



Immunosuppression by L-asparaginase
by Joe Kay Moody

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
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Abstract:

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Intraperitoneal enzyme suppression of the LPS response reduced the plaque forming cells per spleen and per million spleen cells while IV suppression reduced only the plaque forming cells per spleen. Intraperitoneal enzyme treatment conceivably affected the macrophage processing and/ or presentation of antigen, therefore reducing the plaque forming cell per spleen and per million cells.

Intravenous treatment eliminated a non-antibody producing cell ("T" cell) and the macrophage, thereby reducing the total number of cells in the spleen which artificially elevated the antibody producing cells ("B" cells) per million spleen cells. Enzyme induced prolongation of allograft survival also implicated the "T" cell and possibly the macrophage. Immunoglobulin levels were not affected by enzyme which reinforced the idea of a resistant "B" cell.

It was concluded that the macrophage and "T" cells were affected by L-asparaginase in the experiments described, and that "B" cells were not the primary target of the enzyme.

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ABSTRACT

The immunosuppressive effects produced by E. coli L-asparaginase and the cell and/or cell types affected were investigated.

L-asparaginase was shown to suppress the immune responses to SRBC, LPS, SSS III and to prolong allograft survival. The suppressed response to SRBC was reconstituted with normal and SRBC primed peritoneal macrophages. Bone marrow was not able to reconstitute the LPS response in an irradiated, enzyme-treated mouse, but did reconstitute the response in an irradiated recipient which did not receive enzyme. This implied the presence of a radio-resistant cell in the recipient, conceivably the macrophage, which interacted with the donor bone marrow. Intravenous enzyme treatment caused atrophy of the liver, spleen, lymph nodes, and the thymus while IP treatment affected only the thymus.

Intraperitoneal enzyme suppression of the LPS response reduced the plaque forming cells per spleen and per million spleen cells while IV suppression reduced only the plaque forming cells per spleen. Intraperitoneal enzyme treatment conceivably affected the macrophage processing and/or presentation of antigen, therefore reducing the plaque forming cell per spleen and per million cells.

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It was concluded that the macrophage and "T" cells were affected by L-asparaginase in the experiments described, and that "B" cells were not the primary target of the enzyme.

INTRODUCTION

In 1953, Kidd (23,24) observed that certain murine leukemias were inhibited by a component of guinea pig serum, later identified as the enzyme, L-asparaginase. In recent years the enzyme was shown to be a potent producer of immunosuppression by virtue of its cytostatic or cytotoxic effects on lymphocytes (17,18,45).

Most animals and bacteria produce a form of L-asparaginase that causes conversion of asparagine to aspartic acid and ammonia. Only a few forms of the enzyme have been shown to produce the anti-leukemic and/or the immunosuppressive effects.

Initially, small quantities of the active enzyme were obtained from the members of the Superfamily Cavioidae, of which the guinea pig is a member, but bacteria have been the main source of L-asparaginase. Escherichia coli became the main source of L-asparaginase in the early sixties. The enzyme from E. coli has the same molecular weight (138,000) and the same anti-leukemic and immunosuppressive properties as the guinea pig enzyme.

The L-asparaginase from certain strains of E. coli was purified into two distinct forms; E.C. I and E.C. II.

The E.C. I fraction contained very little anti-leukemic or immunosuppressive activity while the E.C. II fraction had maximum activity. These two fractions were separated by varying the concentrations of ammonium sulfate in the protein precipitation steps during purification.

In 1966, Schwartz, et al. (39) showed that the E.C. I fraction was constitutive in E. coli and had a low avidity for its substrate; the E.C. II fraction was inducible and possessed a high substrate avidity.

The proposed mechanisms of anti-leukemic action of E. coli L-asparaginase were provided by the studies of Broome and Schwartz (9), Broome (11, 11a), and Sobin (41). These studies showed that resistant leukemic cells had an increased ability to synthesize asparagine and did not require exogenous amino acid for protein synthesis. Asparagine synthetase, an enzyme facilitating the transfer of an amino group from glutamine to aspartic acid yielding asparagine, was deficient or absent in sensitive cells, while resistant cells maintained relatively high levels. All normal cells possessed synthetase activity and low levels could act as an indicator for sensitivity to L-asparaginase (11,41).

Immunosuppression by L-asparaginase was also believed to be caused by depletion of exogenous asparagine

(18,45). Friedman and Chakrabarty (18) supported this premise by their ability to reverse the immunosuppressive effects by supplementation of exogenous asparagine. Weksler and Weksler (45) showed the same results by using an interesting dialysis system that removed the exogenous asparagine by L-asparaginase without allowing the latter to contact the cells.

Asparaginase was shown to lower the amount of asparagine in the blood for 42 to 72 hours post-treatment. These blood concentrations of asparagine were much less than required for protein synthesis in vitro (12). Other tissues displayed a reduction in asparagine levels, but three hours after treatment there was a gradual increase in asparagine levels in normal tissues. This increase was not seen in the blood or in asparaginase sensitive lymphoma cells. An increase in normal tissues despite low levels of serum amino acid at three hours suggested that synthesis of asparagine was taking place within the resistant cells. Within 48 hours after treatment with asparaginase, normal mitotic indices were obtained in normal tissues but not in sensitive lymphoma cells. Thus, lymphoma cells appeared to be irreversibly damaged by the action of L-asparaginase. Based on

these observations, Becker and Broome (5) postulated that asparaginase interfered with some early stage in cell proliferation.

A controversy yet to be resolved was initiated by the work of Benezra, et al. (6). They alluded to the possibility that asparaginase was not responsible for tumor remissions or immunosuppression. Their data showed, at least in an in vitro system, that glutaminase caused the suppressive effects and that depletion of asparagine did not play a critical role in immunosuppression. This exception can only be explained by differences in experimental protocol and enzyme preparations since many preparations of L-asparaginase causing anti-leukemic and immunosuppressive effects were reported free of glutaminase activity.

In the middle to late sixties, reports appeared which suggested that L-asparaginase from E. coli inhibited lymphocytic blastogenesis (3,16,26,35). Using human lymphocytes, Astaldi, et al. (3) found that upon phytohaemagglutinin (PHA) stimulation, those lymphocytes previously treated with enzyme failed to attain the control levels of mitosis. Thus, human peripheral lymphocytes apparently required L-asparagine for their PHA induced transformation and were not able to synthesize this amino acid in sufficient

quantities when exposed to enzyme. Weksler and Weksler (45) also found that asparaginase markedly inhibited PHA induced lymphocytic transformation and did not interfere with cell viability as measured with trypan blue exclusion.

In a related study, Weiner, et al. (44) showed that protein synthesis in lymphocytes was reduced to 40% of the controls in the absence of L-asparagine. In addition, cells depleted of asparagine with enzyme or cells grown in a medium lacking the amino acid, displayed decreased mitotic indices and reduced levels of protein synthesis.

In 1969, Schwartz (40) suggested the possible relationships between the inhibition of lymphocyte blastogenesis and the immunosuppressive effects caused by L-asparaginase. Using sheep erythrocytes (SRBC) as an antigen and the Jerne Plaque Assay (21) for enumeration of IgM producing cells, Schwartz found that immunosuppression was established over a wide range of enzyme doses, 25 to 1000 International Units (I.U.). Berenbaum (7) predicted a maximum effect would occur two days after contact with the antigen, the time corresponding to a period of maximum cell proliferation after antigenation. Interestingly, the effect was maximum from 48 hours before to 4 hours after asparaginase administration. The effect of the enzyme was temporary and after an interim

of 6 days without treatment, the animals would respond normally to antigens. The importance of timing was also confirmed by Chakrabarty and Friedman (15).

Berenbaum (7) investigated the effectiveness of various enzyme preparations from different sources. The effectiveness of the L-asparaginase varied tremendously with the source from which it was derived. The in vivo half-life in mice differed from 19 hours with guinea pig asparaginase to 30 minutes with yeast enzyme. Berenbaum (7) also showed that yeast asparaginase with ineffective in producing immunosuppression while the guinea pig enzyme was very effective.

Since the pioneering work of Schwartz (40) and Berenbaum (7), numerous studies have confirmed the suppressive effects of the enzyme on the response to SRBC (12,15, 18,19,29,30,31,35,40). These reports demonstrated how both serum titers and antibody producing cells in the spleen were reduced by administration of L-asparaginase. In these studies, enzyme doses were varied from 10 to 1000 I.U., and both enzyme and antigen were given by the intraperitoneal (IP) route.

More recent investigations have included the use of a thymus independent antigen, endotoxin (LPS), to investigate

the effect of L-asparaginase on bone marrow derived or thymus derived lymphocytes. Friedman (18) demonstrated that IP administration of asparaginase suppressed the response to 100 μ gm of LPS administered by the same route.

Kahn and Hill (22) found the response to bovine serum albumen was suppressed by L-asparaginase extending the immunosuppressive effects to a pure protein antigen.

L-asparaginase was found to prolong the survival of allografts (18,25,30,38,42,45). Graft survival was shown to depend on a critical timing of enzyme administration. Since both reduction of the humoral response and homograft survival depended on early contact with the enzyme, the early "processing" phase of the immune response to antigens seemed to be affected. The second set graft rejection was delayed by the presence of asparaginase (18), but Nelson, et al. (30) failed to suppress the secondary response to SRBC. The latter is in contrast to the observations by Friedman and Chakrabarty (18) who were able to obtain suppression of the secondary response to SRBC. Friedman and Chakrabarty (18) showed a greater reduction in the numbers of 7S plaque forming cells than in the 19S producing cells when the secondary response was impaired. This suggested a blockage of the 19S to 7S transition characteristic of a secondary response. On

the other hand, suppression of a primary response to SRBC with L-asparaginase depleted the numbers of both 7S and 19S antibody producing cells.

Since L-asparaginase produced an impaired antibody response to a variety of antigens, it would not be unexpected to find lowered immunoglobulin levels. In 1970, Burgio, et al. (13) measured the gamma globulin levels in children with acute lymphocytic leukemia and rheumatoid arthritis who were under asparaginase treatment. They found the gamma globulin levels slightly increased. The reason for this increase is presently unexplainable.

The cellular site of action of L-asparaginase has recently received attention by several groups of investigators (17,45). In 1971, Friedman (17), using a bone marrow-thymus reconstitution system, showed a cell within the bone marrow to be a target for asparaginase. Thus, Friedman was able to reconstitute the immune capacity of lethally irradiated mice with thymus cells from enzyme treated mice and bone marrow from non-enzyme treated mice.

In 1971, Weksler and Weksler (45) investigated many aspects of L-asparaginase induced immunosuppression. Lymphocytopenia with spleen, thymus, and lymph node atrophy was produced by L-asparaginase treatment. Adrenalectomized and

enzyme-treated mice displayed reduced responses to antigens which indicated that L-asparaginase induced immunosuppression did not require adrenal steroids.

Hobik (20) was able to suppress the graft-versus-host (GvH) reaction by treatment of spleen cells with asparaginase. Weksler and Weksler (45) were also able to suppress the GvH reaction by incubation of donor spleen cells with enzyme in vivo but not in vitro.

Rodney and Good (37) showed that PHA stimulated lymphocytic transformation was dependent upon a population of thymus derived lymphocytes. In view of Weksler and Weksler's (45) observations on PHA stimulated blastogenic inhibition, marked thymic atrophy, and homograft prolongation, he postulated the thymus derived lymphocyte was the affected cell.

Weksler and Weksler (45) also noted an abnormal migration or "homing" of asparaginase treated lymphocytes. Labeled lymphocytes from nodes were incubated in vitro with L-asparaginase and injected into syngenic recipients. The enzyme treated lymphocytes migrated primarily to the liver instead of the spleen and lymph nodes.

The purpose of this work was to investigate the cellular and humoral aspects of L-asparaginase induced

immunosuppression. Major emphasis was placed on the mechanisms underlying the suppressive effect caused by the enzyme and the various cells and/or cell types affected by L-asparaginase.

Recent concepts of cells or cell types affected will be reviewed and discussed. Investigations were carried out to further clarify the total scheme of events involved in L-asparaginase induced immunosuppression.

MATERIALS AND METHODS

I. Mice

Inbred Balb/c (H-2^d) mice obtained from the National Institutes of Health (N.I.H.) were used throughout the study. CBA/J (H-2^k) mice (Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine) were used as donors of skin grafts, and A/Jax (H-2^a) mice were used in some initial weight loss studies. All mice were fed autoclaved Purina 50-10-c fortified mouse chow and acidified-chlorinated water (Appendix I). All cages were cleaned weekly and filled with sterilized corn cob bedding. Litters were weaned at 4 weeks of age and all mice used in the study, with the exception of 4 to 6 month old macrophage donors, were between 4 and 12 weeks of age.

II. L-asparaginase

Lyophilized preparations of E. coli L-asparaginase ("Lyovac") were obtained from Merck, Sharp, and Dohme (West Point, Pennsylvania). Each bottle contained 50,000 International Units (I.U.) of the enzyme plus 200 mg of mannitol. The enzyme was weighed out immediately before

use and rehydrated in 0.25 ml of physiological (0.85%) saline for injection. After the vials were opened, the powdered enzyme was kept at -70°C . until use and was found to maintain its immunosuppressive activity for 40 to 60 days.

The enzyme dosages varied from 50 I.U. to 1000 I.U. per dose, depending on the experimental design.

III. Antigens

Sheep erythrocytes (SRBC) in Alsevers (Colorado Serum Company, Denver, Colorado) were washed three times in saline and resuspended in saline to the desired concentration.

Endotoxin (LPS) (00111:B4, Lot558185) was purchased from Difco Laboratories (Detroit, Michigan) in 100 mg quantities. For injection, the LPS was resuspended in saline to a concentration of 40 μgm per ml and a volume of 0.25 ml was administered. For coating SRBC, the LPS was suspended in 0.01 M phosphate buffer (Appendix I) pH 7.4 and boiled for 2 1/2 hours. A 1:10 dilution was made in phosphate buffered saline (PBS) (Appendix I) and 10 ml of diluted, boiled LPS was added to 0.25 ml of packed, thrice-washed, SRBC. This mixture was incubated at 37°C . for 30 minutes, washed three

times in PBS, and resuspended in Duttons Balanced Salts Solution (DBSS) at a 15% concentration for plaquing.

Pneumococcal Polysaccharide Type III (SSS III), kindly supplied by Dr. P. J. Baker, NIH, Bethesda, Maryland, was rehydrated in saline to a concentration of 1 mg per ml. A volume of 0.8 ml (0.8 mg) of SSS III was added to 1 ml of 0.1% chromium chloride (CrCl_3). Sheep erythrocytes were washed twice in saline and added to the mixture of SSS III and CrCl_3 and allowed to stand at room temperature for 5 minutes. The cells were washed three times in saline and resuspended to a concentration of 20% in saline. This 20% suspension of SSS III coated SRBC was used for plaquing.

IV. Hemagglutination (HS) and Hemolysin (HL) Titrations

For HA titrations, blood was collected from the retro-orbital plexus and allowed to clot at room temperature for one hour. The clot was centrifuged at 3000 rpm for 10 minutes to separate the serum from the cells. The serum samples for the HA and HL titrations were frozen at 5°C. until used. Twofold serial dilutions of the serum were made in saline and the volume, after dilution, was 0.2 ml. Two tenths ml of a 1% suspension of SRBC and 0.1 ml of saline were added making the final volume 0.5 ml. This was

incubated at 37°C. for one hour and held at 5°C. overnight. The tubes were centrifuged for three minutes at 3000 rpm and the reciprocal of the last serial dilution showing agglutination was considered the HA titer of the serum.

Hemolysin (HA) titrations were performed by the same procedure as the HA titrations with two exceptions. One tenth of a ml of 10% guinea pig complement (Colorado Serum Company, Denver, Colorado) was added instead of the 0.1 ml of saline. The tubes were incubated for one hour at 37°C. and read immediately. The reciprocal of the last serial dilution showing complete hemolysis was considered the titer of the serum.

V. Jerne Plaque Technique

A modification of the Jerne Slide Technique, as described by Mishell and Dutton (28), was used to plaque against SRBC. The spleen cell suspensions were not adjusted to 10 million cells per ml before plaquing. Instead, the spleen cells were suspended in 4 ml of DBSS and were plaqued undiluted and as a 1:5 and 1:25 dilution. The number of IgM producing cells was counted from slides incubated with guinea pig complement. The IgG plaques were developed by the addition of rabbit anti-mouse IgG antiserum to the guinea pig

complement. The facilitated plaque counts included both direct and indirect plaques.

The number of cells in the undiluted preparation was counted on a hemocytometer. After incubation with complement, the plaque forming cells were counted and the number of plaque forming cells per million spleen cells and per spleen was calculated.

The procedure for plaquing with endotoxin (LPS) is the same with the SRBC above except the erythrocytes must be coated with the endotoxin before being placed in the overlay agar (See Materials and Methods III). Since mice respond primarily with an IgM response to endotoxin, facilitation with IgG antiserum was not necessary.

The preparation of SRBC for plaquing against SSS III was mentioned earlier (See Materials and Methods III).

Mouse spleen cells for plaquing were suspended in Medium 199 (Microbiological Associates), pH 7.15. Coated SRBC were suspended in Hanks Balanced Salts Solution (HBSS) (Microbiological Associates), pH 7.15. The agarose overlay tubes were prepared with HBSS. Actual preparation for and pouring slides was identical to the SRBC method of Mishell and Dutton (39). The slides were incubated for one hour at 37°C. After incubation, the facilitating antiserum, rabbit

anti- μ antiserum, was applied. This serum was previously titrated to determine the optimum concentration, as too concentrated a serum would inhibit plaque formation. The slides were incubated another hour and diluted guinea pig complement (1:10 in HBSS) was added to the slides. A further two hour incubation at 37°C. was carried out and the slides were scored for plaque forming cells. IgM anti-SSS III plaques were facilitated with a pre-titrated anti-IgM serum.

VI. Skin Grafting

The procedure of Billingham and Silvers was used (8). Inbred CBA/J (H-2^k) mice were used as trunk skin donors and Balb/c (H-2^d) mice served as graft recipients. Mice were anesthetized with sodium pentobarbital (Appendix II) by the method of Pilgrim and DeOme (34).

Trunk skin was removed from mice that had been de-haired with "Nair" (Calcium thioglycolate) the day prior to use. This skin was scraped to remove the piniculus carnosus. The skin was then cut with a cork bore to a diameter of one cm and placed in sterile HBSS, pH 7.15. Mice to be grafted were de-haired with "Nair" one day prior to use. They were anesthetized (Appendix II) and a graft bed approximately one

cm in diameter was prepared immediately above the rib cage. The bed was cut to expose the piniculus carnososa. The graft was held in place by first layering a piece of lawn, impregnated with vaseline and sterilized, over the new graft. A sterile gauze compress was applied and covered with 4 or 5 wraps of one inch gauze. The entire area was covered with three wraps of one inch wide plaster cast.

Six days later, the cast was removed and the progress of graft rejection was observed for two or three weeks. Grafts were considered rejected when 100% of the graft became necrotic.

VII. Macrophage Stimulation and Harvesting

Balb/c mice 4 to 6 months of age were used as macrophage donors. Older mice yielded larger numbers of macrophages. These mice were given three ml of fluid thioglycolate medium IP. Four days later, the mice were sacrificed and the macrophages harvested by flushing the peritoneal cavity with 5 ml of Medium 199, pH 7.15. The cells were centrifuged at 3000 rpm for 10 minutes and resuspended in 4 ml of Medium 199, pH 7.15. The peritoneal exudate cells (PEC) were incubated at 37°C. for two hours in Falcon plastic petri dishes. The non-adherent cells were removed by

pouring off the cells with the supernate, and the adhering macrophages were collected in sterile Medium 199 by scraping the petri dish with a sterile rubber spatula. Cell numbers were determined by the use of the hemocytometer and their viability was evaluated by the use of trypan blue exclusion (See below).

VIII. Cell Viability Determination (Trypan Blue Exclusion)

A 0.5% solution of trypan blue in distilled water was prepared. The cells (0.05 ml) to be tested were mixed with 0.1 ml of the dye and 0.85 ml of saline and allowed to stand at room temperature for 10 minutes. After 10 minutes, the cells were counted in a hemocytometer and only those cells not excluding the dye were considered viable.

IX. Histologic Techniques

All tissues were removed from the mice as soon as possible after death. They were placed in 10% buffered formalin (Appendix I) and allowed to remain for two to three weeks before embedding. The tissues were embedded using the standard procedures and stained with Harris' Hemotoxylin and Eosin according to the procedure of Weesner (43).

X. Immunoglobulin Determinations

Relative levels of the classic immunoglobulins were determined by a modified Masseyelf and Ziswiller technique (27). The sera to be tested were collected from the retro-orbital plexus and frozen at 5°C. until use. One cm discs were cut with a cork bore from a 1.5 mm thick 1% agarose sheet prepared with 0.05 M HCl-phosphate buffer pH 8.4 (Appendix I). To the discs were added 0.03 ml of diluted Meloy (Meloy, Fallschurch, Virginia) goat antiserum specific for one of the mouse immunoglobulins. The discs were incubated in a moist chamber for 12 to 18 hours. At the end of this time interval, a two mm well was placed in the center of each disc. Into the center well were placed 5 lamda of each serial dilution of mouse serum. The discs were allowed to incubate at room temperature for 48 hours and the diameter of the precipitation rings were measured. Ring diameters were compared to a set of standards prepared using serum of normal mice. The results of the treated mice were reported as a relative variation from the normal levels.

XI. Irradiation

Mice were given 675 or 900 rad whole body gamma irradiation using a ^{60}Co source, delivering 97 rad/min. onto a plexiglass cage (H and H Plastics, Lincoln, Nebraska). The cage was placed in a 17.5 x 17.5 cm field on top of a turntable which was rotated at two rpm and placed 60 cm from the source to the center of the mouse. Maximum back-scattering was achieved by placing a 5 inch masonite block between the turntable and the plexiglass cage containing the 12 mice.

XII. Reconstitution Methods

Mice were sacrificed and the two femurs were harvested and cleaned of any muscle tissue. The bones were placed in Medium 199, pH 7.15, and chilled in an ice bath. The ends of the femurs were cut off, and the marrow aspirated from the lumen. The extracted marrow was pipetted in a capillary pipette until a single cell suspension was obtained. Cell numbers were determined and the appropriate dilutions were made in Medium 199, pH 7.15 or HBSS for injection.

Thymuses were removed from young mice and mashed through a number 100 mesh sterile stainless steel screen,

using Medium 199 or HBSS as the suspending medium. After a uniform cell suspension was obtained, the cells were centrifuged at 3000 rpm for 10 minutes and resuspended in sterile medium. The cells were counted, checked for viability by dye exclusion, and diluted to the desired concentration of viable cells for IV injection.

Macrophage stimulation and harvesting were described earlier (Materials and Methods VII). After the viable cell numbers were determined, a dilution yielding 25 to 30 million cells per ml was made. Animals were reconstituted IP with 12.5 to 15 million cells suspended in 0.5 ml of Medium 199, pH 7.15.

XIII. Calculation of "K" Values

The "K" value allows one to assign a significant level of organ weight gain or loss between treated and control groups relative to body weight,

$$\text{where } K = \frac{\frac{\text{Treated Organ Weight}}{\text{Treated Body Weight}}}{\frac{\text{Normal Organ Weight}}{\text{Normal Body Weight}}}$$

Any "K" value greater than 1.3 indicates a significant organ weight increase, and any number lower than 0.7 indicates a significant organ weight decrease.

XIV. Blood Cell Staining

All blood cells were stained with Wrights Stain by the procedure of Bauer, et al. (4).

RESULTS

I. Effect of L-asparaginase Administration on Adult Mice

Balb/c and A/Jax mice served as recipients of 3 - 1000 I.U. doses of L-asparaginase administered IV in 0.25 ml of saline on each of three consecutive days. After the initial three doses, enzyme was given IV every other day for two weeks. The results of total body weight loss in Balb/c and A/Jax mice are shown in Table I.

Balb/c mice, treated with enzyme, displayed a profound body weight loss for the first 5 days of treatment. Continued treatment did not cause any further weight loss. A/Jax mice also displayed a severe weight loss after treatment with 1000 I.U. and death occurred within 5 to 10 days. A/Jax can better tolerate 100 I.U., however, a 40% mortality was observed by 10 days. With a discontinuation of treatment, mortality continued to 80% by day 15. The remaining 20% recovered without any further treatment. Table I illustrates the strain variations in body weight and mortality after L-asparaginase treatment.

Organ weights are also affected by enzyme treatment. Two-month old Balb/c mice were given 1000 I.U. of enzyme IV

on three consecutive days and sacrificed on the fourth day. Table II lists the total body weights, the spleen, thymus, and liver weights and the "K" values of treated and control mice.

Balb/c mice displayed marked reduction of spleen, thymus, and liver weights after IV treatment with L-asparaginase. Lymph nodes also displayed signs of atrophy after mice were treated IV with enzyme. Three to 4 weeks after cessation of enzyme treatment, the organ and body weights had recovered.

The effect on the antibody response of 3000 I.U. of enzyme administered IV was measured by hemolysin (HL) titers, hemagglutination (HA) titers and plaque forming cells in Balb/c mice.

Two-month old mice were injected with 1000 I.U. of L-asparaginase IV on each of three consecutive days. On the second day of enzyme treatment, the mice received 1×10^8 SRBC IV in 0.25 ml of saline. Five days later, the mice were bled from the retro-orbital plexus, sacrificed, and their spleens removed. The results of the HA-HL titrations and plaque forming cell response are reported in Table III.

L-asparaginase was effective, when given IV, in suppressing the humoral response to SRBC given IV to Balb/c

TABLE I

Body Weight in Balb/c and A/Jax Mice
Following Enzyme Treatment

No. & Strain of mice	Treatment route	Average wgt. (gm) at day 0	Average Weight (gm)					
			Days after Initial Enzyme Treatment					
			1	2	3	5	10	14
10 (Balb/c)	Saline (IV)	20.5	21.5	22.8	22.34	23.52	24.1	25.6
10 (Balb/c)	Asp-Ase ¹ (IV)	21.2	21.4	18.06	17.25	15.92	15.5	15.6
10 (A/Jax)	Saline (IV)	18.35	18.4	19.15	19.3	19.92	27.1	--
10 (A/Jax)	Asp-Ase ¹ (IV)	19.23	19.2	17.33	15.1	12.1 (5 mice)	All Dead	--
5 (A/Jax)	Saline (IV)	19.30	19.23	19.91	20.64	21.47	25.2	--
5 (A/Jax)	Asp-Ase ² (IV)	19.75	19.8	18.23	16.62	14.03	12.3 (3 mice)	--

¹Asp-Ase = 1000 I.U. administered IV

²Asp-Ase = 100 I.U. administered IV

TABLE II

Body and Organ Weights, and "K" Values¹
for Balb/c Mice

<u>No. of mice</u>	<u>Treatment</u>	<u>Average² Body wgt. (gm)</u>	<u>Ave. Organ wghts. in mg ("K" Value)</u>		
			<u>Spleen</u>	<u>Thymus</u>	<u>Liver</u>
10	Saline (IV)	23.12	148	71	1573
10	Asp-Ase (IV)	17.93	60 (0.53)	18 (0.315)	700 (0.0572)

¹"K"-Value - See Materials and Methods XIII

²Average Body Weight (gm) - Average body weight on day of sacrifice

TABLE III

Hemolysin, Hemagglutination and Plaque Forming Cell
Responses in Balb/c Mice

No. of mice	Treatment route	Antigen ¹ route	Ave. Spleen weight (mg)	Direct Plaque Forming Cells			
				HA ²	HL ³	per 10 ⁶	per Spleen
15	Saline (IV)	IV	137	960	620	152	4.1 x 10 ⁴
20	Asp-Ase (IV)	IV	52	35	20	18	6.2 x 10 ²
8	Δ-Asp-Ase (IP) ⁴	IV	128	--	--	137	3.75 x 10 ⁴

¹Antigen = 1 x 10⁸ SRBC IV in 0.25 ml Saline

²⁻³HA-HL = Hemagglutination and Hemolysin titers are reported as the reciprocal of the last serial dilution showing agglutinated RBC or complete lysis respectively.

⁴Δ-Asp-Ase = Heat denatured enzyme (30 minutes at 65°C.)

mice. Heat denaturing the enzyme at 65°C. for 30 minutes negated its suppressive effects. Broome (10) showed that heat denaturation destroyed its enzymatic activity. Due to its aggregated state after heating, it had to be given IP.

II. Pathologic Effects of L-asparaginase

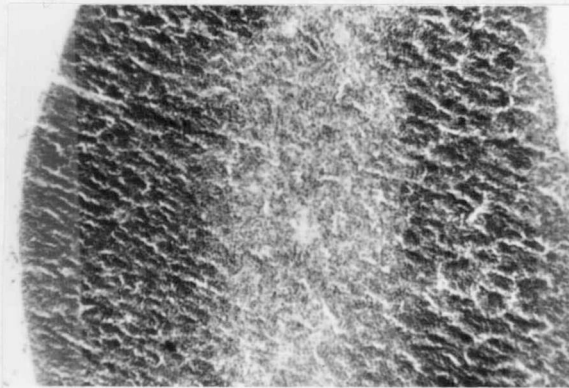
Two-month old Balb/c mice were given 1000 I.U. of enzyme IV on three consecutive days and sacrificed on day 4. The spleens, livers, axillary lymph nodes, and thymuses were removed and processed for histologic examination (See Materials and Methods IX). Plates I-IV are photomicrographs showing the thymus, spleen, lymph node, and liver section from control and enzyme treated Balb/c mice.

PLATE I

Thymus Section from an Enzyme Treated
and a Control Balb/c Mouse

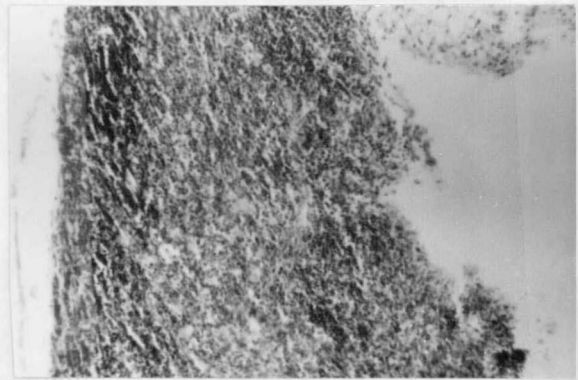
Control Thymus

Treated Thymus



Medulla

Cortex



Medulla

Cortex

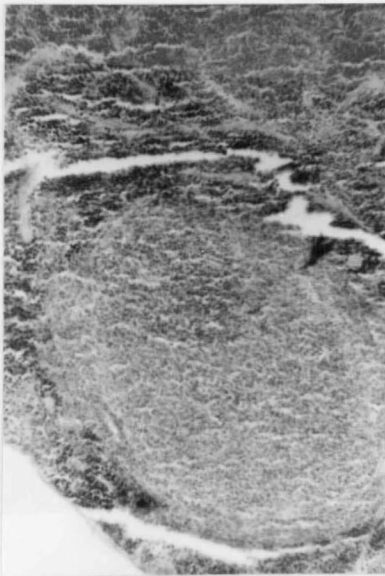
L-asparaginase caused a profound atrophy of the thymic cortex and noticeable atrophy of the medulla leaving no definite discrete boundary between the two areas. The thymic lymphocytes remaining after IV and IP treatment displayed a great affinity for the hemotoxylin stain. A complete lack of eosinophilic staining was observed.

Intraperitoneal treatment with L-asparaginase produced similar effects on the thymus gland.

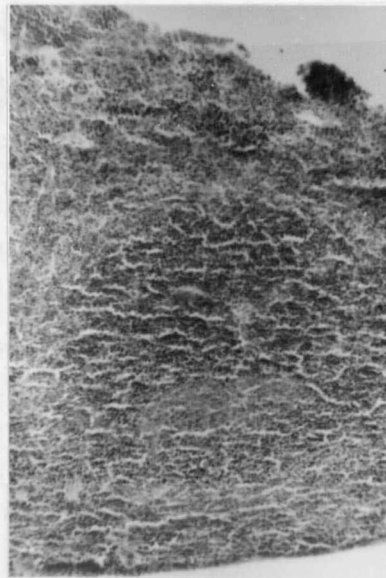
PLATE II

Spleen Section from an Enzyme Treated
and a Control Balb/c Mouse

Control Spleen



Treated Spleen



The spleens from IV treated animals were severely reduced in size. Splenic atrophy seemed uniform throughout the entire organ with equal reduction of the red and white pulp masses. There was a reduction in the numbers of germinal centers in the enzyme treated spleens, but they were not totally lacking. The remaining germinal centers displayed a more diffuse lymphoid cell mass. The treated spleens showed a greater concentration of trabeculae per field indicating these structures were not depleted by L-asparaginase, but concentrated by reduction of the affected nucleated cells. Intraperitoneal enzyme treatment had no noticeable effect on the spleens.

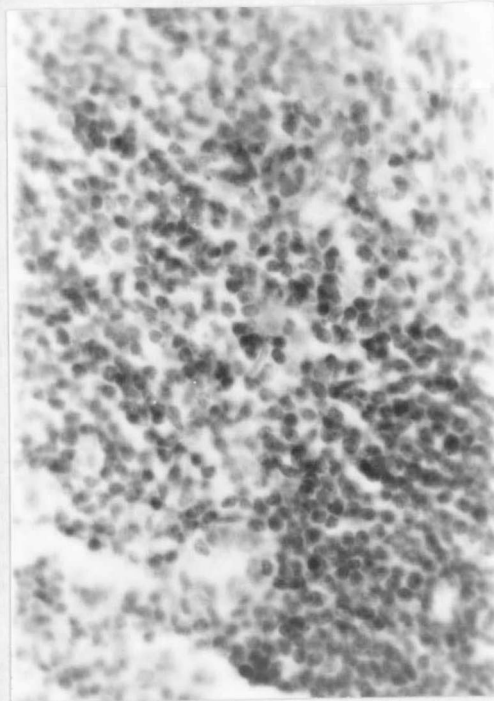
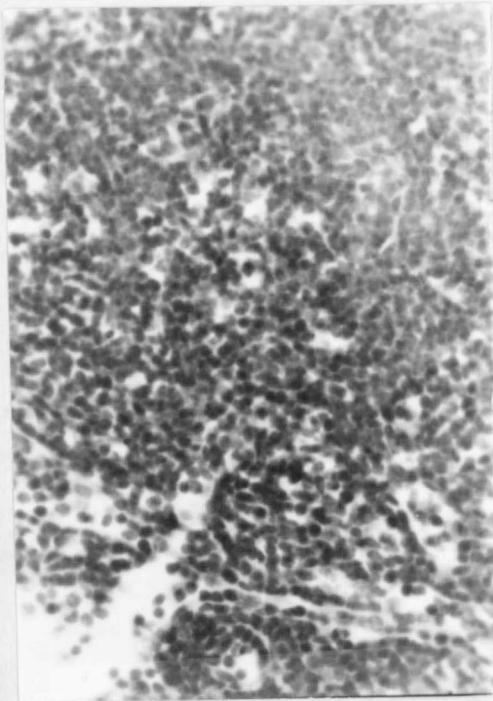
PLATE III

Axillary Lymph Node Sections from an Enzyme Treated
and a Control Balb/c Mouse

Control L.N.

Treated L.N.

Paracortical Area



Medulla

With

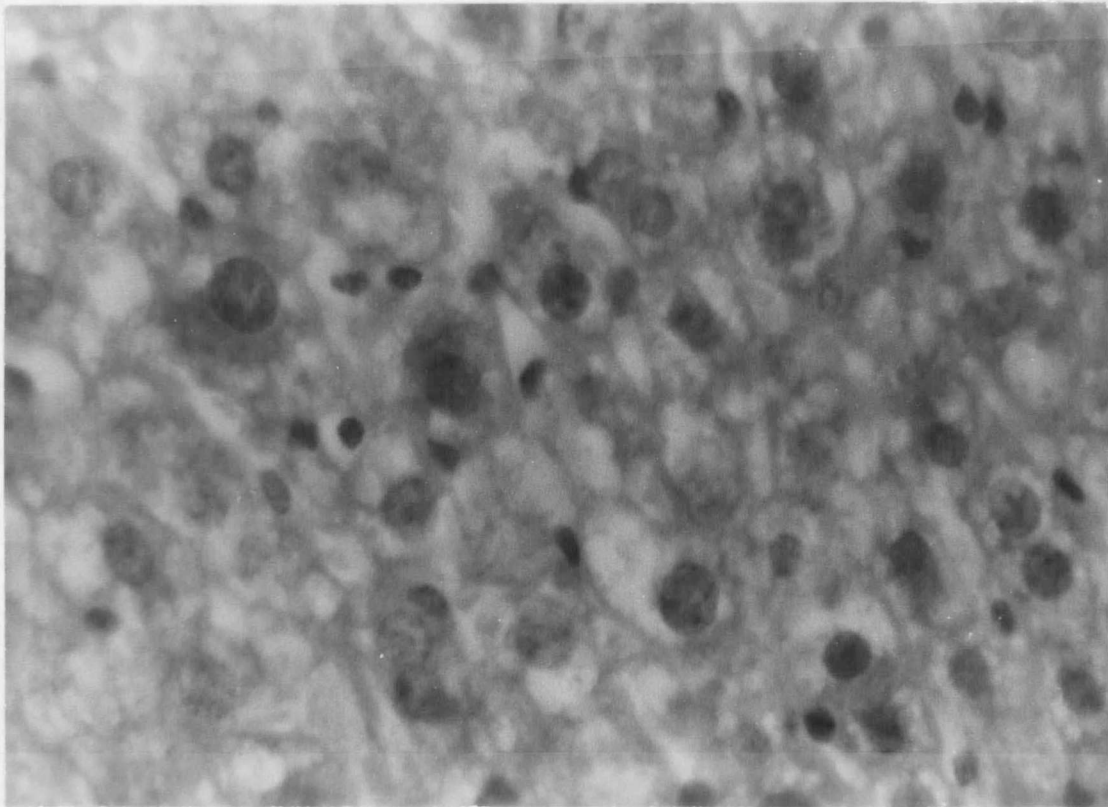
Cords

The axillary lymph nodes from IV enzyme treated mice displayed marked pathology. There was a profound reduction in lymphoid follicles with a depletion of lymphocytes in the cortical, paracortical, and medullary areas of the organ. The boundary between the remaining few follicles and the cortical tissue was very hard to discern. The remaining follicles also displayed a more diffuse concentration of lymphoid cells. There was a considerable amount of cord loss in the medulla of the treated organs. Intraperitoneal treatment with enzyme did not markedly affect these nodes.

The livers of Balb/c mice treated IV with enzyme showed marked atrophy and displayed some of the characteristics of a fatty liver, i.e.: yellow color, rounded margination with a protrusion of cut surfaces. Histologically, the hepatic cells clearly illustrated cloudy swelling with some hydropic degeneration. Intraperitoneal enzyme treatment left the liver normal by gross observations and weight measurements, but histopathologic involvement was not investigated.

PLATE IV

A Liver Section from an L-asparaginase
Treated Mouse



III. Effect of Route and Timing of Enzyme Administration

Since the route of administering the enzyme appeared to influence the pathologic changes in certain organs, the effect of the IV and IP routes of both enzyme and antigen on the humoral response was examined. Balb/c mice were immunized with 1×10^8 SRBC, and the treated mice received 3000 I.U. L-asparaginase given in three doses. The initial dose was given one day prior to antigenation, the second dose 4 to 6 hours before antigenation, and the final dose one day after antigenation. Mice were sacrificed 5 days after antigenation, and the plaque forming cell results are reported in Table IV.

Maximal suppression was obtained when the enzyme and antigen were both given by the same route. When the antigen and enzyme were given by different routes, the degree of suppression was less but still marked. This route effect is more clearly demonstrated later, Tables VIII, IX.

The effect of time of enzyme administration was studied in order to establish the optimum injection time for maximum suppression. The response was monitored by hemolysin titers after enzyme treatment and antigenation with SRBC. Balb/c mice were injected with one or three IV doses of

TABLE IV

Effect of Route of Antigen and Enzyme Administration
on the PFC Response to Sheep Erythrocytes

<u>No. of mice</u>	<u>Route of Enzyme</u>	<u>Route of Antigen</u>	<u>Direct PFC/10⁶</u>	<u>Facilitated PFC/10⁶</u>	<u>Direct PFC/Spleen</u>	<u>Facilitated PFC/Spleen</u>
6	Saline IP	IP	130	142	2.31 x 10 ⁴	2.5 x 10 ⁴
6	Saline IV	IP	123	135	2.26 x 10 ⁴	2.37 x 10 ⁴
6	Saline IP	IV	147	152	3.1 x 10 ⁴	3.15 x 10 ⁴
6	Saline IV	IV	181	200	2.54 x 10 ⁴	2.8 x 10 ⁴
12	Asp-Ase IV	IV	7	9	5.7 x 10 ²	7.3 x 10 ²
12	Asp-Ase IP	IV	21	30	8.7 x 10 ²	1.2 x 10 ³
12	Asp-Ase IP	IP	9	12	7.1 x 10 ²	9.0 x 10 ²
12	Asp-Ase IV	IP	24	28	9.3 x 10 ²	1.1 x 10 ³

1000 I.U. of L-asparaginase at the times indicated in Table V. One hundred million SRBC were given IV on day zero, and the mice were bled 5 days later. The titers are reported as the means with the range given in parentheses.

Suppression was obtained when enzyme was given the day before the day of or the day after antigenic challenge. Maximum suppression was obtained with three injections of 1000 I.U. given one day before, the day of, and one day after antigenation. These data correspond to results published by Berenbaum (7). He found the maximum effect was encountered if enzyme was given one day before antigenation. Berenbaum's use of another enzyme source and an IP challenge route could conceivably account for this slight variation.

IV. Hematologic Changes Following L-asparaginase Treatment

Since L-asparaginase induced atrophy plus pathological changes in the liver, thymus, lymph nodes, and spleens, there was reason to suspect bone marrow involvement. Mice were injected with 1000 I.U. of L-asparaginase IV on each of three consecutive days. On day 4, the mice were sacrificed and bone marrow and peripheral blood smears were made. The smears were stained with Wright Stain and observed.

TABLE V

Effect of Time of Administration of L-asparaginase
Relative to Antigen Challenge in Balb/c Mice

<u>No. of mice</u>	<u>Day of Enzyme Administration</u>	<u>Hemolysin¹ Titer (Range)</u>
10	- 2	768 (256-2048)
10	- 1	563 (256-1024)
10	0	51 (16-128)
10	+ 1	102 (64-256)
10	+ 2	460 (256-1024)
10	- 1, 0, + 1	5 (0-32)

¹Hemolysin Titer - Mean hemolysin titer is expressed as the reciprocal of the last serial dilution exhibiting complete hemolysis

Tables VI and VII show the results of the peripheral differential counts and hematocrits plus the bone marrow differential counts from Balb/c mice.

There was a reduction in peripheral lymphocytes with an increase in neutrophiles per 100 leukocytes in the treated mice. Microscopically, the lymphocytes appeared normal with a shift to the left in the neutrophiles. Eosinophil counts remained at control levels. The hematocrit

values were reduced by 5 volumes percent in enzyme treated mice.

TABLE VI

Differential Blood Counts and Hematocrits
of Enzyme Treated Balb/c Mice

<u>No. of mice</u>	<u>Treatment</u>	<u>Lympho- cytes</u>	<u>Neutro- philes</u>	<u>Eosino- phils</u>	<u>Hct.¹</u>
12	Asp-Ase	50 (41-57)	49 (39-60)	1 (0-4)	46
10	Saline	71 (62-82)	28 (19-35)	1 (0-3)	51

¹Hct. = Hematocrit value (Volumes percent)

Results of the bone marrow differential counts (Table VII) proved an interesting observation.

All of the cell types, excluding the metamyelocytes, myelocytes, lymphocytes, and nucleated red blood cells, did not differ from the control levels.

These data, Tables VI and VII, show that L-asparaginase affected the cellular components of the peripheral blood and also the cells of the bone marrow. There was a reduction in the numbers of lymphocytes of the bone marrow which corresponded to the reduction in lymphocytes in the peripheral count. There was an increase in the myeloid series in the bone marrow of treated animals over the

controls. The immature neutrophils, stabs and segs (See Table VII), did not vary from the control levels in the bone marrow, but the neutrophils were higher in the peripheral blood of treated animals.

V. Effect of a Reduced Enzyme Dose
on the Response to SRBC

Other workers had used the IP route of antigen and enzyme administration and obtained suppression.

Table IV shows that IP administration of enzyme also suppressed antibody production. Table VIII gives the results of the suppressive effects of 400 I.U. of L-asparaginase. Two-month old Balb/c mice were injected with 200 I.U. of enzyme IP on two consecutive days or IV on two consecutive days. The second day, the mice were challenged with 1×10^8 SRBC IP, and they were sacrificed 5 days later and their spleens plaqued.

Mice were suppressed by reducing the enzyme dose to 400 I.U. Five mice received the reduced dosage IV and the degree of suppression was less. The data in Tables IV and VIII reflect the effect of route on spleen weights and antibody production. Enzyme administration IV caused a greater reduction in spleen size but produced a lesser degree of immunosuppression.

TABLE VII

Bone Marrow Differential Counts of Treated
and Control Balb/c Mice

No. of mice	Treatment	Myelo- blasts	Pro- myelo- cytes	Meta- myelo- cytes	Myelo- cytes	Stabs ¹	Segs ²	Eos ³	Baso ⁴	Mono ⁵	Lymph ⁶	Neu ⁷ RBC
5	Saline	1	2	12	5	12.5	31	3.5	0	1	26	6
5	Asp-Ase	2.5	3.5	21	10	9	26	2	0	0	13	12

¹Stabs = Immature Neutrophils

²Segs = Segmented Neutrophils

³Eos + Eosinophils

⁴Baso = Basophils

⁵Mono = Monocytes

⁶Lymph = Lymphocytes

⁷Neu. RBC = Nucleated Red Blood Cells

TABLE VIII

Effect of 400 I.U. of L-asparaginase on Balb/c
Plaque Forming Cells to Sheep Erythrocytes

No. of mice	Treatment	Average Spleen wgt. (mg) (Range)	Direct Plaque Forming Cells	
			PFC/10 ⁶	PFC/Spleen
10	Saline (IP)	143 (100-162)	120	3.2 x 10 ⁴
10	Asp-Ase (IP)	100 (83-120)	33	1.3 x 10 ³
5	Asp-Ase (IV)	80 (62-95)	78	8.7 x 10 ³

VI. Effect of L-asparaginase on E. coli

Lipopolysaccharide (LPS) and Pneumococcal Polysaccharide Type III Produced Plaque Forming Cells

Mice received 150 I.U., 400 I.U., or 3000 I.U. of enzyme in two or three equal doses given on consecutive days. They were immunized with 10 or 100 µgm of LPS or 0.5 µgm of SSS III on the second day. The mice were plaqued 4 days after antigenation. Table IX shows the degree of suppression caused by L-asparaginase on the response to the two thymus-independent antigens, LPS and SSS III.

Enzyme reduces the response to LPS when both antigen and enzyme were given IP. There was a tenfold reduction in plaque forming cells per spleen and a sixfold decrease in plaque forming cells per 10^6 spleen cells. A tenfold reduction was also seen in the plaque forming cells per spleen when the antigen was given IV and the enzyme IV. There was no significant reduction in plaque forming cells per 10^6 spleen cells with the latter experiment.

The response to pneumococcal polysaccharide given IP was reduced by 400 I.U. of L-asparaginase also given IP. Due to a limited supply of this antigen, these studies were used to confirm the ability of the enzyme to reduce the response to a thymus-independent antigen.

The inability of IV L-asparaginase to reduce the plaque forming cells per million spleen cells to IV LPS was confirmed in the next experiment. Balb/c mice were immunized with both LPS and burro erythrocytes IV. They were also treated with 3000 I.U. of enzyme IV. The enzyme was administered in equal doses for three days, and the antigens were given the second day. Mice were plaqued 4 days after antigenation. Table X shows the results of these experiments.

Table IX and X show that IV administration of enzyme and LPS reduced by one log the plaque forming cells per

TABLE IX

Plaque Forming Response to LPS and SSS III
in Balb/c Mice Treated with L-asparaginase

<u>No. of mice</u>	<u>Treatment</u>	<u>Asp-Ase route</u>	<u>LPS and SSS III route</u>	<u>Plaque Forming Cells</u>	
				<u>10⁶</u>	<u>Spleen</u>
10	LPS ¹ + Saline	IV	IV	175	3.2 x 10 ⁴
10	LPS + Asp-Ase ²	IV	IV	157	6.3 x 10 ³
4	LPS + Δ -Asp-Ase	IP	IV	162	3.75 x 10 ⁴
8	LPS ³ + Saline	IP	IP	185	5.1 x 10 ⁴
8	LPS + Asp-Ase ⁴	IP	IP	30	7.0 x 10 ³
4	LPS + Δ -Asp-Ase	IP	IP	163	4.5 x 10 ⁴
4	SSS III ⁵ + Asp-Ase ⁶	IP	IP	2	3.2 x 10 ²
4	SSS III	IP	IP	61	1.2 x 10 ⁴

¹LPS = Difco Lipopolysaccharide, 10 μ gm in 0.5 ml saline

²Asp-Ase = 3000 I.U. administered in 3 - 1000 I.U. doses

³LPS = Difco Lipopolysaccharide, 100 μ gm in 0.5 ml saline

⁴Asp-Ase = 150 I.U. administered in 3 - 50 I.U. doses

Table IX (continued)

⁵SSS III = 0.5 µgm of Pneumococcal Polysaccharide IP

⁶Asp-Ase = 400 I.U. administered in 2 - 200 I.U. doses

TABLE X

Response of Balb/c Mice Treated with Enzyme
to LPS and Burro Erythrocytes (BRBC)

No. of mice	Treatment	LPS PFC/10 ⁶	LPS PFC/Spleen	BRBC PFC/10 ⁶	BRBC PFC/Spleen
8	10µg LPS	113	1.82 x 10 ⁴	--	--
8	Asp-Ase + 10µg LPS	122	2.1 x 10 ³	--	--
8	Asp-Ase + 10µg LPS + 5 x 10 ⁷ BRBC	146	5.35 x 10 ³	2	8.0 x 10 ¹
6	Asp-Ase + BRBC	--	--	4	1.2 x 10 ²
4	BRBC	--	--	43	9.0 x 10 ³

spleen but did not reduce the plaque forming cells per million cells. This indicated a killing of large numbers of non-plaque producing cells, possibly "T" cells. Killing of "T" cells also explained the reduction in plaque forming

cells per spleen and per million cells to BRBC, a thymus dependent antigen, in Table X.

VII. Immunoglobulin Levels after Treatment with L-asparaginase

A modified Masseyeff and Zisswiller technique (27) was used to compare the IgM, IgG₁, IgG₂, and IgA levels in enzyme treated and control Balb/c mice. Mice were treated with 1000 I.U. of L-asparaginase IV on three consecutive days. They were bled from the retro-orbital plexus on the fourth day and the various serum immunoglobulin levels determined. Table XI reflects the results of the immunoglobulin comparison studies.

TABLE XI

Relative Immunoglobulin Levels of Enzyme Treated and Control Balb/c Mice

No. of mice	Globulin	Mean Precipitan Ring Diameter (mm)	
		Enzyme treated mice (range)	Saline Control Mice (range)
12	IgM	3.3 (3.1-3.4)	3.6 (3.1-3.8)
12	IgA	3.9 (3.8-4.1)	3.8 (3.8-4.0)
12	IgG ₁	3.4 (3.0-3.8)	3.8 (3.6-4.2)
12	IgG ₂	5.4 (5.2-5.6)	4.8 (4.6-5.2)

None of the globulin levels presented in Table XI were significantly different from controls ($P < 0.05$ by

Student's T-Test). The various levels were tested for mean and individual variations using Student's "T" Test. The IgG₂ levels of treated mice displayed significant increases over controls (P<0.1).

VIII. Reconstitution of L-asparaginase Treated Mice with Peritoneal Exudate Cells (PEC)

The experimental design was dictated by the in vivo half-life of the enzyme. L-asparaginase retains suppressive activity in vivo for 12 to 18 hours after administration (7,10). Mice were suppressed with 400 I.U. of L-asparaginase given IP in two 200 I.U. doses the day before and the day of IP administration with primed or normal macrophages. Primed macrophages were obtained from mice that had received 1×10^8 SRBC IP the day of PEC stimulation with thioglycolate. Four days later, the PEC were harvested. Normal PEC were stimulated and harvested as above, but with the SRBC priming dose being eliminated. Adherent peritoneal cells were separated from the non-adherent populations (Materials and Methods VII). The lower enzyme dose of 400 I.U. was used to eliminate the possibility of enzyme carryover which would have affected the reconstituting cells. The mice were sacrificed and plaqued 5 days after receiving

the reconstituting macrophages. The data given in Tables XII and XIII show the results of the reconstitution experiments.

Balb/c mice suppressed with enzyme were almost totally reconstituted with 15 million normal peritoneal macrophages. Heat killed cells were unable to reconstitute an enzyme suppressed mouse. Normal macrophages were likewise unable to increase the normal response to SRBC in an untreated mouse.

Primed peritoneal macrophages had the ability to reconstitute enzyme treated mice while heat killed primed cells did not possess this ability. Primed cells given alone to a mouse who had never encountered SRBC gave a very low response.

Thus, the suppressive effect of L-asparaginase toward SRBC antigenized Balb/c mice was overcome with normal and primed peritoneal macrophages. The normal and primed macrophages apparently utilized the residual antigen and cooperated with the functional "B" and "T" cells to produce a normal response.

Thymus cells were totally ineffective in reconstituting suppressed mice in a similar experimental maneuver.

TABLE XII

Reconstitution of L-asparaginase Treated Mice
with Normal Peritoneal Macrophages

No. of mice	Treatment	Normal Macrophages	Plaque Forming cells/spleen		Plaque Forming cells/10 ⁶ spleen cells	
			Direct	Facilitated	Direct	Facilitated
15	SRBC	None	2.2 x 10 ⁴	2.5 x 10 ⁴	137	157
15	Asp-Ase + SRBC	1.5 x 10 ⁷	6.5 x 10 ³	7.4 x 10 ³	81	95
10	Asp-Ase + SRBC	1.5 x 10 ⁷ Δ-killed	7.3 x 10 ²	8.1 x 10 ²	12	15
10	Asp-Ase + SRBC	None	5.7 x 10 ²	7.3 x 10 ²	7	10
5	SRBC	1.5 x 10 ⁷	3.0 x 10 ⁴	2.8 x 10 ⁴	150	142

IX. Bone Marrow Reconstitution of Enzyme Treated Balb/c Mice

Three-month old Balb/c mice were suppressed IP with 400 I.U. of L-asparaginase given in two equal doses on two consecutive days. The same day as the second dose, the mice received 675 r of ^{60}Co whole body irradiation. The following day, the mice received 50 to 200 million syngeneic bone marrow cells IV. Four to 6 hours later, they were given IP 10 μgm of LPS. The mice were sacrificed 4 days later, their spleens removed and plaqued. Table XIV shows the results of these experiments.

The low response of the reconstituted mice was explained by the experimental design employed. Andersson and Blomgren (2) showed a period of three weeks was normally required after irradiation and bone marrow reconstitution before a mouse would respond normally to LPS. Nevertheless, in these studies, mice irradiated and reconstituted with bone marrow gave a tenfold higher response than enzyme treated, irradiated, and bone marrow reconstituted mice. These results would indicate that the irradiation resistant, yet enzyme sensitive population of cells, namely the macrophages, were functionally impaired or eliminated by L-asparaginase. The bone marrow did not contain enough of

TABLE XIII

Reconstitution of L-asparaginase Treated Mice
with Primed Peritoneal Macrophages

No. of mice	Treatment	Primed Macrophages	Plaque Forming cells/spleen		Plaque Forming cells/10 ⁶ spleen cells	
			Direct	Facilitated	Direct	Facilitated
12	SRBC	None	2.47 x 10 ⁴	2.71 x 10 ⁴	206	226
20	Asp-Ase + SRBC	2 x 10 ⁷	3.14 x 10 ⁴	3.3 x 10 ⁴	196	208
10	Asp-Ase + SRBC	Δ-killed 2 x 10 ⁷	1.13 x 10 ³	1.4 x 10 ³	10	15
10	Asp-Ase + SRBC	None	7.1 x 10 ²	9.0 x 10 ²	4	7
5	None	2 x 10 ⁷	4.0 x 10 ²	5.0 x 10 ²	3	3

TABLE XIV

Response of Enzyme Treated Balb/c Mice
Reconstituted with Syngeneic
Bone Marrow Cells

No. of mice	Bone Marrow cells	L-Asp-Ase	IRR.	Direct Plaque Forming cells	
				PFC/10 ⁶	PFC/spleen
6	5 x 10 ⁷	400 I.U.	675 r	12	95
6	5 x 10 ⁷	None	675 r	11	405
8	2 x 10 ⁸	400 I.U.	675 r	4	107
8	2 x 10 ⁸	None	675 r	10	1,009
5	None	400 I.U.	None	22	4,000
5	None	None	None	180	15,000
4	None	None	675 r	0	0

these cells to reconstitute the immune response in 4 days. The bone marrow reconstituted irradiated control mice apparently relied upon the radio-resistant macrophages of the host animal to expedite the processing and/or presentation of antigen to the lymphocytes present in the grafted bone marrow population. When one looked at plaque forming cells per million spleen cells, there was no differences between enzyme treated and control reconstituted mice. It is

conceivable that the function of the macrophages was to stimulate and, at the same time, force an expansion of a specific "B" lymphocyte pool.

X. Effect of L-asparaginase on Allograft Survival

In order to investigate the survival of grafts in enzyme treated animals, two-month old Balb/c mice were used as graft recipients of CBA/J trunk skin. Balb/c mice were injected daily with 1000 I.U. of enzyme IV from one day after grafting until 9 days after grafting in one experiment. In another experiment, mice received 1000 I.U. IV on days 5, 6, and 7 after grafting. The results are illustrated on Figure I.

These studies demonstrate a prolongation of initiation of homografts rejection regardless of time of administration, but a "phasing" phenomenon appeared as a function of the time of enzyme administration. Curve A illustrates the normal rejection sequence when grafting occurred on day zero. Treatment with L-asparaginase on days 5, 6, and 7 (Curve B) prolonged the initiation of rejection, but terminal rejection time was normal. Curve C illustrates how enzyme treatment from day one through 9 produced a curve

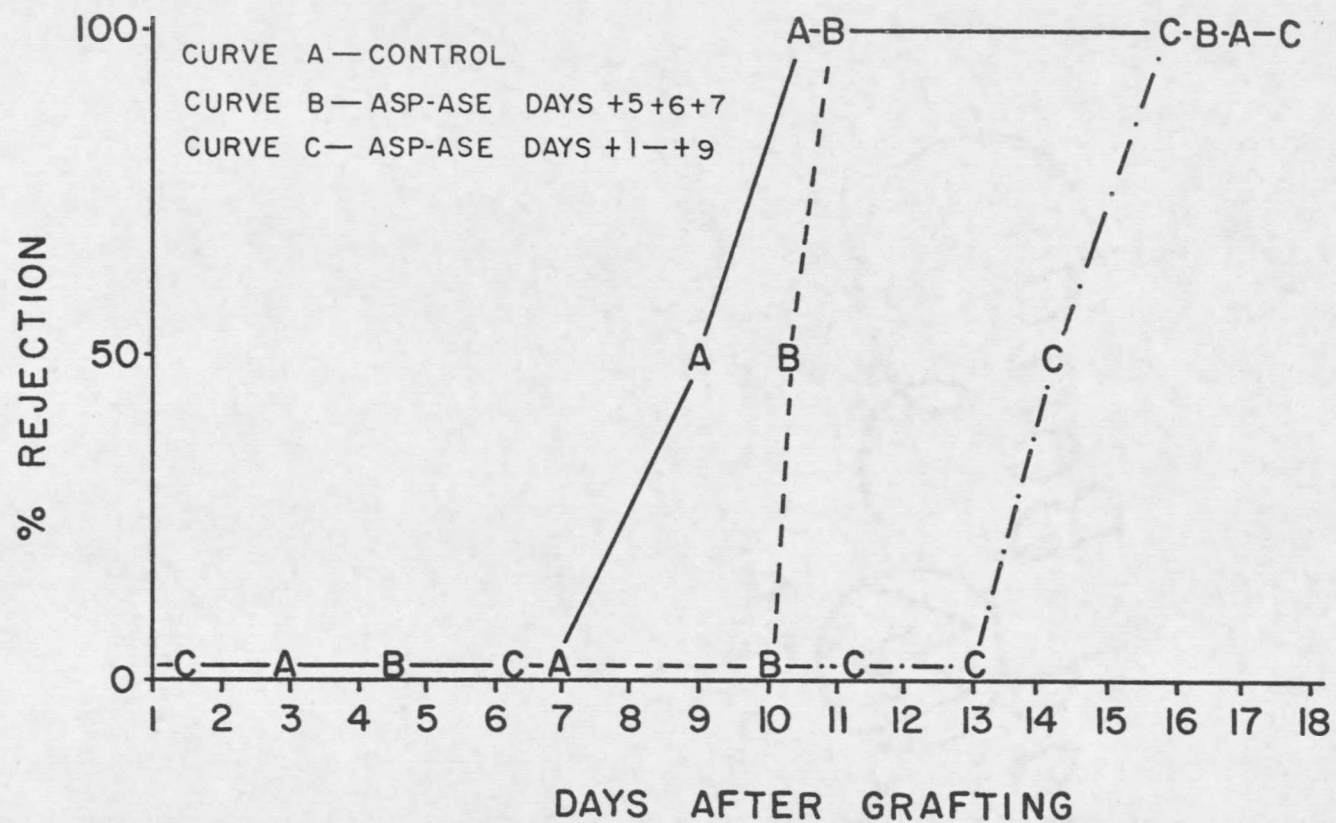


Figure 1. Homograft rejection in asparaginase treated Balb/c mice receiving CBA/J trunk skin.

similar to the normal curve. The only difference was the prolongation of both initiation and terminal rejection.

The "phasing" phenomenon is represented by Curve B. "Phasing" seemed to occur if enzyme was given after "sensitization" to the graft had occurred. Presumably, killer "T" cells were blocked after sensitization, therefore, were not allowed to cause rejection of the allograft. When enzyme treatment was terminated, these primed or sensitized killer cells were released and rejection occurred at an increased rate. Curve C indicates that killer cells were not sensitized by day one and therefore held in a non-sensitized state by the enzyme through day 9. After termination of treatment, the normal rejection sequence occurred. Curve C also illustrates a "leakage" of "sensitized" killer cells during enzyme treatment. The period from day 9 until initiation of rejection was 4 days instead of the 7 days required from grafting until initiation of rejection in the normal Curve A.

The role of the macrophage can also be integrated into the reasons for the rejection phenomenon seen in Curve B and C. Enzyme affected macrophages may not have been able to migrate to the graft site properly or their ability to interact with the "T" cell was affected.

DISCUSSION

The immunosuppressive effects of L-asparaginase have been studied intensively since its discovery in 1953 by Kidd (23,24). Initially, most of the early work was centered around the anti-tumor properties of L-asparaginase (9,11,11a, 12).

Later work was focused on the generalized suppressive effects caused by IP challenges with L-asparaginase. Suppression to SRBC with various IP doses of enzyme occurred (12,15,18,19,29,30,31,35). L-asparaginase was shown to possess the ability to inhibit PHA stimulated blastogenesis (3,16,26,32,44) and normal mitosis in regenerating liver cells (5).

Suppressive events produced by L-asparaginase were shown to encompass some cellular mediated immune phenomenon. Hobik (20) and Weksler and Weksler (45) were able to negate the graft-versus-host (GvH) reaction by treatment of the spleens from donor animals with L-asparaginase. Weksler and Weksler (45) could destroy splenic lymphocytes ability to produce the GvH disease by in vivo treatment with L-asparaginase. Exogenous asparagine concentrations of the culture media may have been greater than the concentrations in the

animal tissues and could have overcome the dose of L-asparaginase used. Evidence suggests that asparagine is more concentrated in some organs than others (45). Enzyme treatment in vivo would have a better opportunity to deplete the asparagine in these tissues. L-asparaginase, being a catalyst, could act more efficiently on a high concentration of substrate rather than a diffuse lower concentration of substrate.

Nelson, et al. (30), Friedman and Chakrabarty (18), St. Pierre, et al. (42), and Levin and Merrill (25) were able to prolong skin and renal allograft survival by the use of L-asparaginase. Kahn and Hill (22) suppressed the delayed skin hypersensitivity reaction in mice that had been treated with enzyme.

None of these studies dealt into the actual mechanisms causing the anomalies. Chakrabarty and Friedman (15) and Friedman and Chakrabarty (18) were able to reverse the suppressive effects of L-asparaginase by the introduction of exogenous asparagine. Broome (11,11a) was able to demonstrate a lack of asparagine synthetase in enzyme sensitive leukemic cells. From these works was formulated the theory that L-asparaginase displayed its immunosuppressive and anti-leukemic properties by reduction of exogenous asparagine.

Protein synthesis is reduced in cells treated in vitro, with asparaginase (12,44). This reinforces the premise that certain cells need exogenous asparagine for protein synthesis and its depletion would functionally inhibit leukemic cells as well as cells of the immune response.

Investigations into the cell or cell types involved in the immune response that were affected by L-asparaginase have been performed by others (17,45). Initially, Rodney and Good (37) showed the dependency of the thymocyte for PHA stimulation of lymphoid cells. This, in conjunction with the study made by Weksler and Weksler (45), implicated the thymocyte as the major cell affected by L-asparaginase. Friedman (17), using irradiation and reconstitution experiments, suggested the bone marrow derived lymphocyte as the cell most likely affected by L-asparaginase.

The data in this thesis serve to confirm and expand the general immunosuppressive effects caused by L-asparaginase and to add further to knowledge of the cell or cell types affected by the enzyme.

Suppression of the humoral response to thymus dependent (SRBC and BRBC) and to thymus independent antigens (LPS and SSS III) was demonstrated in Tables III, IV, V, VIII, IX,

and X of the Results. Another general effect, survival of allografts, was also alluded to in the final section of the Results. The allograft survival data added a new concept not reported before. "Phasing" of the rejection sequence was produced when the enzyme was given on day 5, 6, and 7 after grafting. It is believed that the "T" cells were sensitized over the first 4 days after grafting and the enzyme stopped or severely slowed down the process which converted normal "T" cells to sensitized killer "T" cells or suppressed the migration of killer "T" cells to the graft site. When the effects of enzyme were removed, there was a large concentration of these sensitized killer "T" cells released to cause an accelerated allograft rejection. Enzyme administration on the day after grafting and continued through day 9 verified on the enzyme's ability to prolong allograft survival (18,25,30,42).

The concepts generated by these data toward the cell type or types affected by L-asparaginase failed to coincide entirely with the findings of Friedman (17) and Weksler and Weksler (45). The former reported the bone marrow derived lymphocytes, and the latter reported the thymus derived lymphocytes as the cell type affected. The data in this thesis indicate the peritoneal macrophage and possibly the

"T" cell as the cell types primarily affected by the enzyme. Friedman (17) based his conclusion that bone marrow derived lymphocytes were primarily affected by enzyme on the following observation. Bone marrow derived lymphocytes, obtained from an enzyme treated mouse and placed with radio-resistant in situ macrophages of the recipient and supplied with "T" cells, gave a reduced response. Weksler and Weksler (45) showed that enzyme treated lymphocytes failed to migrate properly. Hence, it seems possible that Friedman stopped the ability of the donor lymphocytes to migrate and did not markedly alter their ability to produce antibody. The experimental design, used in this thesis, that allowed reconstitution of IP enzyme suppressed animals with macrophages did not noticeably affect the spleen or axillary lymph nodes. After IP challenge with enzyme, the thymus was the only gland or organ examined which displayed cellular reduction. Macrophage involvement was implicated when IP challenge with enzyme and antigen did not reduce the spleen size or cell numbers, yet suppressed the response per total and per million cells. Macrophage inhibition did not allow processing and/or proper presentation of antigen to the "T" or "B" cells of the spleen. The ability to reconstitute a mouse, antigenized to SRBC and suppressed IP with enzyme,

with normal or primed macrophages also suggested that "T" and "B" cells were not primarily affected by L-asparaginase.

Intravenous enzyme administration caused a marked reduction in spleen size. Histologic examination revealed fewer cells and some loss of germinal centers. Larger IV doses of enzyme were required to obtain equivalent suppression caused by smaller IP doses. With these large IV doses, there was suppression of the total producing cells per spleen but not per million spleen cells. To obtain this type of suppressive pattern, it would be necessary to produce random killing of the nucleated cells in the spleen, or kill the "T" cells without killing the "B" cells. The first possibility would not seem as logical in this case because the reduction of plaque forming cells per spleen was of the same magnitude as the reduction following IP enzyme treatment. The second possibility, the killing of "T" cells and macrophages preferentially over the "B" cells, is supported indirectly by the data presented in this thesis. There was a reduction in the cells of the paracortical, thymus dependent, area of the lymph nodes with IV treatment plus marked thymic atrophy with either IP or IV enzyme treatment. After enzyme treatment, there was no alteration of the immunoglobulin levels, which would imply the presence of the

producer "B" cells. The reduction in the plaque forming cells per spleen but not per million cells actually showed that when the enzyme killed the macrophages and the "T" cells, it left a proportionately larger number of "B" cells per remaining nucleated spleen cells.

Weksler and Weksler (45) also postulated that "T" cells were affected by enzyme treatment. He supported this theory on the basis that L-asparaginase inhibited lymphocyte transformation and the GvH reaction and prolonged homograft rejection. All of these immunologic reactions were shown to be mediated and dependent upon the "T" lymphocyte.

The conclusions formulated as a result of the data presented in this thesis in combination with the data presented from other sources lead to the following: Macrophages are one cell type affected by L-asparaginase and immunosuppression is a direct result. The "T" cells are also affected after large IV doses of enzyme. "B" cells may also be affected; however, they are more resistant than the macrophage and/or the "T" cells and under our experimental protocol were not markedly affected.

APPENDIX

APPENDIX I

1) Acidified Water

- a) 1 gram of HTH (70%) - Calcium Hypochlorite
(Olin Mathieson Chemical Corporation)
- b) 9 ml of Concentrated HCl
- c) q.s. to 5 gallons with distilled H₂O

2) 10% Buffered Formalin

37%-40% Formaldehyde	100 ml
Distilled Water	900 ml
NaH ₂ PO ₄	4 gm
Na ₂ HPO ₄	6.5 gm

3) Phosphate Buffer for Endotoxin coupling to Erythrocytes

Sol. A KH₂PO₄ 1.36 grams/liter of Distilled Water

Sol. B K₂HPO₄ 1.74 grams/liter of Distilled Water

To prepare .01 M Buffer at pH 7.4, use 19 ml of

Sol. A and 84 ml of Sol. B

4) Phosphate Buffered Saline (pH 7.0)

33 ml of Sol. A above

67 ml B of Sol. B above

8.5 grams NaCl/liter

Appendix I (continued)

5) HCl - Sodium Barbital Buffer (pH 8.4-0.05 M)

Sodium Barbital	20.618 grams
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1 N HCl	30 ml
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Distilled Water	1970 ml
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Store at 4°C.

APPENDIX II

1) Sodium Pentobarbital for Anesthesia (34)

Sodium Pentobarbital (Nembutal-Abbott)	- 20 ml
	(50 mg/ml)
Distilled Water	- 174.0 ml
Ethyl Alcohol (95%)	- 1.8 ml
Propylene glycol	- 3.6 ml
Final concentration of Nembutal	- 5 mg/ml

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