Factor. Fifteen ml of media from 4-day cultures of tumor-bearing spleen cells were dialyzed in the cold for 24 hr with 2 changes of 500 ml of RPMI 1640. The dialyzed media from normal and tumor-bearing spleen cells were then tested for their suppressive activity on stimulated tumor-immune spleen cells. All suppressive activity was lost as a result of dialysis (Chart 10). This could be explained by 2 processes; either the suppressor factor was a small molecule and as a result was lost during dialysis or the suppressor factor could have been inactivated by proteases, the source of which could be the culture media or the dialysis bags. The incorporation of thymidine was increased in cells cultured in the presence of the dialyzed product.

Stability of Suppressor Factor to Heat Inactivation. For evaluation of the lability of suppressor factor, 10 to 15 ml of media from cultures of tumor-bearing spleen cells were aliquoted in centrifuge tubes and incubated at -70, -20, 4, 37, 56, or 80° for 1 hr. The samples were then tested for their capacity to suppress the response of immune spleen cells to PHA and mitomycin-blocked tumor cells. The media from cultures of tumor-bearing spleen cells, after heat inactivation at 80° for 1 hr, were as suppressive as were media incubated at lower temperatures, suggesting that the suppressor factor is heat resistant (Chart 11).

The Concentration of Suppressor Factor in the Media from Spleen Cultures of Tumor-bearing Mice. Spleen cells were stimulated with mitogens in the presence of 2-fold dilutions of media from spleen cultures of tumor-bearing mice or of normal mice. Chart 12 shows that media from spleen cultures of tumor-bearing mice lost their suppressive activity when diluted beyond 1:8, suggesting that suppressor factor was present in the media at a relatively low concentration.

Competitive Inhibition and Metabolic Suppression by Thymidine. For evaluation of the relative roles of competitive inhibition and metabolic suppression by thymidine, spleen cells were stimulated with Con A in the presence of...
Chart 11. Stability of suppressor factor to heat inactivation. Responder spleen cells were stimulated with mitogen or tumor cells in the presence of media from tumor-bearing or normal spleen cells. The media was exposed to different temperatures for 1 hr.

Chart 12. The concentration of suppressor factor in the media from spleen cultures of tumor-bearing mice. Spleen cells were stimulated with mitogens in the presence of 2-fold dilutions of media from cultures of spleen cells from tumor-bearing mice or normal mice. The percentage of stimulation was calculated by dividing cpm from cultures in media containing suppressor factor by that from cell cultures in media from spleens of normal mice. PWM, pokeweed mitogen.

constant amounts of [3H]thymidine, [3H]uridine, [3H]leucine, or [3H]proline but with the addition of increasing concentrations of cold thymidine. Chart 13 shows the effect of various concentrations of cold thymidine on the incorporation of labeled thymidine and other precursors. The direct effect of competition between cold and labeled thymidine is obvious from the steep thymidine curve, with an apparent 50% inhibition of [3H]thymidine uptake by the presence of 0.02 mM cold thymidine. True metabolic inhibition of cell stimulation, shown by inhibition of uptake of the other precursors, was also brought about by cold thymidine, but only at a much higher concentration. About 10 mM thymidine was required to cause 50% inhibition of the uptake of leucine, proline, or uridine.

The Effect of Suppressor Factor on DNA, RNA, and Protein Synthesis of Spleen Cells. For determination of the effect of suppressor factor on aspects of cell metabolism other than thymidine uptake, spleen cells stimulated with Con A in the presence of media from tumor-bearing spleen cultures were labeled with [3H]thymidine, [3H]uridine, [3H]leucine, or [3H]proline. Suppression of 50% or more of the uptake of all of these precursors indicates that syntheses of DNA, RNA, and protein were equally suppressed (Chart 14).

Comparison of Suppressor Factor with Cold Thymidine. In a study of the possibility that spleen cell suppressor factor may be thymidine released by the cells and acting through dilution of the labeled thymidine, spleen cells were stimulated with Con A in the presence of 2.0 mM cold thymidine or media containing suppressor factor from spleen cells of tumor-bearing mice. After 48 hr of incubation, 1 set of spleen cell cultures from each group was washed 3 times with RPMI to remove the added thymidine or suppressor factor, and the cells were then labeled with [3H]thymidine. The response of spleen cells to Con A stimulation in the continuous presence of 2.0 mM cold thymidine was 20% of that of spleen cells cultured in the absence of cold thymidine (Chart 15). However, the uptake

Chart 13. Competitive inhibition and metabolic suppression by thymidine. Spleen cells stimulated by Con A in the presence of constant amounts of [3H]thymidine (Δ), [3H]uridine (Δ), [3H]leucine (Φ), or [3H]proline (O) plus increasing concentrations of cold thymidine.

Chart 14. Effect of suppressor factor on DNA, RNA, and protein synthesis of stimulated spleen cells. Spleen cells stimulated by Con A in the presence of media with suppressor factor and labeled with [3H]thymidine, [3H]uridine, [3H]leucine, or [3H]proline, showing total metabolic suppression.

Chart 15. Comparison of suppressor factor with thymidine in culture. Spleen cells were stimulated with Con A in the presence of 2.0 mM cold thymidine or media from cultures of spleen from tumor-bearing mice. After 48 hr of incubation, part of each set of cultures was washed 3 times with RPMI before addition of [3H]thymidine (Δ), while the other part was exposed to [3H]thymidine in the continuing presence of cold thymidine or suppressor factor (Φ). TBSC-MEDIA, media from spleens of tumor-bearing mice.
of [\textsuperscript{3}H]thymidine following Con A stimulation was completely normal if the cold thymidine was removed by washing prior to exposure to labeled thymidine, indicating that there was no intracellular storage of cold thymidine during incubation and no continuing suppression of cell division. The response of spleen cells to Con A stimulation in the continuous presence of media from spleen cultures of tumor-bearing mice was also significantly suppressed, but this suppression was not reversed when cells were washed prior to exposure to labeled thymidine. Persistence of suppression following removal of the suppressive media from spleen cultures of tumor-bearing mice indicates (a) that the suppression demonstrated was not a result of simple dilution by cold thymidine in the media and (b) that the suppression caused by suppressor factor occurred during the incubation period prior to labeling and lasted into the period of labeling.

**The Effect of Suppressor Factor on Blastogenesis.** For this study lymph node cells were used as responders because spleen cells, with their greater proportion of macrophages and monocytes, had a high level of spontaneous transformation in culture. Lymph node cells from normal mice cultured in growth media (RPMI) with 5% pooled human sera were stimulated with Con A in the presence of media from spleen cultures of tumor-bearing or normal mice. Control cells were grown in growth media alone in the absence of Con A or media from spleen cultures. After 48 hr of incubation, cell smears made by cytocentrifugation were immersed in glycerol:phosphate-buffered saline (1:1) or stained with Giemsa-Wright’s stain, and the percentage of blast lymphocytes was determined (Chart 16). The spontaneous background level of blast transformation of about 18% increased to almost 50% following stimulation with Con A in the presence of media from cultures of normal spleen cells. This was reduced to 32% by media from spleen cells of tumor-bearing mice.

![Chart 16](image)

**DISCUSSION**

Media from the cultures of spleen cells from tumor-bearing mice were shown to contain suppressor factors that reduced the incorporation of [\textsuperscript{3}H]thymidine by stimulated tumor-immune lymphocytes. This was equally effective when stimulation was by mitomycin C-blocked syngeneic tumor cells or nonspecific mitogens. Media from normal lymph node and spleen cells and tumor-bearing lymph node cells did not suppress the incorporation of [\textsuperscript{3}H]thymidine to the same level as did media from “tumor-bearing” spleen cells.

It has been known for some time that media from cultured lymphoid cells can inhibit some mitogen-induced responses of T-cells. As reported by Adler et al. (1) with Smith et al. (16), supernatant fluids from a human lymphoblastoid cell line inhibited DNA, RNA, and protein synthesis in human peripheral blood lymphocytes stimulated by PHA. Hersh et al. (8, 9) reported a factor produced by cultured human lymphoblasts that inhibited the response of lymphocytes to mitogens when measured by the incorporation of [\textsuperscript{3}H]thymidine. Production of inhibitory factor was related to cell density. Both of these factors were identified to be nondialyzable macromolecules, although they differed in their conditions of production.

Significant tumor growth in vivo was required for production of the factor by the spleen cells. The spleen cells from mice bearing tumors for 14 days or more were capable of producing the suppressor factor in vitro, while those from mice bearing the tumor for 10 days or less failed to do so. Prolonged stimulation with tumor antigen in vivo appears to be required to develop the population of cells that produce this material.

A macrophage factor released from cocultures of peritoneal exudate macrophages and spleen cells or thymocytes has been reported by Opitz et al. (13, 14). This factor inhibited [\textsuperscript{3}H]thymidine incorporation but not proliferation of mitogen-stimulated lymphocytes. The factor was subsequently shown to be thymidine released during phagocytosis of dead lymphocytes by macrophages. The suppressor factor reported in this work is released without the exogenous addition of macrophages. Indeed, the nonadherent population of spleens provided stronger suppressor activity than did the adherent cells. Previous work from this laboratory has shown that the total cell content of regional nodes and spleens increased following tumor injection with the relative percentage of macrophages increasing more than that of other cells. However, media from regional lymph nodes increased considerably the incorporation of [\textsuperscript{3}H]thymidine into the responding cells, when compared to media from spleen cells of the same tumor-bearing mice. Media from mitomycin C-treated tumor-bearing spleen cells did not elicit any suppression, indicating that DNA synthesis and subsequent transcriptions steps are probably necessary for production of suppressor factor.

Additional studies were carried out to relate the suppressor factor to thymidine. A comparison between the amount of cold thymidine required to give simple competitive inhibition for the uptake of labeled thymidine and that to “shut down” the target cells, as reflected by reduced uptake of other precursors, showed that vastly greater (500-fold)
C. Subramanian et al.

amounts of thymidine were required for total metabolic inhibition. It was also found that metabolic inhibition by cold thymidine is very transient, essentially requiring its continuous presence. In contrast suppressor factor induced a more lasting inhibition of cell metabolism. Finally, suppressor factor inhibited blastogenesis of lymph node cells, although not to the same extent measured by thymidine uptake. All of these facts suggest that the suppressor factor released by spleen cells in culture is not thymidine.

The suppressor factor was lost on dialysis. It could be a low-molecular-weight substance that escaped in dialysis or it could be larger molecular that was inactivated by proteases (the source of which could have been the media from spleen cell cultures). Nelson et al. (12) reported a partly dialyzable factor released from nonadherent cells that inhibited the DNA-synthetic responses of cultured mouse spleen cells to PHA and Con A. Pertinent to our work is another factor reported by Treves et al. (20) and isolated from cultured tumor-bearing spleen cells that enhanced tumor growth. This was a product of T-lymphocytes and suppressed the protective immune response of the host. Using a disseminated tumor such as leukemia, Stiller and Cerny (17) concluded that the in vitro antibody response was suppressed by direct cell contact and not by an extracellular cell product. Unlike this response mitogen-induced lymphocyte proliferation was suppressed by supernatants from both normal and leukemic spleen cell cultures. However, the suppression was abrogated after dialysis of the supernatant preparations.

There is also evidence of suppressor factors in serum. Recent reports showed that serum from patients with intracranial tumors and several other tumors inhibited the response of normal lymphocytes to PHA (19). Studies from our laboratory, to be reported later, have shown the presence of a suppressor factor in the serum of mice with MC43 tumor for 21 days or more.

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