Guinea Pig Serum Inhibitory Factor and Asparaginase Activity.* (30840)

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The inhibitory effect of normal pooled guinea pig serum (GPS) on the Gardner lymphosarcoma has been shown by Kidd to be highly specific causing regression of the tumor without toxic effect to the host(1,2). Although only certain lymphomas appeared to be affected, the cells of subcutaneous lymphosarcomas were rapidly destroyed and resorbed following intraperitoneal injection of the guinea pig serum(1,2). Rabbit antiserum to Gardner lymphosarcoma caused cell lysis of the tumor *in vitro* in the presence of complement, but its *in vivo* activity against the established tumor was not as great as guinea pig serum(3,4). The GPS factor is not associated with complement activity nor does it appear to work in conjunction with mouse isoantibodies(5).

Several investigations have shown that this inhibitory activity can cross species boundary. Some inhibition of the rat fibrosarcoma ($ACMCA_2$), the Walker carcinosarcoma and the Murphy sturm lymphosarcoma(6-8) has been demonstrated.

The first step in the direction of elucidating the mechanism of action of this material was made by Broome who demonstrated a correlation between the GPS anti-lymphoma activity and asparaginase activity in guinea pig serum (9-11).

The purpose of this report is to provide additional evidence for the hypothesis that asparaginase activity is responsible for the inhibitory effect of GPS on the ascites form of the Gardner lymphosarcoma in mice.

Materials and methods. Asparaginase assay. Assays were carried out on 0.5 ml of enzyme solution in a total volume of 2.0 ml containing 10-40 μmoles of L-asparagine monohydrate in 0.0075 M phosphate buffer pH 8.0. After 90 minutes of incubation at 37°C, the reaction was stopped by addition of 0.5 ml of 15% trichloracetic acid and the precipitate removed by centrifugation. Thirty liters of the supernatant were spotted on a Whatman #1 paper cylinder with aqueous 73.3% phenol as the solvent and then sprayed with 0.5% ninhydrin in water saturated butanol.

Spots of aspartic acid were cut out and eluted in 4 ml of copper nitrate solution (1 ml of saturated $Cu(NO_3)_2$, 0.2 ml of 10% HNO_3, plus 95% ethanol to 100 ml) with absorbance readings taken at 504 mμ on a Zeiss spectrophotometer.

One international unit (U) of L-asparaginase is defined as that amount of enzyme which will liberate from L-asparagine 1 μmole of product(s) per minute. The analysis of aspartic acid as described above was supplemented by determination of the liberated ammonia by nesslerization. Specific activity is expressed as units of the enzyme per milligram of protein. Concentration of the enzyme in solution is expressed as units per milliliter.

Chemical inactivation of asparaginase activity. To inactivate the asparaginase activity of whole guinea pig serum $HgCl_2$ was added to a final concentration of 1 millimole and incubated overnight in the refrigerator(12). Inactivated and control samples were dialyzed separately in cold saline for two days in sodium phosphate buffer, 0.01 M at pH 8.06. 0.2 ml of the guinea pig serum (12.5 mg protein) was assayed as described above. Final dilution of guinea pig serum in the final reaction volume of 2 ml gave 6.25 mg protein per ml.

A 10 ml sample of guinea pig serum was mixed with technical grade polyvinylpyrrolidone (PVP) (Mann Research Laboratories, Inc.) to make a PVP concentration of 3.5%. This was left overnight in the cold and incubated at 37°C for one hour. The content was dialyzed against saline at pH 7.1 for

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3 days. The supernatant after centrifugation was frozen for assay.

Normal pooled rabbit serum (RS) treated in the same manner as above was frozen for assay and served as a negative control.

Guinea pig serum dialyzed only against saline at pH 7.1 also was employed and served as a positive control.

DEAE chromatography of $Ca_3(PO_4)_2$ gel fractions of guinea pig globulins were made according to the method described by Tower (12), with the exception that stepwise elution was employed.

Animal assay of guinea pig serum. C3H/HeJ mice (Heston strain, Jackson Laboratories) were obtained from Bar Harbor, Maine. Animals were injected on day zero with 0.2 ml of ascites cells containing approximately 15×10^6 cells. Varying doses of the serum or fraction samples were injected intraperitoneally on the second, fourth and sixth day after tumor implantation. All animals were observed daily and the experiment was not terminated until 30 days had elapsed. Time of death was recorded and per cent survival in the group was used to determine the efficacy of the treatment.

Results. Effect of normal pooled guinea pig serum on survival of C3H mice bearing lymphosarcoma implants. In the animal assay system the ascites form of Gardner lymphosarcoma tumor administered intraperitoneally was employed. It was found that the C3H mouse strain was unable to resist intraperitoneal tumor implantation. Animals bearing the tumors all die within 15 days after implantation irrespective of being treated with RS, saline, or not treated at all. Fig. 1 gives a representative survival for a composite group of 36 tumor bearing mice treated with 0.5 ml RS (which contained no asparaginase activity) for a total of 3 times on days 2, 5 and 7, after tumor implantation. Most of the deaths occur within 11 to 12 days, *i.e.*, 58% and 78% of the deaths occur at this period, respectively; however, since all the control animals die within 15 days after tumor implantation, any survival beyond this time indicates some protection. When animals bearing such implants are treated with small volumes of GPS, such as 0.2 ml which contained approximately 0.4 unit/dose of enzyme activity and 0.1 ml which contained 0.2 unit/dose of enzyme activity, some slight survival effect can be demonstrated at 11 to 12 days but this effect is negligible. When larger doses of GPS are used, there is prolonged survival and some delaying effect on the capacity of the tumor to kill the host. Animals treated with guinea pig serum containing 2.0 units/dose of enzyme activity consistently protected animals from tumor death as witnessed by 100% survival at 15 days and 90% survival at 30 days. Animals treated with one-half this level of enzyme activity show that over 90% survived 15 days and after 30 days 62% survived (Fig. 1). The effectiveness of prolonging survival appears to depend on the asparaginase activity of the serum. When the asparaginase activity of the serum was inhibited by mercuric chloride, $HgCl_2$, the preparation no longer had any anti-tumor effect (Table I) and all animals treated with this inactive serum died within 10 to 11 days, well within the range of the control animals (Fig. 2). When guinea pig serum asparaginase activity was partially inactivated (90% inactivation of enzyme activity) with 3.5% PVP and used to treat tumor bearing C3H mice, slight prolonging of survival was seen.

A further attempt to correlate enzyme activity with tumor inhibition was made using partially purified asparaginase prepared from guinea pig serum. Sodium sulfate and calcium phosphate gel fractionations from GPS were prepared and protein content adjusted to 7.5 mg/ml. The asparaginase preparation was diluted to contain varying units of enzyme activity. The tumor bearing animals injected with preparations containing varying units of asparaginase showed the following results (Table I). All animals treated with 1.4 units/dose of asparaginase showed longer survival from the tumor implant, 90% at 15 days and 67% at the end of 30 days (Fig. 3). When a smaller quantity of asparaginase activity was injected, progressively less and less protection was afforded. This drop in protection parallels the drop in asparaginase content of the guinea pig serum as well as the calcium phosphate gel enzyme

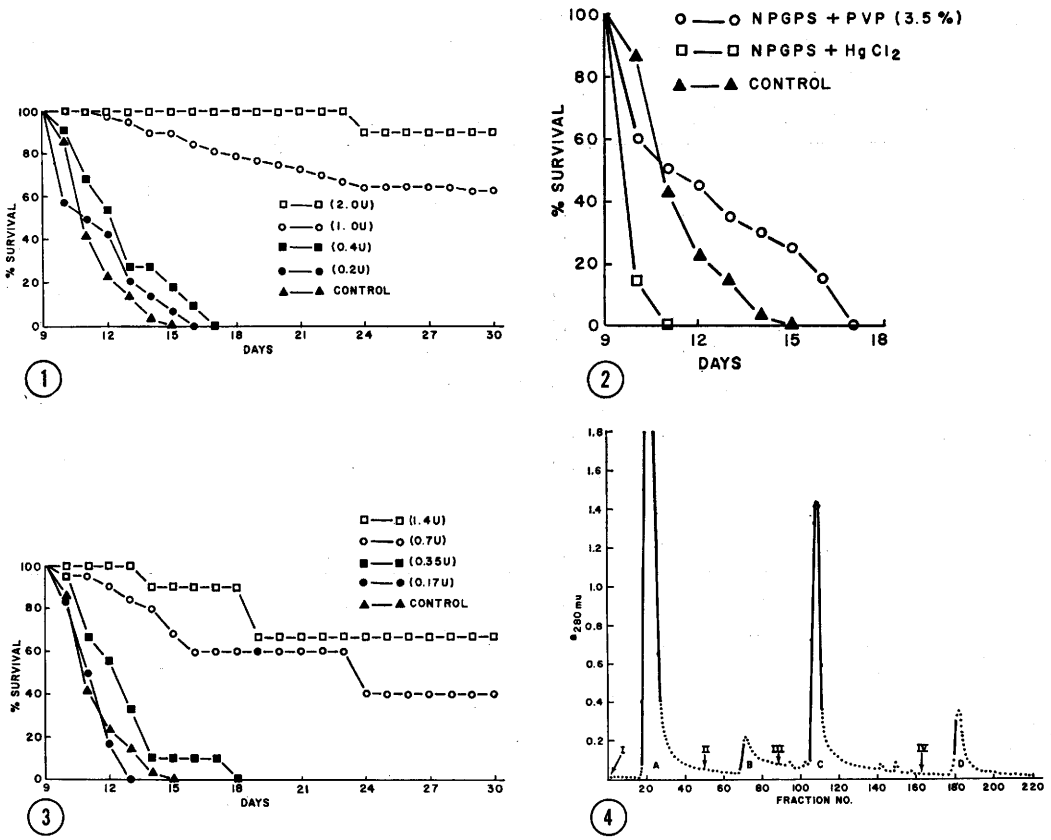

FIG. 1. Survival of tumor bearing mice treated with GPS containing different enzyme units (U). Control animals were treated with 0.5 ml RS.

FIG. 2. Survival of tumor bearing mice treated with GPS inactivated with 3.5% PVP and 1 millimole of $HgCl_2$.

FIG. 3. Survival of tumor bearing mice treated with $CaPO_4$ gel fractions containing varying units of enzyme activity.

FIG. 4. Stepwise elution chromatogram of a partially purified preparation of guinea pig serum L-asparaginase (precipitated with sodium sulfate and negatively adsorbed with calcium phosphate gel) on a DEAE cellulose column. A 32 ml sample of 7.48 mg protein/ml was added to the column (2.5 X 31 cm) conditioned at pH 7.0 with 0.005 M phosphate buffer. The effluent was collected in 6.0 ml aliquots at 0. Elution was carried out by the following buffer changes designated by Roman numerals: (I) 0.005 M phosphate, pH 7 0 (300 ml); (II) 0.02 M phosphate pH 0.9 (300 ml); (III) 0.02 M phosphate + 0.1 M NaCl, pH 5.9 (500 ml); and (IV) 0.02 M phosphate + 0.05 M NaCl, pH 5.9 (500 ml). Major protein peaks are designated by letters A to D. Only peak C showed asparaginase activity.

fraction (compare Fig. 1 & 3). Since the proof of identity that a single protein is responsible for 2 biological activities is difficult to establish, other procedures for purification were undertaken. The material obtained by calcium phosphate gel was purified further by chromatography on DEAE by the method of Tower(12). Results are shown in Fig. 4. The asparaginase activity was found in peak C. Individual peaks were pooled, concentrated and adjusted to contain 2 mg protein/ml. Assay for tumor inhibition showed only peak C to be effective (Fig. 5). The proof that the enzyme activity and the tumor inhibitory action is due to the same protein is difficult to establish because the DEAE isolated fraction still contained more than one component as evidenced by the immunoelectrophoretic studies on this fraction (13). However, the close correlation between enzyme activity and tumor inhibition suggests strongly that both activities are related.

TABLE I. Composite Results of the Various Preparations Used in the Study.

	No. animals tested in group	Enzymes units/dose	Volume injected/ dose (ml)	Mg total protein/dose	Specific activity	% survival at 15 days	% survival at 30 days
GPS	10	2.0	1.0	62.5	.0320 μ/mg	100	90
	40	1.0	.5	31.2		90	63
	22	.4	.2	12.5		18	0
	14	.2	.1	6.25		7	0
$CaPO_4$ gel fractions	9	1.4	1.0	7.5	.19 "	90	67
	19	.7	.5	3.75		78	40
	9	.35	.2	1.5	.23 "	10	0
	6	.17	.1	.75		0	0
(3.5%) PVP + GPS	20	.2	.5	62.5		25	0
$HgCl_2$ + GPS	14	No activity	.5	62.5		0	0
DEAE fractions: (A)	10	<.01	1.0	2.0		0	0
(DEAE preparation #6) (B)	10	<.01	1.0	2.0		0	0
(C)	10	1.0	.85	1.92	.52 "	80	40
(D)	10	<.01	1.0	2.0		0	0
RS	35	<.01	.5	62.0		0	0

The enzyme activity of guinea pig serum and the $Ca_3(PO_4)_2$ gel fractions, and the per cent survival at 15 days and at 30 days are tabulated in Table I. In both cases with a greater enzyme activity, a greater per cent survival is indicated. The protection afforded was a function of enzyme activity irrespective of total protein concentration.

In an attempt to determine if any of the enzyme activity binds directly to the tumor cells, the following experiments were performed. Viable lymphosarcoma cells, 63 $\times$ 10^6 cells/ml were sedimented and the packed cells incubated with GPS containing 2.0 units/ml for 1 hour. The cells were washed and assayed for bound enzyme activity and also tested by immunofluorescence for presence of adsorbed globulin. Rabbit serum with no enzyme activity served as control. In no instance was enzyme activity detectable on the cells as compared with controls. In addition, the enzyme solution used to treat the cells showed essentially no loss of activity in that 99.1% of the enzyme activity was recovered in the supernate.

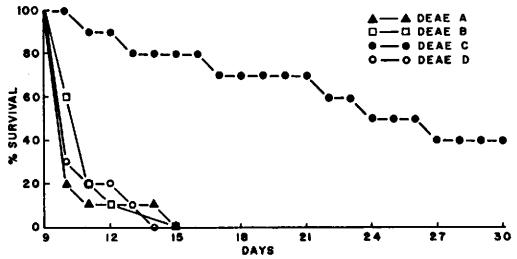

FIG. 5. Survival of tumor bearing mice treated with different DEAE fractions.

Fluorescent antibody studies using rabbit anti-guinea pig globulin showed no staining of the viable tumor cells treated with the guinea pig serum indicating that guinea pig globulin proteins did not adhere appreciably to the tumor preparations.

Discussion. These results show that the ascites form of the Gardner lymphosarcoma can be effectively inhibited by the guinea pig serum or its fractions provided that asparaginase is present. The data support Broome's hypothesis(9-11) and support the observation of others(14,15) that a direct correlation exists between the asparaginase content of guinea pig serum and its capacity to inhibit the Gardner lymphosarcoma.

The inactivation of normal pooled guinea pig serum asparaginase activity by mercuric chloride ($HgCl_2$) and PVP correlated with the loss of its capacity to protect mice bearing the ascites tumor. A comparison of the asparaginase activity of calcium phosphate gel fractions of guinea pig serum and normal guinea pig serum was made with respect to its tumor inhibitory capacity. By varying the enzyme units per dose used to treat tumor bearing animals, these preparations showed

that the enzyme activity paralleled its tumor inhibitory capacity. Further fractionation of the asparaginase preparation by DEAE column chromatography indicates that the activity is associated with the third peak. Comparative *in vivo* assay of all the peaks obtained once more demonstrates that only in the fractions showing enzyme activity was there any tumor inhibitory effect.

Broome has reported that in tissue culture, the 6C3HED cells dependence on L-asparagine is closely related to their sensitivity to guinea pig serum inhibition(11). In light of these observations, it is possible that the mechanism of regression *in vivo* is due to the enzyme converting all available asparaginase to aspartic acid which the tumor cannot utilize in the host. The possibility that the asparaginase enzyme or globulin affects the cells by direct attachment to the tumor cells was investigated. We were unable to detect asparaginase enzyme activity on tumor cells treated with the enzyme. Nor was there any loss of enzyme activity of GPS after such treatment with tumor cells. In addition the fluorescent antibody method was negative for visible attachment of guinea pig globulin components on viable tumor cells.

Summary. The guinea pig serum tumor inhibitory activity was effective for the ascites form of the Gardner lymphosarcoma. The active protein appears to be an enzyme L-asparaginase. Inhibition of the enzyme activity in normal pooled guinea pig serum in all cases caused a loss of the *in vivo* tumor inactivating capacity of the serum. Comparison of enzyme activity of fractionated serum correlates well with tumor destroying ability.

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Fate of Poliovirus in Northern Quahaugs.* (30841)

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Earliest documented outbreaks of infectious hepatitis associated with consuming raw oysters were reported in Sweden in 1956(1,2). Following this, two similar incidences occurred in the United States(3,4) and 4 outbreaks involving approximately 900 cases were traced to consumption of raw quahaugs or hard clams, *M. mercenaria*(5-7). Although the number of such cases is very small as compared with total incidence, it is important in that definite vectors be identified and measures taken to control this mode of transmission.

Studies on interaction of viruses and oysters have been conducted in recent years(8-10). It was shown that several species of shellfish were capable of picking up a significant amount of virus very rapidly and retaining it for as long as 28 days in dry storage.

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