Intracellular Distribution and Extraction of Tumor Homograft-enhancing Antigens*

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SUMMARY

Tumor homograft-enhancing activity was found to be widely distributed in subcellular particulate fractions prepared from Sarcoma I ascites cells but was essentially absent from the soluble fraction. It was most concentrated in the fluffy layer, but, because of the relatively large mass of the nuclei, about half of the total activity of the cells was present in the nuclear fraction.

A procedure for extracting enhancing activity from a particulate fraction prepared from ascites tumor cells was developed. Active extracts contained three components separable by electrophoresis at pH 9.2. The fastest-moving component could be eliminated by incubating the particulate fraction with ribonuclease.

Although the influence of the isoantigens of killed tissues (enhancing antigens) in promoting the survival of tumor homografts is now well established (5, 12), knowledge of the chemical nature of the antigens is still fragmentary. Studies of their stability characteristics indicate that they are labile to protein denaturing agents and to dilute solutions of sodium periodate, properties consonant with those of a protein-carbohydrate complex (6). While the antigens were previously shown to be present in particulate components of cells (6, 11), their distribution within the cell was not determined.

The present report deals with the intracellular distribution of enhancing antigens and with the development of a procedure by which the antigens can be extracted from a particulate fraction of ascites tumor cells.

MATERIALS AND METHODS

The source of the antigens in all experiments was cells of an ascites form of Sarcoma I indigenous to strain A mice. The test for enhancing activity utilized mice of strain B10.D2 (a C57BL/10 subline) as hosts and 0.1 ml. of undiluted Sarcoma I ascites tumor fluid inoculated subcutaneously as the graft. Sarcoma I regresses in all but 5 per cent of untreated mice of the B10.D2 strain. Tumor homograft-enhancing activity was assayed by a procedure previously described (6), wherein the measure of enhancement induced is the number of treated host mice dying as a result of progressive growth of the tumor. In this test system enhancement is probably due entirely to an antigenic component or components determined by the histocompatibility-2 locus.

Isolation of subcellular fractions.—Disruption of the cellular membranes of the ascites tumor cells represented a major problem. Homogenization in cold glycerol (13) was difficult, and in our hands many intact cells remained. A method involving lysis with cold distilled water (7) appeared to give more satisfactory results. Although it is probable that some mitochondria were also lysed by this procedure (9), no evidence for the disruption of nuclear membranes was found. All operations were carried out at 0°–4° C. Washed ascites cells freed of red blood cells (4) were homogenized briefly in a Potter-Elvehjem homogenizer with 2 volumes of distilled water. Eight more volumes of distilled water were added, and the cells were allowed to stand for 30 minutes. Nuclear, mitochondrial, and microsomal fractions were prepared from separate aliquots of the lysate.

Nuclei were sedimented from a medium obtained by adding concentrated solutions of sucrose and calcium chloride to the lysate to final concentrations of 0.25 M and 0.005 M, respectively. The suspension was underlaid with 0.0002 M $CaCl_2$

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and 0.34 M sucrose and centrifuged at 700 *g*[1] for 20 minutes (3). The supernatant fluid was discarded, and the sediment was resuspended in 0.25 M sucrose containing 0.005 M $CaCl_2$ by gentle homogenization. The suspension was underlaid and centrifuged as above. The entire procedure was repeated a third time, after which the nuclei were suspended in 0.14 M NaCl and centrifuged at 1000 *g* to yield the final nuclear fraction. Although examination with a phase contrast microscope showed the nuclei to be relatively free of whole cells (less than 3 per cent), small pieces of cytoplasm were found attached to many of the nuclei.

Mitochondria and the fluffy layer were sedimented from 0.44 M sucrose by centrifugation at 13,000 *g* for 10 minutes after first removing nuclei by centrifugation at 700 *g* for 10 minutes. The fluffy layer was separated from the mitochondria by gentle swirling with 0.44 M sucrose (9) and recovered from the resulting suspension by centrifugation at 13,000 *g* for 10 minutes. Both the mitochondrial fraction and the fluffy layer were washed once more with 0.14 M NaCl, with centrifugation as before.

Microsomes were obtained by sedimentation from 0.25 M sucrose, 0.005 M $MgCl_2$, 0.025 M KCl at 105,000 *g* for 1 hour after first removing larger particles by centrifugation for 30 minutes at 20,000 *g* (8). Suspension and resedimentation were repeated 3 times; the last time 0.14 M NaCl was used as the suspending medium. All fractions were lyophilized and stored in the deep freeze until tested for enhancing activity. The soluble fraction (supernatant fluid after removal of microsomes) was dialyzed for 36 hours against distilled water before lyophilization.

Extraction of enhancing activity.—The usual starting material in experiments aimed at extracting the antigen in soluble form was a particulate fraction of ascites tumor cells. Washed tumor cells free of red blood cells (125 ml. packed cells) were suspended in 2 volumes of distilled water and homogenized for 2 minutes at top speed in a Waring Blendor. After centrifugation for 30 minutes at 48,000 *g* the supernatant fluid was discarded and the sediment again homogenized in the Waring Blendor for 1 minute with 100 ml. of distilled water. When DNA[2] was to be removed, 5 mg. of deoxyribonuclease (Worthington 2 × crystallized) and $MgCl_2$ to .025 M were added to the homogenate, and the mixture was stirred in a covered Erlenmeyer flask with a magnetic stirrer for 1 hour at room temperature. The sediment obtained by centrifuging the mixture for 30 minutes at 48,000 *g* was washed twice with 1 M NaCl, once with 0.14 M NaCl, and finally with distilled water. For each wash, the sediment from the receding centrifugation was suspended by homogenization with a glass homogenizer and centrifuged at 48,000 *g* for 30 minutes. When the DNA was to be retained with the particulate fraction, incubation with DNase was omitted, and the sediment obtained by centrifuging the second Waring Blendor homogenate was washed 3 times with 0.14 M NaCl and finally with distilled water. The preparations were lyophilized and stored in the deep freeze. To extract lipides from the lyophilized preparations, the dry powder was poured into 10 volumes of cold acetone (−20° C.). After the acetone suspension had warmed to room temperature it was centrifuged, and the sediment was extracted once with ether and dried *in vacuo* for 1 hour at room temperature.

Attempts to solubilize the enhancing antigens with detergents or with *n*-butanol were carried out in the following manner: A portion (120 mg.) of the dried particulate fraction was homogenized in a glass homogenizer with water or salt solution. The detergent or butanol was added to the resulting suspension, to give a final volume of about 10 ml., and the mixture was stirred, either at room temperature or in the refrigerator. After the appropriate time interval the mixture was centrifuged at 105,000 *g* for 1 hour or longer at room temperature.

The supernatant fluid was poured into 10 volumes of cold acetone, and the resulting precipitate was washed with ether and dried *in vacuo*. The extract from 120 mg. of particulate fraction was suspended in 18 ml. of saline, 2 ml. of this suspension was diluted to 20 ml., and each of the suspensions was used to treat ten mice. In some experiments the extracted residue was also tested for enhancing activity. RNA, DNA, hexosamine, and nitrogen were determined by methods described previously (6). Electrophoresis in free solution of acetone-precipitated extracts was carried out in a Perkin-Elmer Model 38 A apparatus with a 6-ml. cell.

RESULTS

Intracellular distribution.—Enhancing activity was found associated with all the particulate fractions isolated from the cells but was essentially absent from the soluble fraction (Table 1). The fluffy layer was particularly active, and the microsomal fraction also had a high level of activity when dosage was based on the nitrogen content of the fraction. However, a large part, crudely esti-

[1] All centrifugal forces are average.

[2] The abbreviations used are DNA (deoxyribonucleic acid), DNase (deoxyribonuclease), RNA (ribonucleic acid), and RNase (ribonuclease).

mated at 50 per cent, of the total activity of the cell was present in the nuclear fraction, which contained 73 per cent of the total nitrogen found in all the particulate fractions. There appeared to be some correlation between the concentrations of hexosamine and enhancing activity in the nuclear, mitochondrial, and fluffy layer fractions. In the microsomal fraction, however, the concentration of enhancing activity was high, while the concentration of hexosamine was relatively low.

Extraction of the antigens.—The search for a medium capable of effectively extracting the antigens was carried on in eight different experiments. The data from one such experiment are shown in Table 2. In addition to the agents shown, the effects of repeated freezing and thawing (5 times) and of 1 and 5 per cent solutions of sodium deoxycholate were examined. Negligible amounts of activity were extracted with distilled water or 0.1 M potassium phosphate buffer, pH 8. Extraction with sodium dodecylsulfate resulted in the loss of almost all activity: the extracted residue, like the extract, retained only neglible activity. Repeated freezing and thawing or extraction with sodium deoxycholate resulted in the solubilization of a relatively small portion of the activity. While extraction with butanol or Triton solutions was always the most effective procedure, the amount of activity extracted with these agents varied considerably from one experiment to another. After several attempts to increase the effectiveness of extraction with butanol, this technic was abandoned, and efforts were concentrated on a study of the factors influencing extraction with Triton.

Extraction was more effective at pH 9 than at a lower or higher pH (Table 3). Presumably, the lowered activity in extracts obtained at higher pH is due to the relative instability of the antigens in alkaline solution (6). An 0.02 M borate buffer was as effective as 0.1 M phosphate when extraction was carried out for 4 hours. However, the antigens

TABLE 1

ENHANCING ACTIVITY OF SUBCELLULAR FRACTIONS OF SARCOMA I-A

Fraction	Per cent of total nitrogen	μg. Hexosamine/mg. nitrogen	No. dying out of ten 0.7* mg.N	0.3 mg.N	0.05 mg.N	Per cent of total dying
Whole cells	100.0	16.0	6	7	5	60
Nuclei	21.8	29.0	8	8	2	60
Mitochondria	0.6	23.0	8	4	0	40
Fluffy layer	4.9	57.0	10	10	9	97
Microsomes	2.5	15.0	10	9	2	70
Soluble	70.2	7.0	1	0	0	3

* Dose administered.

TABLE 2

EFFECTIVENESS OF VARIOUS MEDIA IN THE EXTRACTION OF ENHANCING ACTIVITY

Material injected	Per cent dying out of 20
Whole tumor*	50
Particulate (−DNA)*	90
Distilled H_2O extract†	10
0.1 M phosphate (pH 8) extract	15
2% Sodium dodecyl-sulfate extract	10
Butanol-H_2O extract‡	60
Butanol-.1 M phosphate (pH 8) extract‡	65
1% Triton§ extract	60
5% Triton extract	55
5% Triton-phosphate (pH 8) extract	80
None	5

* An amount equivalent to that extracted below (120 mg. dry wt.) was injected into two groups of ten mice each at levels of 10 and 1 mg. per mouse, respectively.

† All extracts were prepared from the particulate fraction (−DNA).

‡ Butanol was added in sufficient amounts to saturate a homogenate of particulate in water or buffer, and the mixture was allowed to stand 20 minutes at room temperature; all other extractions were carried on for 8 hours.

§ Triton × 100, Rohm and Haas Co., Philadelphia, Pa.

TABLE 3

EFFECTS OF BUFFER AND PH ON THE ACTIVITY OF TRITON EXTRACTS*

Buffer	pH	Length of extraction (hr.)	Per cent of mice dying out of 20
0.1 M phosphate	8	4	20
"	8.5	"	50
"	9.0	"	60
0.02 M borate	9.0	"	65
"	9.25	"	60
"	9.5	"	55
0.1 M phosphate	9.0	12	85
0.02 M borate	9.0	"	40
0.1 M borate	9.0	"	45

* Triton added to 5 per cent to a homogenate of particulate fraction (−DNA).

appeared to be more stable in phosphate buffer, since the amount of activity extracted with phosphate buffer after 12 hours increased over that extracted in 4 hours, while extraction with borate buffer for 12 hours resulted in decreased activity. As shown in Table 4, the amount of activity extracted with Triton-phosphate medium pH 9 was greatest after 12 hours, decreasing thereafter. The manner of preparation of the particulate fraction was also an important factor. Removal of DNA greatly increased the amount of activity extracted (Table 4), while removal of both DNA and lipide further improved the effectiveness of extraction. When extractions were carried out in the refrigerator rather than at room temperature, little activity was extracted: 6 hours at room temperature followed by 6 hours in the refrigerator released only as much activity as 6 hours at room temperature alone.

Properties of the extract.—When optimal conditions established by these studies, i.e., particulate fraction free of DNA and lipide as starting material and 12 hours' extraction at room temperature with 5 per cent Triton in 0.1 M phosphate (pH 9) were used, most of the activity of the starting material was obtained in the extract (Table 5). The extract, which was perfectly clear and light tan in color, contained 63 per cent of the total nitrogen originally present in the particulate preparation. After dialysis for 24 hours against distilled water followed by lyophilization, material

TABLE 4

EFFECTS OF LENGTH OF EXTRACTION AND PREPARATION OF PARTICULATE FRACTION ON THE ACTIVITY OF 5 PER CENT TRITON-PHOSPHATE (pH 9) EXTRACTS*

Hours extracted	Particulate fraction	Per cent of mice dying†
4	minus DNA	40 (20)
8	"	35 (20)
12	"	70 (60)
24	"	50 (40)
48	"	35 (20)
96	"	5 (20)
12	minus lipide	50 (20)
12	minus DNA, minus lipide	83 (40)

* Combined results obtained in three different assays.

† The number in parentheses represents the number of mice used.

precipitated from the extract with cold acetone contained 11.3 per cent nitrogen, 6.2 per cent RNA, 1.24 per cent hexosamine, and no detectable DNA. Dialysis of the acetone-precipitated extract against 0.05 M borate buffer, pH 9.2, resulted in an opalescent solution which on electrophoresis showed two major, slow-moving peaks (mobilities, 2.65×10^{-5} and 5.29×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}) and one minor, relatively fast-moving peak (mobility, 10.8×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}) (Chart 1, pattern A). The small, fast-moving peak was not present in a subsequent extract prepared from a particulate fraction from which RNA had been removed by adding 9.5 mg. of RNase (Worthington crystallized, salt-free) with the DNase in the

TABLE 5

EFFECTIVENESS OF OPTIMAL EXTRACTION PROCEDURE*

Material injected	Dose (mg. N)	Per cent of mice dying out of 10
Particulate (−DNA, −lipide)	1.0	100
"	0.6	100
"	0.06	80
Extract	1.2	80
"	0.3	90
"	0.03	70
Extracted residue	.6	50
"	.06	10

* Particulate fraction (−DNA, −lipide) was extracted for 12 hours with 5 per cent Triton in 0.1 M K_2HPO_4.

incubation step (Chart 1, pattern B). Incubation with RNase has been shown to have no effect on enhancing activity (6). Attempts to carry out electrophoresis with acetone-precipitated extracts dialyzed against other buffers or at lower pH were not successful, since the suspensions obtained were too turbid to allow the passage of sufficient light and showed a tendency to sediment with time. This difficulty could be obviated by omitting precipitation with acetone and simply dialyzing the Triton-containing extract. After dialysis against barbital (pH 8.6), phosphate (pH 7.5), and acetate (pH 6.1) buffers, four components were separated by electrophoresis. Three were apparently identical with those shown in Chart 1, while the largest and slowest component was Triton, which passed through the dialysis membrane very slowly, if at all.

DISCUSSION

The wide distribution of enhancing activity in particulate fractions is in general agreement with a hypothesis that the enhancing antigens are distributed throughout the membranes of the cells. On the other hand, the difficulties involved in obtaining clean preparations of nuclei, mitochondria, or fluffy layer, compounded in the present case by the application of methods developed for liver to an ascites tumor, make it impossible to exclude contamination of these fractions as an explanation for their activity. It is of interest that these results bring into question the assumption that enhancing

antigens differ from immunizing antigens in that enhancing antigens are present in cytoplasmic particles, while immunizing antigens are localized exclusively in the nuclei. The intracellular localization of immunizing antigens was determined by comparing the activities of nuclei from spleen with cytoplasmic particles from liver and kidney (1). Since enhancing activity is high in spleen and very

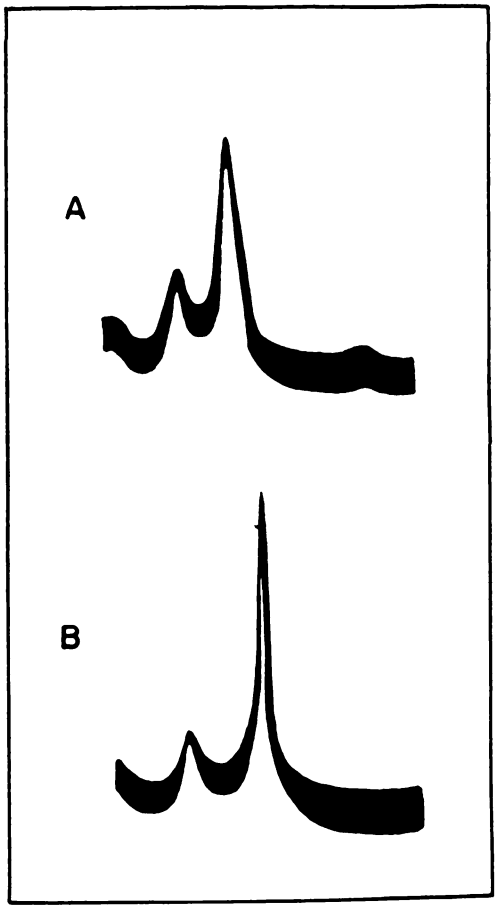

CHART 1.—Electrophoretic patterns of acetone-precipitated extracts in 0.05 M borate buffer, pH 9.2, field strength 7.5 volts/cm.: *A*, extract from a preparation not treated with RNase, protein concentration 1.2 per cent; *B*, extract from a preparation treated with RNase, protein concentration 1.8 per cent.

low in kidney or liver (6), and in view of the amount of activity found in the nuclear fraction in the present study, a distribution of enhancing activity similar to that found for immunizing antigens should be expected in such a comparison. Whether or not enhancing and immunizing antigens actually are localized in different subcellular components may therefore depend upon whether or not liver and kidney are approximately as good immunizing tissues as spleen, a question that has not been clearly answered.

The particulate fractions used as starting material for extracting the antigens may be expected to be composed in a large part of membranous particles (2, 10). Enhancing antigens are concentrated in this fraction and are free from cellular components which are soluble in water or saline solutions and from DNA (also from RNA if desired). A further degree of purification is probably achieved in the step in which the activity is extracted so that the antigens may be obtained in a solution that reveals only two components separable by electrophoresis.

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