SPECIFIC TUMOR IMMUNITY INDUCED WITH SOLUBLE MATERIALS: PURIFICATION OF ANTIGENS INDUCING TUMOR RESISTANCE

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SUMMARY: The tumor-specific transplantation antigen (TSTA) in crude three molar potassium chloride (3M KCl) extracts of a chemically-induced, murine fibrosarcoma was purified by ammonium sulfate salt fraction at 20% saturation (S20S) and by polyacrylamide gel electrophoresis (PAGE). Mice, which had been pretreated with the S20S precipitate, displayed retarded outgrowth of a 100-fold supralethal dose of the corresponding, but not of a non-cross-reactive syngeneic tumor. Analysis of PAGE gels by Coomassie Blue staining revealed at least 30 bands in the crude 3M KCl extract, and only two components (Rf 0.34 and 0.43) in the ammonium sulfate fraction. That these two components bore TSTA activity was demonstrated by the observation that the immunoprotective activity of crude 3M KCl extracts was localized to the Rf 0.25-0.50 region. The two components present in the S20S fraction had isoelectric points of 5.05 and 6.9, and estimated molecular weights of 40,000 and 75,000, thus demonstrating the soluble nature of the active principle. These findings offer the prospect of a chemical dissection of the polymorphic TSTA surface markers on MCA-induced murine tumors.

Two vastly polymorphic systems of cell surface markers are the tumor-specific transplantation antigens (TSTA) present on chemically-induced neoplasms, (1) and the histocompatibility (allospecific) antigens present on all nucleated cells. Solubilization and purification of histocompatibility antigens has yielded immunologically active fractions, which upon amino acid compositional study and tryptic peptide fingerprint analysis revealed differences in primary amino acid sequences (2), The TSTA system displays even greater polymorphism, since each application of the polycyclic hydrocarbon yields a neoplasm bearing distinctive transplantation antigens (3), In the work presented herein TSTA were recognized by the capacity of extracts solubilized from the corresponding tumor, but not from normal tissue or from another, syngeneic MCA-induced neoplasm, to induce specific resistance by syngeneic hosts against viable tumor cell challenges (5-12). This TSTA

activity has been refractory to purification. Based upon the observation that histocompatibility antigens could be partially purified with 50% ammonium sulfate (4), Hollingshead et al. (5), Prager et al. (6) and Meltzer et al. (7) used greater than 30% $(NH_4)_2SO_4$ to obtain biologically active, but chemically complex, tumor antigens. The studies described herein were initiated to obtain soluble, purified materials to dissect the chemical basis of TSTA individuality. Salt fractionation with a 20% saturated $(NH_4)_2SO_4$ solution yielded a material not only capable of inducing tumor-specific transplantation resistance, but also containing only two major components (molecular weight range 40,000 and 75,000 and isoelectric points 5.0 and 6.9) upon analysis in polyacrylamide gel.

METHODS:

Tumors Two sarcomas (MCA-F and MCA-C) were induced with 3-methylcholanthrene and propagated in syngeneic C3H/HeJ mice. The lethal dosage titration curves, parameters of in vivo growth and doses of 3M KCl extracts required to reduce tumor outgrowth by 50% (TG_{50}), namely 1.89 mg and 1.0 mg, for MCA-F and MCA-C were quite similar. Neither the tumors nor their extracts induced cross-protection (8-10).

Solubilization: Solubilization was performed by the method of Reisfeld and Kahan (4), as adapted to murine tumors (8-10). Eighteen billion MCA-F tumor cells, obtained from 30 subcutaneously-propagated, tumor nodules by treatment of finely minced tissue with 0.25% trypsin solution, were washed three times with 0.15M phosphate buffered saline pH 7.2 (PBS) and resuspended in 250 ml PBS, which was adjusted to 3M KCl. Extraction was performed for 16 hours at 4°C with constant stirring. After insoluble material was removed by ultracentrifugation at 164,000g for 60 minutes, the supernate was first concentrated against 50% sucrose, and then dialyzed against 200 volumes of PBS. The 3M KCl crude extract contained 430 mg protein (13), that is, about 20% of the total 2142 mg protein in an 1 N sodium hydroxide digest of 1.8 x 10^{10} intact tumor cells.

Fractionation of the Crude Extract: Two methods were employed to purify TSTA activity from crude 3M KCl extracts: 1) differential solubility in 20% ammonium sulfate solution $(NH_4)_2SO_4$, and 2) polyacrylamide gel electrophoresis (PAGE). Salt precipitation was performed by harvesting the 49,000g sediment following dialysis of 8 ml (40 mg protein), of the 3M KCl extract, against 100 volumes of 20% $(NH_4)_2SO_4$ solution (16 hours, 4°C). This fraction, denoted S20S, was redissolved in PBS to its original volume, and then dialyzed against PBS to remove the $(NH_4)_2SO_4$. It contained 1.4 mg protein per 10^9 cells, that is 6.1% of the protein in the crude extract. Alternatively, 0.5 mg protein of the crude 3M KCl extract was applied to each of 23, 6.5 cm, 7.5% polyacrylamide gels (pH 9.4), run initially for 0.5 hour at 1.5 ma/gel, and then for an additional hour at 2.5 ma/gel (14). One gel was fixed in 12.5% trichloroacetic acid solution, stained with 0.5% Coomasie Blue and scanned for components using a laser densitometer (Biomed Instruments,

Inc., Chicago, Illinois). Twenty-two other gels were cut into four equal regions; corresponding regions from each gel were pooled and eluted with 5 ml of PBS (16 hours, 4°C). A slurry representing the electrophoretic components in each region was prepared by forcing the gels and eluates through a 20 cc syringe bearing a 19 gauge needle. The S20S fraction was subjected to electrophoresis using isoelectric focusing (pH range 3.5 - 10.0), and molecular weight analysis in sodium dodecyl sulfate-polyacrylamide gels, using techniques described in detail elsewhere (15).

Immunoprotection Tests: To perform the immunoprotection test, putative TSTA, in the form of crude 3M KCl extract, S20S fraction, or Regions I, II, III, or IV PAGE slurry was injected subcutaneously into ten C3H/HeJ mice per group, ten days prior to distant, subcutaneous challenge with a single inoculum of 10^4 viable MCA-F, or alternatively, 10^4 antigenically distinct MCA-C, neoplastic cells (100-fold the minimum tumor dose) (9). Tumor growth was monitored by serial measurements of the average tumor diameter (d) using calipers. The mean tumor volume for each group was determined using the formula for spherical volume (v), namely, $v = 4.19\ (d/2)^3$. The reproducibility of this method to measure tumor nodules was tested by twenty independent measurements on a single occasion of two tumors of 2 and a 7.5 cm^3 mean volumes. The standard error for the former was $\pm$ .075 and for the latter $\pm$ 0.236. The mean tumor volume in treated and in control, untreated, hosts was calculated. Differences were analyzed for statistical significance by a two-tailed Student "t" test. In addition each tumor growth curve was subjected to linear regression analysis by obtaining an equation for the plot of log 10 tumor diameter versus days after challenge. Formulae and statistical methods described by Woolf (16) were used. Significant differences between treated and untreated groups were determined by calculating the 0.01 and 0.05 confidence limits using the two-tailed "t" value. Those treatment groups with values less than the 0.01 or 0.05 limits were considered to show significant tumor retardation.

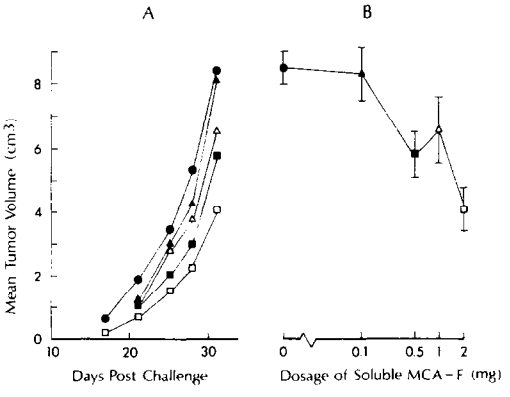

Figure 1. Induction of tumor resistance by treatment with crude, 3M KCl extracts of MCA-F. Groups of ten mice pretreated with 0.0 (●), 0.1 (▲), 0.5 (■), 1.0 (△), or 2.0 (□)mg of crude extract were challenged 10 days afterwards with 10^4 viable MCA-F cells. Tumor growth was monitored by serial measurements of the tumor diameter and expressed as neoplastic tissue volume (Panel A). The dose-response relationship at day 31 after challenge is shown in Panel B. The bars bracketing each point show the standard error, determined as described in the text.

RESULTS AND DISCUSSION: While animals treated with 2 mg of crude extract (Figure 1) displayed tumor resistance ($p < 0.001$, Student "t" test; $p < .05$, linear regression analysis), the S20S (Figure 2A, B) possessed enhanced TSTA activity ($p < 0.001$, "t" test; $p < 0.01$, linear regression). This resistance was immunologically specific: Mice treated with any of these dosages of S20S fraction from MCA-F were equally susceptible to the outgrowth of MCA-C as were untreated controls (Figure 2C). Table 1 compares the yield and immunoprotective activity of soluble fractions with that of irradiated cells.

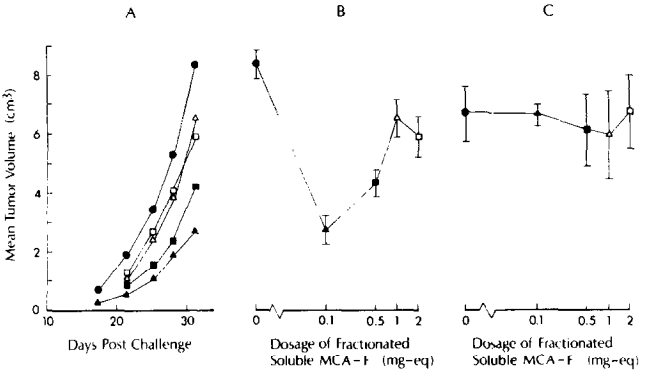

Figure 2. Induction of tumor-specific resistance by treatment of mice with partially purified MCA-F antigen. The crude extract shown in Figure 1 was fractionated by treatment with 20% saturated $(NH_4)_2SO_4$. Precipitated material was redissolved and administered to syngeneic hosts in an immunoprotection test. Groups of twenty mice were pretreated with S20S from MCA-F in dosages equivalent to 0.0 (●), 0.1 (▲), 0.5 (■), 1.0 (△), and 2.0 (□) mg of the crude extract. Ten days later 10 mice from each group were challenged with 10^4 viable MCA-F cells (A,B) and the remainder challenged with 10^4 cells of an antigenically distinct tumor MCA-C (C). Statistical analysis of the dose-response relationship at day 31 is shown for mice challenged with MCA-F (Panel B) and with MCA-C (Panel C).

The dose of x-irradiated tumor cells required to reduce neoplastic outgrowth by 50% (TG_{50}), as determined by a linear regression analysis of the log mean tumor volume plotted against the log cell numbers, was 1.04×10^4 cells, which contained 0.00124 mg protein in the 1N NaOH hydrolysate. The TG_{50} of the crude 3M KCl extract was 1.89 mg; the S20S fraction, 0.03 mg. In

Table 1

Relative yield of Immunoprotective Tumor-specific Transplantation Antigen[a]

Fraction	TG_{50}(mg)[b]	TG_{50} Units[c]	Total Protein per 10^9 Cells	Specific Activity[d]
Irradiated Cells[e]	0.00124[f]	806.72	119	6.77
3M KCl	1.89	0.53	23.50	0.023
S20S	0.03	33.28	1.43	23.27

[a]The preparations were derived from 10^9 cells (containing 119 mg protein).
[b]TG_{50} is the amount of protein (in milligrams) which induces an immune response resulting in a 50% retardation of tumor growth.
[c]TG_{50} Units are the inverse of the TG_{50}(mg).
[d]Specific Activity expresses the ratio of TG_{50} Units to the total amount of protein per 10^9 cells.
[e]Suspensions of tumor cells were irradiated with 12,000 rads using a Clinac 4 linear accelerator set to deliver 889 rads/min.
[f]The TG_{50} for irradiated cells is 1.04×10^4 cells. The TG_{50} was calculated based upon the determination of 119 mg protein in a NaOH digest of 10^9 cells.

order to conveniently express these results, the data were converted to Units by calculating the inverse of each value ($1/TG_{50}$). The S20S fraction displayed a 63-fold increase in TG_{50} units compared to its parent 3M KCl material. Of critical importance to the purification and chemical analysis of TSTA is the specific activity, namely, the TG_{50} units per mg total protein. The S20S material had a 1010-fold increase in specific activity, compared to the crude 3M KCl fraction, and an almost four-fold increase, compared to the very immunogenic, irradiated cells.

PAGE analysis of the crude 3M KCl, MCA-F extract revealed more than 30 components by Coomassie Blue, staining consistent with previous observations upon extracts of MCA-induced guinea pig sarcomas (17). When unstained polyacrylamide gels were cut into four regions, eluted overnight in PBS, and

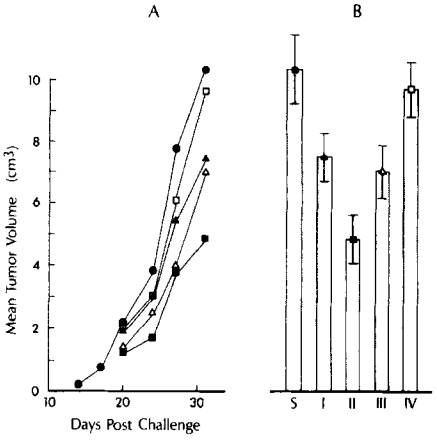

Figure 3. Biological activity of each zone of polyacrylamide gel following electrophoresis of the 3M KCl extract of MCA-F. Ten 7.5% gels were loaded with 100 µl of crude extract containing 1 mg of protein and electrophoresed as described in the text. Gels were each cut into four equal regions; I, Rf 0-0.25 (▲); II Rf 0.26-0.5 (■); III, Rf 0.51-0.75 (△); IV, Rf 0.76-1.0 (□), eluted with PBS, and a slurry made of the gel and eluate. The pooled slurries from each region along with sham slurries (●) were examined in the immunoprotection test. The ability of these preparations to induce resistance to challenge with 10^4 MCA-F cells is illustrated by the serial, mean tumor volume determinations (Panel A) and by statistical analysis of these volumes at day 31 (Panel B).

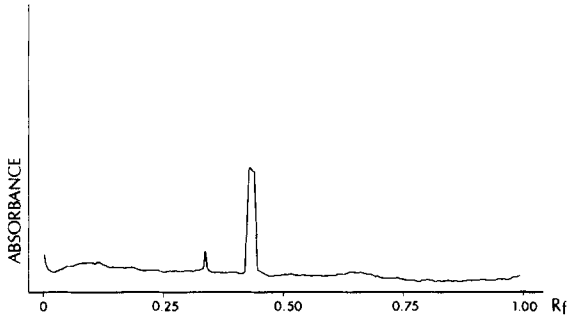

Figure 4. Scan of a 7 1/2% polyacrylamide gel loaded with 45 µg of S20S MCA-F. The gels were electrophoresed for 30 minutes at 1.5 ma/tube, and then for an additional 60 minutes at 2.5 ma/tube. After fixation in 5% trichloroacetic acid, protein-rich regions were visualized by staining with 0.5% Coomassie Blue solution and densitometric analysis performed using a laser gel scanner.

forced through a syringe to make a slurry, the immunoprotective activity was confined to Region II (Rf 0.25-0.50). (Figure 3, $p < .01$, Student "t" test,

and linear regression analysis). When the S20S fraction was subjected PAGE analysis in 7 1/2% gel at pH 9.4, there were two components Rf values of 0.34 and 0.43, both of which were located in Region II, (Figure 4). Furthermore two components were present upon isoelectric focusing (pI values 5.05 and 6.9) and upon SDS-PAGE gels (approximate molecular weights 75,000 and 40,000).

Ammonium sulfate fractionation of 3M KCl extracts of methylcholanthrene-induced sarcomas offers three advantages. First, this method eliminated the need for cumbersome and time-consuming concentration procedures, such as ultrafiltration or lyophilization. Second, the fractions contained relatively few protein components upon PAGE. Third, the specific activity was increased by 1010-fold compared to the parent fraction. This degree of purification may have resulted not only from an enhanced immunogenicity of purified TSTA per se, but also from separation of the immunogenic principle from substances interfering with the induction or expression of a destructive immune response, as previously reported by others (18). The studies presented herein offer the prospect of a molecular dissection of the polymorphic tumor-specific transplantation antigens.

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