In Vivo and in Vitro Effects of the L-Asparaginase Fraction of Guinea Pig Serum on the 6C3HED Ascites Lymphosarcoma

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Summary

The antitumor activity of guinea pig serum fractions was studied in vivo and in vitro by bright field, fluorescence, and electron microscopy observations and by dye permeability and cytidine-H incorporation in 6C3HED lymphosarcoma cells. In C3H mice bearing the ascites tumor treated with the L-asparaginase fraction, there was a decrease in the total number and mitosis of tumor cells, a reduction in the cytoplasmic fluorescence (RNA), and a rise in the number of macrophages and phagocytosis of tumor cells. Anti-6C3HED isoantibodies were not detected in their sera. The L-asparaginase fraction of guinea pig serum had in vitro cytotoxic activity on surviving cell cultures of 6C3HED lymphoma cells in a medium deprived of L-asparagine. Uptake of cytidine-H by tumor cells was found to be a more quantitative and sensitive method than the dye permeability test for measurement of the in vitro cytotoxic activity. A reduction in cytidine-H uptake in most tumor cells after 24 hr of in vitro treatment was found by radioautography. These results indicate that the L-asparaginase fraction of guinea pig serum has in vivo and in vitro antitumor activity. In vitro, this fraction acts directly on the tumor cells; hence, the in vivo host macrophage response and phagocytosis of structurally normal cells appears to be a secondary, nonspecific phenomenon.

Introduction

Normal pooled guinea pig serum (GPS) has shown antitumor activity against several transplantable tumors of mice and rats (1, 11, 13, 15) and certain spontaneous and radiation-induced leukemias (4). The evidence presented by Broome (6, 7) and others (10, 23) indicates that the L-asparaginase activity of GPS is responsible for its antitumor effect. Partially purified asparaginase obtained from guinea pig serum (6, 10), Escherichia coli (16), and guinea pig liver (23) has similar in vivo antitumor activity. In these experiments, the parameters used to measure tumor regression were the size of the tumor graft or animal survival. But the possibility of some host response should be considered when evaluating regression of transplantable tumors induced by antitumor agents without well-defined mechanisms of action.

Studies on the in vitro effects of GPS or GPS fractions on tumor cells may prove useful to gain a better understanding of its mechanism of action. Kidd (13) reported that tumor cells incubated in vitro with GPS for 4-6 hr grew at the same rate as untreated cells when implanted in susceptible mice. A preliminary presentation of evidence for the in vitro cytotoxic effects of GPS on 6C3HED lymphoma cells maintained in short-term cultures was recently made (3). Concurrently, Patterson et al. (17) demonstrated that GPS has an inhibitory effect on the in vitro growth of the Jensen sarcoma.

The aim of the present study was to investigate the reaction of the host during GPS-induced regression of the 6C3HED lymphosarcoma, ascites form, and to extend our previous study on the in vitro cytotoxic effects of the L-asparaginase fraction of GPS.

Materials and Methods

Adult guinea pigs of mixed strain and sex were bled by cardiac puncture. The procedures used to obtain and fractionate the serum have been described (10). The 4 major protein fractions obtained from the diethylaminoethyl (DEAE) column by the method described by Tower (24) are shown in Chart 1. They were designated as GPS Fractions A, B, C, and D. L-Asparaginase activity of GPS, normal pooled rabbit serum (RS), and of each of the fractions was assayed as previously described (10). One unit of L-asparaginase was defined as the quantity of enzyme that catalyzes the formation of 1 μmole of ammonia per min at 37°C. The dilutions of GPS, RS, Fractions A, C, and D used in these experiments had, respectively, the following enzyme activities and protein concentrations: 2.0 units, 62.5 mg/ml; <0.01 unit, 62 mg/ml; <0.01 unit, 2 mg/ml; 1.0 unit, 2 mg/ml; <0.01 units, 2 mg/ml. Fraction B was not used in these experiments because of its low protein concentration.

6C3HED ascites lymphosarcoma, obtained from T. Hauschka, Roswell Park Memorial Institute, Buffalo, New York, was maintained in our laboratory by weekly i.p. transplants in adult male C3H/HeJ mice.

Seventy C3H/HeJ male mice were inoculated i.p. with 30 × 10⁶ 6C3HED lymphosarcoma cells and randomized in 6 groups. Ten mice in Group I received no treatment. The mice of Groups II (15), III (15), IV (10), V (10), and VI (10) received, respectively, GPS, RS, Fractions A, C, and D, 1.0 ml i.p., 6 days after tumor inoculation. Study of the ascitic fluid was performed on Day 6 in the untreated mice and at 20, 40, and 70 hr after treatment in Groups IV and V. The mice were sacrificed, their peritoneal cavities washed with cold Tyrode's medium, the recovered fluid measured in calibrated tubes, and the amount of ascites...
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CHART 1. 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 (25 x 31 cm) conditioned at pH 7.0 with 0.005 M phosphate buffer. The effluent was collected in 0.0-ml aliquots at 0. Elution was carried out by the following buffer changes designated by roman numerals: /, 0.005 M phosphate, pH 7.0 (300 ml); II, 0.02 M phosphate, pH 5.9 (300 ml); III, 0.02 M phosphate + 0.1 M NaCl, pH 5.9 (500 ml); and IV, 0.02 M phosphate + 0.5 M NaCl, pH 5.9 (500 ml). Major protein peaks are designated by letters A-D. Only Peak C showed asparaginase activity.

calculated by subtracting the added Tyrode fluid from the total amount obtained per mouse. Total cell counts were performed with a WBC pipet and a clinical hemocytometer, and aliquots of the fluid were used for bright field, fluorescent, and electron microscopy. Slides for ascites cell differential counts were stained with Wright-Giemsa. On each slide, 300 cells were counted.

Smears of ascites cells on glass slides were fixed in glacial acetic acid-absolute alcohol (1/3) and stained by the acridine orange technic (AO) (2), and examined under the ultraviolet microscope. For electron microscopic (EM) studies, ascites cells were sedimented by gentle centrifugation, fixed in 2% glutaraldehyde in Hanks' balanced salt solution (BSS) (pH 6.4–7.0) for 15 min, and then postfixed for 1 hr in 1% osmic acid in Hanks' BSS. Following rapid dehydration in alcohol, the cells were embedded in Epon by conventional methods. Thin sections were stained with lead citrate and observed in a Siemens Elmiskop I microscope (19).

Blood was obtained from each mouse by cardiac puncture, and the serum tested for cytotoxic antibodies against 6C3HED lymphoma cells by the method of Boyse et al. (5).

For the in vitro experiments, the lymphoma cells were obtained aseptically from the peritoneal cavity of C3H mice 5 days after implant. Spleens from C3H mice were placed in Petri dishes containing TC199, cut in small pieces, and teased with a 21-gauge needle until most of the cells were separated. Ehrlich's ascites cells were obtained aseptically from the peritoneal cavity of Swiss mice 10 days after i.p. inoculation of 20–40 x 10⁶ tumor cells. The same procedure was used for the in vitro cultures of 6C3HED lymphosarcoma, Ehrlich's ascites, and C3H spleen cells. The cells were washed twice with cold TC199 (Difco) and incubated at 37°C in roller tubes containing 2 ml of TC199 (pH 7.4) with 0.29 mg of glutamine, 400 units of penicillin, and 1.5 x 10⁶ cells per ml of medium. In some experiments, 10% calf serum was added.

In all the experiments, L-asparagine was omitted from the media; when calf serum was added, the media was tested for L-asparaginase by 2-dimensional chromatography. To rule out the release of L-asparagine from damaged tumor cells that might be reutilized by intact cells, the following test was performed. 6C3HED cells (2 x 10⁶) were suspended in 2 ml of TC199 media. Repeated freezing and thawing killed 100% of these cells, as demonstrated by their permeability to trypan blue. After centrifugation, the supernatant was tested for L-asparaginase by 2-dimensional chromatography, with negative results.

For examination of the toxic effects of the various fractions, 0.2 ml of RS, GPS, or GPS Fractions A, C, and D was added to 2 x 10⁶ 6C3HED lymphoma, Ehrlich's ascites, or C3H spleen
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To measure the in vitro cytotoxic effects of fractions of GPS, 3 methods were used and compared:

(a) CELL PERMEABILITY TO TRYPAN BLUE. Total cell count of each culture was performed with a WBC pipet and a clinical hemocytometer. An aliquot of the cell suspension was mixed on standard glass slides with a 0.04% solution of trypan blue (5). The % of nonpermeable cells X the number of cells per culture was expressed as number of viable cell cultures.

(b) INCORPORATION OF CYTIDINE IN VITRO. Cytidine-3H in sterile water (2.0 c/m mole) was obtained from New England Nuclear Corporation. Nine ml of TC199 containing 2 μC of cytidine-3H were pipetted into 15-ml centrifuge tubes. At 0, 6, 12, 18, 24, and 72 hr after the in vitro treatment, 1.0 ml of the cell suspension (1.5 X 10⁶ cells) was added to each tube. For control, another 1.0 ml of the cell suspension was added to a tube containing 9 ml of TC199 without cytidine. In the 1st experiments, the tubes were incubated in a water bath at 37°C for 0, 60, 120, and 180 min; thereafter, the incubation was stopped at 180 min and the tubes centrifuged at 2°C and 600 x g for 5 min. The pellet was then washed with 10 ml cold saline, precipitated twice with 5 ml cold 5% trichloroacetic acid (TCA), and washed twice with 5 ml cold methanol. All centrifugations were performed at 2°C and 600 x g for 10 min. The final precipitate was then redissolved in 1 ml of hydroxide of Hyamine (Packard Instrument Co.) and kept at room temperature for 1 hr. Ten ml of a solution of toluene containing dimethyl POPOP, 100 mg/liter, and 2,5-diphenyloxazole (PPO), 5 gm/liter (both obtained from Packard Instrument Co.), were added, the samples kept overnight at 4°C, transferred to plastic vials, and counted for 1 min in a liquid scintillation counter (Packard Model 500D).

(c) RADIOAUTOGRAPHY. The procedure described above in the in vitro incorporation of cytidine-3H was used for radioautography. Following 180 min incubation at 37°C, the cells were washed with 10 ml of TC199. After centrifugation, the supernatant was discarded and 0.5 ml of 50% normal mouse serum in saline added to the pellet. A small drop of this suspension was smeared on regular microscope slides, fixed with acid alcohol, and washed twice with 5 ml cold methanol. All centrifugations were performed at 2°C and 600 x g for 10 min. The final precipitate was then redissolved in 1 ml of hydroxide of Hyamine (Packard Instrument Co.) and kept at room temperature for 1 hr. Ten ml of a solution of toluene containing dimethyl POPOP, 100 mg/liter, and 2,5-diphenyloxazole (PPO), 5 gm/liter (both obtained from Packard Instrument Co.), were added, the samples kept overnight at 4°C, transferred to plastic vials, and counted for 1 min in a liquid scintillation counter (Packard Model 500D).

Results

In Vivo

DETECTION OF HUMORAL ANTIBODIES. We were unable to demonstrate anti-6C3HED lymphosarcoma isohantibodies in the serum of untreated C3H mice with or without tumor (6 days after implant). Neither were humoral antibodies detected in the serum of C3H mice treated with RS, GPS, or Fraction A, C, or D, 20 and 70 hr after inoculation.

LIGHT MICROSCOPY. C3H mice inoculated i.p. with 30 X 10⁶ 6C3HED lymphosarcoma cells developed ascites tumors that killed 100% of the animals in 8–12 days.

Six days after tumor inoculation the amount of peritoneal fluid in mice of the untreated group ranged from 1.5–2.0 ml with 20 X 10⁶ to 35 X 10⁶ tumor cells/mouse. The mean number of tumor cells in mitosis was 2.3 X 10⁷, and the mean number of macrophages was 0.2 X 10⁷/mouse. Macrophages with phagocyted tumor cells, or in the process of phagocytosis, were seen only on careful searching, and calculated to be less than 10% of the total macrophages.

We have reported a direct correlation between the 50% survival of mice bearing the 6C3HED lymphoma, ascites form, and the L-asparaginase activity of GPS fraction. The 50% survival was 10.8 days in the group treated with RS, and 9.7, 10.2, and 9.8 days in the group treated with GPS Fractions A, B, and D, respectively. Neither RS nor Fractions A, B, and D have L-asparaginase activity. The 50% survival of mice treated with GPS or the fraction with L-asparaginase activity (C) was >30 and 24 days, respectively.

In this study, we did not find any significant difference in the number or morphology of the ascites cells of untreated mice, and mice treated with RS or GPS Fractions A and D. The mean number of tumor cells in mitosis was 1.5 X 10⁷ at 20 hr and 0.5 X 10⁷ at 70 hr. The mean number of peritoneal macrophages increased in both GPS and Fraction C treated mice from 1.0 X 10⁷ at 20 hr to 0.3 X 10⁷ at 70 hr. No increase in phagocytosis of tumor cells was observed.

In contrast, in mice receiving GPS i.p., the mean value of tumor cells per mouse was reduced from 17.4 X 10⁶, 20 hr after the 1st injection, to 6.6 X 10⁶, 70 hr later. A similar reduction in the total number of tumor cells was found in the group of mice treated with Fraction C (Table 2). In this group the mean number of tumor cells in mitosis per mouse was 1.5 X 10⁷ at 20 hr and 0.5 X 10⁷ at 70 hr. The mean number of peritoneal macrophages increased in both GPS and Fraction C treated mice from 1.0 X 10⁷ (GPS) and 0.9 X 10⁷ (C) at 20 hr, to 5.0 (GPS) and 4.1 (C) at 70 hr. Phagocytosis of tumor cells became evident after 20 hr, and markedly increased at 70 hr when 1/2 to 1/4 of the macrophages were found to contain tumor cells. In many instances we observed macrophages surrounded by, and in close contact with, several tumor cells. When examined by light microscopy, most of these tumor cells appeared normal and were not permeable to the trypan blue dye.

ACRIDINE ORANGE. When tumor cells from untreated mice were stained by the acridine orange technic and examined under...
the ultraviolet microscope, DNA fluoresced yellow-green and RNA fluoresced bright red. The fluorescence of DNA appeared more intense in dividing cells, which were easily recognized even under low power. Macrophages, in contrast, fluoresced poorly: the cytoplasm with a brownish-red, and the nucleus with a greenish fluorescence. In mice treated with RS or Fraction A or D, the color and intensity of the peritoneal cells were similar even under low power. Macrophages, in contrast, fluoresced poorly: the cytoplasm with a brownish-red, and the nucleus with a greenish fluorescence. In mice treated with RS or Fraction A or D, the color and intensity of the peritoneal cells were similar even under low power.

In Vitro

Lymphoma 6C3HED cells cultured in a medium devoid of L-asparagine (TC199) survived during the 1st 72 hr without significant changes in the number of cells per culture. This initial period was followed by a rapid increase in cell death, fibroblasts and cell debris; but very few tumor cells were seen in most of the cultures by the 5th day. No difference was found in the in vitro uptake of cytidine-^H by lymphoma cells cultured in medium without L-asparagine for 0, 12, 24, or 48 hr, but uptake was reduced markedly (50-90%) in cells cultured for 72 or more hr. This confirmed the results of Broome and indicated that 6C3HED cells cultured in vitro require L-asparaginase for growth.

Broome (7) suggested that the L-asparaginase of GPS acts on the extracellular fluids of the host, lowering the levels of L-asparagine and depriving the tumor cells of an essential nutrient. L-Asparaginase administered in high doses does not, however, reverse the in vitro antitumor activity of GPS L-asparaginase. The following experiments were performed to determine whether GPS or any of its fractions has antitumor activity in the absence of extracellular L-asparagine.

It was found that GPS and GPS-Fraction C had in vitro cytotoxic activity on lymphoma 6C3HED cells cultured in a medium deprived of L-asparagine. There was no significant difference in the number of dye-permeable cells in control or GPS-Fraction C-treated cultures during the 1st 18 hr of treatment; but of the GPS-Fraction C-treated tumor cells, 30-40% were dye-permeable at 24 hr and 80-90% after 48 hr.

In an attempt to develop a more quantitative and objective test for evaluation of cell damage, particularly during the 1st 24 hr, we measured the in vitro uptake of cytidine-^H by tumor cells and compared these results with the dye permeability test. In the 1st experiment, we compared the uptake of cytidine at 60, 120, and 180 min in tumor cells treated with GPS-Fraction C and in control tumor cells treated either with Fraction A, D, RS, or normal saline for 24 hr. Because the incorporation of cytidine was similar in all the controls, Fraction A was used as control in all the following experiments. As seen in Chart 2, there was a significant difference in the uptake of cytidine between cells treated with Fraction C and control tumor cells. The mean cpm/ml of cultures treated with Fraction C was 30% of the cpm/ml of cultures treated with Fraction A at 60 min and 25% at 180 min.
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**Chart 2.** *In vitro* uptake of cytidine-\(^{3}H\) by 6C3HED lymphoma cells after 24-hr incubation at 37°C with 0.1 ml of GPS Fractions A or C/1.5 \(\times 10^6\) cells. • — •, Fraction A; ○ — ○, Fraction C. Each point represents the mean of 4 cultures. Bars indicate standard errors.

**Chart 3.** Correlation between permeability to trypan blue dye and cytidine-\(^{3}H\) uptake in 6C3HED lymphoma cells treated *in vitro* with Fraction C (0.4 mg of protein and 0.2 unit of L-asparaginase/\(3 \times 10^6\) cells). Control cells were treated with Fraction A (0.4 mg of protein and 0.0 unit of L-asparaginase/\(3 \times 10^6\) cells). • — •, (cpm/ml Fraction C culture)/(cpm/ml Fraction A culture) \(\times 100\); ○ — ○, (unstained cells/ml Fraction C culture)/(unstained cells/ml Fraction A culture) \(\times 100\). Each point represents the mean of 4 cultures.
Permeability to trypan blue and cytidine uptake were measured and compared in tumor cells treated with Fractions A or C for 6, 12, 18, 24, 32, and 48 hr. The results are shown in Chart 3. There was a correlation between the values obtained by both methods, the uptake of labeled cytidine being more sensitive. Its incorporation was significantly reduced in 6C3HED lymphoma cells treated with Fraction C for 12 hr, although no cytotoxicity was demonstrated by the trypan blue test. Dye permeability and % reduction in cytidine uptake rapidly increased between the 18th and 24th hr. Cells treated with Fraction C for 32 hr did not incorporate cytidine, but 30% were nonpermeable to trypan blue.

Ehrlich's ascites cells and normal C3H spleen cells, which are not sensitive to Fraction C in vivo, were not affected when treated in vitro for 48 hr (Chart 4). This provided further evidence of a correlation between the in vivo and in vitro antitumor activity of Fraction C and demonstrated that the in vitro cytotoxicity is not due to a nonspecific contaminant in this fraction.

Reduction of cpm in cultures treated with Fraction C could be explained by 2 mechanisms: (a) no uptake in a certain % of cells and normal incorporation by the rest; or (b) mild to marked uptake impairment in most tumor cells. By radioautography it was found that most of the cells treated with Fraction C for 24 hr incorporated less than the control (Chart 5). More than 40 grains/cell were counted in 67% of the control cells and in only 13% of those treated with Fraction C.

Discussion
The results of our experiments confirmed that the fraction of GPS with antitumor activity also possessed L-asparaginase ac-
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Our findings revealed an early and very active phagocytosis of apparently normal tumor cells by peritoneal macrophages indicating participation of the host in the process of tumor regression. The specificity of this response seems, however, questionable. A reduction in the total number of tumor cells, or tumor cells in mitosis, was demonstrable 20 hr after the first injection of GPS or Fraction C. Changes in the intensity and color of the cytoplasmic fluorescence (RNA) were easily observed at 40 hr in most of the tumor cells. These changes indicate that the tumor cells were primarily damaged by the effects of GPS or Fraction C, and suggest that the host macrophage response is secondary and nonspecific, but nevertheless contributes to the rapid regression of the tumor.

Tumor cells ingested by host macrophages from mice treated with GPS or Fraction C, appeared, in many instances, to have an intact fine structure. Increased phagocytosis of tumor cells was seen only in mice treated with GPS or the antitumor fraction. Based on these observations it appeared that early changes induced by the L-asparaginase fraction were not detectable at the cytologic level, but were sufficient to make the tumor cells susceptible to phagocytosis by host macrophages.

A direct effect of Fraction C on the tumor cells was indicated by our in vitro experiments. It was shown that in very high doses, Fraction C is cytotoxic to 6C3HED lymphoma cells in the absence of any host factor. Broome (7) and Patterson (17) suggested that the L-asparaginase of GPS acts not directly on the tumor cells, but on the L-asparagine of the extracellular fluid. However, the in vitro antitumor activity was not reversed by high doses of L-asparaginase (7). We presented evidence that Fraction C had in vitro cytotoxic activity in the absence of L-asparaginase in the medium. It is possible that L-asparaginase of GPS may act directly in an intracellular pool of L-asparagine.

This intracellular pool could also explain the survival and normal cytidine uptake for 48–78 hr of the asparagine-dependent 6C3HED lymphoma cells cultured in a medium deprived of L-asparagine. L-Asparaginase that could be liberated into the medium by dead 6C3HED cells was not detected by 2-dimensional chromatography; but this may be related to cell permeability or sensitivity of the method.

The incorporation or release of labeled cellular components by damaged cells has been used to quantitate antitumor cells' antibodies or to measure cellular antigens. Hansen demonstrated uptake inhibition of 14C-labeled amino acids in 6C3HED lymphoma and 6-180 cells treated with specific antisera (8). The release of radioactivity from target cells labeled with thymidine-3H was correlated with the antibodies' cytotoxic effect, as determined by supravital staining (14). Haughton measured tumor-specific antigen by an inhibition test using the same principles and determined the effects of anti-Moloney tumor antibodies by the liberation of 3Cr from labeled cells (9). Our findings demonstrated that the measurement of cytidine-3H uptake by 6C3HED lymphoma cells can be used as an objective and quantitative assay for the in vitro cytotoxic activity of the GPS, L-asparaginase fraction. This method may be utilized in 24-hr cultures and is more sensitive than the trypsin blue assay. For these reasons it may prove useful, in combination with radioautography, to study further the mechanism of action of the L-asparaginase.

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fraction of GPS and its in vitro effects on animal and human tumors.

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References

7. ———. Evidence that the L-Asparaginase of Guinea Pig Serum is Responsible for its Antilymphoma Effects. II. Lymphoma C3H/He Cells Cultured in a Medium Devoid of L-Asparagine lose their Susceptibility to the Effects of Guinea Pig Serum In Vivo. Ibid., 118: 121-48, 1963.
Fig. 1. A section to illustrate some typical features of the 6C3HED ascites tumor cell. The single, lobulated nucleus often appeared binucleate because of the angle of thin sectioning. The Golgi apparatus, usually with distended sacculles, was located near the nucleus. Tubular elements of the endoplasmic reticulum were sparse, but free ribosomes were densely packed in the cytoplasm. X 24,000.
Fig. 2. This plate represents part of a 6C3HEI tumor cell showing annulate lamellae structures which are frequently found in normal embryonic tissues as well as tumor cells. × 24,000.

Fig. 3. Portion of an ascites tumor cell with the nuclear envelope evident in the upper right of the plate. Numerous virus-like particles are seen in the cytoplasm and, in some instances, appear to lie within the cisternae of the endoplasmic reticulum. × 30,000.
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