PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **184**, 172–178 (1987)

Purification of a Syngeneic Cross-Reactive Autotumorolytic Factor (42463)

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Abstract. An autotumorolytic fraction (ATF) derived from sera of mice bearing spontaneous mammary adenocarcinoma has been purified by sequential chromatography on Sephacryl 200 SF, DEAE Sephacel, hydroxylapatite, and Sephacryl 200 SF. At each stage of the purification, tumorolytic activity was assessed by the ability of the active material to induce lysis of a 2-week-old mammary tumor, and verified by histopathological analysis. Active fractions obtained during the stages of purification were capable of inducing lysis of tumors in both the original donor mice and the syngeneic tumor-bearing mice. Lytic activity was specific only for tumor tissue. This syngeneic crossreactivity of partially purified materials permitted pooling of serum samples for purification purposes. The resultant final preparation of ATF is an homogenous protein fraction, as verified by gradient SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and high-performance liquid chromatography. The molecular weight of active ATF is approximately 50,000 Da, as determined by gradient SDS–PAGE.

It has been reported previously that a serum-derived fraction from mammary tumor-bearing mice possesses selective autologous tumorolytic activity (1, 2). The injection of this material (autotumorolytic factor (ATF)) induces a significant reduction of the palpable mass of mammary adenocarcinoma within 48–72 hr. This crude autolytic tumor preparation has been active only when reinjected into the original tumor-bearing mouse from which the serum was derived.

Injection of the initial preparation (SF III) resulted in a reproducible set of histopathological changes within the tumor mass (2). The induced tumorolysis began with the infiltration of the intraalveolar connective tissue by macrophages, lymphocytes, and plasma cells. Infiltration was followed by breakdown of the connective tissue, the appearance of debris and hemorrhagic foci, and the formation of cystic "holes" within the tumor tissue. Normally, young untreated tumors rarely showed massive degenerative changes. Yet older tumors (30–60 days) can display some breakdown and necrosis. The induced tumorolysis appeared to be a quantitative enhancement of the abortive attempts at self destruction seen in most tumors of the C3H(T/F) strain and reported (3) in the type B mammary adenocarcinoma of the BALB/cfRIII mouse. Similar destructive changes (sclerotic and cystic lobules) also have been observed, though to a minor extent, in the human cancerous breast after radical mastectomy (4). Comparable histopathological changes have been reported in patients who have been exposed to plasma perfusion therapy (5).

Spontaneous destruction of murine mammary tumors has been reported (6); however, it is extremely rare in the C3H(T/F) strain. Tumor necrosis has been induced in tumor-transplanted animal systems by tumor necrosis factor (TNF) (7–11) and in spontaneous tumors by plasma perfusion over Protein A (5, 12–14). The action of autotumorolytic factor may differ considerably from other factors previously mentioned.

The purpose of these studies was to isolate and purify ATF from sera of tumor-bearing C3H(T/F) mice.

Methods and Materials. The C3H(T/F) blood-donor and assay animals used in these studies comprise a strain in which every female develops Type A adenocarcinoma of the mammary gland(s), usually between 5 and 12 months of age (1, 2). The acquisition of individual serum samples and subsequent processing to crude, active SF III preparations

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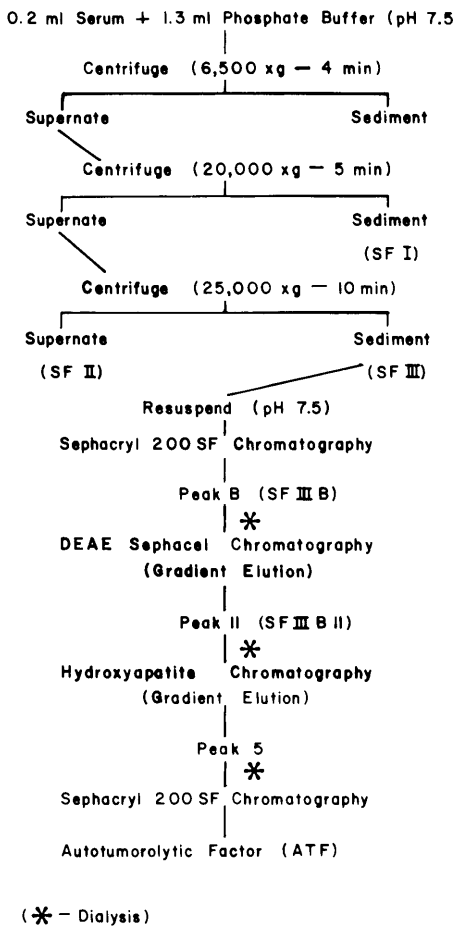

FIG. 1. Flow chart for ATF isolation.

(Fig. 1) has been reported previously (2). When pooled sera was used as source material for purification, 1.5 ml of serum was initially diluted with an equal volume of 0.05 *M* of sodium–potassium phosphate buffer containing 0.9% NaCl, pH 7.5. SF III sediments were suspended in a volume of this buffer equal to one-third the volume of crude serum. All eluted column fractions were scanned at 280 nm and each resultant peak was assayed for protein content by a modification of the method of Warburg and Lowry *et al.* (15, 16) and for tumorolytic activity.

Tumorolytic activity was assessed by injection of sample materials into at least 10 mice bearing 2-week-old tumors, from the time of initial palpation of a mass, at a site distant from the tumor. Measurement of tumorolytic activity was monitored by the reduction of total tumor volume (length × width × depth) and verified by histopathological examination. Volume measurements were performed immediately prior to the subcutaneous injection of 0.5 ml of sample into assay mice. Gross reduction in tumor volume by a minimum of 50% and a change in palpable consistency from firm to soft and boggy within a 24-hr period was considered indicative of activity for a given fraction. Lysis was confirmed by histopathological examination of all tumors and comparison to the criteria established (2) for the 24-hr stage of the induced lytic process.

Purification techniques for the isolation of ATF followed the pattern of sequential chromatographic procedures shown in Fig. 1. SF III (1 ml) was applied to a Sephacryl 200 SF (Pharmacia, Uppsala, Sweden) 0.9 × 60-cm column. The column was eluted with the suspension buffer at a flow rate of 12.0 ml/hr and collected in samples of 0.6 ml per tube.

The active peak from the Sephacryl 200 SF column was dialyzed (4°C) overnight against 0.05 *M* Na–K phosphate buffer, pH 7.5, and concentrated against polyethylene glycol (mol wt 20,000) to a volume of 0.5 ml. This sample was applied to a DEAE-Sephacel (Pharmacia) column and eluted with a linear gradient of 0.05 *M* Na–K phosphate buffer, pH 7.5, to 0.50 *M* Na–K phosphate buffer, pH 5.5, in aliquots of 1.0 ml per tube. After protein assay, sample peaks were concentrated to a volume of 0.5 ml and analyzed for tumorolytic activity. For further purification, active samples were dialyzed at 4°C overnight against 0.01 *M* Na–K phosphate buffer (pH 6.8), concentrated to 0.5 ml, and applied to an hydroxylapatite column. This column was eluted with a linear gradient of 0.01 *M* (pH 6.8) to 0.30 *M* (pH 6.8) Na–K phosphate buffer (1.0 ml per tube). Each peak was dialyzed against 0.05 *M* Na–K phosphate buffer containing 0.9% NaCl (pH 7.5) at 4°C for 18 hr and concentrated to 0.5 ml. All samples were assayed and the active peak was reapplied to a Sephacryl 200 SF column and eluted with 0.05 *M* Na–K phosphate buffer containing 0.9% NaCl, pH 7.5.

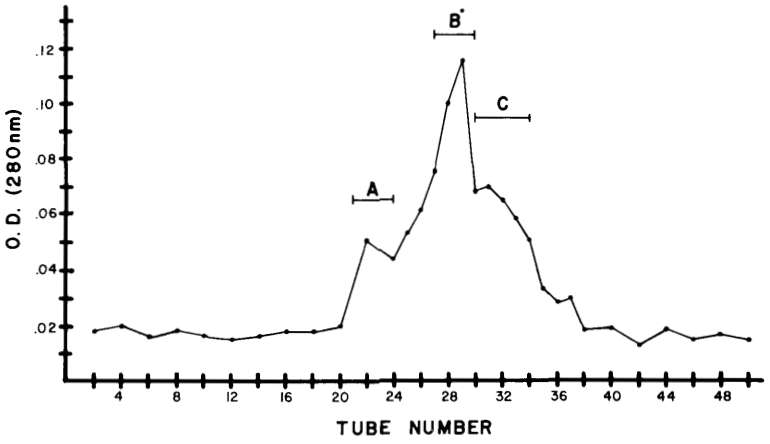

FIG. 2. Sephacryl 200 SF chromatography of the SF III fraction. SF III was suspended and eluted with a 50 m*M* sodium–phosphate buffer containing 0.9% NaCl (pH 7.5). Samples of 0.6 ml were collected. Biologically active ATF was found in Peak B.

Molecular weight determinations were performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a gel gradient of 10–15% according to the PhastGel procedure (17). Gel slabs are approximately 0.45 mm thick. The buffer system in the gels is 0.112 *M* acetate and 0.112 *M* Tris, pH 6.4. The buffer system in the PhastGel SDS buffer strips is of 0.20 *M* tricine, 0.20 *M* Tris, and 0.55% SDS (analytical grade), pH 7.5. The buffer strips are made of 2% agarose. This procedure requires only 10 μl of sample and will detect as low as 0.3–0.5 ng of protein using the silver staining techniques developed for the PhastSystem (18). Gels were run at 250 V, 10.0 mA, 3 W, 15°C, for 60 V-hr. The entire procedure, from sample application to acquisition of silver-stained gel slab usually is concluded in under 2 hr. High-pressure liquid chromatography (HPLC; Perkin–Elmer Series 4) also was utilized to determine the homogeneity of the final preparation. A reverse-phase column (C-18) was utilized and eluted with 0.05 *M* Na–K phosphate buffer, pH 7.5. The final preparation was collected from this column and its activity was determined.

Results. A typical elution pattern of SF III preparations chromatographed on Sephacryl 200 SF was resolved into three main peaks (Fig. 2). Tumorolytic activity was determined to be in the second peak (protein concentration 0.12 mg/ml). This peak is referred to as SF IIIB.

In contrast to SF III, SF IIIB preparations were shown to be active in both autologous and syngeneic animals (19). Typical histopathological changes were observed using these preparations. Therefore pooled sera from tumor-bearing animals were used as source material in subsequent purification experiments.

Chromatography on DEAE-Sephacel resulted in a number of peaks (Fig. 3). Only one of these (0.09 mg protein/ml) displayed tumorolytic activity and was designated Peak 11. When this peak was applied to an hydroxyl-

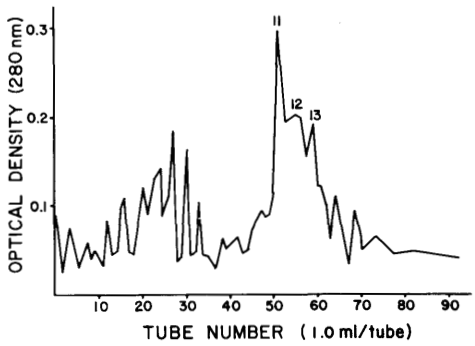

FIG. 3. DEAE-Sephacel chromatography of active Peak B from the Sephacryl 200 SF column. The active peak was designated as Peak 11.

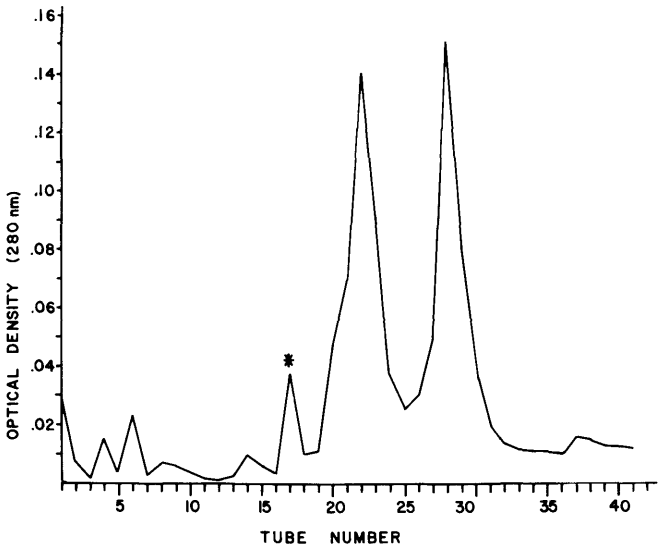

FIG. 4. Hydroxylapatite chromatography of the active material (Peak 11) from the DEAE-Sephacel column. Biological activity was found in only one peak (asterisk).

apatite column, it was resolved into four peaks, one of which (HP) contained ATF activity (Fig. 4) and had a protein content of 0.06 mg/ml. This active material was reapplied to a Sephacryl 200 SF column and the second of two resolved peaks contained all of the tumorolytic activity (Fig. 5). This final purified ATF had a protein content of 0.021 mg/ml.

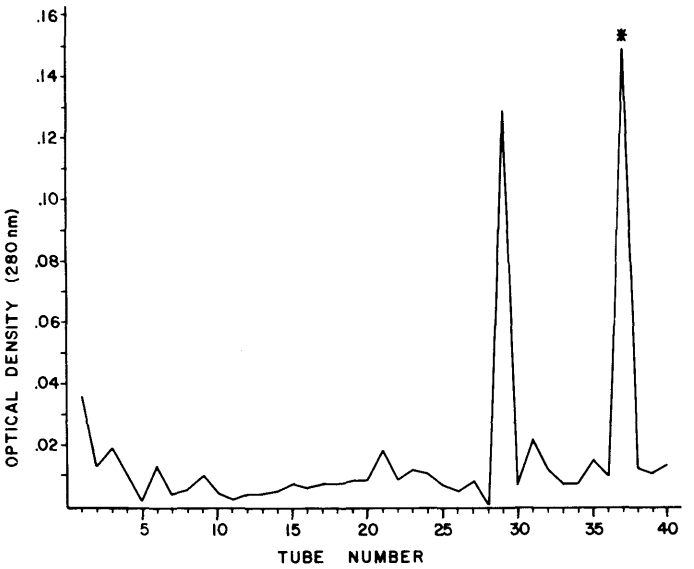

FIG. 5. Sephacryl 200 SF chromatography of the active fraction from the hydroxyapatite column. Only the second major peak (asterisk) contained biologically active ATF.

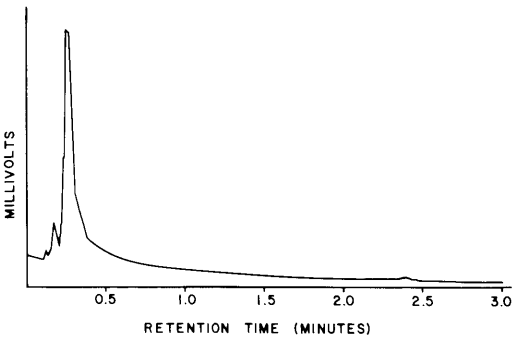

FIG. 6. HPLC of purified ATF. A 3-cm reverse-phase Perkins–Elmer C-18 column was utilized. The ATF sample was eluted with 0.05 *M* sodium–phosphate buffer containing 0.9% NaCl. The purified and biologically active sample had a retention time of 0.36 min.

HPLC of the final preparation indicated that only one peak was present (Fig. 6). SDS–gradient polyacrylamide gel electrophoresis revealed the presence of a homogeneous preparation (Fig. 7). Molecular weight determinations of purified sample ATF indicated a molecular weight of approximately 50,000 Da.

Tumorolytic activity was seen in all animals injected with active fractions. The degree of tumor volume reduction, within 24 hr, varied between 50 and 80%. The protein concentrations and the amounts of protein injected at assay are given in Table I. Using these values, an average ATF protein concentration of 0.010–0.011 mg/tumor-bearing mouse was calculated, based on a body weight range of 20–25 g, an average hematocrit of 40% VPRC, and a blood volume range of 6.5–8.0 ml/100 g body wt.

Discussion. The results presented indicate that a serum-derived autotumorolytic factor has been purified to a homogeneous species with a molecular weight of approximately 50,000 Da. This preparation has the capacity to induce lysis of spontaneous mammary neoplasia in autologous or syngeneic C3H(T/F) mice without apparent cytotoxicity to other tissues. Regardless of serum source (autologous or syngeneic), comparable histopathologic effects of ATF are demonstrable in tumor tissues of autologous and syngeneic hosts. This tumorolytic activity is maintained throughout the purification process. ATF is an endogenous proteinaceous entity in the spontaneous tumor-bearing mice of the C3H(T/F) strain.

In recent years, there have been reports of selective destruction of tumor tissue by various regimes. One of these is tumor necrosis factor, which is derived from the serum of mice primed with Bacillus Calmette–Guerin (BCG) and later challenged with endotoxin (bacterial lipopolysaccharide). TNF has the capacity to cause hemorrhagic necrosis of methylcholanthrene-induced tumors syngeneicly transplanted in BALB/c mice *in situ* (7, 8). It also has been shown to inhibit the growth, but not induce lysis, of other tumors *in vivo* (20, 21). TNF is cytotoxic to several cell lines *in vitro* (9) and cytostatic for others (10, 22). To our knowledge, TNF has not been found in the serum of nonstimulated tumor-bearing animals. Further, primary mammary tumors of mice show no reaction to TNF (23). Another type of tumorolytic regime involved the injection, into tumor-bearing hosts, of their own plasma after perfusion over Protein A (5, 12–14). This procedure resulted in some autologous destruction of tumor tissue. ATF is similar to these in that it can specifically target

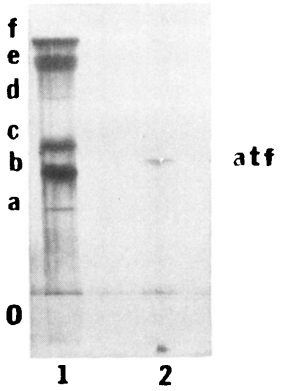

FIG. 7. Discontinuous gradient gel electrophoresis pattern of ATF. Channel 1 represents a standard mixture of proteins. The standard proteins are identified by letters a through f: a, phosphorylase B, mol wt 90,000; b, albumin, mol wt 67,000; c, ovalbumin, mol wt 43,000; d, carbonic anhydrase, mol wt 30,000; e, trypsin inhibitor, mol wt 20,100; f, α lactalbumin, mol wt 14,400. Channel 2 = ATF-MW, approximately 50,000.

TABLE I. PROTEIN CONCENTRATIONS OF ACTIVE FRACTIONS

Fraction	Protein concentration (mg/ml)	Volume (ml)	Total protein (mg)	Volume injected (ml)	Protein injected (mg)
Serum[a]	30.0	3.0	90.0	NA[b]	NA
SF III[c]	1.75	1.0	1.75	1.0	1.75
SF IIIB	0.12	2.0	0.24	0.5	0.06
Peak 11	0.09	1.0	0.09	0.5	0.045
HP	0.06	1.0	0.06	0.5	0.03
ATF	0.021	0.6	0.0126	0.5	0.0105

[a] Diluted 1:1 with buffer.
[b] NA, nonactive.
[c] Nonsyngeneicly active.

tumor tissue while apparently not affecting other nontumorous tissues. ATF, however, appears to differ from these other tumorolytic materials in that (i) it is derived from the blood of spontaneous tumor-bearing hosts, (ii) it does not require a prior stimulatory manipulation, and (iii) it causes the lysis of spontaneous tumors *in situ*.

The availability of purified ATF will enable us to develop a quantitative assay, and characterize its structure, site(s) of production, and site(s) and mode(s) of action.

This work was supported in part by grants from The Arde Bulova Foundation, The Laidlaw Foundation, and by BRS Grant S07 RR05332 from the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

The authors express their thanks to Matthew J. Scanlon, John Grew, Ephraim Bartfeld, and Colleen Lowman for their assistance and to Alba Velez for the preparation of histological sections.

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Received May 19, 1986. P.S.E.B.M. 1987, Vol. 184.
Accepted October 24, 1986.