COMPARATIVE STUDIES ON THE EFFECTS OF L-ASPARAGINASE SOLUTION AND L-ASPARAGINASE IMMOBILIZED WITHIN SEMIPERMEABLE MICROCAPSULES IN NON-IMMUNIZED AND IMMUNIZED MICE

by

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Short Title:

STUDIES ON THE EFFECTS OF FREE AND MEC-L-ASPARAGINASE

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ABSTRACT

In non-immunized mice, significant levels of L-asparaginase activity appeared in the blood after i.p. injection of L-asparaginase solution (AS), whereas no blood L-asparaginase activity appeared after i.p. injection of L-asparaginase microcapsules (AM). "Body" L-asparaginase levels fell very rapidly after injection of AS, but not after AM. AM were no longer physiologically active in-vivo after 8 days post injection, probably because of a decreased permeability of the microcapsules. AM maintained a zero plasma L-asparagine concentration for a significantly longer period of time than the equivalent dose of AS. AM were more effective than AS in inducing complete regression of established 6C3HED lymphosarcoma in non-immunized mice. However, both AM and AS lost their antitumor activity in immunized mice. In-vitro, anti-L-asparaginase serum inhibited the catalytic activity of AS but not AM. In immunized mice, much lower levels of L-asparaginase activity were assayed in the "body" and in the blood after injection of AS, as compared to non-immunized mice. Blood L-asparaginase activity was cleared much more rapidly also. Neither AS nor AM could lower the plasma L-asparagine concentration significantly, although the "body" L-asparaginase levels remained high for up to 2 days after injection of AM. With the aid of Erwinia carotovora L-asparaginase, further studies were carried out to examine possible mechanisms that may account for these observations in immunized mice.

RESUME

Chez des souris non-immunisées, nous avons pu observer l'apparition dans le sang de niveaux significatifs d'activité de L-asparaginase après injection intrapéritonéale d'une solution de L-asparaginase (SA). Nous n'avons cependant noté aucune activité sanguine de L-asparaginase après injection intrapéritonéale de microcapsules de L-asparaginase (MA). Les taux "somatiques" de la L-asparaginase ont rapidement diminué après injection de SA, cependant on n'a pas noté la même variation après injection de MA. Huit jours après l'injection, MA ne possédaient plus aucune activité physiologique in vivo, probablement à cause d'une perméabilité diminuée des microcapsules. MA maintiennent la L-asparagine à des concentrations plasmatiques égales à zéro durant une période de temps significativement plus longue que ne le fait SA à des taux semblables. MA ont été plus effectives que SA dans l'induction d'une régression complète de lymphosarcomes 6C3HED prouvés chez les souris non-immunisées. Cependant, chez les souris immunisées, les deux, MA et SA, ont perdu leur activité antitumorale. In vitro, un sérum anti-L-asparaginase inhibe l'activité catalytique de SA mais non celle des MA. Chez les souris immunisées, par opposition aux résultats obtenus chez les non-immunisées, après injection de SA, on retrouve de très bas niveaux d'activité sanguine et "somatique" de L-asparaginase. L'activité de la L-asparaginase s'est négativé beaucoup plus rapidement aussi chez ces mêmes souris immunisées. Quoique les taux "somatiques" de L-asparaginase soient demeurés élevés jusqu'à deux jours après injection des MA, ni SA ou MA n'ont pu diminuer significativement la concentration plasmatique de la L-asparagine. Avec l'aide de la L-asparaginase de Erwinia carotovora, d'autres études furent entreprises afin d'examiner les mécanismes possibles expliquant les observations obtenues chez les souris immunisées.

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FOREWORD

In this thesis, I have taken advantage of the option provided by section 4.2.7. (h) of the regulations of the Faculty of Graduate Studies and Research that allows the candidate to present his thesis in the form of original papers which would be suitable for submission for publication. Chapter II was submitted for publication in May 1973, and has already been published by the journal, Enzyme (Volume 18:218 - 239, 1974). Chapters III and IV have been written in somewhat more detail to comply with usual thesis requirements. These latter two chapters in appropriately modified form will be submitted shortly for publication. Appendices have been added to Chapters II, III and IV, wherever it is believed that they will be of value to the reader. Since Chapters II, III and IV were written as separate articles, there is of necessity some degree of repetition in the text.

CHAPTER I

GENERAL INTRODUCTION

IA - INTRODUCTION

The treatment of cancer by chemotherapy is based on the fundamental premise that cancer cells differ in some way from normal cells, and thus can be destroyed by chemicals which will be less toxic to normal cells. The demonstration that some cancers, most notably choriocarcinoma and Burkitt's lymphoma, can be cured by chemotherapy provides definitive proof that this basic premise is a valid one. Successful chemotherapy to date, however, has not been qualitatively specific for tumor cells, but is based largely on quantitative differences between tumor cells and normal cells, for example the more rapid growth of some tumor cells. As an unfortunate corollary of this, normal tissues, such as those of the bone marrow, lymphatic system, gastrointestinal epithelium, skin, and hair, that also have a high growth fraction are also susceptible to the cytotoxic effects of most of the currently used anticancer drugs.

The discovery of the therapeutic effects of the enzyme L-asparaginase represents one of the nearest approaches to success in the search for an exploitable, qualitative, metabolic difference between normal and neoplastic cells. It was proposed that administration of L-asparaginase would result in selective starvation of tumor cells by depleting their exogenous supply of L-asparagine upon which the tumor cells have an absolute nutritional dependence. At the same time, normal tissues would be spared because they, in contrast to these tumor cells, are capable of synthesizing sufficient quantities of endogenous

L-asparagine which is normally considered to be a non-essential amino acid. The reaction of cancer chemotherapists to the potential of L-asparaginase as an antitumor agent was appropriately summed up by Zubrod (1) who wrote in 1970, "the excitement generated over the early announcements (of the therapeutic activity of L-asparaginase) was greater than for any other antitumor drug. Here, seemingly, was the first antitumor drug, which, penicillin-like, killed the invading cells without harming the host..."

The initial hopes that the nutritional dependence of some cancer cells on L-asparagine may represent an absolute biochemical difference between neoplastic and normal cells have been dampened by findings that L-asparaginase is not without toxicity to normal cells. However, L-asparaginase has found a place in the armamentarium of drugs used clinically by cancer chemotherapists for the treatment of acute lymphocytic leukemia.

L-asparaginase differs uniquely from all the other clinical antineoplastic agents in that the rationale for its use is based on a nutritional defect of certain cancer cells. The induction of a selective nutritional deficiency for tumor cells by L-asparaginase represents the first practical demonstration of the clinical use of an enzyme in cancer therapy. The future success of a "selective nutritional deprivation" approach to cancer therapy may be influenced profoundly by the results of research on L-asparaginase as a model of this approach. From experience with this enzyme, certain basic, important concepts have emerged concerning the use of enzymes for cancer therapy in general.

For example, such experience suggests that if other potential antitumor enzymes are to be clinically active, they should possess the following prerequisite properties: stability and catalytic activity at physiologic pH, a low K_m value, and an appreciable plasma half-life. Negative results obtained have also been important in so far as it is only when present limitations are clearly defined that future enzymic approaches to cancer therapy could be developed with increased hopes for success. In this regard, a variety of other amino acid-degrading enzymes have since been suggested as possible antitumor agents. These include: glutaminase (2 - 9), serine dehydratase (10, 11), arginase (12), phenylalanine ammonia-lyase (13, 14), and most recently, leucine dehydrogenase (15).

In the following section of this thesis, a brief review on L-asparaginase will be presented. Since well over 500 papers on L-asparaginase have been published in the last decade (16), no attempt has been made to provide an exhaustive review of this wide-ranging topic. Several review articles (16 - 28) and proceedings from two international symposia (29, 30) on L-asparaginase are available in the literature. These have been used as general references. More specific and detailed references have also been provided in the text.

IB - L-ASPARAGINASE

Historical introduction

In 1953, Kidd (31, 32) reported that injection of normal guinea pig serum caused the complete regression of established Gardner 6C3HED lymphosarcoma and the Lorenz lymphoma II in mice, and delayed the appearance of the Murphy-Sturm lymphosarcoma in rats. Sera from several other species, namely the horse, rabbit and human, were without effect. Also, susceptibility to the effects of guinea pig serum was found to be confined to certain tumor cell lines. It was at first thought that guinea pig serum acted through its high complement activity, thus augmenting an immunological reaction between the host and the implanted tumor. However, no fraction of complement could be identified with tumor-inhibitory properties and rabbit antisera which also contain high degree of complement activity, did not have any antitumor effects. Independent work by Neuman and McCoy (33) in 1956 and Haley et al (34) in 1961 showed that cells of the Walker carcinosarcoma 256 and the L5178Y mouse leukemia had an absolute nutritional requirement for growth in vitro for L-asparagine which is generally considered to be a non-essential amino acid for normal tissues. Any relationship between the reports by Kidd, Neuman and McCoy, and Haley et al, was not recognized at the time, and was left unnoticed.

Broome, while working in Kidd's laboratory, began a search to find constituents in guinea pig serum which were not present in other animal sera found to be ineffective against tumors. He fortunately came across a paper published in 1922 by Clementi (35) who

was studying the distribution of amino acid-degrading enzymes in the tissues of different animal species in relation to diet. Clementi observed that L-asparaginase was present in the liver and kidney of many species, but occurred in the blood of only one of the animals he studied, the guinea pig. Broome therefore compared the antitumor properties of guinea pig serum with its L-asparaginase activity, and in a series of papers (36 - 38) convincingly provided evidence that the L-asparaginase activity of guinea pig serum was responsible for its antilymphoma effects. On the basis of these and other extensive studies that will be referred to later, the antineoplastic activity of L-asparaginase is now well-established.

A very important advance in the development of L-asparaginase as a cancer chemotherapeutic agent was the report by Mashburn and Wriston (39) that L-asparaginase present in Escherichia coli had tumor-inhibitory properties. This discovery made it possible to obtain larger quantities of enzyme for extensive investigation and evaluation. Subsequent studies showed that E. coli L-asparaginase was also effective against several other types of murine leukemias and solid tumors (17, 40), and spontaneous dog lymphosarcoma (41). These latter findings in such diverse animals as the mouse, the rat, and the dog, encouraged hopes that E. coli L-asparaginase would be of clinical value.

Before adequate supplies of E. coli L-asparaginase became available, De Barros et al (42) in Brazil treated one patient with melanoma using crude agouti serum L-asparaginase, and Dolowy et al (43)

in the United States treated one patient with acute lymphocytic leukemia using crude guinea pig serum L-asparaginase. They both noted slight and transient therapeutic effects. When adequate supplies of L-asparaginase from E. coli did become available, clinical trials were carried out in 1967 in the United States by Hill et al (44) at the Wadley Institutes of Molecular Biology, Dallas, and by Oettgen et al (45) at the Memorial Sloan-Kettering Cancer Center, New York. These studies were the first to demonstrate significant therapeutic responses to L-asparaginase in the treatment of human cancer, particularly acute lymphocytic leukemia. Since then, intensive research on this enzyme has been carried out both at the basic and clinical levels of investigation, and an impressive literature has developed on this subject.

Distribution and sources of L-asparaginase

L-asparaginase is widely distributed, being found in animal, plant and microbial sources.

It was first found in guinea pig serum by Clementi (35) who also reported its absence from sera of other common mammals. It has since been found in the sera of 4 other members of the superfamily Cavioidea to which the guinea pig belongs, namely the capybara, the Patagonian hare, the paca, and the agouti (46 - 48). With the exception of a report of traces of L-asparaginase activity in rabbit serum (49) and its presence in the sera of 2 species of New World monkeys (18, 50), L-asparaginase appears to be absent from the sera of all

other mammalian species.

The enzyme is, however, present in the tissues of several animals. It has been found in the liver of the rat (51 - 54), chicken (55), and guinea pig (56 - 58), and in the liver and kidney of certain birds (35). It is also present in the tissues of fish (17). A recent detailed report by Milman and Cooney (59) on the distribution of L-asparaginase in the principal organs of representative mammals and birds indicate that L-asparaginase is present in the pancreas, liver, brain, ovary, spleen, lung, testes and kidney of many of the animals surveyed.

L-asparaginase has also been found in plants sources, barley rootlets (17).

L-asparaginase fortunately is present in many microorganisms, especially bacteria, making these the major sources of today's supply of L-asparaginase. It should be noted that only some of these bacterial L-asparaginases possess antineoplastic activity, most notably, Escherichia coli, Erwinia carotovora, and Serratia marcescens. A review of the various types of microorganisms in which L-asparaginase has been found is available in the literature (16, 60 - 63).

<u>Some properties of E. coli L-asparaginase EC-2 (L-asparagine amidohydrolase; EC. 3.5.1.1)</u>

The properties of this enzyme have been described in detail in several papers (16, 22, 64 - 67). The enzyme is quite stable in aqueous solution in the pH range of 5-9. At room temperature under

sterile conditions, enzyme activity is preserved for weeks. At 60°C, the enzyme loses its catalytic activity within 1 hour. The enzyme is relatively stable in the presence of organic solvents such as acetone, alcohol, and others. The enzyme has an isolectric point of 4.6 - 5.5. It has a broad pH optimum between pH 5 - 8.5. K_m values for E. coli L-asparaginase have been reported to be 1.15×10^{-5} M - 1.25×10^{-5} M. Although there is some discrepancy in the literature with respect to the exact molecular weight of E. coli L-asparaginase, the generally accepted value is 130,000.

The enzyme has a tetrameric structure composed of 4 identical subunits. The subunits are structurally similar and possess "222 pseudosymmetry". A crystal lattice structure with spherical subunits (diameter 40 Å) account satisfactorily for the X-ray data. Electron microscopy reveals a generally globular appearance for the whole molecule and supports the existence of a tetrameric structure with 4 approximately spherical subunits forming a square with a centre hole. Circular dichroism studies on the conformation of the enzyme show that the native enzyme consists of 10% α-helix, 45% β-structure, and 45% disordered. Amino acid analysis of L-asparaginase after acid hydrolysis indicates that L-aspartic acid and L-asparagine make up the largest number of amino acid residues in the enzyme molecule. Very low concentrations of cystine, histidine, and tryptophan are present in the molecule. Neither carbohydrate nor metallic moieties could be detected in the structure.

The substrate specificity of L-asparaginase appears to be

restricted to 4 and 5-carbon L - or D - α-amino acids with an ω-nitrogen function. The hydrolysis of L-glutamine by L-asparaginase is an intrinsic property of the enzyme and not due to the presence of contaminating L-glutaminase. E. coli L-asparaginase has 3 - 4% of the activity toward L-glutamine that it has toward L-asparagine. Most of the known L-asparaginases, with the notable exception of guinea pig serum L-asparaginase, have inherent L-glutaminase activities. The hydrolysis of L-asparagine and L-glutamine occur at the same active site in E. coli L-asparaginase; the lesser hydrolytic activity toward L-glutamine is probably due to an imperfect fit of the substrate at the active site of the enzyme. In this thesis, it should be noted that only the L-asparagine-depleting effect of L-asparaginase has been emphasized.

Spectrum of sensitivity of tumors to L-asparaginase

Many animal tumors have been shown to be inhibited by L-asparaginase (17, 18, 22). L-asparaginase is effective against over fifty neoplasms in the mouse, including the L5178Y and EARAD1 leukemias, the 6C3HED and P1798 lymphosarcomata, and the Lorenz lymphoma (31, 40, 68), and the Murphy-Sturm lymphosarcoma, the Walker 256 carcinosarcoma, the ACNC42 and the Jensen sarcomata in the rat (31, 69, 70), and spontaneous lymphosarcoma in the dog (41, 44).

Since the initial clinical trials mentioned earlier (42-45), more extensive clinical trials with L-asparaginase have been conducted in many centres in well over 1,000 cancer patients (29, 30, 71-81).

These studies have shown that L-asparaginase is most effective in the treatment of acute lymphocytic leukemia, producing complete remissions in about 60% of patients treated, most of whom were resistant to conventional antileukemic drugs. The response rate in patients with acute myelocytic, myelomonocytic, or monocytic leukemia has been much less (10 - 15%). Occasional responses have been obtained in patients with malignant melanoma, chronic myelocytic leukemia, lymphosarcoma and reticulum cell sarcoma. No significant responses have been observed in patients with other types of solid tumors.

Mechanism of antitumor action

Although the precise mechanism of the antitumor activity of L-asparaginase is not fully understood, the most widely accepted hypothesis is based on the catalytic action of the enzyme in the hydrolysis of L-asparagine to L-aspartic acid and ammonia. L-asparaginase-sensitive tumors, in contrast to most normal cells and L-asparaginase-resistant tumors, require an external source of L-asparagine for optimal growth. Thus, administration of L-asparaginase depletes the plasma of circulating L-asparagine, and so deprives dependent tumor cells of their extracellular source of L-asparagine (38, 40, 82-84). It has been shown that these L-asparagine-dependent cells cannot synthesize sufficient endogenous L-asparagine due to very low levels of the enzyme, L-asparagine synthetase, or the inability of these cells to increase L-asparagine synthetase activity after L-asparaginase administration (68, 85-88).

At the present time, the only known pathways of L-asparagine

metabolism in animal tissues are conversion to L-aspartate by hydrolysis or to α-ketosuccinamic acid by transamination, and utilization for protein synthesis (17, 89). Because no major route of L-asparagine utilization, other than protein synthesis, is known, it is believed that a lack of L-asparagine results in the inhibition of synthesis of certain proteins necessary for the growth of neoplastic cells. On the other hand, it is possible that there may exist other routes of L-asparagine utilization, which though not yet detected, are essential to the survival of tumor cells. Sobin and Kidd (83, 84) and Broome and Schwartz (85) have shown that protein synthesis is inhibited when sensitive cells are deprived of L-asparagine. Bossman and Kessel (90) studied the effect of L-asparaginase on glycoprotein synthesis in L5178Y cell suspensions and suggested that hydrolysis of cell membrane glycoproteins, as well as inhibition of their synthesis by the enzyme, cause rapid cell lysis.

Following inhibition of protein synthesis, DNA synthesis, then RNA synthesis are inhibited (84, 91). Although the inhibition of DNA synthesis has been regarded as a secondary effect of disturbed protein metabolism, Hirschmann et al (92) have suggested that L-asparaginase has a direct influence on DNA metabolism, perhaps by inhibition of de novo purine synthesis via some unknown mechanism. A preliminary report by Woods and Dixon (93) also suggests that the C-skeleton of L-asparagine may serve as a direct precursor in the biosynthesis of certain pyrimidine nucleotides isolated from lymphosarcoma cells. It has been proposed by Ellem et al (91) that L-asparaginase could stop

histone synthesis and thereby inhibit DNA replication. Another in vivo effect of L-asparaginase treatment that may relate to the mechanism of tumor death is the results by Mashburn and Wriston (94) and Mashburn and Landin (95) who found an increase in both acid and alkaline ribonuclease activities after L-asparaginase treatment of the P1798 lymphosarcoma.

In addition to its effects on protein and nucleic acid metabolism, L-asparaginase treatment may also result in other indirect effects which are harmful to neoplastic tissue. For example, Broome (82) has suggested that the high levels of L-aspartic acid observed after L-asparaginase treatment may be toxic or may significantly alter metabolic control mechanisms in the tumor cells. Miller et al (96) proposed that the inherent L-glutaminase activity of L-asparaginase may enhance its antitumor effect by lowering the circulating levels of L-glutamine which is the nitrogen donor for the biosynthesis of L-asparagine in mammalian cells. Ryan and Dworak (97a) and Ryan and Sornson (97b) have shown that administration of L-asparaginase depresses the glycine level in susceptible but not resistant 6C3HED tumors, and suggested that the loss of cellular glycine may be more important than loss of L-asparagine because of the requirement of glycine for purine synthesis. The latter findings may be related to preliminary in vitro results obtained in a recent study by Chattopadhyay et al (98) who reported that glycine inhibits the activity of L-asparaginase from guinea pig liver.

The relation of all these possible effects to the tumor-

inhibitory properties of L-asparaginase have not yet been fully evaluated, but inhibition of protein synthesis appears to be of the most significance.

Factors that influence the tumor-inhibitory effectiveness of L-asparaginase

There are 2 major factors that determine the tumor-inhibitory properties of a particular L-asparaginase preparation. These are:

(a) The affinity of the enzyme for substrate or its K_m; and

(b) The clearance rate of the enzyme from the host's circulation or its plasma half-life.

(a) The affinity of L-asparaginase for L-asparagine: A low K_m, i.e. a high affinity for substrate, at physiological pH is an absolute requirement for antitumor activity. This is especially important since L-asparagine concentration must be lowered to at least 10^{-5} M (10 nmoles/ml) in the medium to become rate-limiting in protein synthesis (82, 83, 85, 99). Indeed, the difference in the tumor-inhibitory activity of 2 distinct L-asparaginases isolated from E. coli (39, 64, 100, 101) has been related to their K_m values: EC-1 enzyme with low affinity has no tumor-inhibitory action, whereas EC-2 has high affinity and is a potent antitumor agent (18). Broome (18, 99) has similarly attributed the relative tumor-inhibitory effectiveness of L-asparaginases from E. coli and agouti serum to the difference in their K_m values, the more potent E. coli enzyme having a lower K_m. It should be noted that Erwinia carotovora L-asparaginase which is clinically active has a (low) K_m of

1.0×10^{-5} M (102). This enzyme has been used by us in studies to be described later in Chapter IV of this thesis.

(b) <u>The clearance rate of L-asparaginase from the host's circulation:</u>

The more rapid the removal of injected enzyme from the host's circulation, the lesser the antitumor activity of the enzyme. The clearance rate of the enzyme itself is influenced by at least 5 factors:

(1) The source of the enzyme. Thus, the lack of tumor-inhibitory activity of L-asparaginases from yeast (103), Bacillus coagulans (104), chicken liver (55), and other sources, have been related to the short plasma half-lives of these enzymes, whereas guinea pig (103) and agouti (99) serum L-asparaginases, for example, have long half-lives and are potent antitumor agents. Even different commercial preparations derived from a common source, for example E. coli, vary in their plasma half-lives to some extent (16, 105).

(2) The isoelectric point of the enzyme. Mashburn and Landin (105) have suggested that the rate of clearance of circulating injected L-asparaginase was directly related to the extent of departure of the isoelectric point of the enzyme from neutrality. More detailed studies by Rutter and Wade (106) led these authors to suggest that the isoelectric point of L-asparaginase influences the phagocytosis of the enzyme from the plasma, perhaps by increasing the degree of opsonisation of the enzyme.

The half-life of the enzyme in the plasma not only depends on the

properties of the enzyme itself, but also on the physiological state of the host, as discussed below.

(3) The presence of the lactic dehydrogenase-elevating virus (LDH virus). This virus is present as a contaminant in most, if not all, of the mouse tumors generally employed for L-asparaginase studies. Studies by Riley's group (107 - 109) have conclusively demonstrated the profound beneficial influence this virus may have on the therapeutic response of the tumor-bearing host to L-asparaginase therapy. When the virus was eliminated from infected mouse tumors, the therapeutic potency of L-asparaginase was markedly diminished. When the tumor-bearing mice were intentionally infected with the LDH virus, long-term remissions were reobtained following single doses of L-asparaginase. The virus alone had no antitumor effect. The potential value of the LDH virus in test systems for determining the therapeutic value of a given enzyme has been emphasized by more recent studies (110, 111) which showed that the glutaminase-asparaginase enzyme isolated by Roberts et al (4, 6) was totally without therapeutic effect in the absence of the LDH virus, whereas it was a potentially valuable antitumor agent in the presence of the virus. The observed beneficial influence of this benign replicating agent as an adjunct to L-asparaginase therapy has no theoretical antecedent and the basis of its action is the induction of an impairment of the capacity of the infected host to clear protein from the blood, thus prolonging the plasma

half-life of the enzyme.

(4) The immune status of the host. Many studies (100, 112-118) have conclusively demonstrated that the presence of circulating, humoral anti-L-asparaginase antibodies, inhibit the catalytic activity of injected enzyme and cause a dramatically accelerated removal of the enzyme from the bloodstream of immunized hosts, thus reducing the therapeutic value of the enzyme. This will be discussed more fully later.

(5) Finally, estrogen pretreatment of tumor-bearing mice have been shown to result in increased clearance rate of injected L-asparaginase (103). This is consistent with the observation of Kim (119), later confirmed by Broome (103), that estrogen treatment decreases the tumor-inhibitory effectiveness of guinea pig serum. It has been proposed that estrogen treatment may cause an increase in the phagocytic activity of the reticuloendothelial system for removing injected substances (103).

Advantages of the use of L-asparaginase in acute lymphocytic leukemia therapy

L-asparaginase has been considered to be a valuable addition to the chemotherapeutic regimen for the management of acute lymphocytic leukemia because of the following major properties (1, 24, 29, 30, 120):

(a) It has a good remission rate even when used singly.

(b) It has a fairly rapid speed of response.

(c) It has the highest therapeutic index (1000) among all clinically available antileukemic agents. All other drugs used in the treatment

of acute leukemia have therapeutic indices which are invariably < 10 and are usually given in near toxic doses.

(d) Its lack of cross-resistance with other antileukemic agents makes it often effective at a very late stage of the disease even when the patient has become resistant to all other chemotherapeutic agents. This is probably related to the fundamental difference in its mechanism of action.

(e) Its lack of significant bone marrow depression in a situtation where both disease and other antileukemic drugs depress hematopoiesis makes it a prime candidate for use in combination with a variety of other antileukemic but myelosuppressive drugs.

Problems associated with L-asparaginase therapy

Two major drawbacks have arisen during the course of clinical trials that deter a more widespread use of L-asparaginase. These are:

(a) A wide spectrum of adverse side effects; and

(b) The development of resistance to repeated L-asparaginase therapy.

(a) Toxicity: A large variety of untoward side effects have been observed with the clinical use of L-asparaginase (1, 23, 28, 29, 81, 121 - 123). Some of the general side effects reported include anorexia, nausea, vomitting, chills, fever, diarrhea and weight loss. Liver toxicity was manifested by increased levels of serum glutamic-oxaloacetic transaminase, alkaline phosphatase, 5'-nucleotidase, bilirubin, increased retention of bromsulphalein, and decreased levels of serum albumin (causing generalized edema and low serum calcium),

fibrinogen and other coagulation factors. Fatty metamorphosis of the liver has also been observed. Hypolipidemia with decreased serum levels of cholesterol, phospholipids, and total lipids was found in most patients, but hyperlipidemia has also been observed. Pancreatic dysfunction was manifested as pancreatitis, and by increased levels of serum amylase, lipase and blood glucose, with decreased levels of serum insulin (causing diabetes to develop in some cases). Renal dysfunction was manifested by increased levels of blood urea nitrogen and acute renal failure in some cases. Hematological changes include granulocytopenia, lymphocytopenia, and thrombocytopenia. Effects on the central nervous system include headache, drowsiness, lethargy, depression, disorientation, and confusion. Immune reactions to L-asparaginase have developed with hypersensitivity reactions occurring in approximately one third of treated patients. These reactions were usually of the anaphylactic type, characterized by urticaria, abdominal cramps, hypotension, cyanosis, respiratory distress and facial edema. Coma and even fatal anaphylaxis has also been reported, as a result of immunological complications.

This wide spectrum of effects may be attributed to at least 3 factors:

(1) They may be the direct or indirect result of L-asparagine depletion causing, for instance, inhibition of protein synthesis in the liver, pancreas and brain.

(2) They may arise because of the antigenic nature of the foreign

enzyme preparation. Thus, repeated injections of enzyme may evoke hypersensitivity reactions and other immunological complications in the host. Several groups have reported the presence of anti-L-asparaginase antibodies in the sera of L-asparaginase-treated patients, using the techniques of precipitation (117, 118, 122, 124), complement fixation (122), radioimmunoassay (125) and passive hemagglutination (77, 118). The presence of reagin-type antibodies (Ig E) has also been detected in patients who experienced anaphylaxis (77, 118, 125).

(3) They may originate from the possible contamination of the enzyme preparation with bacterial substances such as endotoxins. Indeed, bacterial endotoxins are known to cause side effects which resemble many of those observed after L-asparaginase therapy: nausea, chills, fever, vomitting, weakness, hypotension, respiratory disturbances, fibrinogenopenia, coagulopathies, hepatic injuries, renal failure, and delayed hypersensitivity reaction (73, 126 - 128).

The latter 2 factors represent problems which would generally be encountered when using enzymes derived from heterogenous sources, and presumably are not peculiar to L-asparaginase.

(b) Resistance. The other major limitation of L-asparaginase treatment is development of clinical resistance to the antileukemic effect of the enzyme. There have been 4 major mechanisms proposed to explain the origin of resistance to L-asparaginase therapy:

(1) A marked increase in L-asparagine synthetase levels has been

shown to occur in tumor cells, probably by a mechanism of derepression of L-asparagine synthetase, so that the tumor cells no longer have to depend on an external supply of L-asparagine (68, 85 - 88, 129). In this regard, Gallo et al (130 - 132) have shown that L-asparaginase-resistant cells contain reduced levels of an asparaginyl-tRNA species which they proposed normally acts as a co-repressor for L-asparagine biosynthesis.

(2) Phenotypically stable L-asparagine-independent mutant cells may arise spontaneously in a population of parent L-asparagine-dependent cells even when L-asparagine is continuously available in the growth medium. This possibility was demonstrated by Handschumacher's group (133, 134) who reported a mutation rate in vitro of L5178Y mouse leukemia cells from L-asparagine-dependence to L-asparagine-independence of about 1×10^{-6} /cell/ generation, properties of this change being consistent with a spontaneous, random, mutational event. Thus, mutation followed by selection for L-asparagine-independent mutant cells in an L-asparagine-free medium could lead to the development of an L-asparaginase-resistant cell population.

(3) Another mechanism of resistance might involve the development of more efficient means by tumor cells of extracting L-asparagine from the plasma, erythrocytes, or other normal host cells (82, 135).

(4) Because bacterial L-asparaginase is a large protein which is

foreign to the recipient host, it is not surprising that repeated injections of the enzyme will result in the production of humoral antibodies against the enzyme. This has been reported to occur in several studies (77, 100, 112 - 118, 122, 124, 125). Humoral antibodies have been shown to inhibit the catalytic activity of injected L-asparaginase and cause an accelerated in vivo removal of the enzyme from the host's circulation (100, 112 - 118), thus reducing the antitumor effectiveness of the enzyme. Of these 2 factors, the shortened plasma half-life of the enzyme appears to be the more important, especially since the maximum inhibition of catalytic activity by immune serum has been found to be 45 - 53% (100, 113, 116 - 118).

Combination chemotherapy

In attempts to reduce the problem of tumor cell resistance to L-asparaginase, while increasing the total tumor cell kill at the same time, L-asparaginase has been studied for use in combination with other chemotherapeutic agents. There are 2 basic concepts which form the rationale for the use of several chemotherapeutic agents in combination against a particular neoplasm.

(a) If 2 or more drugs have different toxicities for normal tissues and are effective against a particular type of neoplasm, the antitumor effects may be potentiated without concomitant additive toxic effects for any given normal tissue.

(b) The use of drugs with different mechanisms of action may decrease the development of resistance by reducing the chances for survival of tumor cells resistant to a single agent.

Three properties of L-asparaginase in particular, namely its high therapeutic index, different expression of host toxicity, and lack of cross-resistance with other antitumor agents, led to extensive investigation of its use in combination with other drugs. Indeed, Burchenal (136), paraphrasing the well-known statement of Sir Winston Churchill, appropriately stated, "never have so many compounds with so many different mechanisms of action potentiated the antileukemic activity of one agent". Agents that have been shown to have synergistic effects when used in various combinations with L-asparaginase include: prednisone, vincristine, daumomycin, cytosine arabinoside, thioguanine, cyclophosphamide, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), azotomycin, 5-fluorouracil, 5-azacytidine and others (29, 30, 137 - 145). No attempt will be made here to describe the results obtained in these studies. However, 2 interesting findings which have emerged from these reports will be mentioned in this regard:

(a) The addition of other antileukemic agents to L-asparaginase therapy does not necessarily result in any therapeutic advantage. For example, L-asparaginase + azaserine, a glutamine antagonist, did not yield better results than L-asparaginase alone in animals (137), or in patients with acute lymphocytic leukemia (138, 139). Also, the addition of a third drug, 6-azauridine, to the 2-drug combination of L-asparaginase + cytosine arabinoside, did not show any

advantage in animals (140), or in patients with acute lymphocytic leukemia (141).

(b) Therapeutic synergism for a given combination of drugs may be critically schedule-dependent, that is, whether the drugs are given simultaneously or sequentially. For example, Capizzi et al (142) reported that there was no therapeutic advantage over the use of methotrexate and L-asparaginase when these agents were given simultaneously. Subsequent reports by Connors and Jones (143) and by Vadlamudi et al (144), however, showed potentiating anti-tumor effects when these same agents were given sequentially but not simultaneously.

The use of L-asparaginase in combination with other antitumor agents as a more optimal means of cytoreductive chemotherapy represents one attempt to overcome the problem of cell resistance experienced during L-asparaginase therapy. Several other approaches have been tried in an effort to improve L-asparaginase therapy. These have been described in Chapter II. One of these alternate approaches involves the entrapment of the enzyme within "semipermeable microcapsules" prior to its administration.

As mentioned earlier in section IB of this Chapter, the wide spectrum of side effects observed during L-asparaginase therapy may be partly attributable to the antigenic nature of the enzyme and the possible presence of accompanying bacterial contaminants such as endotoxins. The latter represent fundamental problems which limit the

therapeutic usefulness of enzymes in general since most enzymes used in clinical therapy are derived from heterogeneous, e.g. bacterial, sources. Moreover, one of several mechanisms responsible for the loss of therapeutic potency of L-asparaginase upon repeated injections of the enzyme, has been shown to be the rapid in vivo removal and inactivation of the enzyme by circulating, humoral antibodies present in the host. If some of these difficulties could be circumvented or overcome, the use of enzymes in clinical medicine, in general, and L-asparaginase, in particular, would be extended to their full potential importance. A major advance in this direction has come from Chang's pioneering work on the entrapment of enzymes within semipermeable microcapsules ("artificial cells"). In the following section of this thesis, the subject of semipermeable microcapsules will be reviewed with particular emphasis on their biomedical applications. Chang has published a recent monograph (146) and has written several more recent review articles (147 - 151) on this subject.

IC - SEMIPERMEABLE MICROCAPSULES

Introduction

The term "artificial cell" as proposed by Chang is not a physical entity but a concept involving the preparation of artificial structures of cellular dimensions for various possible biomedical applications such as the replacement or supplement of deficient cell functions (146). These artificial structures have also been described as semipermeable microcapsules (146, 152 - 156). In 1957, Chang (152) reported his first attempt to produce an artificial cell system consisting of red blood cell hemolysate enclosed within spherical ultrathin collodion membranes of cellular dimensions. This simplified model of a red blood cell, in which the biological cell membrane was replaced by an artificial cell membrane, was found to behave like its natural counterpart in many ways. Tests showed that carbonic anhydrase and catalase in the hemolysate retained their catalytic activity after encapsulation, and the hemolysate partly retained its ability to combine reversibly with oxygen diffusing in and to catalyse the hydration of CO_2. Although the initial artificial cell model of the red blood cell had limited functional properties, results were sufficiently interesting to prompt Chang to investigate the potential of the "artificial cell" concept in more detail. Since the initial approach, Chang (153 - 156) has prepared semipermeable microcapsules by various other techniques which have subsequently been modified and updated (146). These newer procedures result in the preparation of microcapsules with great variations in contents, size, configuration, membrane material, permeability

characteristics and other properties (146, 154 - 156). For example, Chang has applied the artificial concept to the encapsulation of several individual enzymes, multiple enzyme systems, cell homogenates of liver and endocrine cells, intact cells, smaller artificial cells, cell organelles, ion exchange resins, and activated charcoal. Membrane properties have also been altered so that desired properties may be obtained, including variations in the pore size, thickness, charge, polymer material, biocompatability, and incorporation of protein, lipid, polysaccharides and even transport carrier molecules into the artificial cell membrane.

Basic features of artificial cells

Artificial cells, in general, possess at least 4 major features of biological cells (146, 154 - 156):

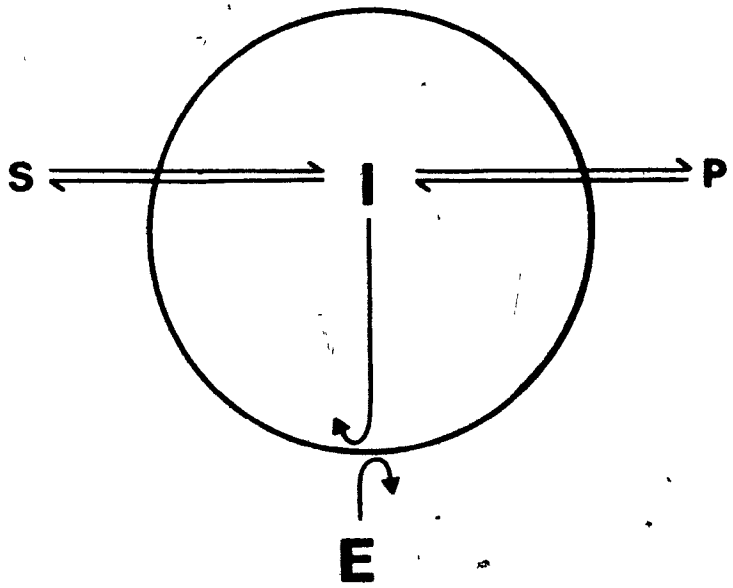
(a) They are aqueous compartments of microscopic dimensions which are enclosed by an ultrathin membrane. Because of the small size, there is a large surface area : volume relationship for the microcapsules. Chang (146, 157) has calculated that 10 ml of 20 μm - diameter microcapsules have a total surface area of 2.5 m^2, which is more than twice the surface area present in a standard hemodialysis machine (1 m^2). The thickness of the enclosing membrane is about 200 Å (146, 156) which is of the same order of magnitude as biological cell membranes. Because of the enormous surface area of the microcapsules and the fact that the microcapsule membrane is at least 100 times thinner than standard hemodialysis membranes, the potential transport

rate of 10 ml of 20 μm-diameter microcapsules is at least 200 times higher than for a whole hemodialysis machine (146, 157 - 159). In addition, the small diameter of the artificial cells, like their biological counterparts, allows for an extremely rapid equilibration of permeant solutes throughout the small intracellular space of the microcapsules.

(b) Microcapsules have been prepared with enclosing membranes of protein, polysaccharide, lipids, lipid and cross-linked protein complex, or lipid and polymer, in an attempt to mimic more closely the complex lipoprotein structure, coated externally with mucopolysaccharide, of biological membranes (146, 154 - 156, 160, 161).

(c) Artificial cells also partially mimic biological cells in that they create an intracellular environment which differs from the extracellular environment. Through a mechanism of passive restriction (Figure 1), intracellular macromolecules and organelles are conserved within the artificial cells and prevented from coming into direct contact with macromolecular substances in the external environment. Also, external impermeant materials are prevented from entering the intracellular environment. At the same time, however, permeant molecules can equilibrate rapidly across the membranes by simple diffusion or special carrier mechanisms to be acted upon by the cell contents.

(d) Variations in the permeability characteristics and other properties of the artificial cell membranes have also been made so as to



I - INTRACELLULAR IMPERMEANT MATERIALS

E - EXTRACELLULAR IMPERMEANT MATERIALS

S,P - PERMEANT MOLECULES

FIGURE 1

Schematic representation of biological and artificial cells.

(from Chang, T.M.S. (146)).

allow a more selective permeation of various types of molecules, as occurs in natural cells. Examples of these variations include alterations in the polymer material, protein, lipid and polysaccharide content, charge, pore size, thickness, surface properties, and even incorporation of special carrier molecules (161, 162) into the artificial cell membranes.

Methods of preparation of semipermeable microcapsules

Many methods now exist for the preparation of semipermeable microcapsules (146, 149, 151, 154, 156). The general procedure involves 3 basic steps (Figure 2):

(a) Emulsification of the aqueous protein solution in an organic liquid with the aid of a suitable detergent;

(b) Formation of a permanent polymer membrane around each microdroplet by the addition of a suitable material to the continuous phase; and

(c) Transferral of newly formed microcapsules from the organic phase with the aid of a suitable detergent, and suspension in an aqueous medium.

This general procedure has been modified and extended to prepare microcapsules with many variations in size, configuration, membrane materials, permeability characteristics, etc.

In the method of interfacial coacervation (interfacial precipitation or organic phase separation), the formation of the membrane is a physical process which depends on the lower solubility of the polymer, dissolved in a water-immiscible fluid, at the

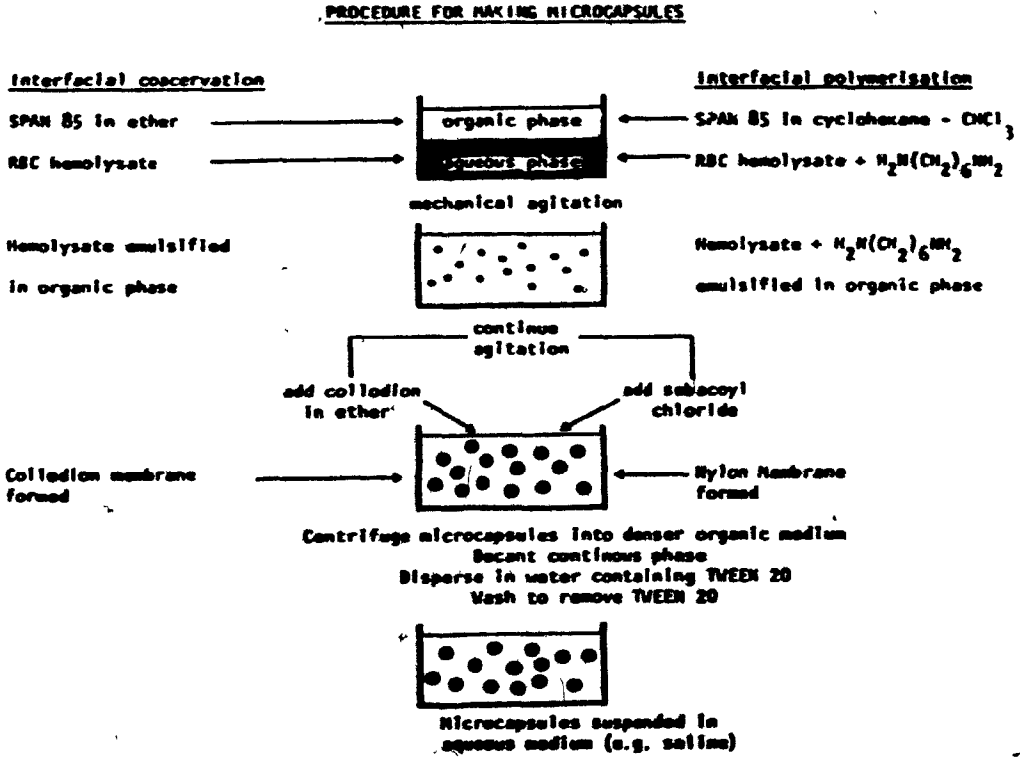


FIGURE 2

Schematic diagram of the two basic procedures for preparing collodion (interfacial coacervation) and nylon (interfacial polymerization) microcapsules.

(from Chang, T.M.S. (154)).

interface of each aqueous microdroplet in an emulsion. The use of collodion (cellulose nitrate) as the polymer material with erythrocyte hemolysate as the internal aqueous phase is most commonly employed. The detailed preparation of collodion microcapsules has been described in Chapter IV. With appropriate modification of this procedure, polymers other than cellulose nitrate can be used: for example, cellulose acetate and polystyrene (146, 154, 156). Mueller and Rudin (172) have also described the preparation of microspherical bilayer lipid membranes enveloping red blood cell hemolysate, using a variation of this approach.

Morgan et al (163) introduced interfacial polymerization, as a method for preparing a large class of polymers, including nylon. This method depends on the chemical reaction (polycondensation) between a diamine (e.g. hexamethylenediamine) in the aqueous phase and a dicarboxylic acid halide (e.g. sebacoyl chloride) in the organic phase, to form a polymer (nylon - polyamide 610) at the interface. The chemical reaction that takes place may be represented as follows:

$$\underset{\text{1,6 Hexamethylenediamine}}{NH_2(CH_2)_6NH_2} + \underset{\text{Sebacoyl chloride}}{ClCO(CH_2)_8COCl} \xrightarrow{-HCl} \underset{\substack{\text{Nylon - Polyamide 610}\\ \text{(Polyhexamethylene sebacamide)}}}{\left\{NH(CH_2)_6NH\text{-}CO(CH_2)_8CO\right\}}$$

Chang et al (153 - 156) used this principle to modify the first method of interfacial coacervation for preparing semipermeable microcapsules with nylon membranes. The procedure for the preparation of nylon microcapsules has been described in detail in Chapter II. Membranes made of polymers other than nylon can also be prepared by this procedure: for example, cross-linked protein, polyureas, polyphthalamides, polyurethanes, polysulfonamides and polyphenylesters (146, 154 - 156, 161, 164 - 168).

A third method for the preparation of semipermeable microcapsules is by secondary emulsion (146, 157). In this approach, a fine emulsion of aqueous microdroplets is dispersed in an organic phase containing polymer. The organic phase containing the fine aqueous microdroplets is then itself dispersed into larger droplets in an aqueous phase. Thus, each of the large organic phase droplets will contain a number of smaller aqueous microdroplets. The organic polymer solution is allowed to solidify to form solid spheres, each containing varying numbers of aqueous microdroplets inside. It should be noted that this method, unlike the other two described above, results in the production of microcapsules consisting of a large amount of polymer matrix. Microcapsules made of silicone rubber (silastic), ethyl cellulose, polystyrene, and other polymer materials, have been prepared by this method (146, 157, 169). An extension of the principle of secondary emulsion is the emulsification of aqueous solution in a polymer organic solution as above, but instead of forming microspheres, the polymer solution is extruded in the form of fibers (170). Another variation of the double emulsion technique is to emulsify aqueous microdroplets in a water-immiscible phase consisting of surfactant, additives, and a high molecular weight paraffin, and then emulsifying the organic phase. This results in the encapsulation of aqueous microdroplets within liquid-surfactant membrane microcapsules (171).

Properties of semipermeable microcapsules

These properties have been described in detail by Chang (146, 154 - 156). Unless otherwise specified, "microcapsules" (or "microencapsulated") referred to in the text from here on relate to those prepared either by interfacial coacervation (collodion) or interfacial polymerization (nylon), inasmuch as these have been studied and characterized in more detail than others. Microcapsules stored in an aqueous medium are generally spherical due to the high colloidal osmotic pressure gradient generated by the enclosed protein. Nylon microcapsules usually have a clear interior (see Figure 1, Chapter II), but collodion microcapsules always show considerable precipitation of the enclosed protein. The mean diameter of microcapsules prepared by the most convenient (standard) method is approximately 100 μm. The diameter of the microcapsules can be varied by changes in the speed of the mechanical emulsifier, the concentration of the emulsifying agent, and the viscosity of the organic phase. Larger microcapsules can also be prepared one at a time by interfacial coacervation or polymerization, using a drop technique. Thus, microcapsules have been prepared with mean diameters ranging from less than 5 μm to 5 mm by the interfacial coacervation or polymerization techniques.

The membrane thickness of nylon microcapsules prepared by the standard method has been estimated by electron microscopic studies to be 200 Å; this is thinner than the collodion microcapsule membranes (500 Å). Although the nylon membranes are very thin and flexible, Jay and Edwards (173) have reported that they, unlike biological membranes, can withstand a membrane tension of up to 2520 ± 20 dynes/cm. Thus, even when suspended in distilled water, the high internal pressure

generated does not result in rupture of the microcapsule membrane.

Studies on the electrical properties of microcapsules show that there is no net surface charge measurable by electrophoresis for nylon microcapsules. The electrophoretic mobility of collodion microcapsules has been measured to be 0.93 μm ± 0.06 μm/sec/volt/cm (160). The electrophoretic mobilities of microcapsules with variously modified membrane compositions have also been described (146, 160, 167). Studies by Jay and Burton (175) show that a potential difference of 0.52 ± 0.02 mV exist across the membranes of nylon microcapsules, probably because of the phenomenon known as Donnan equilibrium, resulting from the presence of the nondiffusible hemoglobin inside the microcapsules. In further studies, Jay and Sivertz (176) calculated that the resistivity of the nylon microcapsule membrane is 3 x 10 ohm-cm.

When microcapsules are placed in a hypertonic solution, water is rapidly withdrawn osmotically and the microcapsules assume a folded appearance, somewhat analagous to the phenomena of crenation in erythrocytes or plasmolysis in plant cells. Reversible crenation and swelling of microcapsules, when placed in a hypertonic or hypotonic medium respectively, illustrate the general property of semipermeability of the microcapsule membrane. Detailed studies by Chang and Poznansky (177) on the permeability characteristics of nylon microcapsules provided an estimate of the equivalent pore radius of the microcapsule membrane to be 18 Å. Conceptually, a functional pore radius of 18 Å means that the microcapsule membrane is impermeable to macromolecules, such as hemoglobin, enzymes and other proteins,

but are permeable to smaller molecules. The permeability studies by Chang and Poznansky (177) show that there is an extremely rapid equilibration of permeant molecules across the microcapsule membrane. For example, the half-time for equilibration was found to be 4.3 seconds for urea, and 35.5 seconds for sucrose. As stated earlier, this extremely rapid permeation rate may be attributable to the ultrathiness of the membrane and the large surface area of microcapsules. The equivalent pore radius of the membrane can be varied, for example, by changing the polymerization reaction time (in the case of nylon microcapsules) or using membrane materials with different permeability characteristics. The permeability characteristics of microcapsules are very important factors in the multitudinous applications of artificial cells.

Microencapsulated enzymes

Chang (146, 149) and Zaborsky (178) have published comprehensive reviews on the different types of enzymes that have been microencapsulated by several laboratories using the various techniques described earlier. The enzymes that have been microencapsulated include: enzymes present in erythrocyte hemolysate, urease, carbonic anhydrase, uricase, trypsin, catalase, asparaginase, lipase, lactase, α-glucosidase, zymase complex (yeast), muscle extract, phenolase, nitrate reductase, β-fructofuranosidase. More recently, Campbell and Chang microencapsulated hexokinase and pyruvate kinase (179) and also alcohol dehydrogenase and malic dehydrogenase (180). A general

feature of such microencapsulated enzymes is that they do not leak out because the surrounding membrane is impermeable to macromolecules; however, permeant substrates can be efficiently acted on by the enclosed enzyme. One exception to this that deserves brief mention is the recent work by Gregoriadis and his colleagues (181 - 183) on the encapsulation of enzymes within liposomes (lipid spherules) for possible enzyme replacement therapy. In these cases, the enzymes are entrapped within aqueous compartments between concentric lipid bilayers. Unlike other types of microencapsulated enzymes, however, liposomally entrapped enzymes can only act on substrate after release from the liposomes.

In theory, since microencapsulated enzymes are in free solution in the aqueous phase, there should be no inherent changes in the properties of the entrapped enzymes themselves after encapsulation. However, diffusional effects of the membrane and changes in the intracellular microenvironment may cause changes (apparent) in the V_{max}, K_m, pH-activity profile, specificity, and other kinetic parameters, of the enzyme, as compared to its native counterpart. A review of the diverse literature on the various properties of different types of microencapsulated enzymes is not appropriate in this thesis and will not be attempted. However, one important property, that of stability, will be discussed in the following as this directly relates to some of the in vitro and in vivo observations made with relation to L-asparaginase in the present studies. A detailed account of some of the kinetic and other data specifically relating to microencapsulated

L-asparaginase will be presented in a later section.

All enzymes microencapsulated with a high concentration of protein, have shown, to date, the general feature of increased stability relative to the native enzyme in free solution (146, 149, 151; 184). For example, when stored at 4°C, catalase in free solution lost 50% of its initial activity after 15 days, whereas microencapsulated L-asparaginase retained more than 90% of its initial activity for more than 100 days (184). When stored at 37°C, the stability of microencapsulated catalase was also more stable than catalase in free solution (184). Studies on the stability of L-asparaginase in the solution and microencapsulated forms will be described later. Increased stability was also observed by Chang for storage at 4°C of carbonic anhydrase, urease and lactase (146, 151, 154 - 156). Chang (146, 184) has attributed the increased stability of microencapsulated over the solution form of enzymes to the fact that, since proteins cannot leak out, the entrapped enzymes remain at all times in an intracellular environment containing a high concentration of protein. This is the case even if the microcapsules themselves are dispersed in a very dilute suspension. Chang (184) has also shown that microencapsulated enzymes can be stabilized even further if the enzyme - containing microcapsules, after formation, are treated with glutaraldehyde to cross-link the enzymes within the microcapsules. In this way, glutaraldehyde-treated catalase microcapsules were found to be significantly more stable than untreated catalase microcapsules after storage at 37°C (184). Another method to increase enzyme stability is to microencapsulate enzymes which have already been "immobilized" (see

section ID) by various techniques (146, 185). The feature of increased stability of microencapsulated enzymes over their native counterparts offers the advantage that there may be less need for frequent, repeated injections of microencapsulated enzymes since they retain their catalytic activity for a longer period of time.

In addition to a high diffusion rate of permeant substrates and increased stability, there are other features of biomedical significance which are conferred by the microencapsulation of enzymes. These include the following (146, 151, 154, 178, 185).

As a result of the small amount of polymer matrix required to entrap a given quantity of enzyme, there is negligible accumulation of polymer material after in vivo introduction of microencapsulated enzymes into the host.

Although an ideal membrane material has not yet been developed, Chang et al (146, 160, 186, 187) have shown that microcapsules with a more biocompatible membrane could be obtained if the membrane is coated with albumin or benzalkonium-heparin complex collodion, or if the membrane is prepared with benzalkonium-heparin complex collodion instead of collodion in the organic phase. This would allow enzyme-containing microcapsules to come into contact with biological fluids without causing serious adverse effects on the formed elements of blood. Earlier this year, reports from this laboratory (188, 189) also described the preparation of an albumin-coated, heparin-grafted cellulose membrane which had improved biocompatible properties on in vitro and in vivo testing.

Since most enzymes used in clinical therapy are derived from heterogeneous, e.g. bacterial, sources, the administration of soluble enzymes into the body pose a number of problems which limit their therapeutic usefulness (146, 185). Such problems, for example, have already been described in detail in the case of L-asparaginase in section IB of this thesis. A significant feature of microencapsulated enzymes is that enzymes and any accompanying macromolecular contaminants, such as endotoxins, once entrapped within the microcapsules, do not leak out into the circulation of the host (146, 154- 156, 185). Thus, there may be reduced immunological and other toxic problems arising in the host. Furthermore, if humoral antibodies are present in the surrounding milieu, they cannot cross the microcapsule membrane to inactivate the entrapped enzyme (146, 154 - 156, 185). These latter considerations are fundamental to the therapeutic use of microencapsulated enzymes and will be discussed at length in subsequent sections of this thesis with particular reference to microencapsulated L-asparaginase.

It should be noted that some care must be taken in selecting the appropriate procedure for micrpencapsulation of a given enzyme. For instance, the reagents used in the interfacial polymerization procedure do not affect enzymes like L-asparaginase and urease but may inactivate other enzymes such as catalase (190) and uricase (191). Catalase and uricase, however, can be successfully microencapsulated by the interfacial coacervation procedure (190, 191). Another important point that deserves comment is that enzymes entrapped within semiper-

meable microcapsules can act only on external substrates which can diffuse across the membrane (146, 154 - 156, 178). Permeant substances include substrates up to the size of polypeptides but not macromolecules. In the case of interfacial polymerization, it should also be pointed out that the enzyme may be incorporated into the membrane structure of microcapsules (146, 154, 156, 167).

Biomedical applications of microencapsulated enzymes

Because of the fundamental importance of enzymes in all biological processes, their biomedical applications, both realized and potential, are multitudinous. The following section of this review will be restricted to 3 examples of model enzyme systems that have been investigated in this laboratory to illustrate the principle of using microencapsulated in lieu of native enzymes for biomedical purposes.

The basic demonstration of the feasibility of using microencapsulated enzymes for in vivo administration came from studies in this laboratory (155, 192) using microencapsulated urease for i.p. injection into dogs as a test model enzyme system. In these initial studies, it was found that the enzyme did not leak out into the blood, but remained inside the microcapsules in the peritoneal cavity where it acted efficiently on systemic urea diffusing in. The conversion of blood urea into ammonia resulted in a quantitative increase in blood ammonia levels. These results led to further studies to examine the feasibility of using microen-

capsulated enzymes for various biomedical applications, as illustrated by the 3 examples described next.

(a) Enzyme replacement therapy for congenital enzyme deficiency diseases

The first studies on microencapsulated enzyme replacement therapy were carried out by Chang and Poznansky (190), using a special strain of mice (Feinstein's acatalasemic Cs^b mice) congenitally deficient in the enzyme catalase (193). Feinstein (194) showed that intravenous injection of beef catalase solution can reverse hydrogen peroxide toxicity in acatalasemic mice and suggested that this mutant strain of mice and catalase were useful as a model system for the study of enzyme replacement therapy of inborn errors of metabolism. Feinstein found that although injection of catalase solution could replace the enzyme deficiency, repeated injections caused the production of antibodies to the enzyme.

Since Chang had earlier successfully microencapsulated catalase in collodion microcapsules (191), Chang and Poznansky (190) began investigating various aspects of enzyme replacement therapy using artificial cells containing catalase for injection into these acatalasemic mice. They showed that i.p. injection of microencapsulated catalase, like catalase in solution, was effective in protecting acatalasemic mice from toxic doses of injected sodium perborate. Analysis showed that this protection of acatalasemic mice by microencapsulated catalase was due to the effective removal

of perborate by the microencapsulated enzyme. This demonstration that microencapsulated catalase successfully replaced the deficient enzyme led to further studies by Poznansky and Chang (195). These showed that catalase activity could be assayed in the blood after i.p. injection of catalase solution, but not after injection of microencapsulated catalase which remained in the peritoneal cavity where it continued to act efficiently on perborate. Microencapsulated catalase was also found to be more stable in vivo than catalase in solution. Immunological studies showed that repeated injections of catalase solution, but not catalase microcapsules, resulted in the production of anti-catalase antibodies. It was further reported that in mice immunized to catalase, catalase solution was ineffective in reducing body perborate levels whereas microencapsulated catalase continued to act efficiently.

The experimental studies described above, therefore, encourage the hope that microencapsulated enzymes might eventually be extended for use in enzyme replacement therapy in certain enzyme deficiency conditions in man. The experimental model used in these studies is of particular interest because a similar congenital deficiency disease, acatalasemia, exists in man (196). It should be emphasized here that the work described above serves only as an experimental model for investigating the feasibility of using microencapsulated enzymes for supplementing certain enzyme deficiency conditions. Thus, the i.p. route of injection may not be the optimal route of administration for use in patients. Other routes of administration of

artificial cells have been reviewed recently by Chang (146, 147). In this regard, catalase microcapsules have been used successfully in an extracorporeal shunt system for the removal of perborate injected into acatalasemic mice. Microencapsulated catalase has also been applied directly to oral lesions in acatalsemic mice and was found to act efficiently (197).

(b) Enzyme therapy of substrate-dependent tumors

Having demonstrated in preliminary studies the feasibility of using microencapsulated L-asparaginase for suppressing the growth of the L-asparagine-dependent 6C3HED lymphosarcoma in mice (200), Chang proposed that another biomedical application of microencapsulated enzymes is their potential use for chemotherapy of substrate-dependent tumors. Since then, the use of microencapsulated L-asparaginase for the treatment of the Gardner 6C3HED lymphosarcoma has been studied in this laboratory as a model system of this approach to cancer chemotherapy.

In vitro studies

In vitro studies show that ^{14}C-labelled L-asparagine equilibrated rapidly across the microcapsule membrane (199). The assayed enzyme activity of L-asparaginase after microencapsulation has been reported by Chang (200, 201) and Siu Chong and Chang (202) to vary from 33% to 40% of the same amount of enzyme in free solution. These values are in agreement with that reported by Mori et al (168) of 37% using a slight modification of Chang's technique. The V_{max} of microencapsulated L-asparaginase has been

reported to be about 1/3 the V_{max} of the enzyme in free solution (168, 200). The available data on the K_m of microencapsulated L-asparaginase, however, are not in agreement. Whereas Chang (199) has reported that the K_m for microencapsulated L-asparaginase was the same as L-asparaginase in solution, Mori et al (168) has found that the K_m for microencapsulated L-asparaginase (1.3×10^{-3} M) was about 100 times higher than for L-asparaginase in solution (1.8×10^{-5} M).

Studies on the pH - activity profile (168) indicate that although the optimum pH was 8.0 for both L-asparaginase in solution and microencapsulated L-asparaginase, the range of activity of microencapsulated L-asparaginase was more restricted by changes in the pH of the incubating medium. The optimum temperature for activity has also been found to be lower by 10^{o}C for microencapsulated L-asparaginase, as compared to L-asparaginase in solution (168). The reason(s) for changes in the pH profile and optimum temperature as a result of microencapsulation are presently not clear.

Stability studies by Chang (201) showed that collodion L-asparaginase microcapsules, when stored at 4^{o}C, retained 90% of their original catalytic activity after 100 days, whereas L-asparaginase solution stored at this temperature lost 50% of their initial activity after 20 days. Chang (201) also found that the microencapsulated enzyme kept at 37^{o}C was much more stable than the enzyme in solution. Mori et al (203) reported that there

was no loss of activity after repeated use of nylon L-asparaginase microcapsules in vitro and that the activity of these microcapsules was more stable than L-asparaginase solution when stored at 4°C. However, when stored at 30°C, they found that the activity of both forms of enzyme decreased in a similar manner.

L-asparaginase microcapsule suspensions do not show any leakage of enzyme on in vitro testing of the supernatant for enzyme activity (146, 199, 201, 202) or by immunological testing using the Ouchterlony double diffusion technique (203). Other in vitro evidence showing the absence of leakage of properly prepared L-asparaginase microcapsules will be discussed in more detail in Chapter III of this thesis.

In vivo studies

The principle behind the in vivo action of i.p. injected L-asparaginase microcapsules is the same as for other microencapsulated enzymes (146). This principle is shown schematically in Figure 3. Briefly, the physical size of the microcapsules prevents the microcapsules from leaving the peritoneal cavity. As a result of the equivalent pore radius of the microcapsule membrane, entrapped macromolecules, such as hemoglobin, L-asparaginase, and endotoxins, cannot leak out from the microcapsules nor can immunospecific antibodies enter the microcapsule to inactivate the enclosed enzyme. Permeant molecules can, however, diffuse efficiently across the microcapsule membrane. Thus, L-asparagine diffuses from the blood into the peritoneal

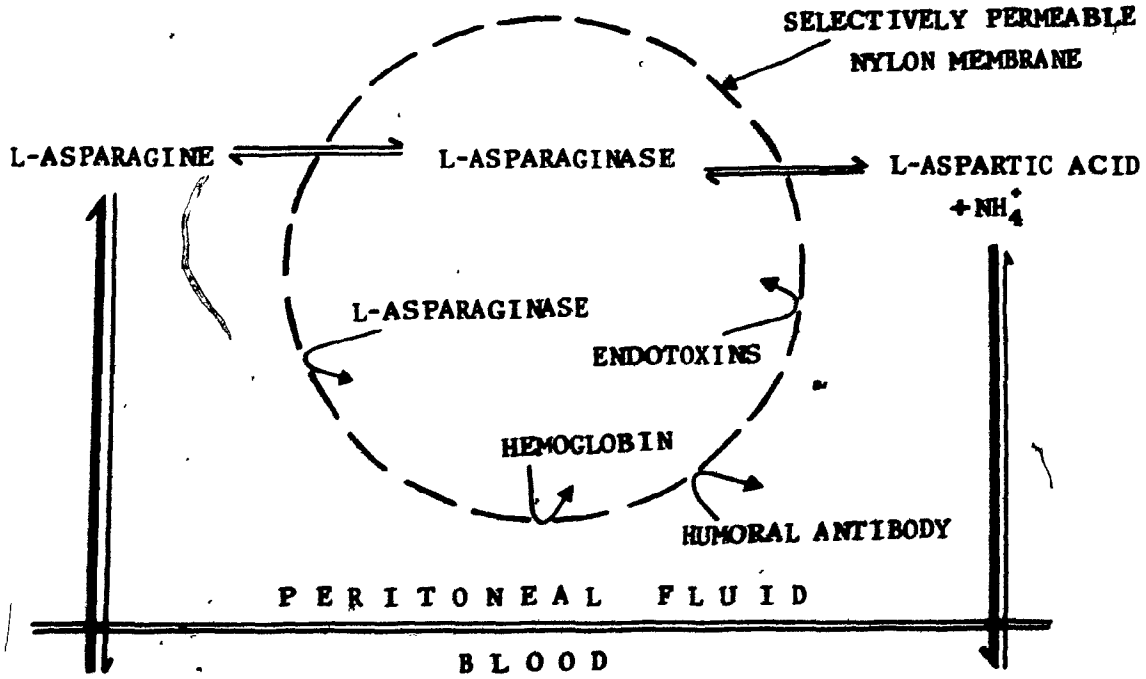


FIGURE 3

Schematic diagram to illustrate the mode of action of i.p. injected semi-permeable L-asparaginase microcapsules.

(modified from Chang, T.M.S. (146)).

fluid and then across the microcapsule membrane along its concentration gradient to be acted on by the enclosed L-asparaginase. The products, L-aspartic acid and ammonia, of the reaction then diffuse along their concentration gradients from inside the microcapsule back to the blood in the reverse direction. It should be noted that the peritoneal cavity is somewhat similar to the interstitial space between tissue cells in that ultrafiltrate and diffusible molecules from the blood capillaries can equilibrate rapidly across the peritoneal cavity. In this way, the plasma levels of L-asparagine can be lowered without the enzyme having to enter the host's circulation.

Chang (200) in 1969 first described preliminary in vivo studies showing that microencapsulated L-asparaginase was effective in suppressing the growth of the 6C3HED lymphosarcoma in mice. In a more detailed report, Chang (146, 199) presented data which showed that when mice were given a single i.p. injection of 0.06 IU/gm (calculated from Chang's data) immediately after tumor implantation, microencapsulated L-asparaginase was more effective than L-asparaginase solution in delaying the appearance of the tumor. He showed that the time when the tumor first appeared was 14.0 ± 4.5 days for those given L-asparaginase solution, and > 69 days for those given L-asparaginase microcapsules. The values obtained for control animals were 9.0 ± 0.9 days and 8.7 ± 1.6 days for those given saline or control microcapsules containing no L-asparaginase, respectively. Furthermore, whereas

all control and L-asparaginase solution-treated animals died as a result of the tumor, 50% of those treated with L-asparaginase microcapsules were tumor-free for more than 120 days after tumor implantation. Subsequent to these studies, concomitant studies were carried out by Chang (201) using collodion L-asparaginase microcapsules and by Siu Chong and Chang (202) using nylon L-asparaginase microcapsules, on the effects of i.p. injected L-asparaginase microcapsules on the plasma L-asparagine levels in normal C3H/HEJ mice. It was shown by both these studies that microencapsulated L-asparaginase was more effective than L-asparaginase solution in suppressing the plasma L-asparagine concentrations in these mice. A more detailed description of the results obtained in these and other follow-up studies will be reserved for fuller discussion in Chapters II to IV.

In addition to the i.p. route of administering L-asparaginase microcapsules, Chang (147) recently described preliminary studies using a combination of L-asparaginase-loaded artificial cells with synthetic capillary fibres in a shunt system. In these experiments, Chang used a Bio-fibre 50 Minibeaker (developed by the Dow Chemical Co.) which is a device containing synthetic capillary fibers in the form of a bundle. These capillary fibers act very much like the blood capillaries, allowing ultrafiltration and dialysis to occur. By adding artificial cells containing L-asparaginase to the fluid bathing the external surface of the capillary fibers and recirculating L-asparagine solution through

the capillary fiber bundle, Chang showed that the artificial cells can efficiently convert the L-asparagine into L-aspartic acid and ammonia. With prolonged use of this system, L-asparaginase in the artificial cells was found to remain active for more than 2 months in the suspending fluid bathing the capillary fiber bundle. This is in marked contrast to the observation that the catalytic activity of L-asparaginase solution added to the external fluid decreased rapidly after prolonged use. Chang has proposed that the similarity in design of this combined system of artificial cells and synthetic capillary fibers to the physiological and functional organization of organs and tissues may be promising in the future construction of artificial organs.

(c) Removal of accumulated metabolities

Finally, the third example that will be discussed is the possible use of a combination of microencapsulated urease with ammonia adsorbent in the treatment of uremia that accompanies renal failure. Chang (157) in 1966 showed that 10 ml of 90 μm microcapsules placed in an extracorporeal chamber were efficient in lowering the urea content of blood allowed to flow directly over the microcapsules (more than 50% of its pretreatment level within 90 minutes of hemoperfusion). Concomitant with this fall in blood urea level, the blood ammonia rose to levels well above values characteristic of terminal hepatic coma. In this same study (157), it was further demonstrated that the ammonia so generated can be

removed by the use of microencapsulated ammonia adsorbent (Dowex-50 W x 12). The feasibility of this urease-ammonia adsorbent model system for the removal of systemic urea has been confirmed and extended by subsequent work in this (204) and other laboratories (205 - 207). In addition to their use in extracorporeal shunts, microencapsulated urease and ammonia adsorbents have also been used for oral ingestion (204, 206, 207). In this regard, oral ingestion of microcapsules containing other adsorbents have also been shown to be effective in the removal of urea (208). The work described above for the removal of urea is still in an experimental stage and has not yet been developed for clinical use in patients with renal failure.

Clinical demonstration of artificial cell concept

As a result of experiments performed in 1966 using urease, ammonia adsorbent and activated charcoal (157), Chang proposed the theoretical basis for the construction of a compact artificial kidney using microencapsulated charcoal as the adsorbent for the removal of uremic toxins (157). After initial, basic, laboratory work (157, 160, 209 - 211) demonstrated the feasibility of using microencapsulated charcoal as a compact artificial kidney, Chang (211) in 1970 reported its first clinical use in a patient with chronic renal failure.

The rationale behind the use of microencapsulated charcoal as a compact artificial kidney may be stated briefly as follows (146, 157, 212, 213). It had been shown by several workers (214 - 217) that free activated charcoal granules could efficiently remove many uremic

metabolites and drugs from perfusing blood. Unfortunately, however, the charcoal causes a significant reduction in the platelets of the perfusing blood and releases embolizing charcoal particles (215, 126). Chang (157, 160) therefore suggested that microencapsulated charcoal within blood-compatible membranes may avoid this problem, since the enclosing membranes would prevent any free powder from going into the patient's circulation, and at the same time, prevent the blood platelets from coming into direct contact with the surface of the charcoal. However, permeant uremic metabolites would still be able to diffuse through the microcapsule membrane and be removed by the enclosed adsorbent. In its present form, the microcapsule artificial kidney consists essentially of a silicone-coated chamber containing a packed column of 300 gm of 1-5 mm activated charcoal granules which had been coated first with a collodion membrane then with human albumin. Because of the ultrathin membrane (500 Å) and the large surface area (2.25 m^2) of these microencapsulated charcoal granules, Chang (146, 148, 213) has calculated that the theoretical transport rate of permeant molecules across the microcapsule membrane is at least 200 times faster than in a standard hemodialyser.

Using the above albumin-coated collodion microencapsulated activated charcoal (ACAC) microcapsule kidney, Chang (146, 211, 213, 218 - 226) has carried out extensive clinical trials since 1970. Such experience has shown that the clearance of creatinine, uric acid, and other uremic metabolites is significantly higher than in the standard hemodialyzer. More importantly, Chang (221 -223) has recently found that

the ACAC microcapsule artificial kidney efficiently removes molecules in the range of 300 - 1500 MW ("middle molecules") which have been implicated by Babb et al (227, 228) as the molecules principally responsible for toxicity in uremia. Furthermore, most recent clinical assessment of combined ACAC hemoperfusion with Dow (model 4) hemodialysis or the Amicon ultrafiltrator indicate that in addition to creatinine, uric acid, and "middle molecules", the rapid removal of urea, water, and electrolytes are now also possible (222, 223).

The microcapsule artificial kidney has also been shown to be more efficient than the standard hemodialyzers for the removal of drugs such as salicylate, barbiturates and glutethimide in animals with acute intoxication (210, 211) and for the removal of glutethimide, methaqualone, methyprylon and phenobarbital in overdosed patients (224, 225).

At present, no artificial liver is available for treating patients with liver failure. Chang (221, 226) has also carried out ACAC hemoperfusion successfully in patients with hepatic coma and has attributed the clinical improvement of the patient to efficient removal of large toxic molecules (> 2000 MW) and protein-bound molecules by the ACAC microcapsule system.

These extensive studies carried out using the ACAC microcapsule artificial kidney demonstrated the safety and efficiency of microencapsulated systems in an extraporeal shunt for use in patients. With further progress in other areas of research, the clinical success of the artificial cell concept encourages the development of microencapsulated enzyme systems for analogous uses in patients.

It is obvious from the foregoing that research on the artificial cell concept covers a wide spectrum of studies and is not confined to enzyme therapy. The entrapment of enzymes within semipermeable microcapsules has recently been recognized to be one of 4 major techniques for the "immobilization" of enzymes (see section ID). L-asparaginase has also been immobilized by these other techniques in an effort to reduce the severity of some of the problems associated with L-asparaginase therapy. In the final section of this introduction, the work done on these other types of immobilized L-asparaginase will be reviewed.

ID - OTHER TYPES OF IMMOBILIZED L-ASPARAGINASE

Introduction

The immobilization of enzymes represents one area of research in enzyme technology which together with the production, isolation, purification and use of enzymes in a variety of reactor geometries, make up the new area of specialization referred to as "enzyme engineering" (229, 230). The term "immobilized enzymes" has been used to describe all enzyme preparations in which the enzyme is constrained one way or another within the limited confines of a supporting polymer (230). Several excellent and detailed reviews on the subject of immobilized enzymes are available in the literature (146, 178, 229 - 237). As a result of the Enzyme Engineering Conferences held in 1971 and 1973 (229, 230), the following classification of immobilized enzymes has been recommended, based on the technique used for immobilization (see Figure 4). Immobilized enzymes should first be divided into entrapped and bound enzymes. Entrapped enzymes should then be sub-divided into matrix-entrapped and microencapsulated enzymes, while bound enzymes should be sub-divided into adsorbed and covalently bound enzymes. In addition to its microencapsulation within semipermeable microcapsules, L-asparaginase has also been immobilized by each of these other techniques.

Adsorbed L-asparaginase

Nicolaev and Mardashev were the first to immobilize L-asparaginase. They adsorbed L-asparaginase onto carboxymethyl cellulose (238)

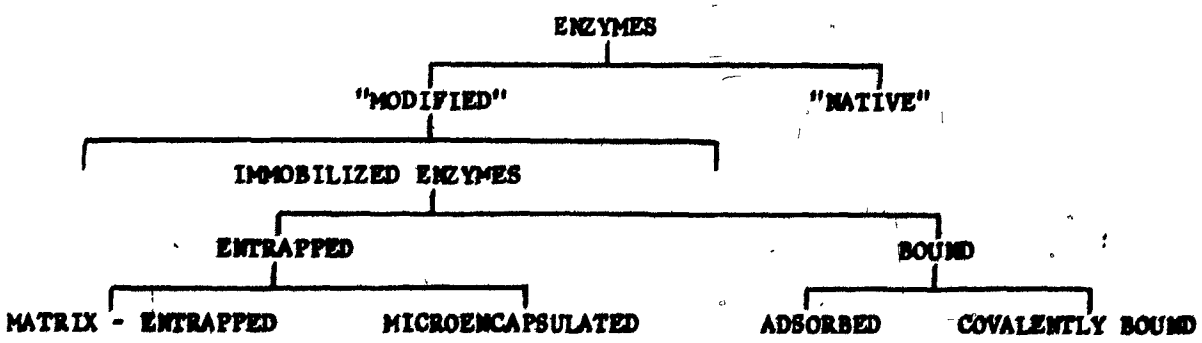


FIGURE 4

General classification of immobilized enzymes.

(from Sundaram, P.V., and Pye, E.K. (230)).

and later onto diethylaminoethyl cellulose (239) for in vitro kinetic studies on the hydrolysis of L-asparagine. They found that the column of adsorbed L-asparaginase retained catalytic activity. These studies were not in any way related to interests in L-asparaginase as a potential antitumor agent. Indeed, they preceded Broome's discovery (36 - 38) of the antineoplastic effects of L-asparaginase. This immobilization technique, however, has not been since investigated as a means of improving L-asparaginase therapy, probably because of the relative ease with which the enzyme can reversibly adsorb and desorb from the supporting matrix.

Covalently-bound L-asparaginase

Weetal (240) was the first to immobilize L-asparaginase by covalent linkage. He covalently linked L-asparaginase to a Dacron vascular prosthesis and tested its in vitro and in vivo stability in a dog. In vitro studies showed that at the end of 18 days storage at 23°C in saline, 27% of the initial activity remained. In vivo studies showed that the enzymatically active graft implanted in the abdominal aorta of a dog retained 44.5% of its initial activity. Weetal (241) later also covalently bound L-asparaginase to carriers such as glass or methacrylate and tested these systems in vivo. These studies showed that the L-asparagine level could successfully be lowered in blood allowed to perfuse over it. Leakage of enzyme into the blood was however reported in these studies.

Hasselberger et al (242) described 4 methods for covalently binding L-asparaginase to insoluble matrices of microcystalline cellulose,

carboxymethyl cellulose and carboxymethyl dextran. Except for one preparation, L-asparaginase immobilized to these supports was found to be more stable at 37°C than the enzyme in free solution. No in vivo testing of these derivatives was reported, although their use in an extracorporeal shunt system was suggested.

Allison et al (243) covalently attached L-asparaginase to partially hydrolyzed nylon tubing, and carried out in vitro studies on stability and kinetic parameters of the immobilized enzyme. They found that the L-asparaginase nylon tubing, after 2 months of storage, retained 100% of its initial activity at 4°C and 50% at room temperature. An increase in the K_m (apparent) was observed upon immobilization. Although the pH optimum of both the immobilized and solution form of the enzyme was the same, the range of activity was somewhat more restricted by pH changes in the case of the immobilized enzyme. The immobilized L-asparaginase was also found to be more resistant to thermal inactivation than enzyme in free solution. No in vivo work has been reported as yet by this group. However, they suggested the use of L-asparaginase nylon tubing in an extracorporeal shunt for leukemia therapy. More recently, Bunting and Laidler (244) have also studied the flow kinetics of L-asparaginase covalently attached to nylon tubing.

Horvath et al (245) covalently bound L-asparaginase to a polycarboxylic gel layer which itself was attached to the inner wall of nylon tubes. In vitro studies showed that the L-asparaginase activity of the tubes was quite stable, and there was no detectable

leakage of enzyme from the tubes into the perfusate. Studies in which this enzyme reactor system was connected in series with isolated rat liver, showed that the normally steady-state L-asparagine level in the hepatic output could be effectively lowered depending on the enzymic activity of the L-asparaginase tube. On the basis of these studies, this design of nylon tubes was suggested for use as extracorporeal shunt systems for maintenance of a low plasma L-asparagine. It was proposed that this type of reactor design was different to the earlier one used by Allison et al (243) because of the added advantage of the presence of the gel layer which protects the embedded enzyme from external proteolytic agents.

Sampson et al (246 - 248) have described the use of L-asparaginase covalently coupled to polymethylmethacrylate plates in an extracorporeal chemotherapeutic unit for in vivo studies in dogs, baboons and man. They showed that hemoperfusions over the L-asparaginase plates caused a sharp decline in the serum L-asparagine concentrations. Significant immunosuppression, manifested by decreased lymphocytic reactivity to various vegetable mitogens, was observed during hemoperfusion. Assays of sera in initial studies (246) for L-asparaginase activity revealed that there was about 33% leakage of the enzyme from the plates during perfusion. In later studies (248) however, enzyme leakage from the plates was reduced to only 2%. No major toxicity was observed in animals during a month of study after one or more hemoperfusions. Recent clinical studies (248) on 2 patients showed that this type of hemoperfusion also did not cause any obvious deleterious effects.

It is interesting to note that serum L-asparagine levels remained low as long as perfusion continued, but promptly returned to normal within 3 hours after cessation of perfusion. On the basis of these studies, Sampson et al (248) suggested that this extracorporeal approach might be of potential value in the management of human allograft recipients, while obviating some of the toxic effects of L-asparaginase. Hersh, who was collaborating with Sampson's group, covalently coupled E. coli and Erwinia carotovora L-asparaginases to polymethylmethacrylate plates and described the in vitro properties of these immobilized preparations in a subsequent paper (249).

The most detailed studies on covalently-bound L-asparaginase have been those of Cooney et al (250), using the Dacron vascular prostheses described earlier (240). In vitro studies showed that amidohydrolysis by this "enzyme graft" was dependent on the flow rate, character of the perfusate, temperature, substrate concentration and pH. Immunological studies showed that mouse anti-L-asparaginase serum inhibited the catalytic activity of the enzyme graft by 30 - 52% in vitro. In vivo studies showed that mice implanted i.p. with the enzyme graft (Dacron circlet bearing 1 IU L-asparaginase) developed humoral antibodies to the enzyme (measured by the technique of passive hemagglutination). The immunogenicity of the immobilized L-asparaginase implant was attributed to release of enzyme from the support into the body, as a result of gradual rupture of the covalent bond between the enzyme and the polymer. No increase in the survival time of L5178Y leukemic mice was found after i.p. implantation of the

L-asparaginase graft. Autopsy studies showed that fibrous adhesions were present on the intimal surface of the enzyme graft. Removal of these fibrous adhesions revealed that the graft still retained 15% of its original activity (after 2 weeks of implantation). On the basis of these studies, Cooney et al proposed that the protein accumulation on the surface of the graft limits accessibility of substrate to the enzyme and thus impairs its catalytic activity in vivo. It is interesting to note, in this regard, that Siu Chong and Chang (202), in an earlier report, also described similar findings with i.p. injected L-asparaginase microcapsules. They found that progressive fibrosis occurred around the surface of i.p. injected L-asparaginase microcapsules, this reaction becoming very marked around the 8th day post injection. They proposed that this likely formed a relatively impermeable barrier to external L-asparagine, thus accounting for their finding that L-asparaginase microcapsules, though containing catalytically active L-asparaginase, exhibited limited duration of physiological activity in vivo (202). Microencapsulated L-asparaginase, however, was found to retain significant tumor inhibitory properties in vivo (146, 199, 202, Chaper III of this thesis).

Venkatasubramanian et al (251) used a biological protein, collagen, as a carrier for the immobilization of L-asparaginase by covalent linkage. In this preliminary study, L-asparaginase was mixed with collagen, and the L-asparaginase-collagen mixture cast as a membrane, dried, and then cross-linked with glutaraldehyde. This

membrane was wound to form a modular reactor. In vitro tests showed that the immobilized L-asparaginase was very stable for over 4 months upon storage at 4°C. An increase in the K_m (apparent) was also observed (about 80 times higher than the value for the enzyme in solution). The pH profile in this case was very similar for L-asparaginase in the immobilized or solution form. Other in vitro work and possible clinical evaluation of collagen-immobilized L-asparaginase were suggested.

Lastly, Paillot et al (252) described the rather interesting technique of binding L-asparaginase to an inert carrier protein, human albumin, by cross-linking it with glutaraldehyde to form a soluble, immobilized enzyme system. L-asparaginase immobilized by this technique was shown to retain as high as 80% of its initial activity after cross-linking, and was more resistant to proteolytic attack by trypsin in vitro. In vivo studies on mice did not show any overall toxicity for this type of L-asparaginase polymer. This type of immobilized enzyme system was suggested for use via intravenous injection.

Matrix-entrapped L-asparaginase

Updike's group (253 - 255) has immobilized L-asparaginase by entrapping the enzyme in a hypoallergenic polyacrylamide gel which was then mechanically fragmented into smaller gel particles. In vitro studies showed that the gel-entrapped enzyme was protected from attack by proteolytic enzyme (pronase), bacteria, and specific antibody (253, 254). Although the average size of the gel could be made smaller (by increasing the polymer concentration) than the physical size of the

entrapped protein, it was reported that leaching out of protein always occurred from the matrix (253, 254). In vivo studies showed that i.p. injected gel-entrapped L-asparaginase particles had an activity half-life of about 9 days, and that L-asparaginase activity could still be measured in particles recovered from the peritoneal cavity for up to 4 weeks after injection. As a result, serum L-asparagine levels were suppressed to nearly zero for at least 2 days after i.p. injection (255). It was also shown that intravenously injected L-asparaginase particles into rats produced a more prolonged depression of the serum L-asparagine level than observed after similar injection of L-asparaginase solution. Particles so injected caused plugging of the pulmonary microcirculation, and wheezing and hyperventilation were observed. These particles were removed by the reticuloendothelial system, and the spleen was found to increase to double its normal size during the first 10 days after injection. Updike has suggested the use of a more biodegradable polymer so as to avoid possible iatrogenic polymer storage disease as a result of repeated injections of this type of immobilized enzymes.

Mori et al (256) have also immobilized L-asparaginase by entrapping it in a polyacrylamide gel lattice and studied the resultant properties of the preparation in vitro. They found that, compared to L-asparaginase in free solution, the pH optimum of gel-entrapped L-asparaginase was lower and that the K_m (apparent) was 200 times higher. They, like Updike's group, also found that gel-entrapped L-asparaginase was resistant to attack by chymotrypsin, trypsin and Pronase-P, and

was more stable than L-asparaginase solution when kept at 37°C. Gel-entrapped L-asparaginase packed in a column retained 40% of the original activity after 15 days of continuous operation at 37°C. In these studies, no leakage of L-asparaginase activity could be detected from such a column which efficiently lowered the L-asparagine content to zero in blood flowing through it. No in vivo studies were reported.

Ohnuma et al (257) immobilized L-asparaginase by entrapping it in 2-hydroxymethylmethacrylate gel which was then dried, crushed to obtain smaller gel particles, and then rehydrated. In vivo studies showed that after i.p. injection of these gel-entrapped L-asparaginase particles, a peak L-asparaginase activity appeared in the plasma which was 5% of that observed after similar injection of an equivalent dose of L-asparaginase solution. The clearance rate of the enzyme from the plasma after i.p. injection of gel-entrapped L-asparaginase was also found to be about 7 times slower than that of the native enzyme. This was probably due to enzyme leakage from the gel matrix in vivo. Gel-entrapped L-asparaginase suppressed the plasma L-asparagine level to the same degree as L-asparaginase solution. Preliminary studies on the tumor-inhibitory effect of the 2 enzyme preparations, however, suggested that gel-entrapped L-asparaginase was slightly more therapeutic than L-asparaginase in solution.

More recently, an interesting technique for immobilizing L-asparaginase was described by Inada et al (258) who entrapped the enzyme into a matrix of bovine fibrin formed from fibrinogen in the presence of thrombin. They proposed that coagulation factor XIII

caused a cross-linking effect between fibrin molecules and between fibrin and L-asparaginase molecules. No significant leakage of L-asparaginase from the fibrin polymer was reported, even after washing the immobilized enzyme preparation for 2 days. The use of such a low immunogenic material, especially human fibrinogen in lieu of bovine fibrinogen, was suggested for clinical application.

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* Abbreviated hereon as TASALO

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CHAPTER II

IN-VIVO EFFECTS OF INTRAPERITONEALLY INJECTED L-ASPARAGINASE SOLUTION AND L-ASPARAGINASE IMMOBILIZED WITHIN SEMIPERMEABLE NYLON MICROCAPSULES WITH EMPHASIS ON BLOOD L-ASPARAGINASE, "BODY" L-ASPARAGINASE, AND PLASMA L-ASPARAGINE LEVELS

(from Siu Chong, E.D., and Chang, T.M.S.
Enzyme 18:218-239 (1974)).

ABSTRACT

C3H/HEJ mice were given i.p. injections of one of the following: L-asparaginase solution, microencapsulated L-asparaginase, saline or control microcapsules. After injection of L-asparaginase solution, enzyme activity appeared in the blood very rapidly with the highest concentration occurring after 4 hours, and was then cleared from the circulation with a half-life of 4.4 hours. In marked contrast, when microencapsulated L-asparaginase was injected, no significant L-asparaginase activity appeared in the blood for the entire duration of this study. "Body" L-asparaginase levels declined very rapidly with a half-life of 2 hours after injection of L-asparaginase solution, whereas it took 60-72 hours for the "body" L-asparaginase to decrease to 50% of the original activity after injection of microencapsulated L-asparaginase. The microencapsulated L-asparaginase still retained about 20% of its original activity up to 16 days after injection. Plasma L-asparagine was maintained at zero concentration for 3 days after injection of L-asparaginase solution, compared to 8 days after injection for microencapsulated L-asparaginase. Liver L-asparaginase activity was found to increase after injection of L-asparaginase solution but not after injection of microencapsulated L-asparaginase. The response of the host to i.p. injection of nylon microcapsules is described. Preliminary experiments indicate that the half-life for clearance of the free enzyme from the circulation of 6C3HED lymphosarcoma-bearing mice was 13.2 hours as compared to 4.4 hours of normal mice, and that microencapsulated L-asparaginase was capable of causing regression of the tumor in the advanced, well-established stage.

INTRODUCTION

Kidd's observations (1, 2) that guinea pig serum inhibited the growth of certain murine tumors and Broome's (3-5) identification revealing that the anti-tumor properties of this serum were due to its L-asparaginase content have resulted in a great deal of interest in this enzyme. Mashburn and Wriston (6) discovered that L-asparaginase with tumor-inhibitory activity could be obtained from Escherichia coli thereby making this enzyme more readily available for extensive investigation. Further studies showed that L-asparaginase was also effective against several other types of murine leukemias and solid tumors (7, 8) and against spontaneous dog lymphosarcoma (9). Independent work by Neuman and McCoy (10) and Haley et al (11) showed that cells of the Walker Carcinosarcoma 256 and the L5178Y mouse leukemia would not grow in culture medium devoid of L-asparagine which is generally considered to be a non essential amino acid for normal tissues. Although the exact mechanism of the anti-tumor action of L-asparaginase is not fully known, the most widely accepted thesis is that administration of this enzyme depletes the extracellular source of L-asparagine, thereby selectively destroying L-asparagine-dependent tumor cells (5, 7, 12-14). These L-asparaginase-sensitive tumors, in contrast to most normal cells and L-asparaginase-resistant tumors, cannot synthesize sufficient endogenous L-asparagine due to the lack of L-asparagine synthetase or their incapacity to increase L-asparagine synthetase activity after L-asparaginase administration (15-19).

The first attempt to evaluate L-asparaginase clinically was carried out by Dolowy et al (20) who used a partially purified preparation of guinea pig serum on a patient with acute lymphoblastic leukemia. Hill et al (21) and Oettgen et al (22) reported that E. coli L-asparaginase also had significant

therapeutic effects in man. Subsequent and more extensive clinical trials carried out by several groups (23-32) demonstrated that L-asparaginase is a very promising remission-inducing agent in acute lymphoblastic leukemia in children, but is less successful in the treatment of non-lymphocytic leukemia, and produces only occasional responses in lymphosarcoma and reticulum-cell sarcoma. There are a number of side effects associated with E. coli L-asparaginase therapy. The side effects which have been observed in some patients include: anorexia, nausea, vomitting, chills, fever, diarrhea, weight loss, hypotension, cerebral dysfunction, hypolipidemia, hyperlipidemia, various manifestations of hypoproteinemia, coagulopathy, abnormal liver function tests, fatty metamorphosis of the liver, pancreatic dysfunction, azotemia, granulocytopenia, lymphocytopenia, thrombocytopenia, and hypersensitivity reactions with possible life-threatening anaphylaxis (32-36). This wide spectrum of side effects may be attributed to at least three factors. Firstly, they may be the direct or indirect result of the enzymatic removal of L-asparagine causing, for instance, a depression or cessation of protein synthesis. Secondly, they may arise because of the antigenic nature of the foreign enzyme preparation. Thus with repeated injections of enzyme there may be problems such as immunological reactions, rapid removal and inactivation of the foreign enzyme by the host. Thirdly, they may originate from the possible contamination of the enzyme preparation with bacterial endotoxins or other bacterial substances. The latter two possibilities represent problems which one would encounter when using enzymes derived from heterogeneous sources.

Employing the concept of 'artificial cells' in the form of semi-permeable microcapsules (37-39), this type of enzyme technology has been used for the experimental suppression of the Gardner lymphosarcoma in C3H/HEJ mice (39-41).

Semipermeable microcapsules (37-39) are spherical, ultrathin, semipermeable polymer membranes of cellular dimensions, each enveloping a microdroplet of an aqueous protein solution or suspension. Both the equivalent pore radius and mean diameter of the microcapsules can be varied at will over a wide range. In addition to having ultrathin membranes, the small size of the microcapsules offers the advantage of having an extremely large surface area/volume ratio, not unlike their biological counterparts. The enclosing membranes of these microcapsules are such that they are selectively permeable to small molecules (e.g. L-asparagine, L-aspartic acid, ammonia), but are impermeable to macromolecules (e.g. enzymes and other proteins) or cells. In this way, therefore, L-asparaginase and any accompanying macromolecular contaminants, once loaded inside such microcapsules, are prevented from interacting directly with the external environment and from leaking out to give rise to unwanted immunological and other toxic reactions, or be rapidly removed and inactivated by the host. At the same time, however, external permeant molecules can dialyse across the semipermeable membrane to be acted on by the enzyme which has been entrapped in the intracellular environment of the microcapsule. Thus, while still being capable of acting efficiently on the L-asparagine in the surrounding medium, immobilization of the enzyme in semipermeable microcapsules now permits it to be administered in such a form that it is both non-immunogenic and non-toxic with respect to contaminating bacterial substances. Furthermore, microencapsulation of L-asparaginase within an environment containing a high concentration of proteins results in an increased stability of the enzyme (42, 43), and thereby may reduce the need for frequent, repeated injections of the enzyme. Another significant advantage is that continued L-asparaginase therapy is now made possible using the microencapsulated form of the enzyme even in patients who

have developed a high antibody titre against L-asparaginase previously administered in the free form. This circumvents the problems associated with rapid removal and inactivation of the enzyme or the risk of anaphylaxis and other immunological complications.

This paper analyses in detail some of the effects of i.p. injections of L-asparaginase in free solution and in the microencapsulated form in C3H/HEJ mice. In particular, we have examined the in vivo stability of the two forms of the enzyme in the body, the associated changes that take place in relation to blood L-asparaginase activity and plasma L-asparagine levels and the fate of the i.p. injected microcapsules. Preliminary findings on the regression of the 6C3HED lymphosarcoma in tumor-bearing mice given free or microencapsulated L-asparaginase are also reported. The concomitant changes in endogenous liver L-asparaginase activity will be published in greater detail in a later paper.

MATERIALS AND METHODS

Animals

Normal male C3H/HEJ mice weighing about 25 gm were used in these studies. These originated from Jackson Laboratories, Bar Harbor, Maine. They were maintained on a diet consisting of Purina Mouse Chow and water ad libitum.

Enzyme

E. coli L-asparaginase (EC-2, L-asparagine amidohydrolase, EC 3.5.1.1) was obtained in vials of 1000 IU from Nutritional Biochemical Corporation, Cleveland, Ohio. The specific activity of the preparation was 312.3 IU/mg. One IU of L-asparaginase has been defined as that amount of enzyme which will liberate 1.0 µmole of ammonia per minute at 37°C.

Preparation of L-asparaginase microcapsules

Semipermeable nylon microcapsules having a mean diameter of 80 µm were prepared by interfacial polymerization using the updated procedure (39). Briefly, to a beaker surrounded by ice the following are added: 0.3 ml of L-asparaginase solution (1000 IU/ml), 2.5 ml of 10 gmX hemoglobin solution (hemoglobin substrate, Worthington Biochemical Corporation, Freehold, New Jersey) and 2.2 ml of a freshly prepared alkaline 1,6-hexamethylenediamine solution containing 5.0 gmX 1,6-hexamethylenediamine, 1.8 gmX sodium hydrogen carbonate and 7.5 gmX sodium carbonate.* Using a "Jumbo" magnetic stirrer (Fisher Scientific Co., Montreal, Quebec) at a speed setting of 5, this aqueous solution was immediately emulsified for 1 minute at 4°C with 25 ml of a "mixed solvent" (chloroform-cyclohexane, 1:4, containing 1% v/v "Span 85" (Atlas Chemical Industries, Brantford, Ontario). Without stopping the stirring, 25 ml of

* See Appendix 1.

0.018 N sebacoyl chloride solution-prepared immediately before use by adding 0.1 ml of pure sebacoyl chloride (Eastman Kodak Co.) to 25 ml of the mixed solvent - were added and the reaction mixture stirred for another 3 minutes at the same speed. The reaction was then quenched by the addition of 50 ml of the "mixed solvent" to the stirred suspension which is then stirred for a further 15 seconds. The supernatant was then removed by sedimentation and discarded. 50 ml of a detergent, Tween 20 solution (Atlas Chemical Industries, 50% v/v in water), were then added to the suspension, and the microcapsules were dispersed by stirring at a speed setting of 8 for 1 minute. The speed was then decreased to a setting of 4 and 50 ml of distilled water were added. The suspension was stirred for 30 seconds more and then poured into a beaker containing 200 ml of distilled water. The microcapsule suspension was then washed 10 times with saline to remove any excess Tween 20. The microcapsules were then resuspended in saline to produce a 50% microcapsule suspension. Microcapsules prepared in this way have an activity of about 12 IU/ml of 50% microcapsule suspension. Prior to use, the exact activity of the preparation was always determined. The supernatant was tested for any enzyme activity to ensure that there was no leakage of enzyme from the microcapsule preparation, and the microcapsules were examined under the microscope to ensure that their membranes were well formed. Only properly prepared microcapsules (Figure 1) that satisfied these criteria were used in these experiments.

Experimental procedure

Groups of mice receiving L-asparaginase solution or L-asparaginase microcapsules were given i.p. injections of 0.5 IU/gm body weight.

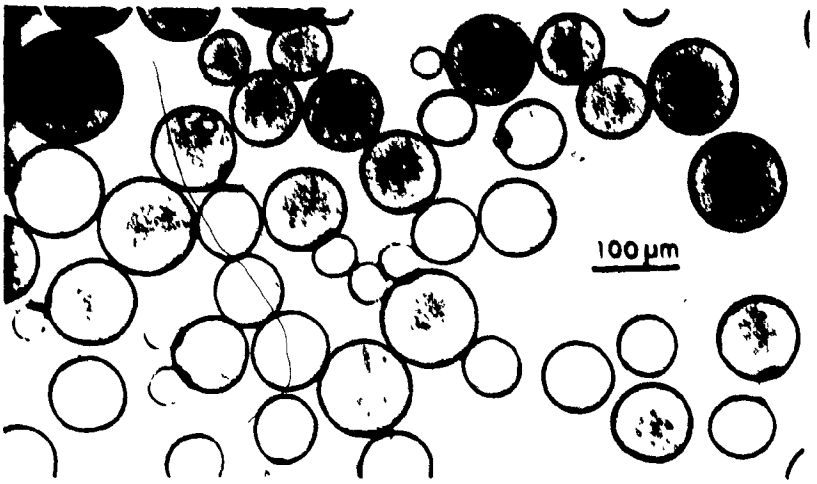


FIGURE 1

Photograph of typical L-asparaginase-loaded nylon microcapsules. The microcapsules shown here were prepared by interfacial polymerization, as described under Materials and Methods. The nylon microcapsules formed are of 80 µm mean diameter and have membranes which are 200 Å thick with an equivalent pore radius of 16 Å.

Control mice were given i.p. injections of 1 ml saline or 1 ml 50% microcapsule suspension containing no L-asparaginase.

Preparation of tissues for L-asparaginase assay

(a) Blood. Blood was drawn into fire-polished, heparinized Pasteur pipettes from the subclavian artery of ether-anesthetized mice. The blood was then transferred into small containers which were immediately stored at -20°C until ready for assay in the next few hours.

(b) "Body". Having exsanguinated the mice, the skins were removed and the livers dissected. The remaining portions of the animals were then homogenized 1/4 (w/v) in ice-cold saline using a Waring blendor. The homogenates were filtered through triple-layered cotton gauze and the filtrate immediately kept at -20°C until ready for assay. Any subsequent mention of the amount of L-asparaginase activity remaining in the "body" after enzyme injection refers to the enzyme activity assayed in the homogenates prepared as described here.

L-asparaginase assay

Assays of enzyme activity were carried out using a slight modification of the automated continuous flow method developed by Schwartz et al (44) (See Appendix 2 for details). This method of assay is based on the Berthelot reaction in which enzyme hydrolysis of L-asparagine results in the release of ammonia which can be detected colorimetrically by its reaction with sodium hypochlorite and phenol to form indophenol. The major changes in our procedure were 2-fold:

(1) 0.1 M sodium borate buffer, pH = 8.5, was used instead of 0.1 M veronal buffer, pH = 8.6.

(2) The incubation coil was made longer so that the reacting time of the buffered enzyme-substrate mixture at 37°C was increased from 5 to about 12 minutes, thereby allowing a greater amount of ammonia formation. This permits more accurate measurements of lower concentrations of L-asparaginase. The exact period of incubation was determined with a stop watch prior to each set of analyses.

Preparation of plasma for L-asparagine assay

Freshly drawn blood was centrifuged at 1000 g for 10 minutes. The plasma was removed and added to a tube containing 10% TCA (2 vol plasma to 1 vol TCA). The contents of the tube were mixed well with a vortex mixer and then centrifuged at 1000 g for 20 minutes. The supernatant was extracted 5 times with ether. After the final extraction and removal of ether, any residual ether in the supernatant was removed by vacuum.

L-asparagine assay

This was performed according to the fluorimetric technique of Cooney et al (45), which is based on the enzymatically coupled oxidation of reduced pyridine nucleotide (See Appendix 3 for details). In our case, four solutions were sequentially prepared as follows:

(a) 2.5 mg NADH (Calbiochem, Los Angeles, Calif.) was dissolved in 100 ml Tris buffer (0.005 M, pH 8).

(b) 10 ml of the solution prepared in (a) was diluted with 290 ml of the same Tris buffer.

(c) 4 mg α-ketoglutaric acid (Sigma Chemical Co., St. Louis, Mo.) was added to 250 ml of the solution prepared in (b).

(d) 52 IU GOT (glutamic oxaloacetic transaminase, porcine heart; Sigma Chemical Co.) and 126 IU MDH (malic dehydrogenase, porcine heart; Calbiochem) was added to the solution prepared in (c).

4 ml of the reaction mixture prepared in (d) were pipetted into glass fluorimeter rubes (12 x 75 mm) (G.K. Turner Associates, Palo Alto, Calif.). 0.05 ml of the sample was added to the reaction mixture. After incubation at 22°C for 30 minutes, a reading was taken on the fluorimeter (Turner Model III). After this, 0.1 IU L-asparaginase was added to the reaction mixture which was then incubated at 22°C for 1 hour, and then read on the fluorimeter.

Fate of injected microcapsules

C3H/HEJ mice were injected i.p. with nylon microcapsules and sacrificed at various time intervals up to 16 days. Microcapsules were recovered from the peritoneal cavity and observed under the microscope. Microcapsule aggregates were also fixed in 10% formalin and embedded in paraffin wax (See Appendix 4 for details). 5 μm thick sections were then cut and stained with hematoxylin-eosin.

RESULTS

Blood L-asparaginase activity

Figure 2 shows that after i.p. injection of L-asparaginase solution, peak enzyme activity appeared in the blood after 4 hours, and the enzyme was then cleared rapidly from the blood. The elimination of this L-asparaginase preparation followed first-order kinetics with a half-life of 4.4 hours (Figure 3), enzyme activity being no longer detectable in the blood 48 hours after injection. In contrast, after i.p. injection of microencapsulated L-asparaginase, no detectable L-asparaginase activity appeared in the blood for the entire duration of this study (16 days, Figure 2), showing that the microencapsulated L-asparaginase did not leak out into the vascular compartment. Injection of saline or control microcapsules did not result in any changes in blood L-asparaginase levels (Figure 2).

Concomitant with the administration of L-asparaginase solution or L-asparaginase microcapsules, there were associated elevated base-line blood ammonia levels of 0.30 - 0.50 μmoles/ml as compared to control values of 0.15 - 0.20 μmoles/ml in untreated mice. This was probably due to the amidase action of the enzyme resulting in an increased liberation of ammonia. This increase in blood ammonia level was not observed when saline or control microcapsules were injected.

"Body" L-asparaginase activity - In vivo stability

When the mice were given i.p. injections of enzyme in free solution, the L-asparaginase activity remaining in the "body" (as defined in Materials and Methods) was found to decline exponentially (Figure 4) with a half-life of 2 hours (Figure 5). Enzyme activity became undetectable after 16 hours by

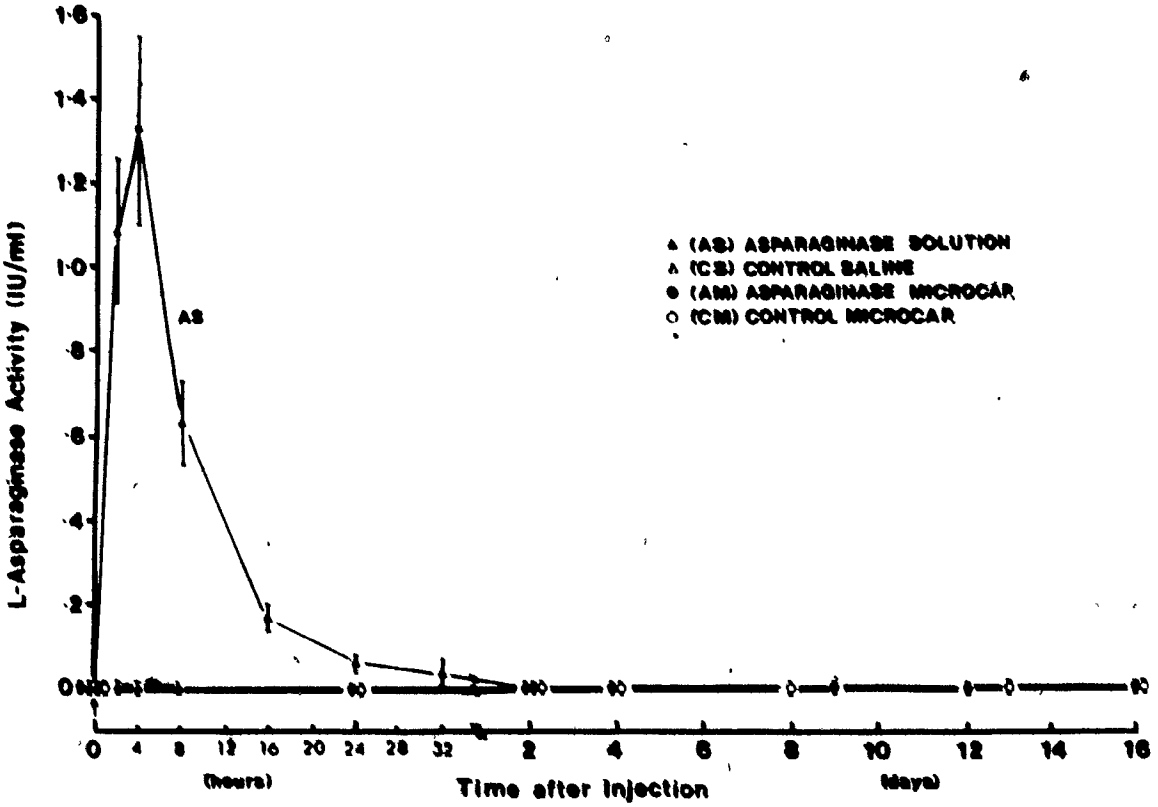


FIGURE 2

Time course of L-asparaginase activity in the blood of normal C3H/HEJ mice after one of the following intraperitoneal injections:

Each ▲ = 8 mice injected with AS and sacrificed at the indicated time interval.

Each △ = 3-4 mice injected with CS and sacrificed at the indicated time interval.

Each ● = 4-8 mice injected with AM and sacrificed at the indicated time interval.

Each ○ = 3-4 mice injected with CM and sacrificed at the indicated time interval.

All values represent means and standard deviations.

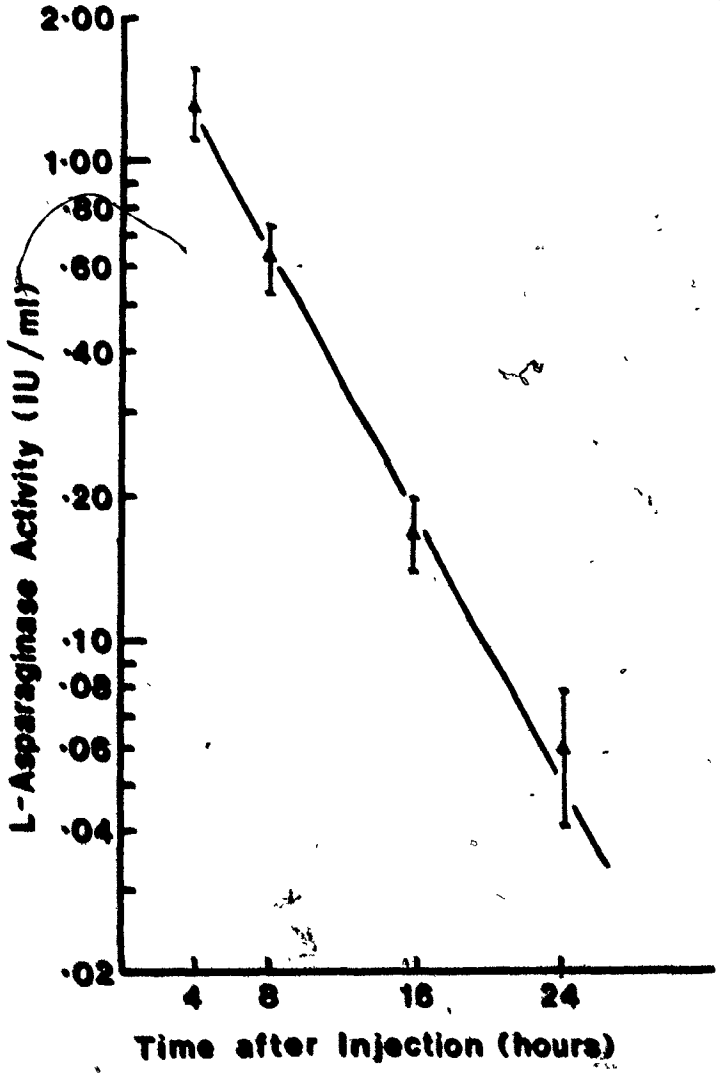


FIGURE 3

Semilogarithmic plot of the clearance of L-asparaginase from the blood with time after i.p. injection of L-asparaginase solution.

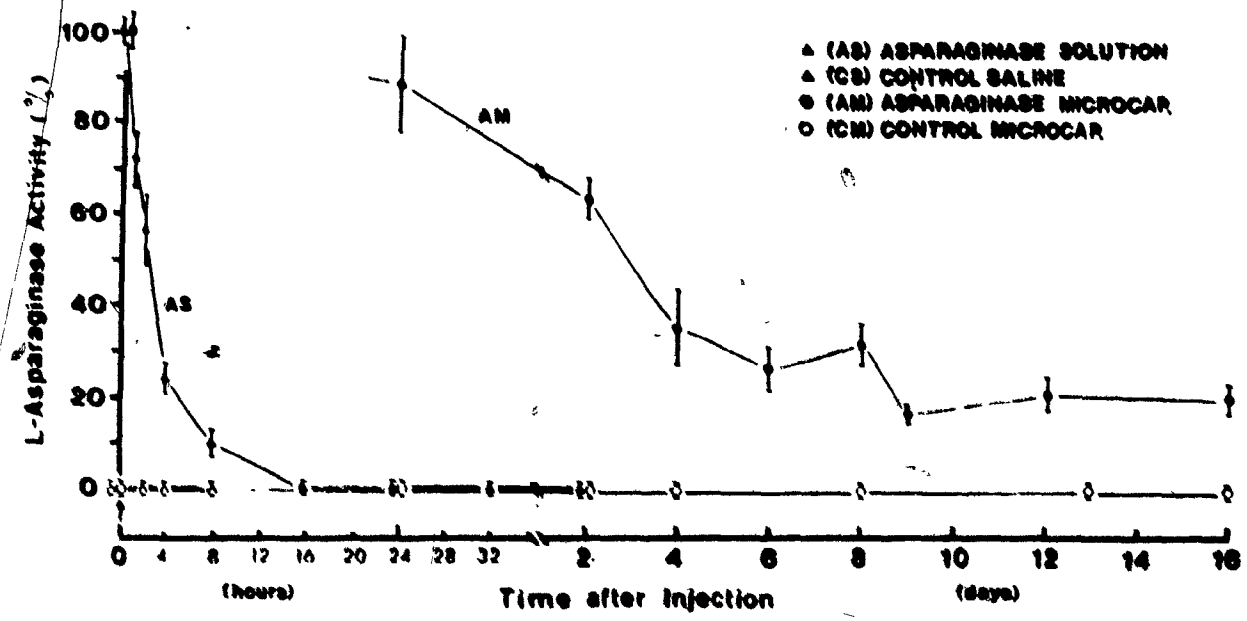


FIGURE 4

Time course of L-asparaginase activity remaining in the "body" of normal C3H/HEJ mice after one of the following intraperitoneal injections:

Each ▲ = 8 mice injected with AS and sacrificed at the indicated time interval.

Each △ = 3-4 mice injected with CS and sacrificed at the indicated time interval.

Each ● = 4-8 mice injected with AM and sacrificed at the indicated time interval.

Each ○ = 3-4 mice injected with CM and sacrificed at the indicated time interval.

All values represent means and standard deviations.

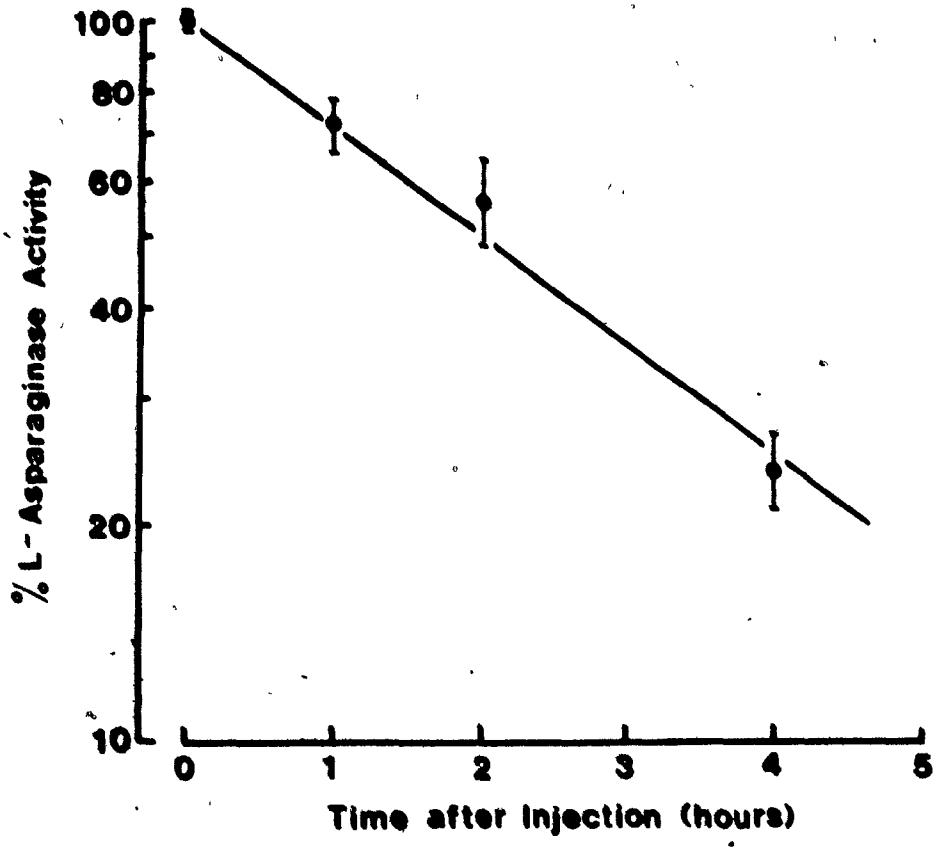


FIGURE 5

Semilogarithmic plot of the decrease in the "body" L-asparaginase activity after i.p. injection of L-asparaginase solution.

the method of assay used (Figure 4). On the other hand, when the mice were given microencapsulated L-asparaginase i.p., the L-asparaginase activity remaining in the "body" declined much more slowly (Figure 4). It took 60 - 72 hours for the L-asparaginase activity to decrease to 50% of the initial activity. This represents a marked increase in the in vivo stability of the microencapsulated form of the enzyme compared to the enzyme in free solution. Furthermore, microencapsulated L-asparaginase stayed in the peritoneal cavity for the duration of the period followed. At the end of 16 days, 19.9 ± 3.5% of the original activity was still retained. Injection of saline or control microcapsules in the control groups of mice did not contribute to total body L-asparaginase activity (Figure 4).

Plasma L-asparagine levels

Figure 6 shows that after the i.p. injection of L-asparaginase solution, the plasma L-asparagine concentration fell from 31.6 ± 3.2 nmoles/ml at zero time to 0 nmoles/ml 1 hour after injection. After this, plasma L-asparagine remained at zero concentration for 3 days, rose to 3.6 ± 2.6 nmoles/ml on day 4 and was back to normal, 35.8 ± 5.1 nmoles/ml, on day 5. It should be noted here that although L-asparaginase activity disappeared completely from the blood 2 days after injection (Figure 2), plasma L-asparagine did not rise until day 4. In the case when microencapsulated L-asparaginase was injected, plasma L-asparagine dropped from 32.6 ± 2.5 nmoles /ml at zero time to 0 nmoles /ml when the first sample of plasma was taken 4 hours later (Figure 6). Plasma L-asparagine remained at zero concentration for 8 days, rose to 23.5 ± 3.5 nmoles/ml on day 9, and was normal, 32.0 ± 2.0 nmoles/ml, on day 12. Attention is drawn to the observation that although

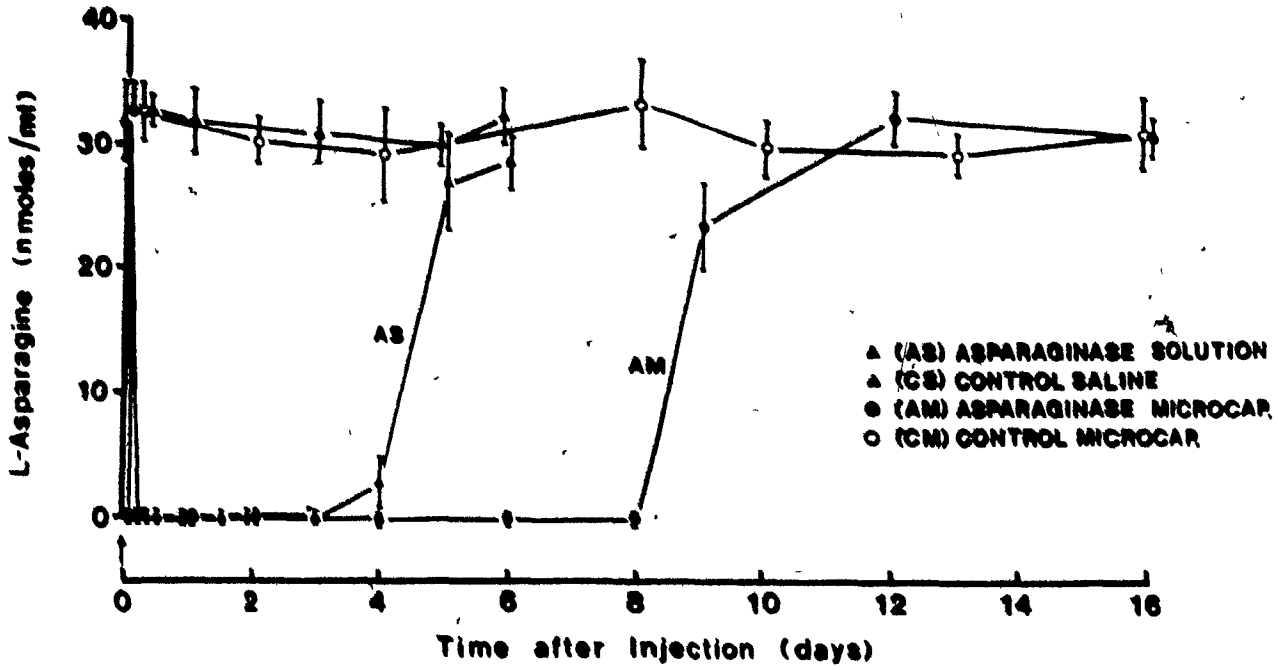


FIGURE 6

Time course of plasma L-asparagine levels in normal C3H/HEJ mice after one of the following intraperitoneal injections:

Each ▲ = 4 mice injected with AS and sacrificed at the indicated time interval.

Each △ = 3–4 mice injected with CS and sacrificed at the indicated time interval.

Each ● = 4 mice injected with AM and sacrificed at the indicated time interval.

Each ○ = 3–4 mice injected with CM and sacrificed at the indicated time interval.

All values represent means and standard deviations.

20% of the injected L-asparaginase activity still remained in the "body" after 8 days (Figure 4), plasma L-asparagine levels rose back towards normal on day 9 (Figure 6). Injection of saline or control microcapsules did not cause any significant change in plasma L-asparagine levels (Figure 6).

Response of the host to intraperitoneal injection of nylon microcapsules

There were no external signs of toxicity associated with the i.p. introduction of control or L-asparaginase loaded microcapsules, and the animals remained active and healthy without any abdominal tenderness or rigidity or significant changes in weight.

24 hours after injection, the microcapsules appeared to be covered by a sticky film. These microcapsules tended to form loose, sticky clumps or aggregates, which were found in large numbers dispersed throughout the peritoneal cavity, and especially in the upper parts of the peritoneal cavity. On day 2, the microcapsules tended to form small masses and locate mostly in the regions of the spleen and upper surfaces of the liver. These microcapsule aggregates could easily be dispersed at this stage. Examination of the microcapsules under the microscope showed that they were intact (Figure 7). There is a progressive decrease with time as regards the ease with which individual microcapsules could be dispersed from the aggregates, so that by day 6 the microcapsules could only be separated with great difficulty (Figure 7). However, it can be seen that the microcapsules are still intact.

Histological sections show a typical foreign body reaction with an infiltration of neutrophils, mononuclear phagocytes and foreign body giant cells. By day 8, there was an increase in the presence of mononuclear phagocytes, a decrease in the neutrophilic infiltrate and vascular fibroblastic

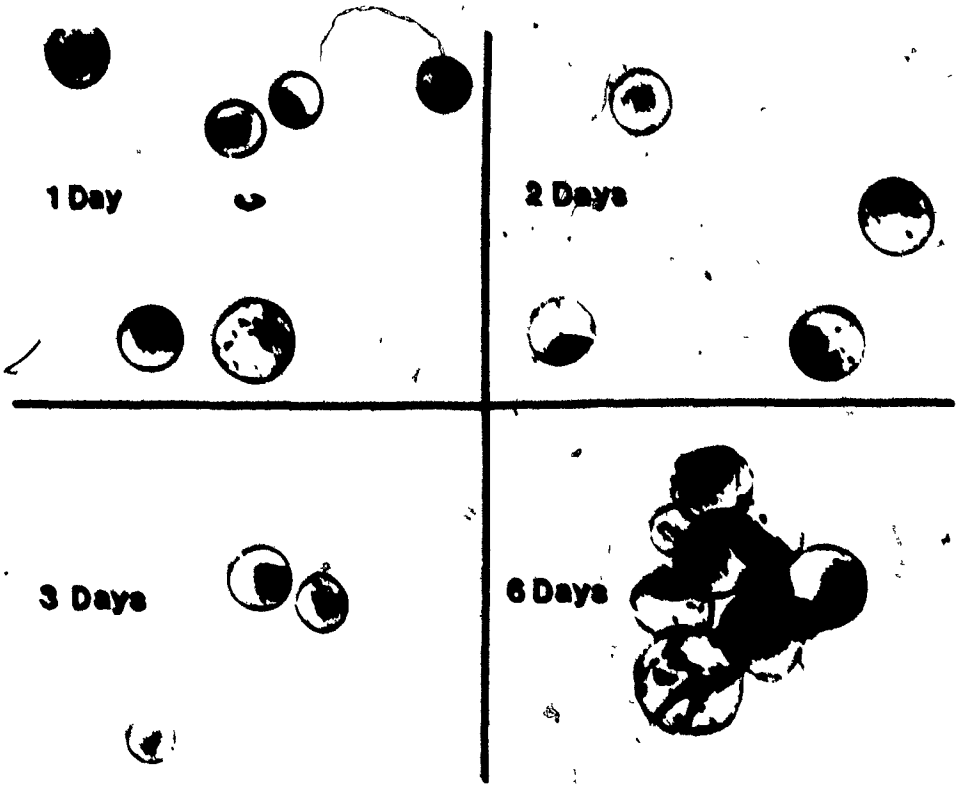


FIGURE 7

Photograph of i.p. -injected nylon microcapsules recovered from the peritoneal cavity after the indicated time intervals.

proliferation beginning from the periphery of the aggregates. Increased organization of the periphery (as shown in Appendix 5) occurs with time as progressive collagenous fibrosis advances inwards from the periphery to the center of the microcapsule aggregates.

Tumor regression in mice bearing the Gardner°lymphosarcoma 6C3HED

Preliminary studies were initiated using male C3H/HEJ mice implanted subcutaneously in their flanks with the 6C3HED lymphosarcoma. After growth of the tumor to about 3 cm in diameter, the mice were given a single i.p. injection of L-asparaginase solution or L-asparaginase microcapsules (0.5 IU/gm). The mean body weights of these tumor-bearing mice prior to injection were 38.6 ± 2.9 gm for those that were injected with L-asparaginase solution and 34.8 ± 3.5 gm for those injected with L-asparaginase microcapsules. Results obtained showed that injection of L-asparaginase solution or microencapsulated L-asparaginase both resulted in the almost complete regression of the large tumor mass within 5 days. This decrease in tumor size was quite drastic, the tumor being no longer visible to the eye. The mean body weights at this time were then measured to be 25.3 ± 2.4 gm for those which received L-asparaginase solution and 24.4 ± 1.7 gm for those given L-asparaginase microcapsules. Normal male mice of the same age weigh about 27.1 ± 4.3 gm.

DISCUSSION

The results reported here show that after i.p. injection of L-asparaginase solution, L-asparaginase activity appeared very rapidly in the blood (Figure 2); in marked contrast, there was no significant leakage of L-asparaginase into the circulation from i.p. injected L-asparaginase microcapsules in the C3H/HEJ mice for at least 16 days after injection, the duration of the experiment (Figure 2). Whether or not there are minute quantities of L-asparaginase leaking out from the microcapsules in vivo but in insufficient amounts to be detected by the present assay technique cannot be entirely dismissed. The following observations however, would seem to indicate that this is not likely to be important. In vitro evidence for the absence of leakage of L-asparaginase from microcapsules is provided by the present and earlier (40, 41) observation that there was no detectable L-asparaginase activity in the supernatant of the microcapsule suspension prior to injection, and by the finding of Mori et al (46) using the Ouchterlony technique, that there was no precipitin line detectable between L-asparaginase-loaded microcapsules and antiserum prepared against free L-asparaginase. Earlier studies here (39, 41) also demonstrated that ^{51}Cr-labelled hemoglobin in the microcapsules did not leak out after i.p. injection into rats; since L-asparaginase is a larger molecule than hemoglobin, it is likely that encapsulated L-asparaginase also would not leak out from the microcapsules. Furthermore, the present study shows that microcapsules recovered from the peritoneal cavity appeared to be intact (Figure 7).

It is well known that one of the factors affecting the therapeutic usefulness of a particular L-asparaginase is its biological half-life in the host's circulation, which in turn depends on the source of the preparation

and the methods used for its isolation and purification, L-asparaginase prepared by NBC used in the present study was cleared from the circulation in an exponential manner with a half-life of 4.4 hours (Figure 3), and became undetectable 48 hours after injection (Figure 2). On the other hand, the action of L-asparaginase microcapsules does not depend on its half-life in the circulation, but is based on an entirely different mechanism. As long as L-asparagine can dialyze across the microcapsule membrane and the encapsulated enzyme is still catalytically active, L-asparagine will continue to be removed from the extracellular fluid by the L-asparaginase microcapsules in the peritoneal cavity. The barrier to L-asparagine diffusion and the in vivo stability of the microencapsulated enzyme are therefore important parameters determining the effectiveness of L-asparaginase-loaded microcapsules instead of the half-life of the enzyme in the circulation.

After i.p. injection of L-asparaginase solution, the decrease in "body" L-asparaginase activity occurred exponentially (Figure 4) with a half-life of 2 hours (Figure 5); in marked contrast, in the case of the microencapsulated L-asparaginase the fall in enzyme activity to 50% occurred only after 60 - 72 hours (Figure 4). It is noteworthy that although L-asparaginase activity disappeared from the blood with a half-life of 4.4 hours after i.p. injection of L-asparaginase solution (Figure 2), yet plasma L-asparagine level was kept at zero concentration for 3 days, rose to 11% of normal on day 4, and was back to the normal pre-injection level on day 5 (Figure 6). This suggests that some mechanism exists in vivo whereby the effect of injected L-asparaginase solution continues for some time even after its removal from the blood. Broome (47) has suggested that i.v. - injected L-asparaginase may be removed by the reticuloendothelial system as evidenced by an increase in liver L-asparaginase activity. In studies

to be published, we have also found an increase in liver L-asparaginase activity following the i.p. injection of L-asparaginase solution. However, no such increase in liver L-asparaginase activity was found after the i.p. injection of L-asparaginase microcapsules. It has been proposed that sequestered enzyme in the reticuloendothelial system may still be capable of acting on plasma L-asparagine, thereby continuing to maintain it at zero concentration for 3 days. Injection of microencapsulated L-asparaginase i.p. on the other hand, resulted in a significantly prolonged lowering of L-asparagine level with L-asparagine being kept at zero concentration for 8 days, rising back to 72% of normal on day 9 (Figure 6). The superiorly prolonged ability of a single injection of equivalent activity of microencapsulated L-asparaginase may be due to both the increased stability of the microencapsulated form of the enzyme and the protective function of the microcapsule membrane in preventing the removal and inactivation of the enzyme as a foreign protein (39, 42, 43). Although there was still 20% of injected L-asparaginase activity remaining in the body after 8 days (Figure 4), the plasma L-asparagine rose back to 72% of the normal on day 9. Control experiments showed that the microencapsulated enzyme was still present in an active form in the microcapsules in amounts which are sufficient to lower effectively the plasma L-asparagine level from normal values. Perhaps the rise of L-asparagine concentration may be explained by the possibility that in some manner, plasma L-asparagine was now being prevented from being acted on by the still active enzyme within the microcapsules. This hypothesis was supported by the following histological findings.

Histological studies showed that there was a progressive foreign body reaction with time beginning from the periphery of the microcapsule aggregate and becoming very marked around the 8th day after injection. This

is likely to form an impermeable barrier around the microcapsules rendering the catalytically active L-asparaginase in the microcapsules inaccessible to external L-asparagine (See Appendix 5). In this way, the microcapsules, though still containing active L-asparaginase, were no longer physiologically active after the 8th day. Thus, the prolonged depression of L-asparagine levels after microencapsulated L-asparaginase administration not only reflects the increased stability and protection afforded the enzyme, but also the permeability of the microcapsules to L-asparagine.* It should be pointed out that the formation of the impermeable fibrosed wall around the microcapsule aggregates also serves as an additional reinforcement and prevents possible subsequent release of microcapsule contents directly into the body. The type of foreign body reaction described here is similar to that reported for other forms of nylon materials like surgical suture material (48). The i.p. injection of L-asparaginase nylon microcapsules employed in these studies is not meant to represent the optimal route of administration nor the optimal polymer membrane for ultimate clinical use. It serves only as a model system to demonstrate some of the basic principles and the feasibility of using microencapsulated L-asparaginase. Further research needs to be done with regard to, for example, the development of a more biocompatible membrane for clinical use. Possible ways to improve the biocompatibility of a variety of microcapsules have been discussed elsewhere (39).

Microencapsulated L-asparaginase has already been shown to be effective in inhibiting the growth of the 6C3HED lymphosarcoma when given at the same time as tumor implantation (39 - 41). Preliminary results in the present paper indicate that this form of the enzyme is also capable of causing regression of the advanced, well-established lymphosarcoma. In these studies

* See Appendix 6 for subsequent work

it was also shown that the half-life for the clearance of L-asparaginase from the blood of mice bearing the Gardner lymphosarcoma was found to be 13.2 hours as compared to 4.4 hours for normal mice. This 3-fold increase in the half-life of the enzyme in the blood of tumor-bearing mice supports the idea that the presence of the LDH-elevating virus carried as a contaminant in the 6C3HED lymphosarcoma might in fact be a critical factor in determining the therapeutic effectiveness of L-asparaginase solution in inhibiting tumor growth (49). Since the effectiveness of i.p. injected microencapsulated L-asparaginase into tumor-bearing mice does not depend on L-asparaginase activity in the blood, the role of the LDH virus is probably not important in this case as an adjunct to effective microencapsulated L-asparaginase therapy.

By microencapsulation of the enzyme, one avoids problems associated with immunological reactions, bacterial contaminants, especially endotoxins, rapid in vivo removal and inactivation of the foreign enzyme by the body's defense mechanisms (39-41). Furthermore, microencapsulation also increases the in vitro and in vivo stability of the enzyme (42, 43). Recovery of enzyme activity after microencapsulation is also greater than with other methods of immobilization (39). Since the ratio of the amount of polymer used to immobilize the enzyme is very small compared to other immobilization methods, there is a reduced problem of polymer material retention in the body in cases where immobilized L-asparaginase is used either for injection or as an implanted graft (39, 43). Another important factor that should be noted is that 27 ml of microcapsules of 80 μm mean diameter would offer a surface area of approximately 2 m^2, which is larger than the surface area of standard hemodialyzers (39, 50). This, plus the fact that the ultrathin membrane of the semipermeable microcapsules is at least 100 times thinner than

that of standard hemodialzer membranes, means that the rate of diffusion of permeant molecules across 27 ml of microcapsules 80 μm in diameter would be at least 100 times greater than that available in a standard hemodialyzer (39, 51). It is apparent, therefore, that the use of L-asparaginase microcapsules has the features of increased stability, extremely large surface area:volume ratio, dialysis of substrate across a semipermeable membrane without the risk of introducing free enzyme into the body, and non immunogenicity. In patients with a high L-asparaginase antibody titer (52), there is a further advantage since antibody is prevented from entering the microcapsules to cause antigen-antibody reactions (37-41). This approach of microencapsulation is a simpler and more effective way to circumvent or reduce the incidence of side effects and/or the severity of some of the problems encountered in L-asparaginase therapy, as compared to the approaches proposed by others. These alternative approaches others have proposed include:

(a) The administration of immunologically distinct forms of tumor-inhibitory L-asparaginases derived from sources other than E. coli, e.g. Erwinia carotovora, in patients who had previously developed anaphylaxis from the E. coli enzyme (53).

(b) Modification of the enzyme dialysis approach (39, 50) in extracorporeal hemodialysis with L-asparaginase added to the dialysis bath of the hemodialyzer (54).

(c) The use of L-asparaginase immobilized by gel entrapment (55) or covalent linkage (56-59) either for injection, as an internal implant, or for use in an extracorporeal shunt.

Because of the virtually unlimited possible variations in the membrane properties and materials that may be enclosed in microcapsules,

microcapsules can be prepared to have a wide range of biological activities in biomedical and clinical applications (39). Detailed studies in this laboratory have demonstrated the potential of the principle of "artificial cells" in biomedical research and its actual clinical applications. These have included their use for replacement therapy in congenital enzyme deficiency in experimental animals (39, 60), for the suppression of the growth of the 6C3HED lymphosarcoma in mice (39-41), for the construction of a compact artificial kidney (39, 50) presently in its third year of clinical trial for the treatment of patients with chronic renal failure (39, 61), and more recently, for the treatment of hepatic coma (62) and drug intoxication in patients (63). These results support the feasibility of using microencapsulated enzyme in an extracorporeal shunt system to alleviate some of the problems discussed here, while obviating the need for introduction or accumulation of microcapsules in the patient. With further advances in the field of biocompatible membranes and progress in the use of microencapsulated enzymes in an extracorporeal shunt system (39, 50), perhaps such an envisaged use of microencapsulated L-asparaginase may become a clinical reality as part of the therapeutic treatment of acute lymphoblastic leukemia. With improved schedules of intensive cytoreductive chemotherapy with microencapsulated L-asparaginase in combination with other conventional agents, followed by immunotherapy (64, 65), the percentage of 5-year survivors and perhaps of permanent remissions in acute leukemia may likely increase (66). L-asparaginase has also been found to impair a number of immune reponses in the host (67-69). The discovery of the immunosuppressive properties of L-asparaginase has opened up an entirely new area of research with the demonstration of the use of this enzyme as a potential immunosuppressive agent. However, its usefulness is limited again by the nature of the heterogeneously derived foreign preparation.

Presumably, the rationale behind the use of microencapsulated L-asparaginase, seemingly a more favourable immunosuppressive agent, as an alternative to L-asparaginase solution may also be applicable here.

APPENDIX 1

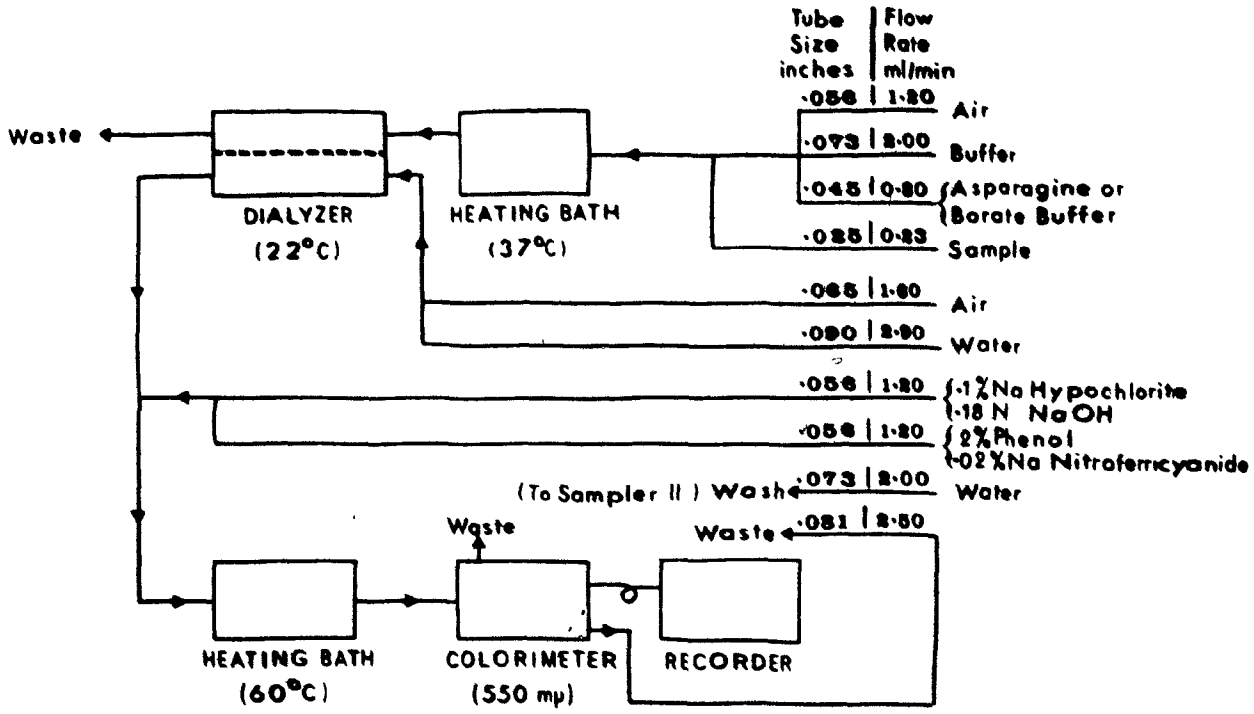
Preparation of L-asparaginase nylon microcapsules

The specific activity of the L-asparaginase preparation used in the present study was high and the enzyme was available commercially in very small vials containing 1000 IU or 3.2 mg protein per vial. Because of the difficulty of weighing out 0.96 mg protein (= 300 IU) from these vials, it was experimentally more convenient to dissolve the contents of the vial in 1 ml distilled water and pipette from this solution a volume of 0.3 ml which contained the desired quantity of L-asparaginase (300 IU). To compromise the dilution effect caused by the addition of 0.3 ml of L-asparaginase solution to the buffered hemoglobin hexamethylenediamine mixture at the start of the preparative procedure, it was found necessary to adjust the concentrations of the various components of the buffered hexamethylenediamine solution to the values given in Materials and Methods. The net final concentrations therefore of hemoglobin and hexamethylenediamine, after the addition of L-asparaginase solution, were the same as that described in the standard procedure (39). After this adjustment, microcapsules which satisfied the criteria outlined in Materials and Methods could easily be prepared. The necessity of this modification emphasizes the critical importance of the concentrations of hemoglobin and the hexamethylenediamine at the start of the procedure, if satisfactory microcapsules are to be formed. This is especially important in the case of hexamethylenediamine which must be present in a ratio of 22:1 of hexamethylenediamine : sebacoyl chloride. The importance of the choice of the optimal reactant concentrations has been discussed in Chang's monograph (39).

APPENDIX 2

Automated assay method for L-asparaginase activity

A Technicon autoanalyzer (Technicon Co., Tarrytown, New York) was used for the L-asparaginase assays by the continuous-flow, automated method shown schematically in the figure below.



Briefly, the procedure was as follows. Samples of specimens were aspirated at a rate of 60 samples/hour, mixed with 0.1 M borate buffer, pH 8.5, and 0.04 M L-asparagine in borate buffer (or borate buffer alone for blank determinations). The buffered enzyme-substrate reaction mixture was segmented with bubbles of air, and was passed into an incubation coil immersed in a water bath kept at 37°C to allow the enzyme reaction to

proceed optimally. After incubation for about 12 minutes, the reaction mixture was passed into a dialysis module at room temperature. NH_3 present in the reaction mixture was separated from the protein-containing solution by dialysis into a recipient flowing stream of water. The ammonia-containing solution in the recipient stream was mixed with alkaline sodium hypochlorite and phenol containing sodium nitroferricyanide as a catalyst. The mixture was passed into a heating bath which was heated to 60°C to allow for the color reaction to develop. The solution was then passed through a colorimeter equipped with 550 mμ filters, and the optical density of the blue color of the solution was then recorded on a recorder.

$(NH_4)_2 SO_4$ standards ranging from 0.25 μmoles of nitrogen/ml to 16 μmoles of nitrogen/ml were assayed in a similar fashion.

The specimens and standards were run twice, first with L-asparagine and then with buffer substituted for L-asparagine to correct for endogenous ammonia. It was essential to assay an additional set of ammonia standards with the blank run because L-asparagine depresses the color reaction.

From the corresponding standard curves, the μmoles of ammonia/ml present in the specimen were determined.

The L-asparaginase activity in the specimen was calculated as follows:

Let x = μmoles ammonia/ml in the specimen assayed in the presence of L-asparagine

y = μmoles ammonia/ml in the specimen assayed with buffer substituted for L-asparagine (blank)

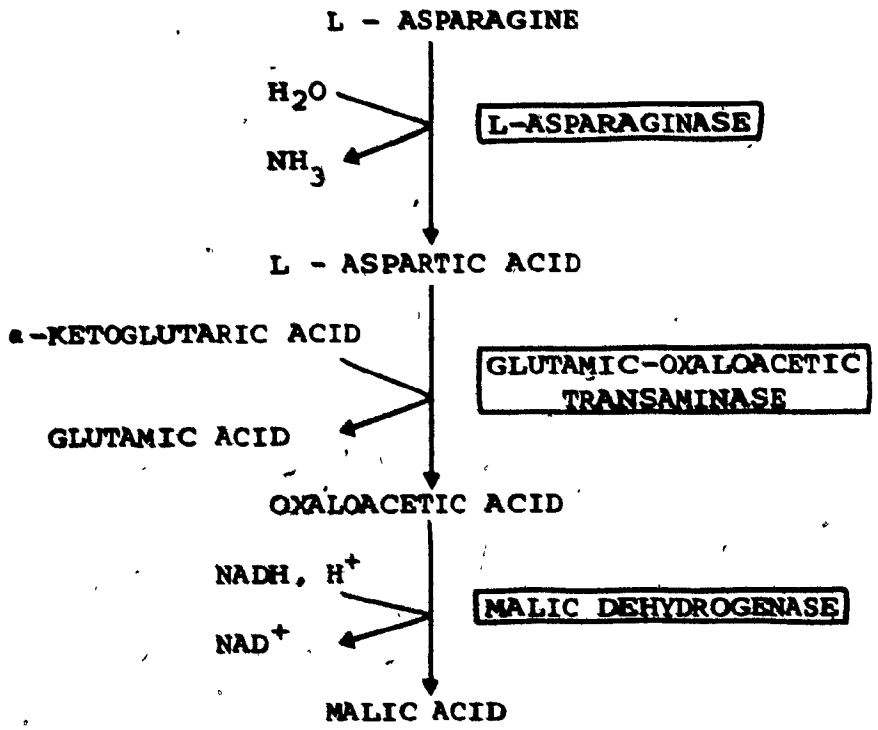
t = time of incubation of enzyme with buffered substrate at 37°C (minutes).

Then, L-asparaginase activity (IU/ml) = $\left[\frac{(x - y)}{t}\right]$ $\frac{\mu moles/ml}{min.}$

APPENDIX 3

Fluorimetric technique for L-asparagine assay

The fluorimetric technique for L-asparagine assay is based on the following enzymatically coupled sequence of reactions that results in the oxidation of NADH. The latter reaction is manifested as a decrease in fluorescent emission.



The details for preparation of the assay reaction mixture are given under Materials and Methods. From the net change in fluorescent emission, in arbitrary units, the L-asparagine concentration present in the original sample was calculated as follows:

Let A_1 = fluorescence units after 30 minutes incubation of sample with reaction mixture.

A_2 = fluorescence units after 1 hour incubation of reaction mixture containing sample to which L-asparaginase has been added.

S_1, S_2 = corresponding readings in which L-asparagine standard (35 nmoles/ml) was substituted for sample.

B_1, B_2 = corresponding readings in which water was substituted, as a blank, for sample.

Then L-asparagine concentration (nmoles/ml) = $35 \times \left[\frac{(A_1 - A_2) - (B_1 - B_2)}{(S_1 - S_2) - (B_1 - B_2)}\right]$.

APPENDIX 4

Method of preparation of specimens for histological studies

Specimens were first fixed with sodium phosphate buffered 10% formalin for at least 24 hours at room temperature (22°C). The sequential procedures of dehydration, clearing and infiltration with embedding medium were then performed, using an automated "Fisher Tissuematon Model 60" (Fisher Scientific Co.,Pittsburgh, Pennsylvania). The methodology used for dehydration,clearing and infiltration was as follows:

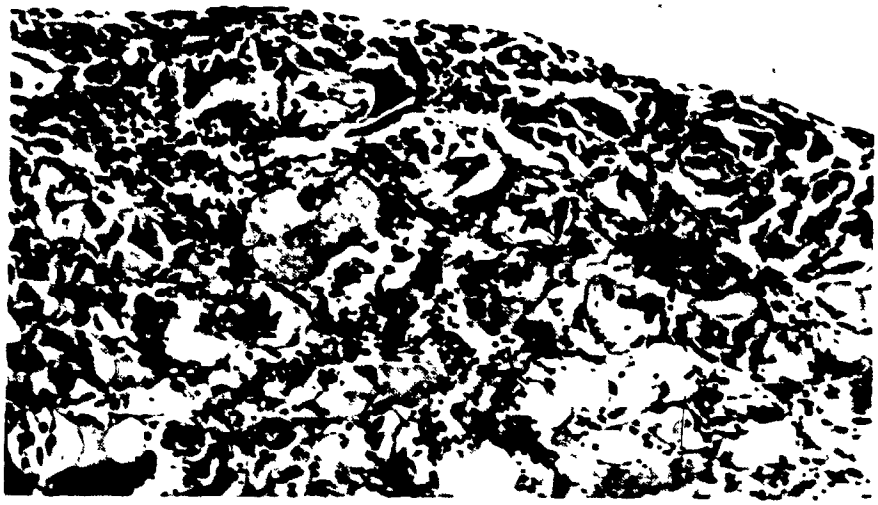
10% buffered Formalin	15 minutes
Distilled Water	15 minutes
70% Alcohol I	1 hour
70% Alcohol II	1 hour
Absolute Alcohol I	1 hour
Absolute Alcohol II	1 hour
50% (v/v) Absolute Alcohol in Dioxane I	2 hours
50% (v/v) Absolute Alcohol in Dioxane II	2 hours
Dioxane I	1 hour
Dioxane II	1 hour
Paraffin (m.p. 56.5°C) I	2 hours
Paraffin II	6 hours

The specimens were then embedded in paraffin blocks. Sections, 5 μm in thickness, were cut using a rotary microtome, mounted on slides and dried overnight at 37°C. These were deparaffinized with xylene, stained with Harris' hematoxylin - eosin and then coverslipped.

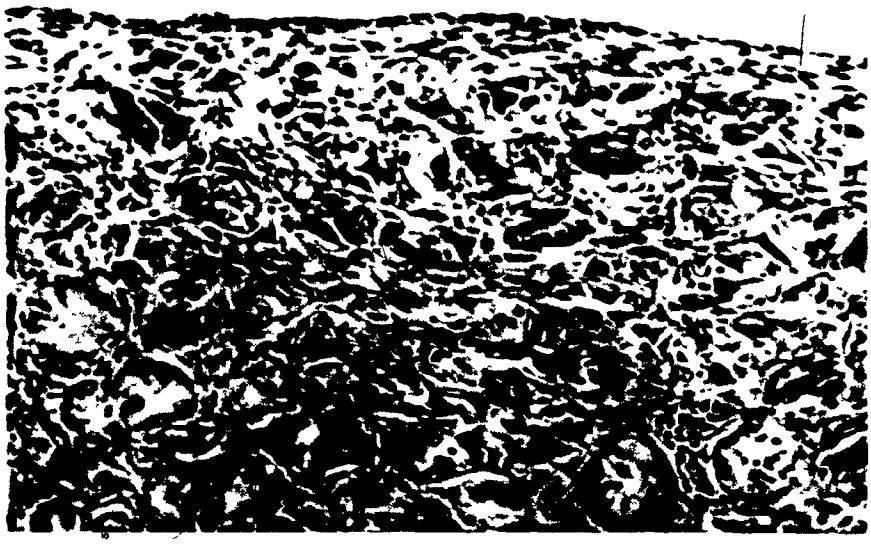
APPENDIX 5

Histological sections showing progressive tissue reaction around the periphery of microcapsule aggregates recovered from the peritoneal cavity on day 8 (Appendix 5A) and on day 16 (Appendix 5B) after i.p. injection of nylon microcapsules. It should be noted that the distorted appearance of the microcapsules (indicated by the arrows) are a result of fixing and other various procedures employed in the preparation of the specimens for histology, as described in Appendix 4.

APPENDIX 5



(A)



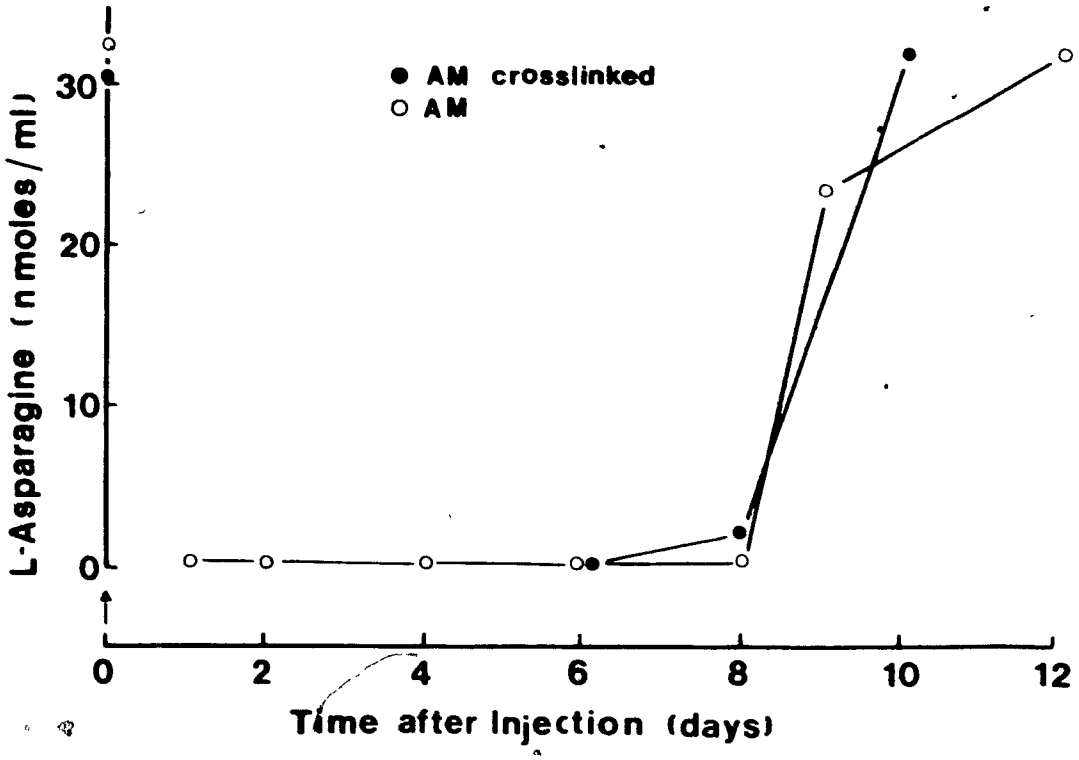
(B)

APPENDIX 6

Plasma L-asparagine levels after i.p. injection of L-asparaginase microcapsules cross-linked with glutaraldehyde

Subsequent to the publication of the results described in this chapter, the following experiment was performed to further investigate the problem of decreased permeability of L-asparaginase microcapsules. The question was asked, if microcapsules do become impermeable after day 8 post i.p. injection, then increasing the in vitro stability of the microencapsulated L-asparaginase should have little, if any, effect on the duration of its in vivo action, assuming that the biocompatibility properties of the microcapsules themselves are not altered by the cross-linking procedure. This hypothesis was tested, using L-asparaginase microcapsules that had been cross-linked with glutaraldehyde.

The method used in the present study for cross-linking L-asparaginase microcapsules was modified from the technique described by Chang (42), as suggested to me by Dr. John Campbell in this laboratory. L-asparaginase microcapsules were first prepared as described in Materials and Methods. Glutaraldehyde (0.1% v/v) was added to 5 ml L-asparaginase microcapsules suspended in a total volume of 25 ml in 0.1 M borate buffer, pH = 8.5, at $4^{o}C$. The reaction mixture was agitated for 20 minutes at $4^{o}C$, centrifuged, and washed quickly with tris buffer, pH = 8.5, at $4^{o}C$, then with borate buffer. The suspension of L-asparaginase microcapsules, now cross-linked with glutaraldehyde, was washed repeatedly, then assayed for its enzyme activity. The increased stability of these glutaraldehyde L-asparaginase microcapsules over untreated L-asparaginase microcapsules, was confirmed by in vitro assays of the enzyme activities of both enzyme



APPENDIX 6

Plasma L-asparagine levels in normal C3H/HEJ mice after i.p. injection of L-asparaginase microcapsules crosslinked with glutaraldehyde.

Each ● = the mean of 2 mice sacrificed at the indicated time interval.

Values for mice injected with untreated L-asparaginase microcapsules were taken from Figure 6.

preparations, kept at $4^{o}C$ and $37^{o}C$, over a period of 3 weeks. Normal C3H/HEJ mice were injected i.p. with the cross-linked L-asparaginase microcapsules at a dose of 0.5 IU/gm body weight.

Assays of the plasma L-asparagine concentrations at the time intervals indicated in the accompanying figure show that there was no significant difference in the plasma L-asparagine levels after i.p. injection of untreated L-asparaginase microcapsules versus L-asparaginase microcapsules that had been cross-linked with glutaraldehyde. These preliminary results are interpreted to mean that increasing the in vitro stability of L-asparaginase microcapsules by cross-linking the enzyme with glutaraldehyde after microencapsulation for example (42), does not increase the in vivo ability of microencapsulated L-asparaginase to maintain a zero concentration of plasma L-asparagine. This is in accord with the earlier findings that i.p. injected L-asparaginase microcapsules, although containing catalytically active L-asparaginase, were no longer physiologically active in vivo after 8 days post injection. The results described here are probably also related to a decreased permeability of the microcapsules consequential to the bioincompatible properties of the present microcapsule membrane. If the problem of bioincompatibility of the microcapsule membrane could be solved, presumably, then, increasing the in vitro stability of the microencapsulated enzyme might result in a prolongation of its in vivo action.

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CHAPTER III

A COMPARISON OF THE TUMOR INHIBITORY EFFECTIVENESS OF INTRAPERITONEALLY INJECTED L-ASPARAGINASE SOLUTION AND L-ASPARAGINASE MICROCAPSULES IN NON-IMMUNIZED AND IMMUNIZED MICE BEARING THE 6C3HED LYMPHOSARCOMA

ABSTRACT

1×10^6 Gardner 6C3HED lymphosarcoma cells were implanted subcutaneously into the lower back of each of ninety non-immunized and sixty-six immunized C3H/HEJ mice. Ten days after tumor implantation, the mice were injected with one of the following: L-asparaginase solution in varying doses; L-asparaginase microcapsules in varying doses; saline, as control; or microcapsules containing no L-asparaginase, as control.

At a dose of 0.500 IU/gm, all non-immunized mice given either L-asparaginase solution or L-asparaginase microcapsules were alive and tumor-free 100 days after tumor implantation. However, at doses of 0.250 IU/gm and 0.125 IU/gm, very striking differences were observed between the groups receiving equivalent doses of L-asparaginase solution or L-asparaginase microcapsules. For non-immunized mice treated with enzyme of equivalent assayed activity, the microencapsulated form of the enzyme was significantly more effective as a tumor-inhibitory agent than the enzyme in solution. All non-immunized mice injected with saline or microcapsules containing no L-asparaginase died with a median survival time of about 18 days.

In mice which had previously been immunized against L-asparaginase, the results obtained showed that both L-asparaginase solution and L-asparaginase microcapsules were ineffective in inducing regression of the established lymphosarcoma. The median survival time of immunized mice treated with L-asparaginase solution or L-asparaginase microcapsules was found to be about 18 days, which was not significantly different from immunized mice injected with saline or microcapsules containing no L-asparaginase, as control.

INTRODUCTION

E. coli L-asparaginase has been used as an anti-neoplastic agent, both experimentally and clinically (1 - 5). Two major drawbacks have arisen during the course of clinical trials that deter a more routine use of L-asparaginase. These are:

(a) a wide spectrum of adverse side effects, and

(b) the development of resistance to repeated L-asparaginase therapy.

These side effects may be partly attributed to the antigenic nature of the enzyme preparation and the presence of bacterial contaminants, such as endotoxins, in the enzyme preparation (1, 2, 4, 6). The production of humoral anti-L-asparaginase antibodies in animals (7 - 10) and in man (11, 12) when the tumor-bearing hosts have previously been treated with L-asparaginase represents one of several mechanisms deemed to be responsible for the development of resistance to continued L-asparaginase therapy. Antibodies have been shown (7 - 12) to:

(a) inhibit the catalytic activity of the enzyme and, more importantly,

(b) cause a dramatically accelerated clearance of any injected L-asparaginase from the host's circulation.

It has been proposed that prior immobilization of L-asparaginase in semipermeable microcapsules may alleviate or circumvent some of the problems associated with L-asparaginase therapy, as compared to its use in free solution (13 - 16). Semipermeable microcapsules (13, 17, 18) are spherical ultrathin, semipermeable polymer membranes of cellular dimensions, each enveloping a microdroplet of an aqueous protein solution or suspension. The small size together with the ultrathin membrane of the microcapsules allow for a very rapid diffusion of permeant substances. Moreover, the equivalent

pore radius (16 Å) of the microcapsule membrane is such that the microcapsule is selectively permeable to small molecules, such as L-asparagine, L-aspartic acid, and ammonia, but is impermeable to macromolecules, such as L-asparaginase. Thus, L-asparaginase and any accompanying macromolecular contaminants, once physically entrapped in the intracellular environment of the microcapsules, would not leak out to give rise to various toxic manifestations and are protected from rapid removal and inactivation by the host. L-asparaginase can, however, still act efficiently on the external permeant substrate diffusing into the microcapsules. Microencapsulated L-asparaginase has already been shown to be more effective than L-asparaginase solution in suppressing the growth of the 6C3HED lymphosarcoma when it is administered immediately after tumor implantation (13, 14), as well as being able to induce complete regression of established lymphosarcoma implants (16).

The studies presented here were done to compare and analyze in more detail the tumor inhibitory effectiveness of various doses of free and microencapsulated forms of L-asparaginase in bringing about tumor regression of established 6C3HED lymphosarcoma implants and to correlate these findings with earlier knowledge of the in vivo effects of these two enzyme preparations. It was also considered desirable to determine whether microencapsulated L-asparaginase would be therapeutically active against the L-asparaginase-sensitive 6C3HED lymphosarcoma borne in hosts previously immunized to L-asparaginase.

MATERIALS AND METHODS

Animals

Normal female C3H/HEJ mice, weighing about 25 gm, were purchased from Jackson Laboratories, Bar Harbor, Maine. Female C3H/HEJ mice implanted with the lymphosarcoma were also obtained from Jackson Laboratories and used as donors of 6C3HED lymphosarcoma cells. All mice were maintained on a diet consisting of Purina Mouse Chow and water ad libitum.

Enzyme

(a) E. coli L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) with a specific activity of 312.3 IU/mg (Lot No.4905) was purchased as a lyophilized preparation in vials of 1000 IU from Nutritional Biochemical Corporation, Cleveland, Ohio, and reconstituted in sterile saline for injection of L-asparaginase solution into animals. One IU of L-asparaginase has been defined as that amount of enzyme which will liberate 1.0 μmole of ammonia per minute at 37°C.

(b) Microencapsulated E. coli L-asparaginase was prepared by the process of interfacial polymerization, according to the procedure and criteria described in detail elsewhere (16).

Each enzyme preparation was always assayed for its exact activity just prior to injection.

Blood L-asparaginase

Blood from the severed subclavian artery of ether-anesthetized mice was drawn into fire-polished, heparinized Pasteur pipettes. The blood was then stored at -20°C in small containers and assayed within 2 hours.

L-asparaginase assay

L-asparaginase activity was measured using the automated continuous-flow method of Schwartz et al (19), with the modifications as described previously (16).

Immunization procedure

Mice were each given a single i.p. injection of 32 µgm (10 IU) of L-asparaginase in sterile saline at weekly intervals for 4 weeks. The presence of L-asparaginase antibody in the serum of these animals was confirmed by the techniques of double diffusion (Ouchterlony) on agarose slides, passive hemagglutination, and the ability of antiserum to inhibit enzyme activity when incubated in vitro with L-asparaginase solution. These techniques are described in detail in Chapter IV of this thesis.

Technique of tumor implantation

The technique of tumor implantation used in this study is similar to that described by Broome (20). Donor mice bearing the 6C3HED lymphosarcoma were sacrificed on the 12th day after the tumor was transplanted by Jackson Laboratories. Pieces of the solid subcutaneous tumor were removed under sterile conditions and gently forced through a stainless 250 mesh steel screen (63 µm diameter) into mammalian Ringer's solution kept at $4^{o}C$. The tumor cell suspension was poured into a measuring cylinder and placed in the refrigerator at $4^{o}C$. In about 10 minutes, clumps of tissue and the larger particles in the suspension would have settled leaving a suspension of single cells. This cell suspension was transferred into a flask and the concentration of viable tumor cells determined in a hemocytometer by the method of Schrek (21). (See Appendix 1 for details). The volume of the

tumor cell suspension was adjusted with Ringer's solution so that it contained 2×10^6 cells/ml. 0.5 ml of cell suspension containing 1×10^6 tumor cells was then injected subcutaneously by means of a 1 ml syringe and a 25 gauge needle into the lower back of each animal. It should be noted that the tumor "take" in all groups of non-immunized and immunized mice was 100% and the average size of the tumors before treatment was about 3 cm in diameter.

Experimental procedure

(a) Non-immunized mice. Prior to tumor implantation, mice were randomly divided into eight groups with 10 - 12 mice/group. The mean body weights of the mice in each group were determined. Each mouse was then implanted with 1×10^6 cells as described above (day 0). Ten days after tumor implantation, each group of mice received one of the following i.p. injections: (i) 1 ml saline, as control (11 mice); (ii) 0.125 IU/gm body weight of L-asparaginase solution (12 mice); (iii) 0.250 IU/gm L-asparaginase solution (12 mice); (iv) 0.500 IU/gm L-asparaginase solution (12 mice); (v) 1 ml 50% suspension of microcapsules containing no L-asparaginase, as control (11 mice); (vi) 0.125 IU/gm L-asparaginase microcapsules (11 mice); (vii) 0.250 IU/gm L-asparaginase microcapsules (11 mice); or (viii) 0.500 IU/gm L-asparaginase microcapsules (10 mice).

(b) Immunized mice. Mice which had been immunized as described above were each given a booster dose of 8 µgm (2.5 IU) L-asparaginase solution in 0.5 ml saline 5 days before tumor implantation. On the day of tumor implantation (day 0), the plasma L-asparagine level of boostered mice was assayed and found to

be normal. These mice were randomly divided into six groups with 11 - 12 mice/group and the mean body weights of the mice in each group were determined. Each mouse was then implanted with 1×10^6 6C3HED cells, using the technique described above. Ten days after tumor implantation, each group of mice was given one of the following i.p. injections: (i) 1 ml saline, as control (12 mice); (ii) 0.250 IU/gm L-asparaginase solution (11 mice); (iii) 0.500 IU/gm L-asparaginase solution (11 mice); (iv) 1 ml of 50% suspension of microcapsules containing no L-asparaginase, as control (12 mice); (v) 0.250 IU/gm L-asparaginase microcapsules (11 mice); or (vi) 0.500 IU/gm L-asparaginase microcapsules (11 mice).

The growth or disappearance of the tumors, days of death and survival time of the mice in each experimental group were followed for 100 days after tumor implantation.

RESULTS

Blood L-asparaginase activity in non-immunized tumor-bearing mice after injection of L-asparaginase solution and L-asparaginase microcapsules

After i.p. injection of L-asparaginase solution, enzyme activity appeared quickly in the blood with the highest levels occurring between 8-12 hours post injection (Figure 1). After this, the blood L-asparaginase level fell with a half-life of 13.2 hours, as compared to 4.4 hours for normal mice. In contrast to the injection of L-asparaginase solution, there was no detectable L-asparaginase activity in the blood after i.p. injection of L-asparaginase microcapsules. There was also no detectable L-asparaginase activity in the blood after i.p. injection of saline or microcapsules containing no L-asparaginase, given as controls.

Tumor regression in non-immunized mice bearing the 6C3HED lymphosarcoma after treatment with L-asparaginase solution and L-asparaginase microcapsules

After implantation, the tumors grew progressively larger so that the average size was 3 cm in diameter on day 10 after tumor implantation. Although the initial rate of tumor regression was more rapid in mice given L-asparaginase solution than in those given L-asparaginase microcapsules, three days after the administration of either L-asparaginase solution or L-asparaginase microcapsules, the tumors were no longer visible in all of these mice, at all doses given. However, tumors reappeared more quickly in general in animals that received 0.125 IU/gm L-asparaginase solution or L-asparaginase microcapsules than in these given 0.250 IU/gm L-asparaginase solution or L-asparaginase microcapsules. Tumors also reappeared more quickly in these given L-asparaginase solution than in those given the equivalent dose of

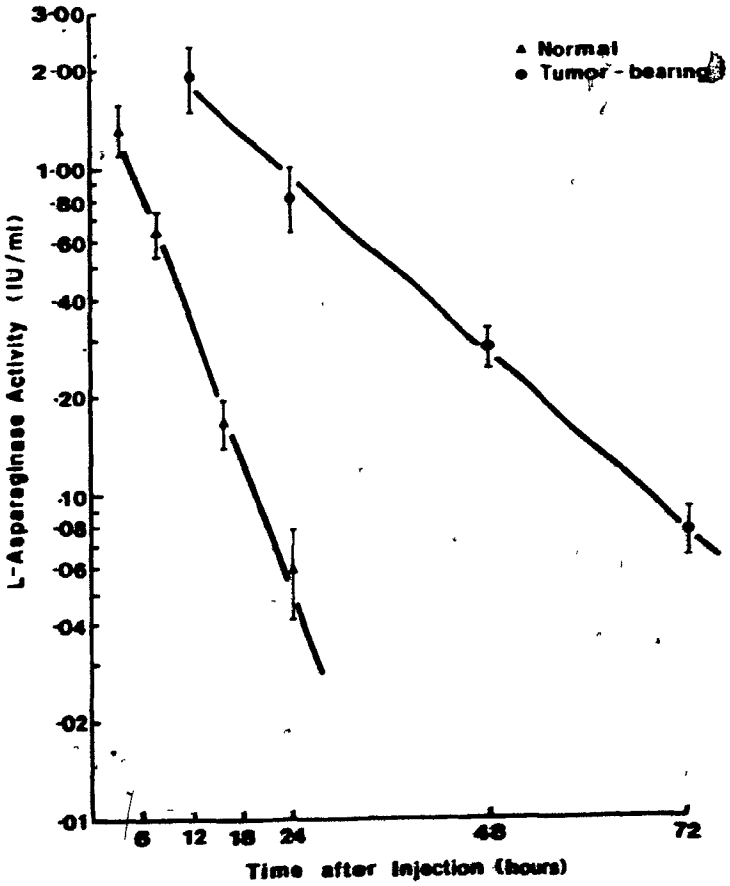


FIGURE 1

Semi-logarithmic plot of the clearance of L-asparaginase from the circulation of normal and tumor-bearing non-immunized mice after i.p. injection of L-asparaginase solution.

L-asparaginase microcapsules. In all the mice that were treated with 0.500 IU/gm L-asparaginase solution or L-asparaginase microcapsules, tumors completely regressed and did not reappear up to 100 days after tumor implantation. Figure 2 shows typically the tumor regression observed in mice before and three days after treatment with L-asparaginase solution or L-asparaginase microcapsules. (See Appendix 2 for further details). Figure 3 shows that there is no gross evidence of the presence of a tumor at the original site of tumor growth, six days after treatment with 0.500 IU/gm L-asparaginase microcapsules. Similar results were also obtained after the administration of 0.500 IU/gm L-asparaginase solution.

Comparison of the effects of different doses of L-asparaginase solution and L-asparaginase microcapsules on the survival time of non-immunized 6C3HED lymphosarcoma-bearing mice

The results obtained have been summarized in Figures 4 - 6. (A tabulation of this data is given in Appendix 3).

No L-asparaginase given: It may be seen that all mice given saline or microcapsules containing no L-asparaginase died, with a median survival time of 18 days for those injected with saline and 19 days for those injected with control microcapsules. (Figures 4 & 5).

Dose of 0.125 IU/gm: There was a general tendency for mice treated with L-asparaginase solution to die earlier than those treated with L-asparaginase microcapsules. It may be noted, for instance, that on day 24 after tumor implantation, 50% (6/12) of those receiving L-asparaginase solution had already died whereas only 9.1% (1/11) had died in the L-asparaginase microcapsules-treated group (Figures 4 & 5). More significantly, 100% (12/12) of the animals treated with L-asparaginase solution died by day 35 after tumor implantation, whereas 27.3%

FIGURE 2

Photographs of a 6C3HED lymphosarcoma-bearing, non-immunized mouse before treatment (day 10 after tumor implantation) and the same mouse 3 days after treatment with an i.p. injection of 0.500 IU/gm body weight of L-asparaginase microcapsules.

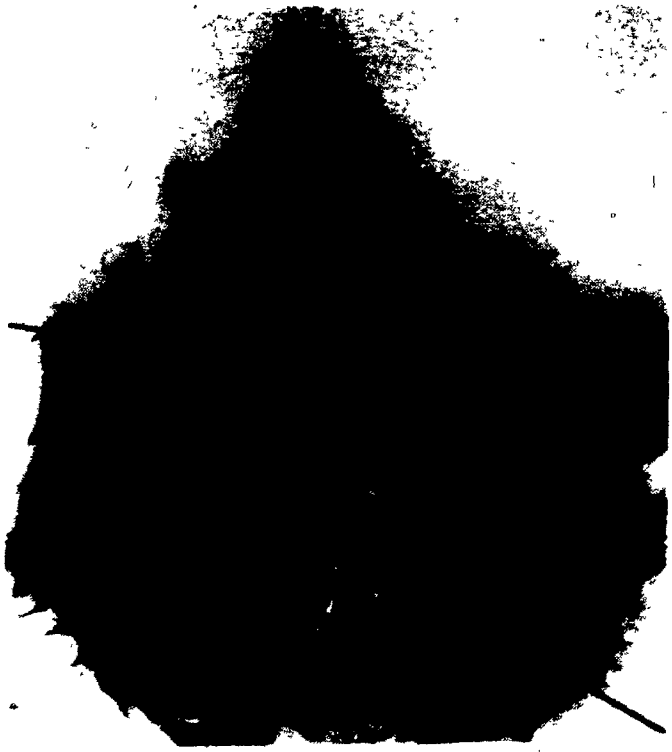
FIGURE 3

Photographs of the gross appearance of the tumor-implanted sites before and 6 days after treatment with an i.p. injection of 0.500 IU/gm body weight of L-asparaginase microcapsules.

Figures 3A & 3C were taken before treatment (on day 10 after tumor implantation). It can be seen that there is local invasion of the tumor into adjacent tissues from the primary site of tumor growth. The implanted lymphosarcoma is indicated in each figure by the arrow.

Figures 3B & 3D were taken 6 days after treatment with L-asparaginase microcapsules. There was no evidence of lymphosarcoma either at the original site of tumor implantation or at other sites in the body. Similar results were also obtained when the tumor-bearing hosts were treated with 0.500 IU/gm body weight of L-asparaginase solution. In contrast, post mortem examinations of those tumor-bearing mice given saline or control microcapsules revealed an enlarged tumor mass and the occurrence of wide-spread metastases. An accumulation of large quantities of edema fluid was also characteristically observed in these animals.

FIGURE 3



(A)

(B)

FIGURE 3

(C)

(D)

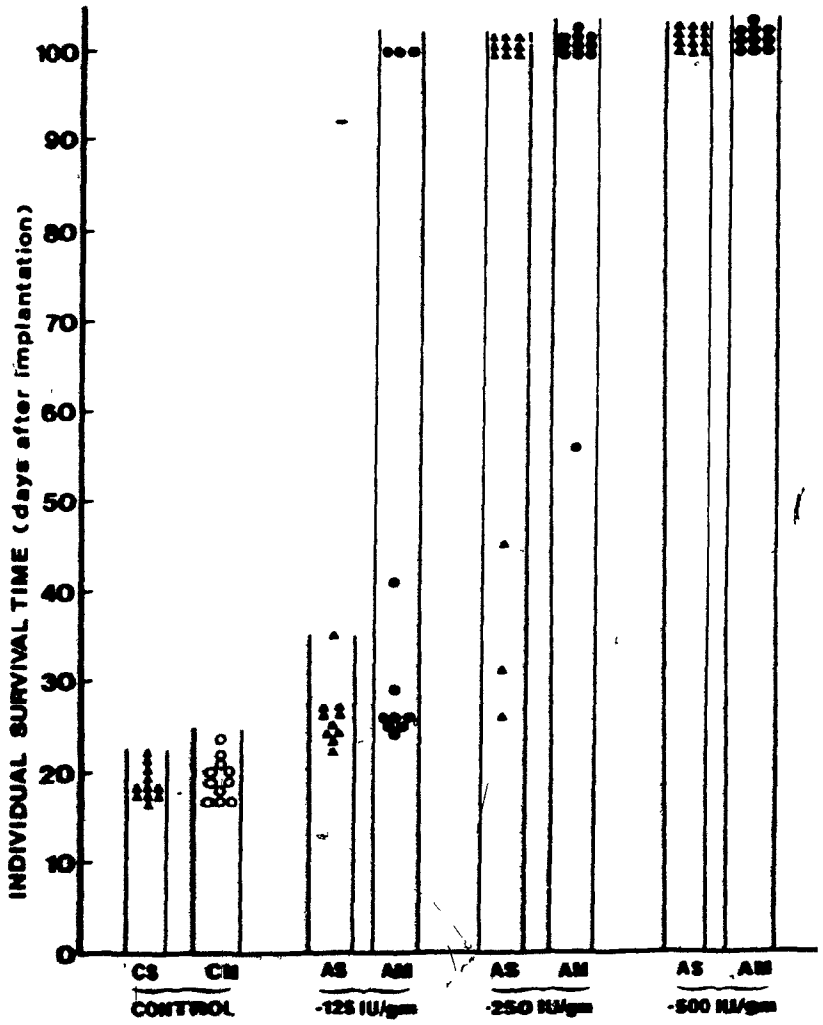


FIGURE 4

Comparison of the therapeutic effectiveness of different doses of L-asparaginase solution (AS) and L-asparaginase microcapsules (AM) against the established 6C3HED lymphosarcoma in non-immunized mice.

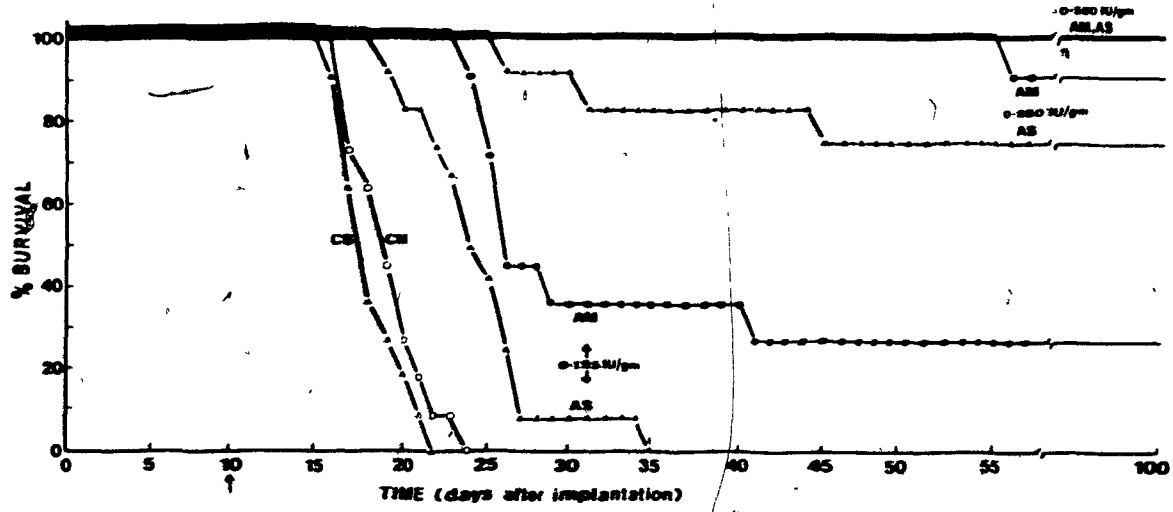


FIGURE 5

Survival time curve for 90 non-immunized mice bearing the 6C3HED lymphosarcoma and treated with saline (CS), different doses of L-asparaginase solution (AS), control microcapsules (CM), or different doses of L-asparaginase microcapsules (AM).

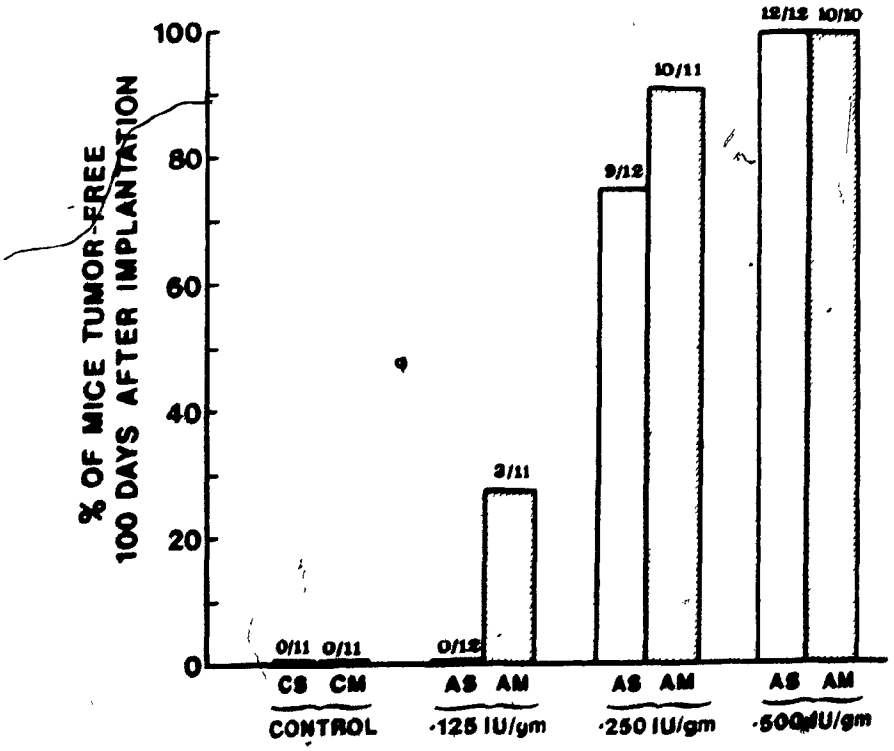


FIGURE 6

Comparison of the number of mice tumor-free and still alive ("cured") 100 days after tumor implantation.

(3/11) of the mice that received L-asparaginase microcapsules were still alive and tumor-free up to 100 days after tumor implantation (Figure 6).

Dose of 0.250 IU/gm: As in the groups above, deaths among the non-survivors occured earlier in mice treated with L-asparaginase solution rather than L-asparaginase microcapsules (Figures 4 & 5). 75% (9/12) of the mice receiving L-asparaginase solution were tumor-free 100 days after tumor implantation, as compared to 90.9% (10/11) for those treated with L-asparaginase microcapsules after the same period (Figure 6).

Dose of 0.500 IU/gm: There was no reoccurrence of the tumor in any of the animals treated at this dose. There was 100% survival rate in both groups of animals treated, and all animals were tumor-free up to 100 days after tumor implantation (Figures 4 - 6).

Effect of different doses of L-asparaginase solution and L-asparaginase microcapsules on the survival time of 6C3HED lymphosarcoma-bearing mice previously immunized against L-asparaginase

In marked contrast to the results described above for non-immunized mice, there was no significant tumor regression in immunized mice treated with either L-asparaginase solution or L-asparaginase microcapsules at both dose levels of 0.250 IU/gm and 0.500 IU/gm. Tumors progressed in an essentially similar manner for those treated with L-asparaginase solution or L-asparaginase microcapsules at doses of 0.250 IU/gm or 0.500 IU/gm, as for those injected with saline or control microcapsules. The results obtained in this series of experiments have been summarized in Figures 7 & 8. (A tabulation of this data is given in Appendix 4). It can be seen that all the mice in the six experimental groups died with a median survival time of 17.5 days

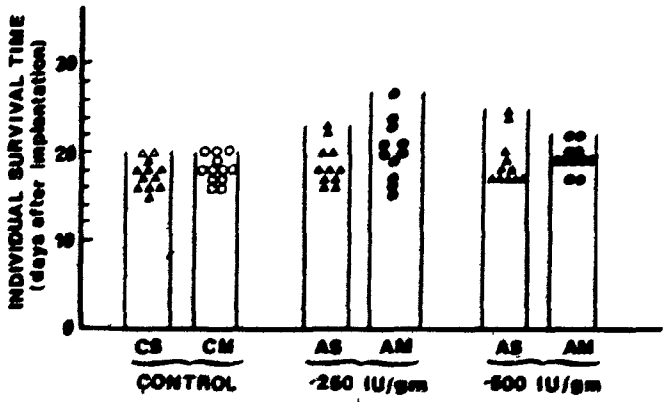


FIGURE 7

Comparison of the therapeutic effectiveness of different doses of L-asparaginase solution (AS) and L-asparaginase microcapsules (AM) against the established 6C3HED lymphosarcoma in mice previously immunized against L-asparaginase.

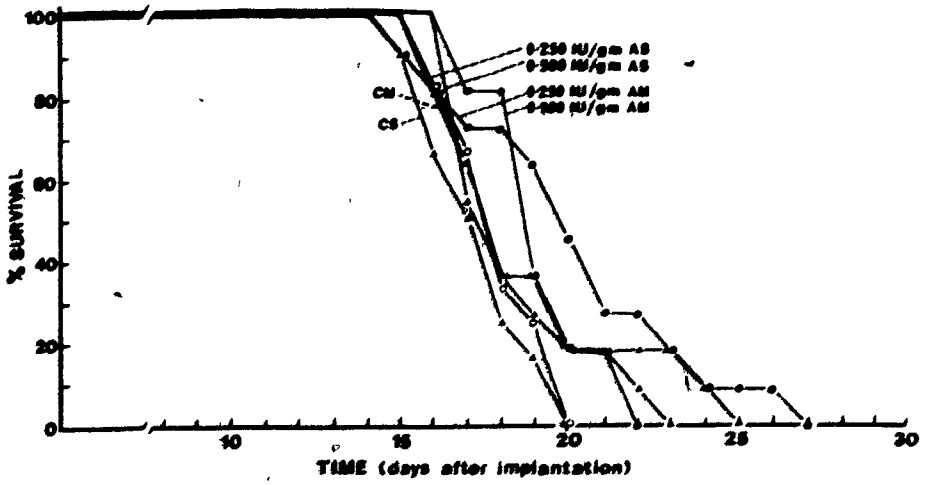


FIGURE 8

Survival time curve for 66 immunized mice bearing the 6C3HED lymphosarcoma and treated with saline (CS), different doses of L-asparaginase solution (AS), control microcapsules (CM), or different doses of L-asparaginase microcapsules (AM).

for those injected with saline, 18 days for the 0.250 IU/gm L-asparaginase solution group, 18 days for 0.500 IU/gm L-asparaginase solution group, 18 days for the group which received microcapsules containing no L-asparaginase, 20 days for the 0.250 IU/gm L-asparaginase microcapsules group, and 19 days for the 0.500 IU/gm group (Figure 7). Thus, these results indicate that both L-asparaginase microcapsules and L-asparaginase solution are ineffective in inducing regression of the 6C3HED lymphosarcoma in mice previously immunized to L-asparaginase, even at a dose level of 0.500 IU/gm which is curative in non-immunized mice bearing equivalent tumor loads.

DISCUSSION

The results presented here (Figures 4 - 6) demonstrate that in non-immunized mice, L-asparaginase microcapsules at the lower doses of 0.125 IU/gm and 0.250 IU/gm are therapeutically more effective than the equivalent dose of L-asparaginase solution against the established 6C3HED lymphosarcoma. At a dose of 0.500 IU/gm, both L-asparaginase solution and L-asparaginase microcapsules are curative. These observations are related to earlier knowledge of the physiological properties and in vivo effects of these two enzyme preparations in non-immunized mice. It has been shown that a single i.p. injection of L-asparaginase microcapsules lowers the plasma L-asparagine concentration to zero for a significantly longer period of time than a single i.p. injection of an equivalent dose of L-asparaginase solution (15, 16). This may be an important factor underlying the observed difference in tumor-inhibitory effectiveness of free versus the microencapsulated form of L-asparaginase, at subcurative doses of the enzyme. This enhanced efficacy in the physiological action of L-asparaginase microcapsules may be explained by the increased stability of the microencapsulated over the solution form of the enzyme, and the protective function of the microcapsule membrane in preventing the rapid removal and inactivation of the foreign enzyme by the body's defense mechanisms (13, 15, 16, 22).

Riley has demonstrated (23) that the clearance of L-asparaginase from the circulation of tumor-bearing mice is significantly prolonged in the presence of the LDH virus which is carried as a contaminant in the 6C3HED lymphosarcoma. As a result of this finding, Riley credited the presence of this virus during L-asparaginase therapy as being an important contributing factor to the cures of established, L-asparaginase-sensitive neoplasms. Our results (Figure 1) also show that after i.p. injection of

L-asparaginase solution, the half-life of L-asparaginase in the blood of tumor-bearing mice was 3 times longer than that in non-tumor-bearing mice. However, the presence of the LDH virus probably is not an important consideration in the case of microencapsulated L-asparaginase therapy wherein success depends on a different mechanism of action: as long as L-asparagine can dialyze efficiently into the microcapsules to be acted on, L-asparagine will continue to be removed from the extracellular fluid by the L-asparaginase microcapsules in the peritoneal cavity. Our demonstration (16) that there is no significant L-asparaginase activity in the blood of animals treated with L-asparaginase microcapsules i.p. suggests that the LDH virus probably plays a small role, if any, in effective microencapsulated L-asparaginase therapy.

Four major mechanisms have been proposed to account for the development of resistance to L-asparaginase therapy. The first three mechanisms involve changes in the tumor cell population as a result of L-asparagine deprivation:

(a) There is marked increase in L-asparagine synthetase levels in the tumor cells, probably by a mechanism of derepression of L-asparagine synthetase, so that the tumor cells are no longer dependent on an exogenous supply of L-asparagine (24 - 27). In this regard, Gallo et al (28, 29) have shown that L-asparaginase-resistant cells contain reduced levels of an asparaginyl-tRNA species which they proposed normally acts as a co-repressor for L-asparagine biosynthesis.

(b) The process of mutation whereby L-asparaginase-sensitive cells convert from L-asparagine-dependence to L-asparagine-independence may occur. This followed by selection in an L-asparagine-free medium may ultimately lead to the development of L-asparaginase-resistant cell populations (30,31).

(c) Another mechanism of resistance might involve the development of more efficient means by malignant cells of extracting L-asparagine from the plasma, erythrocytes, or other normal host cells (32, 33).

However, in the study reported here L-asparaginase-sensitive 6C3HED lymphosarcoma cells which have never been exposed to L-asparaginase treatment were used for transplantation to recipient animals which had already been immunized by repeated injections of L-asparaginase solution. This experimental procedure makes it very unlikely that development of resistance to L-asparaginase therapy observed in the immunized mice could be a consequence of alterations in the properties of the tumor cells themselves.

The possibility existed, however, that the repeated injections of enzyme in the animals may have brought about increased levels of L-asparagine synthetase activity in normal host cells, particularly those of the liver and pancreas which have been implicated as possible regulatory sites for the homeostasis of plasma L-asparagine levels (34, 35). Thus, host cells may respond to plasma L-asparagine depletion by increasing synthesis of sufficient quantities of endogenous L-asparagine to effectively counteract L-asparaginase therapy. Three lines of evidence seem to suggest that this was probably not an important consideration in the present study.

(1) We have shown (36) that injection of an equivalent dose of Erwinia carotovora L-asparaginase, which is immunologically different from E. coli L-asparaginase, was able to deplete plasma L-asparagine concentration to zero level in C3H/HEJ mice immunized to the E. coli enzyme.

(2) Other studies (9) have demonstrated that tumor-bearing mice, previously immunized to E. coli L-asparaginase, received similar benefit from the therapeutic activity of Erwinia carotovora L-asparaginase as did non-immunized, tumor-bearing mice.

(3) There have also been several clinical reports (37 - 39) that L-asparaginase therapy can be successfully continued with Erwinia carotovora

L-asparaginase in patients who had developed resistance to the E. coli enzyme.

(d) The fourth mechanism responsible for the loss of potency of anti-tumor activity of L-asparaginase solution is the development of host immunity to the enzyme after repeated injections of the enzyme (7 - 12). Humoral antibodies to L-asparaginase have been attributed to cause a reduction in the catalytic activity of the enzyme and, more importantly, a drastic increase in the clearance of enzyme from the circulation.

The latter mechanism would also seem to explain the loss of therapeutic effectiveness of L-asparaginase solution in the present study. In the case of L-asparaginase microcapsules, however, results to date indicate that microencapsulated L-asparaginase stays within the microcapsule where it acts on L-asparagine diffusing in from the extracellular medium. L-asparaginase does not leak out into the circulation where it may be bound by circulating antibodies and removed rapidly. For L-asparaginase microcapsules to be physiologically effective even in the presence of circulating anti-L-asparaginase antibodies therefore, it is fundamentally imperative that:

(a) microencapsulated L-asparaginase does not act via a mechanism that necessitates leakage of enzyme from the microcapsules into the circulation; and

(b) the presence of humoral anti-L-asparaginase antibodies does not directly affect the catalytic activity of the microencapsulated enzyme.

That microcapsule contents do not leak is supported by the following findings. Permeability studies in this laboratory (13, 40) have demonstrated that microcapsules are not permeable to macromolecules. Earlier observations (13, 14, 16) indicate that there is no detectable L-asparaginase activity in the supernatant of microcapsule suspension, prepared as described, when the suspension is left to stand. Microencapsulated ^{51}Cr-labelled hemoglobin (M.W. = 68,000), a much smaller protein than L-asparaginase (M.W. = 130,000), does not leak out after i.p. injection into animals (13, 14). Using the Ouchterlony double-diffusion technique, no precipitin line is detectable between L-asparaginase microcapsules and antiserum prepared against free L-asparaginase (41). In vivo evidence for the absence of leakage of microcapsule contents is provided by the absence of assayable levels of L-asparaginase activity in the blood after i.p. injection of L-asparaginase microcapsules (16). L-asparaginase microcapsules recovered from the peritoneal cavity up to 6 days post injection when observed under the microscope have an intact membrane (16). It has been shown (See Appendix 6 of Chapter II) that plasma L-asparagine can be maintained at zero concentration for a significant period of time after i.p. injection of L-asparaginase microcapsules which had been cross-linked with glutaraldehyde. Since glutaraldehyde treatment insolubilizes the encapsulated enzyme by cross-linking it to other encapsulated proteins, the possibility of enzyme leakage from the microcapsules is highly unlikely. All these observation suggest that a leakage mechanism is not a sine qua non to explain the in vivo action of L-asparaginase microcapsules in non-immunized mice.

Mori et al (42) also found that, in marked contrast to L-asparaginase in solution, microencapsulated L-asparaginase did not lose any

catalytic activity after incubation with various proteases, thus supporting the idea that microcapsules are not permeable to large molecules such as enzymes. In the case of microencapsulated catalase, in vitro studies (43) using ^{125}I-labelled antibody and the technique of complement fixation showed that antibodies do not enter microcapsules. Furthermore, more recent experiments (36) have shown that when L-asparaginase microcapsules are incubated in vitro with plasma containing anti-L-asparaginase antibodies for 30 minutes at 37°C, the L-asparagine content in the plasma was found to fall to zero. Assay of the L-asparaginase activity of the microencapsulated L-asparaginase after incubation with anti-L-asparaginase serum showed that it did not lose any catalytic activity, in marked contrast to similarly treated L-asparaginase solution. These results may be interpreted to mean that:

(a) there is no leakage of L-asparaginase from the microcapsules; and

(b) the presence of humoral antibodies outside the microcapsules does not affect the catalytic activity of L-asparaginase inside the microcapsules which in vitro continued to act effectively on plasma L-asparagine in the surrounding medium.

In the light of all the above data and the demonstration that i.p. injected L-asparaginase microcapsules are physiologically and therapeutically effective although the microencapsulated enzyme stays confined within the microcapsule membrane in the peritoneal cavity, it seemed reasonable to expect that the use of microencapsulated L-asparaginase might circumvent the problem posed by the presence of circulating antibodies. However, results obtained in the present study (Figures 7 & 8) indicate that L-asparaginase treatment did not induce the regression of the 6C3HED

lymphosarcoma transplanted into hosts previously immunized to L-asparaginase according to the schedule described. Both L-asparaginase solution and L-asparaginase microcapsules were found to be ineffective in causing tumor regression in immunized mice, even at L-asparaginase doses which are curative in non-immunized animals. Because of the unexpected nature of these observations, a detailed investigation into the physiological basis and possible mechanisms underlying the loss of therapeutic activity of L-asparaginase microcapsules in immunized hosts was initiated. A report of these studies will be presented in the following chapter.

APPENDIX 1

Method of counting viable cells in tumor cell suspension

The method of counting the concentration of viable tumor cells was as follows. To 0.2 ml of cell suspension was added 4.8 ml of a 1:2000 solution of methylene blue in mammalian Ringer's at pH = 7.6. The mixture was shaken, a drop placed in a hemocytometer chamber and the number of unstained (viable) cells counted within 2 minutes of adding the methylene blue solution. The number of viable cells in the tumor cell suspension was calculated as follows:

Let n = number of viable cells counted in a volume of 0.9 cub. mm. in the hemocytometer chamber.

Then concentration of viable cells present in the original tumor cell suspension = n x 25 cells/0.9 cub. mm.

$$= n \times 25 \times \frac{10}{9} \text{ cells/cub. mm.}$$

$$= n \times 25 \times \frac{10}{9} \times 10^3 \text{ cells/ml.}$$

(where 25 = dilution factor).

APPENDIX 2

It can be seen that there was no tumor regression in non-immunized mice after injection of saline or microcapsules containing no L-asparaginase, as control. However, there was complete disappearance of any palpable tumor after treatment with L-asparaginase solution (0.500 IU/gm body weight). Similar tumor regression was also seen after treatment with L-asparaginase microcapsules, as shown in Figure 2. It should be noted that for each type of treatment, the photographs above were taken of the same mice before and after treatment. Also, it was difficult to have the animals "stand still" while being photographed. Thus, photographs of the mice could not be obtained from ideal similar angles before and after treatment to allow a fairer comparison of the tumor size to be made. These photographs have been presented, nevertheless, so as to give some idea of the results observed after treatment with a given experimental preparation.

APPENDIX 2

BEFORE TREATMENT

3 DAYS AFTER TREATMENT

SALINE

CONTROL MICROCAPSULES

L-ASPARAGINASE SOLUTION

Experimental group (Non-immunized)	Days of death of non-survivors (Tumor implanted on day 0)	No. of survivors on day 100[a] ("cures")
I (a) Saline (11)[b]	16, 17, 17, 17, 18, 18, 18, 19, 20, 21, 22	0
(b) 0.125 IU/gm AS (12)	19, 20, 22, 23, 24, 24, 25, 26, 26, 27, 27, 35	0
(c) 0.250 IU/gm AS (12)	26, 31, 45	9
(d) 0.500 IU/gm AS (12)		12
II (a) Control microcapsules (11)	17, 17, 17, 18, 19, 19, 20, 20, 21, 22, 24	0
(b) 0.125 IU/gm AM (11)	24, 25, 25, 26, 26, 29, 41	3
(c) 0.250 IU/gm AM (11)	56	10
(d) 0.500 IU/gm AM (10)		10

APPENDIX 3

Comparison of the therapeutic effectiveness of L-asparaginase solution (AS) and L-asparaginase microcapsules (AM) against the established 6C3HED lymphosarcoma in non-immunized mice.

a. Experiment was terminated 100 days after tumor implantation, and all surviving animals were tumor-free at this time.

b. Numbers in parentheses represent number of mice injected in that group.

Experimental group (Immunized)	Days of death of non-survivors (Tumor implanted on day 0)	No. of survivors ("cures")
III (a) Saline (12)[a]	15, 16, 16, 16, 17, 17, 18, 18, 18, 19, 20, 20	0
(b) 0.250 IU/gm AS (11)	16, 16, 17, 17, 18, 18, 18, 20, 20, 22, 23	0
(c) 0.500 IU/gm AS (11)	17, 17, 17, 17, 17, 18, 18, 19, 20, 24, 25	0
IV (a) Control microcapsules (12)	16, 16, 17, 17, 18, 18, 18, 18, 19, 20, 20, 20	0
(b) 0.250 IU/gm AM (11)	15, 16, 17, 19, 20, 20, 21, 21, 23, 24, 27	0
(c) 0.500 IU/gm AM (11)	17, 17, 19, 19, 19, 19, 19, 20, 20, 22, 22	0

APPENDIX 4

Comparison of the therapeutic effectiveness of L-asparaginase solution (AS) and L-asparaginase microcapsules (AM) against the established 6C3HED lymphosarcoma in mice previously immunized against L-asparaginase.

a. Numbers in parentheses represent number of mice injected in that group.

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CHAPTER IV

INFLUENCE OF IMMUNIZATION ON THE IN-VIVO EFFECTS OF INTRAPERITONEALLY INJECTED L-ASPARAGINASE SOLUTION AND L-ASPARAGINASE MICROCAPSULES

ABSTRACT

In vitro studies showed that incubation of hyperimmune anti-L-asparaginase serum with L-asparaginase solution inhibited the catalytic activity of the L-asparaginase solution by 70%, whereas there was no inhibition in the catalytic activity of L-asparaginase microcapsules after similar incubation with hyperimmune anti-L-asparaginase serum. C3H/HEJ mice, immunized to E. coli L-asparaginase, were each given an i.p. injection of E. coli L-asparaginase solution or E. coli L-asparaginase microcapsules, or were not injected with any L-asparaginase preparations, as control. After injection of L-asparaginase solution in immunized mice, very low levels of blood L-asparaginase activity were attained, which then rapidly disappeared from the circulation. No L-asparaginase activity appeared in the blood after injection of microencapsulated L-asparaginase into immunized mice. "Body" L-asparaginase levels were lower in immunized mice than in non-immunized mice after injection of L-asparaginase solution. There was no significant difference in the "body" L-asparaginase levels in immunized and in non-immunized mice after injection of L-asparaginase microcapsules for up to 2 days post injection; after this period, "body" L-asparaginase levels were lower in immunized than in non-immunized mice. The plasma L-asparagine concentration in immunized mice could not be lowered significantly after injection of L-asparaginase solution or L-asparaginase microcapsules, even at a dosage of 5.0 IU/gm body weight. Injection of collodion L-asparaginase microcapsules also was unable to lower the plasma L-asparagine concentration to zero in immunized mice. In mice immunized to E. coli L-asparaginase, whereas injection of E. coli L-asparaginase solution or E. coli L-asparaginase microcapsules was unable to lower the plasma L-

asparagine level to zero, injection of equivalent doses of Erwinia carotovora (abbreviated - Erwinia) L-asparaginase solution or Erwinia L-asparaginase microcapsules was effective in depleting the plasma L-asparagine content to zero. Reciprocal experiments in mice immunized to Erwinia L-asparaginase showed that injection of equivalent doses of E. coli L-asparaginase solution or E. coli L-asparaginase microcapsules could lower the plasma L-asparagine level to zero whereas injection of Erwinia L-asparaginase solution or Erwinia L-asparaginase microcapsules could not. Injection of Erwinia L-asparaginase microcapsules previously exposed to E. coli L-asparaginase solution decreased the plasma L-asparagine level to zero in mice immunized to E. coli L-asparaginase, but not in mice immunized to Erwinia L-asparaginase. Injection of microcapsules containing both E. coli and Erwinia L-asparaginase lowered the plasma L-asparagine level to zero in mice immunized to E. coli L-asparaginase and in mice immunized to Erwinia L-asparaginase. The type of cellular response to injection of variously modified microcapsules was compared in non-immunized and immunized mice. Possible mechanisms that may explain the inability of microencapsulated L-asparaginase to lower the plasma L-asparagine concentration in mice specifically immunized to the encapsulated enzyme are discussed in light of the present data.

INTRODUCTION

The rationale behind the suggested use of L-asparaginase microcapsules in lieu of L-asparaginase solution for enzyme therapy of the L-asparagine-dependent 6C3HED lymphosarcoma has been discussed in previous reports from this laboratory (1 - 5). It has been shown that because of the increased in vitro and in vivo stability of the microencapsulated over the solution form of the enzyme, plasma L-asparagine level can be maintained at zero concentration for a significantly longer period of time in mice injected i.p. with L-asparaginase microcapsules than in those injected i.p. with an equivalent dose of L-asparaginase solution (3, 4). Microencapsulated L-asparaginase has been shown to be more effective than L-asparaginase solution in suppressing the growth of the 6C3HED lymphosarcoma when it is given to non-immunized mice immediately after tumor implantation (1, 2). More recent experiments demonstrated that microencapsulated L-asparaginase at subcurative doses, is also more effective than L-asparaginase solution in the treatment of established 6C3HED lymphosarcoma implants in non-immunized mice (5). However, further studies indicated that L-asparaginase microcapsules, like L-asparaginase solution, were therapeutically ineffective in inducing regression of established tumors borne in mice which had previously been immunized with repeated injections of L-asparaginase solution (5). As discussed in detail in Chapter III of this thesis, this loss in therapeutic potency of L-asparaginase microcapsules in immunized mice was somewhat unexpected and could not be explained by the experimental results available to date.

This chapter describes detailed studies that were carried out to determine the physiological basis for the loss of therapeutic activity of L-asparaginase microcapsules in immunized mice and to examine possible mechanisms that may explain this.

MATERIALS AND METHODS

Animals

Normal female C3H/HEJ mice, weighing about 27 gm, were purchased from Jackson Laboratories, Bar Harbor, Maine and maintained on a diet consisting of Purina Mouse Chow and water ad libitum.

Enzyme

(a) *E. coli* L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) was purchased in vials of 500 IU and 1000 IU as a lyophilized preparation from Nutritional Biochemical Corporation, Cleveland, Ohio. The specific activity of this enzyme was 312.3 IU/mg for Lot No.4905 and 320 IU/mg for Lot No.7819.

(b) *Erwinia carotovora* L-asparaginase was obtained in vials of 10,000 IU as a generous gift of Dr. G. Strauss of the National Cancer Institute, Bethesda, Maryland. The specific activity of the lyophilized preparation (Batch No. MRE 11) was 730 IU/mg.

The contents of these vials were reconstituted in sterile saline before injection of L-asparaginase solution into animals. One IU of L-asparaginase has been defined as that amount of enzyme which will liberate 1.0 μmole of ammonia per minute at 37°C. Unless otherwise specified, L-asparaginase mentioned in these studies refers to L-asparaginase derived from *E. coli*.

Preparation of nylon microcapsules

(a) *E. coli* L-asparaginase or *Erwinia carotovora* L-asparaginase microcapsules: These microcapsules were prepared by the standard method (1, 6, 7) with minor modifications as described in detail in Chapter II of this thesis.

It should be noted that unless otherwise specified, L-asparaginase microcapsules mentioned in the text refer to nylon microcapsules containing E. coli L-asparaginase, prepared exactly as described in Chapter II.

(b) E. coli L-asparaginase microcapsules for injection of 10 times the usual L-asparaginase dose of 0.5 IU/gm body weight: 1000 IU of lyophilized E. coli L-asparaginase was dissolved directly in 2.5 ml 10 gm% hemoglobin solution to which was then added another 500 IU E. coli L-asparaginase solution in a volume of 0.3 ml water. The rest of the procedure was carried out according to the methodology described in Chapter II. Microcapsules prepared in this way have approximately 5 times the L-asparaginase activity per ml than when prepared by the standard procedure.

(c) Control nylon microcapsules containing no L-asparaginase: Control nylon microcapsules were prepared by the standard method except that 0.3 ml of water instead of 0.3 ml of enzyme solution was added to the hemoglobin-hexamethylenediamine solution before the emulsification step.

Preparation of microcapsules with different surface properties

(a) Collodion L-asparaginase microcapsules: Semipermeable collodion microcapsules were prepared by interfacial precipitation using the updated procedure (1). Only the volumes of reagents were altered so as to prepare a larger yield of microcapsules each time. (See Appendix 1 for details).

(b) Erwinia carotovora L-asparaginase microcapsules exposed to E. coli L-asparaginase solution: Erwinia carotovora L-asparaginase microcapsules were first prepared as described earlier. The volume

of the 50% microcapsule suspension was noted and the preparation was assayed for its exact L-asparaginase activity. The microcapsule suspension was centrifuged, the supernatant removed, and an equal volume of E. coli L-asparaginase solution (150 IU/ml) was added. The suspension was incubated at room temperature for 30 minutes with continuous agitation to allow E. coli L-asparaginase to adsorb onto the surface of the microcapsules. The suspension was centrifuged and the supernatant containing the E. coli enzyme retained for future use. The Erwinia carotovora L-asparaginase microcapsules exposed to E. coli L-asparaginase solution were then washed once with saline and resuspended to the original volume. These microcapsules were injected at a dose corresponding to the original assayed activity of the freshly prepared Erwinia carotovora L-asparaginase microcapsules.

(c) Microcapsules containing both E. coli and Erwinia carotovora L-asparaginase: Separate solutions of E. coli and Erwinia carotovora L-asparaginase were prepared, each having an L-asparaginase activity of 2000 IU/ml. 0.15 ml (= 300 IU) of each enzyme solution was dissolved in the hemoglobin solution and the standard method for preparing L-asparaginase microcapsules was followed. A 50% microcapsule suspension so prepared had approximately 2 times the L-asparaginase activity per ml than when prepared by the standard procedure.

All microcapsules used in these studies were examined under the microscope to ensure that their membranes were well formed, and the supernatant of each preparation was always tested for L-asparaginase activity to ensure that there was no leakage of enzyme from the microcapsules.

All preparations of L-asparaginase solution and L-asparaginase microcapsules were assayed for their exact enzyme activity prior to injection.

Experimental procedure

Immunized mice were injected i.p. with L-asparaginase solution or L-asparaginase microcapsules at a dose of 0.5 IU/gm body weight in these studies, unless otherwise noted in the text. Control immunized mice were not injected with any L-asparaginase preparations ("untreated").

L-asparaginase assay

Assays of the L-asparaginase activity in the blood, "body" and enzyme preparations were determined according to the methods described in detail in Chapter II.

L-asparagine assay

Assay of plasma L-asparagine was also performed according to the procedure outlined in Chapter II.

Immunization procedure

Mice were each given a single i.p. injection of 32 µgm (10 IU) of E. coli L-asparaginase or 13.7 µgm (10 IU) of Erwinia carotovora L-asparaginase dissolved in 0.3 ml sterile saline, at weekly intervals for 4 weeks. 10 days before the start of all experiments, they were each boostered with the corresponding immunizing antigen solution - 8 µgm

(2.5 IU) of E. coli L-asparaginase solution or 3.4 µgm (2.5 IU) of Erwinia carotovora L-asparaginase solution. Mice which were not used for experiments after immunization were boostered with 2.5 IU of L-asparaginase solution every 3 months.

Collection of anti-L-asparaginase serum

10 days before the collection of antiserum, immunized mice were boostered with 2.5 IU of the immunizing L-asparaginase solution. In these studies, hyperimmune antisera were obtained from mice which had been immunized as described above over a period of about one year. Antisera so collected were stored at -20°C until ready for use.

Demonstration of anti-L-asparaginase antibody in serum

The presence of L-asparaginase antibody in the serum of immunized animals was always confirmed by one or more of the following techniques.

(a) Ouchterlony technique of double diffusion in two dimensions: This test is based on the formation of a distinct precipitation line in a semi-solid gel medium when an antigen and its homologous antibody combine in optimal proportions. The procedure used was similar to the one described by Campbell et al (8). A 1% SeaKem agarose solution (Behring Diagnostics Hoechst Pharmaceuticals, Montreal, Quebec) was prepared in 0.01 M phosphate-buffered saline (PBS) (pH = 7.4) and the solution heated in a beaker of boiling water until the agarose was completely dissolved. With a 10 ml serological pipette that had been heated in a Bunsen burner flame immediately prior to use, 8 ml of the hot agarose solution was spread evenly on 6 precoated agarose slides which were secured in a LKB electrophoresis slide holder (Fisher

Scientific Co., Montreal, Quebec) with vaseline. The agarose was allowed to gel by standing at room temperature for 30 minutes. Wells were then cut in the agarose medium with a die and the agarose from the wells was removed by suction. With a separate Pasteur pipette for each different sample, the centre well was filled with undiluted test serum, and varying concentrations of the antigen solution (ranging from 200 µg/ml to 12.5 µg/ml) were placed in the peripheral wells. The reactants were allowed to diffuse in a LKB humidified chamber (Fisher Scientific Co.) at room temperature for 24 hours to allow for the development of precipitation lines. The slides were immersed in PBS (pH = 7.4) to wash out unbound protein from the agarose layer. This washing was carried out twice a day for 2 days at 4°C. The slides were then immersed in distilled water for 2 hours, changing the water 3 times during this period. A wet piece of filter paper, the same size as the slides, was placed over the surface of the slides and the paper was allowed to dry undisturbed. The filter paper was removed and the surface of the slides was rinsed with distilled water to remove any adhering fibers of the paper. The precipitation lines were stained with a 0.5% solution of Coomassie Blue in 10% v/v acetic acid and 45% v/v ethanol in distilled water. The slides were washed, then destained with the aqueous acetic acid - ethanol solvent. The slides were allowed to dry in the air at room temperature. A typical stained slide showing the presence of anti-L-asparaginase antibody in the test serum is shown in Appendix 2.

(b) Passive ("indirect") hemagglutination: This procedure is a very sensitive technique for antibody detection and is based on the observation that erythrocytes coated with soluble antigens can be agglu-

tinned with homologous antisera directed to these antigens on the erythrocyte surface. The following method, modified from Stavitsky (9), was used in these studies for the preparation of 20 ml of a 0.5% suspension of fresh or glutaraldehyde - fixed sheep red blood cells, tanned and coated with L-asparaginase. Briefly, freshly drawn or fixed sheep red blood cells were washed 3 times with PBS (pH = 7.4) and 10 ml of a 1% v/v suspension of the washed cells were prepared. 10 ml of freshly prepared tannic acid (British Drug House, Montreal, Quebec) diluted 1:30,000 with PBS were added to the 10 ml of 1% cell suspension in a centrifuge tube. The contents of the tube were mixed and incubated at 37°C for exactly 10 minutes. The tanned cell suspension so prepared was centrifuged and washed 2 times with PBS. The supernatant was removed and PBS added to resuspend the cells to the original concentration 1% (v/v). To coat the tanned red blood cells with antigen, 10 ml of the tanned cell suspension were mixed with 10 ml of L-asparaginase solution (100 µg/ml, diluted with PBS) and incubated for 20 minutes at room temperature. The contents of the tubes were mixed 3 times during the incubation period. The tanned cells coated with L-asparaginase were centrifuged and washed 2 times with cold 1% normal rabbit serum (NRS) (Grand Island Biological Co., Grand Island, New York) in PBS (pH = 7.4). The cells were resuspended in 1% NRS to give a 0.5% suspension of "sensitized" cells. 1 drop of the suspension of "sensitized" sheep red blood cells was mixed with 1 drop of anti-L-asparaginase antiserum on a slide to check for cell agglutination. If the slide agglutination test was positive, then titration of the test sera was carried out. For each sample of test serum, one row

of 15 tubes (12 x 100 mm) was set up. A doubling dilution series of the test serum in 0.5 ml volumes of 1% NRS were prepared, starting from a 1:10 dilution in tube 1. Tube No. 15 contained 0.5 ml of the diluent, 1% NRS, instead of serum, as control. Thus dilutions of test serum ranged from 1:10 to 1:81,920. To each of the tubes was then added 0.1 ml of the 0.5% "sensitized" sheep red blood cell suspension. The contents of the tubes were mixed thoroughly and left undisturbed, at room temperature overnight. The patterns of hemagglutination were read according to the pattern of settling of the cells in the tubes. Appendix 3 shows a typical appearance of patterns of hemagglutination. The endpoint chosen was the last tube showing an even carpet of cells with a slight ring at the edge. The hemagglutination titre was expressed as the reciprocal of the dilution of antiserum present at the end point. Freshly immunized mice gave antibody titres of about 5,120 to 20,480 by this technique.

(c) Inhibition of L-asparaginase activity: 0.1 ml of the antigenic solution (L-asparaginase solution with activity of 2.5 - 3.0 IU/ml) was incubated with 0.1 ml of test serum or 0.1 ml normal mouse serum at 37°C. The mixture was agitated for 30 minutes and then assayed directly for its L-asparaginase activity. The presence of anti-L-asparaginase antibody in the test serum is revealed by a decrease in the L-asparaginase activity of the mixture containing the test serum, as compared to the L-asparaginase activity of the mixture containing normal mouse serum.

<u>In vitro studies with anti-L-asparaginase serum</u>

Aliquots of 0.1 ml of L-asparaginase solution with activity of

about 3.0 IU/ml or 10% L-asparaginase microcapsule suspension with activity of about 2.5 IU/ml were incubated at 37°C for 30 minutes with different volumes of either "stock" hyperimmune antiserum, collected from mice immunized to L-asparaginase over a period of one year, or normal mouse serum. The reaction mixtures were then assayed directly for their L-asparaginase activity after the incubation period. The volumes of sera used in these studies ranged from 0.010 ml to 0.400 ml. With the smaller volumes of stock antiserum or normal serum, 0.010 ml, 0.025 ml or 0.050 ml, saline was added to make up a total volume of 0.100 ml in each case before incubation with the L-asparaginase preparations. This was done because it was necessary to have a total sample volume of at least 0.200 ml for the continuous-flow assay method for L-asparaginase activity. The % inhibition of L-asparaginase activity by anti-L-asparaginase serum was calculated as follows:

Let n = L-asparaginase activity after incubation with normal mouse serum,

a = L-asparaginase activity after incubation with antiserum,

then % inhibition $= \left[\frac{(n - a)}{n} \times 100\right]$

Histological studies

Normal mice and mice immunized against E. coli L-asparaginase were injected i.p. with one of the following:

(a) E. coli L-asparaginase nylon microcapsules;

(b) Erwinia carotovora L-asparaginase nylon microcapsules;

(c) Erwinia carotovora L-asparaginase nylon microcapsules previously exposed to a solution of E. coli L-asparaginase;

(d) control nylon microcapsules containing no L-asparaginase.

The mice were sacrificed one day after injection, dissected and the microcapsules recovered from the peritoneal cavity. Microcapsules that had aggregated were fixed in 10 % formalin and histological slides were prepared as described in Chapter II.

RESULTS

In vitro incubation of L-asparaginase solution and L-asparaginase microcapsules with anti-L-asparaginase serum

The results obtained with L-asparaginase solution are presented in Table 1. It can be seen that the residual L-asparaginase activity in the reaction mixture at each dilution was always lower for L-asparaginase solution that had been incubated with anti-L-asparaginase serum than with control normal mouse serum. The maximum inhibition of L-asparaginase activity obtained with hyperimmune serum was 70% in these studies, even in antibody excess (Figure 1). Inhibition of L-asparaginase activity ranged from 41% to 70% over the volumes of antiserum employed in these studies. It should be noted that maximum inhibition obtained for anti-L-asparaginase serum collected from mice which had received repeated injections of L-asparaginase solution over a period of one month, instead of one year, was 45%.

The results obtained with L-asparaginase microcapsules are shown in Table 2. In marked contrast to the results in Table 1, there is no significant difference in the L-asparaginase activity of L-asparaginase microcapsules after incubation with anti-L-asparaginase serum or normal mouse serum. Figure 1 shows that there was 0% inhibition in the case of L-asparaginase microcapsules at all dilutions with antiserum.

Blood L-asparaginase activity

Figure 2 shows that after i.p. injection of L-asparaginase solution into immunized mice, very low levels of L-asparaginase activity were assayed in the blood up to 2 hours after injection. The maximum L-aspara-

Volume of serum (ml)	Enzyme activity (IU/ml) of L-asparaginase solution after incubation with		
	Normal serum (n)	Anti-L-asparaginase serum (a)	% inhibition $\left[\frac{(n-a)}{n} \times 100\right]$
0.010 (+0.090 ml saline)	1.50	0.88	41
0.025 (+0.075 ml saline)	1.57	0.87	45
0.050 (+0.050 ml saline)	1.50	0.59	61
0.100	1.58	0.48	70
0.200	1.04	0.31	70
0.400	0.64	0.19	70

TABLE 1

L-asparaginase activity after incubation of 0.1 ml of L-asparaginase solution with different volumes of anti-L-asparaginase serum or normal mouse serum.

Volume of serum (ml)	Enzyme activity (IU/ml) of L-asparaginase microcapsules after incubation with		
	Normal serum (n)	Anti-L-asparaginase serum (a)	% inhibition $\left[\frac{(n-a)}{n} \times 100\right]$
0.010 (+0.090 ml saline)	1.30	1.30	none
0.025 (+0.075 ml saline)	1.20	1.28	none
0.050 (+0.050 ml saline)	1.30	1.35	none
0.100	1.25	1.28	none
0.200	1.00	1.05	none
0.400	0.58	0.62	none

TABLE 2

L-asparaginase activity after incubation of 0.1 ml L-asparaginase microcapsule suspension with different volumes of anti-L-asparaginase serum or normal mouse serum.

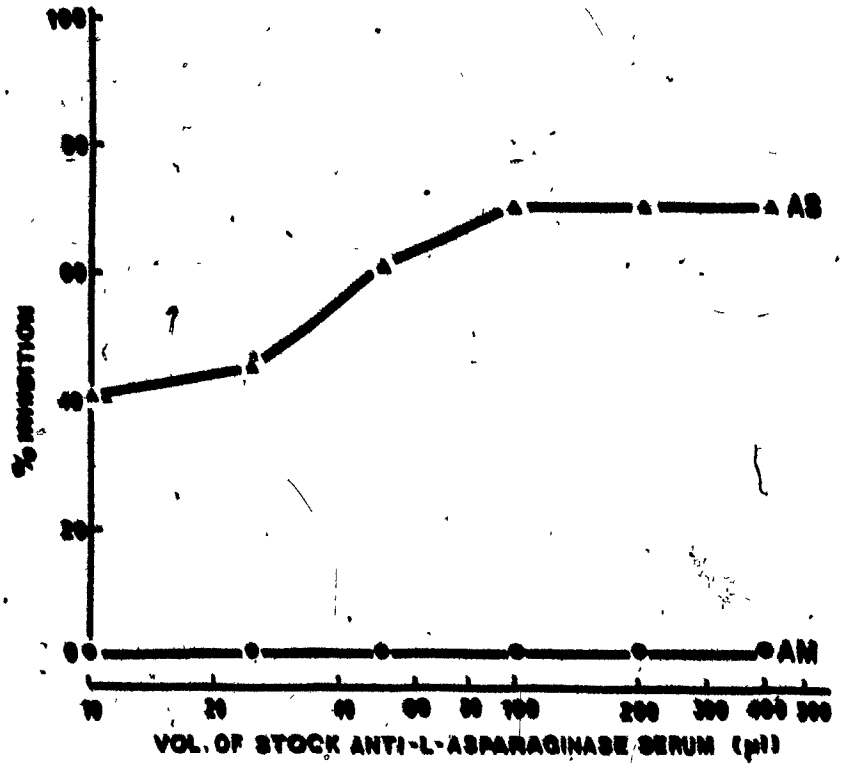


FIGURE 1

% inhibition of L-asparaginase activity after incubation of L-asparaginase solution or L-asparaginase microcapsules with anti-L-asparaginase antiserum.

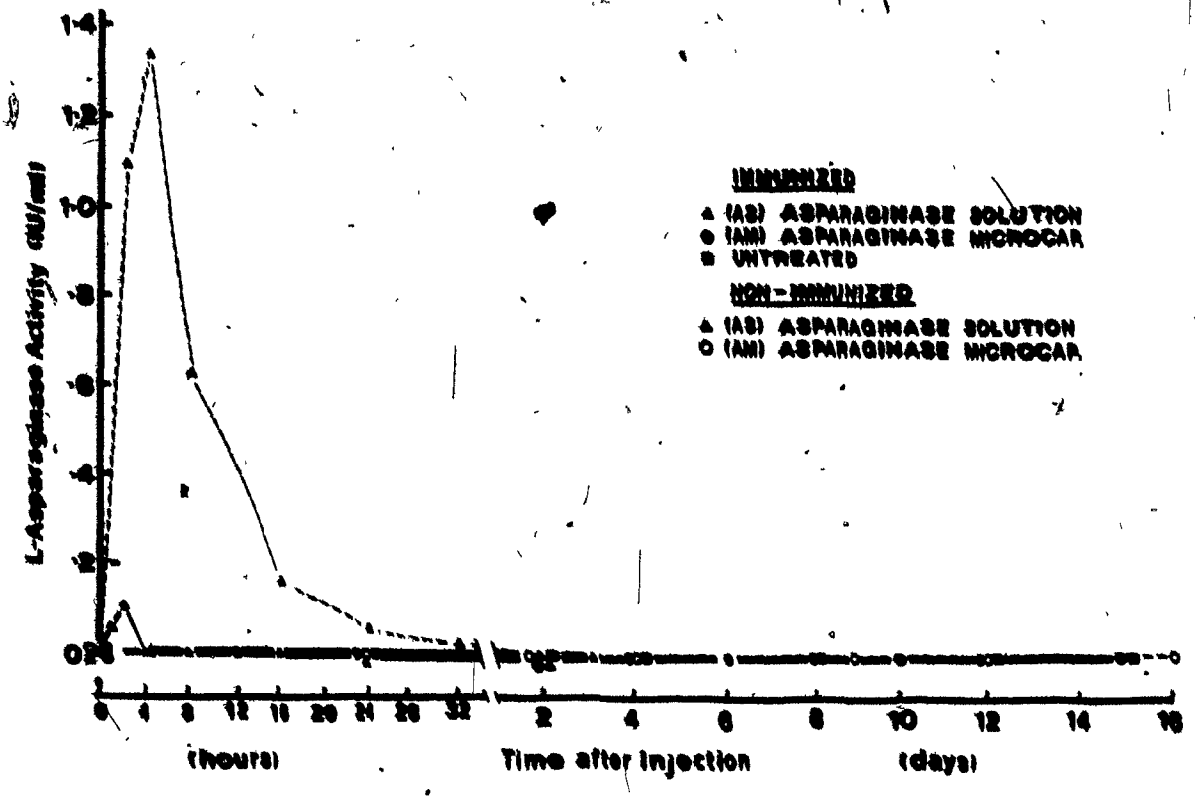


FIGURE 2

Time course of L-asparaginase activity in the blood of immunized mice after one of the following i.p. -injections:

Each ▲ = the mean of 2-4 immunized mice injected with AS and sacrificed at the indicated time interval.

Each ● = the mean of 2-4 immunized mice injected with AM and sacrificed at the indicated time interval.

Each ■ = the value for 1 untreated, immunized mouse sacrificed at the indicated time interval.

Each △ = the mean of 8 non-immunized mice injected with AS and sacrificed at the indicated time interval.

Each ○ = the mean of 4-8 non-immunized mice injected with AM and sacrificed at the indicated time interval.

Values for non-immunized mice were taken from Figure 2 in Chapter II.

ginase activity in the blood was 0.10 IU/ml, occurring in the 2 hour sample. There was no L-asparaginase activity detectable in blood samples taken 4, 8, 16, 24, 48 or 72 hours after injection (Figure 2). These observations are in marked contrast to results obtained in non-immunized mice. The maximum L-asparaginase activity in the blood after i.p. injection of an equal dose of L-asparaginase solution per gm body weight in non-immunized mice was 1.32 IU/ml, and L-asparaginase activity could still be detected in the blood up to 32 hours post injection (Figure 2). As for non-immunized mice, there was no detectable L-asparaginase activity in the blood of immunized mice injected i.p. with L-asparaginase microcapsules for the duration of these studies (15 days, Figure 2). No L-asparaginase activity was found in the blood of control immunized mice which were not injected with L-asparaginase ("untreated"), during these studies (Figure 2).

"Body" L-asparaginase activity

The L-asparaginase activity remaining in the "body" homogenates (prepared as described under Materials and Methods in Chapter II) of non-immunized mice sacrificed immediately after injection of L-asparaginase solution or L-asparaginase microcapsules was taken as 100% in these studies. Figure 3 shows that the L-asparaginase activity assayed in the "body" of immunized mice sacrificed immediately after i.p. injection of L-asparaginase solution was only 63% of the "body" L-asparaginase activity in non-immunized mice injected with an equivalent dose of L-asparaginase solution and sacrificed after a similar time interval. On the other hand, after i.p. injection of L-asparaginase microcapsules, there was no significant difference in the L-asparaginase activity remaining in

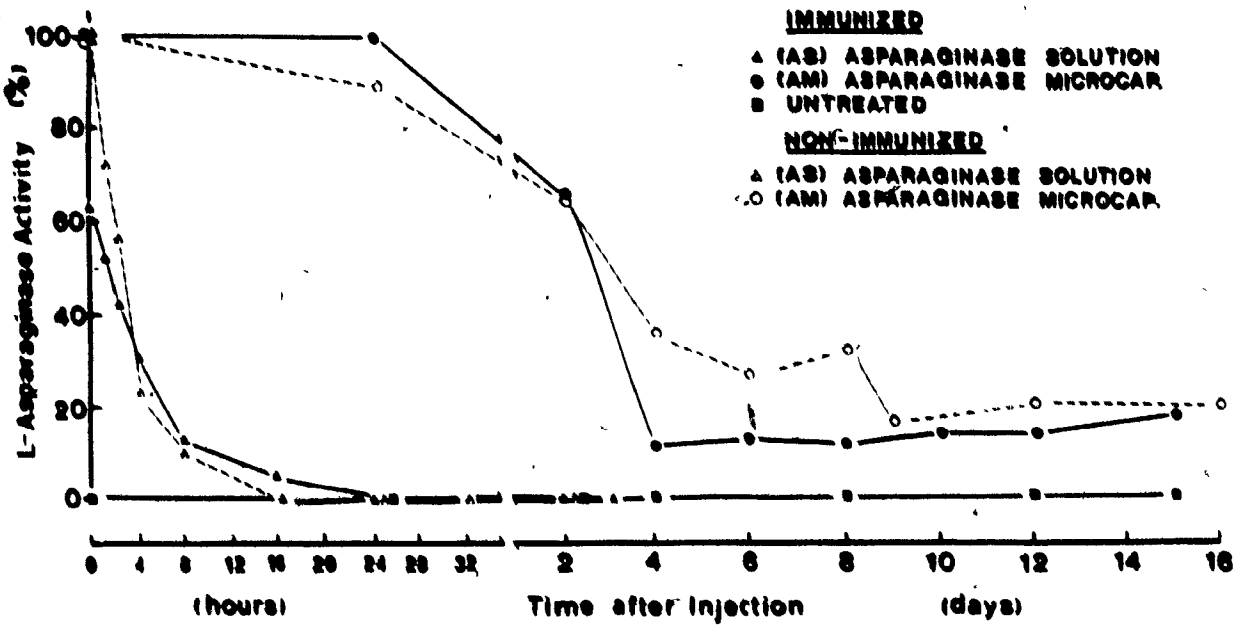


FIGURE 3

Time course of L-asparaginase activity remaining in the "body" of immunized mice after one of the following i.p. -injections:

Each ▲ = the mean of 2-4 immunized mice injected with AS and sacrificed at the indicated time interval.

Each ● = the mean of 2-4 immunized mice injected with AM and sacrificed at the indicated time interval.

Each ■ = the value for 1 untreated, immunized mouse sacrificed at the indicated time interval.

Each △ = the mean of 8 non-immunized mice injected with AS and sacrificed at the indicated time interval.

Each ○ = the mean of 4-8 non-immunized mice injected with AM and sacrificed at the indicated time interval.

Values for non-immunized mice were taken from Figure 4 in Chapter II.

the "body" of immunized mice as compared to non-immunized mice injected with an equivalent dose of L-asparaginase microcapsules for up to 2 days post injection (Figure 3). However, in the post injection period of 4 to 8 days, L-asparaginase activity remaining in the "body" appeared to be lower in immunized mice than in non-immunized mice at the corresponding time intervals (Figure 3). Thereafter, the "body" L-asparaginase levels were similar in immunized and in non-immunized mice (Figure 3). There were no assayable levels of endogenous L-asparaginase activity remaining in the "body" of control immunized mice for the duration of these studies (Figure 3).

Plasma L-asparagine levels

Figure 4 shows that after i.p. injection of L-asparaginase solution into immunized mice, the L-asparagine concentration in plasma samples was 0 nmoles/ml after 1 and 2 hours, 13.8 nmoles/ml after 4 hours, and 20.6 nmoles/ml after 8 hours. The plasma L-asparagine concentration was back to normal 16 hours after injection, and remained at this level for 3 days (Figure 4). It should be pointed out that the zero L-asparagine content in the 1 hour and 2 hour plasma samples probably represents the result of ongoing L-asparaginase activity still present in the blood at these times (Figure 2). Injection of L-asparaginase microcapsules i.p. into immunized mice did not result in a lowering of the plasma L-asparagine concentration to zero in any of the plasma samples taken after injection (Figure 4). A transient decrease, however, to 19.0 nmoles/ml was observed in the 4 hour sample (Figure 4). Apart from this, the plasma L-asparagine level remained normal for the duration of these studies (15 days, Figure 4). There was no significant change in the plasma

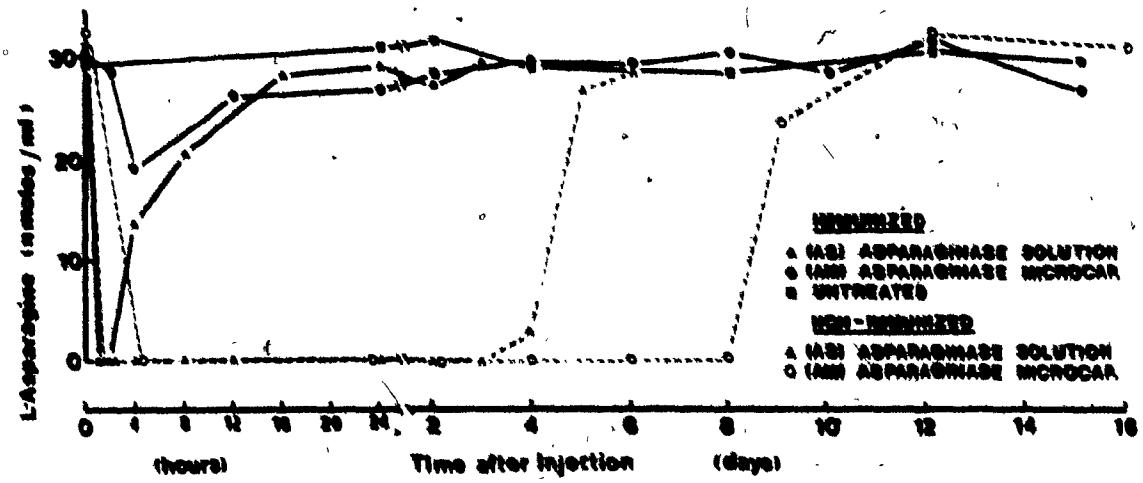


FIGURE 4

Time course of plasma L-asparagine levels in immunized mice after one of the following i.p. -injections:

Each ▲ = the mean of 2-4 immunized mice injected with AS and sacrificed at the indicated time interval.

Each ● = the mean of 2-4 immunized mice injected with AM and sacrificed at the indicated time interval.

Each ■ = the value for 1 untreated, immunized mouse sacrificed at the indicated time interval.

Each △ = the mean of 4 non-immunized mice injected with AS and sacrificed at the indicated time interval.

Each ○ = the mean of 4 non-immunized mice injected with AM and sacrificed at the indicated time interval.

Values for non-immunized mice were taken from Figure 6 in Chapter II.

L-asparagine levels in untreated, immunized mice during this period also (Figure 4). Concomitant control studies with non-immunized mice confirmed that i.p. injections of these enzyme preparations depleted the plasma L-asparagine content to zero.

Preliminary studies showed that neither L-asparaginase solution nor microencapsulated L-asparaginase injected i.p. at a dose of 5.0 IU/gm body weight (which is 10 times the usual dose given) could lower the plasma L-asparagine level to zero in immunized mice (Figure 5). A transient decrease to 13.4 nmoles/ml was observed, however, when L-asparaginase solution was injected at this dose (Figure 5).

Preliminary studies showed that there was no significant difference in plasma L-asparagine levels in immunized mice after i.p. injection of collodion L-asparaginase microcapsules instead of nylon L-asparaginase microcapsules (Figure 6). Control studies showed that i.p. injection of collodion L-asparaginase microcapsules into non-immunized mice lowered the plasma L-asparagine concentration to zero.

Studies using L-asparaginase derived from E. coli and Erwinia carotovora

It was shown in Figure 4 that in mice immunized to E. coli L-asparaginase, i.p. injection of E. coli L-asparaginase solution or E. coli L-asparaginase microcapsules could not lower the plasma L-asparagine concentration significantly. However, i.p. injection of an equivalent dose of Erwinia carotovora (abbreviated hereon as Erwinia) L-asparaginase solution was found to be effective in depleting the plasma L-asparagine level to zero for 2 days in these mice immunized to E. coli L-asparaginase (Figure 7). After this, the plasma L-asparagine rose to 6.3 nmoles/ml on day 3, and was normal on day 4 (Figure 7). Also,

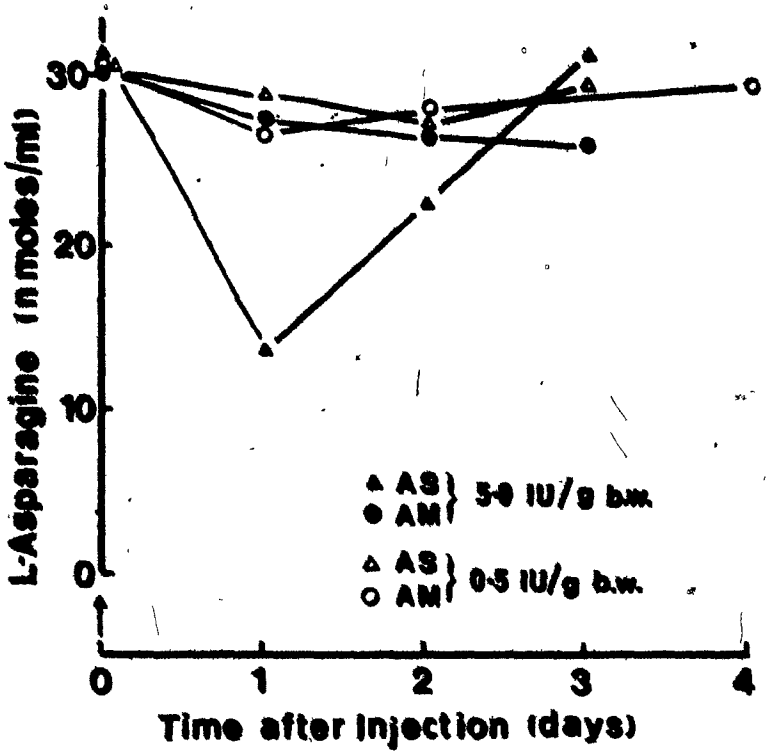


FIGURE 5

Plasma L-asparagine levels in immunized mice after i.p. injection of L-asparaginase solution (AS) or L-asparaginase microcapsules (AM) at a dose of 5.0 IU/gm body weight.

Each ▲ or ● = the mean of 2 immunized mice sacrificed at the indicated time interval.

Values for immunized mice injected with AS or AM at a dose of 0.5 IU/gm body weight were taken from Figure 4.

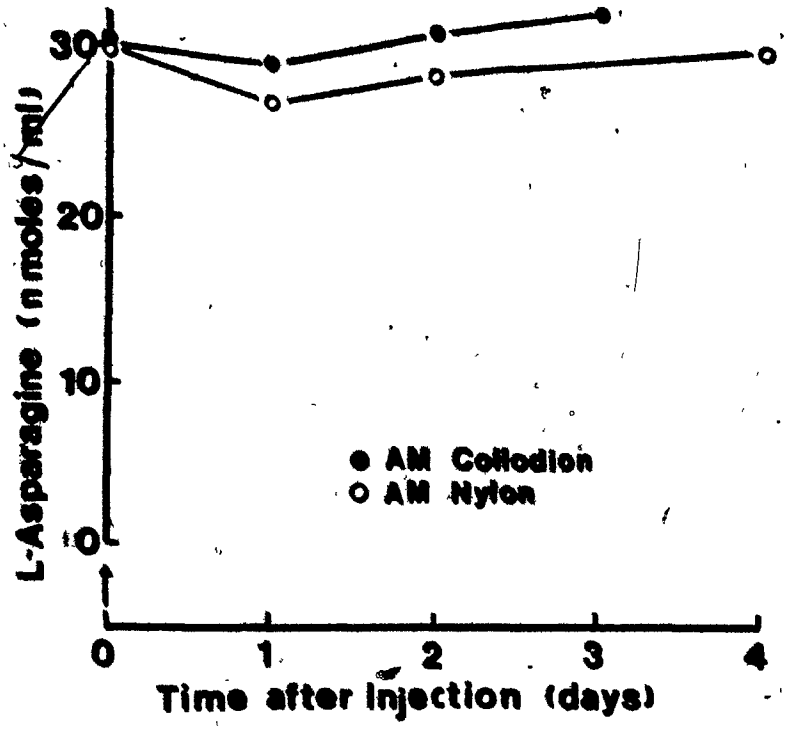


FIGURE 6

Plasma L-asparagine levels in immunized mice after i.p. injection of collodion L-asparaginase microcapsules (AM).

Each ● = the mean of 2 immunized mice sacrificed at the indicated time interval.

Values for immunized mice injected with nylon AM were taken from Figure 4.

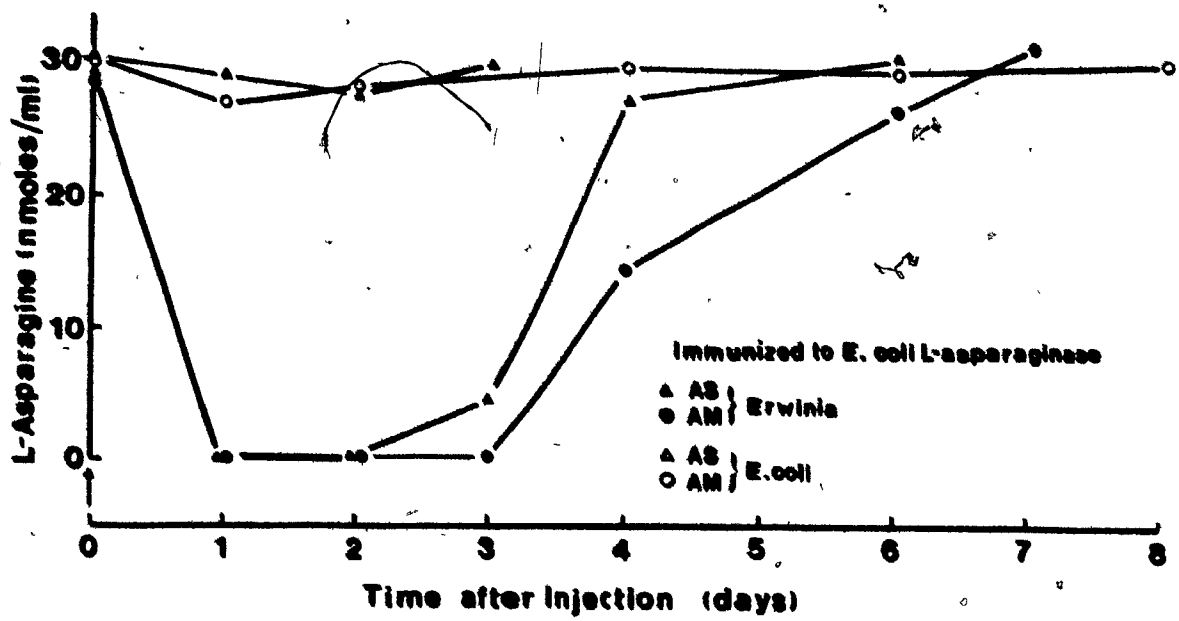


FIGURE 7

Plasma L-asparagine levels in mice immunized to E. coli L-asparaginase after one of the following i.p. injections:

Each ▲ = the mean of 2-4 immunized mice injected with Erwinia carotovora L-asparaginase solution (AS) and sacrificed at the indicated time interval,

Each ● = the mean of 2 immunized mice injected with Erwinia carotovora L-asparaginase microcapsules (AM) and sacrificed at the indicated time interval.

Values for immunized mice injected with E. coli AS or E. coli AM were taken from Figure 4.

injection of an equivalent dose of microencapsulated Erwinia L-asparaginase lowered the plasma L-asparagine to zero concentration for 3 days in these mice (Figure 7). After this, the plasma L-asparagine rose to 14.5 nmoles/ml on day 4, and was normal when the next plasma sample was taken on day 6 (Figure 7).

In reciprocal experiments with mice immunized to Erwinia L-asparaginase, preliminary studies indicated that neither i.p. injection of Erwinia L-asparaginase solution nor Erwinia L-asparaginase microcapsules was able to lower the plasma L-asparagine level to zero on day 1 after injection (Figure 8). On the other hand, i.p. injection of an equivalent dose of either E. coli L-asparaginase solution or E. coli L-asparaginase microcapsules was effective in depleting the plasma L-asparagine content to zero in this group of mice (Figure 8).

Control experiments with non-immunized mice, carried out concomitantly with the above experiments, showed that i.p. injection of E. coli L-asparaginase solution, E. coli L-asparaginase microcapsules, Erwinia L-asparaginase solution, or Erwinia L-asparaginase microcapsules, lowered the plasma L-asparagine level to zero in all cases on day 1 post injection.

In mice which had been immunized to E. coli L-asparaginase, preliminary studies showed that i.p. injection of Erwinia L-asparaginase microcapsules which had previously been exposed to E. coli L-asparaginase solution (according to the procedure described in Materials and Methods) caused a decrease in plasma L-asparagine level to zero on day 1 after injection (Figure 9). On the other hand, in mice which had previously been immunized to Erwinia L-asparaginase, injection

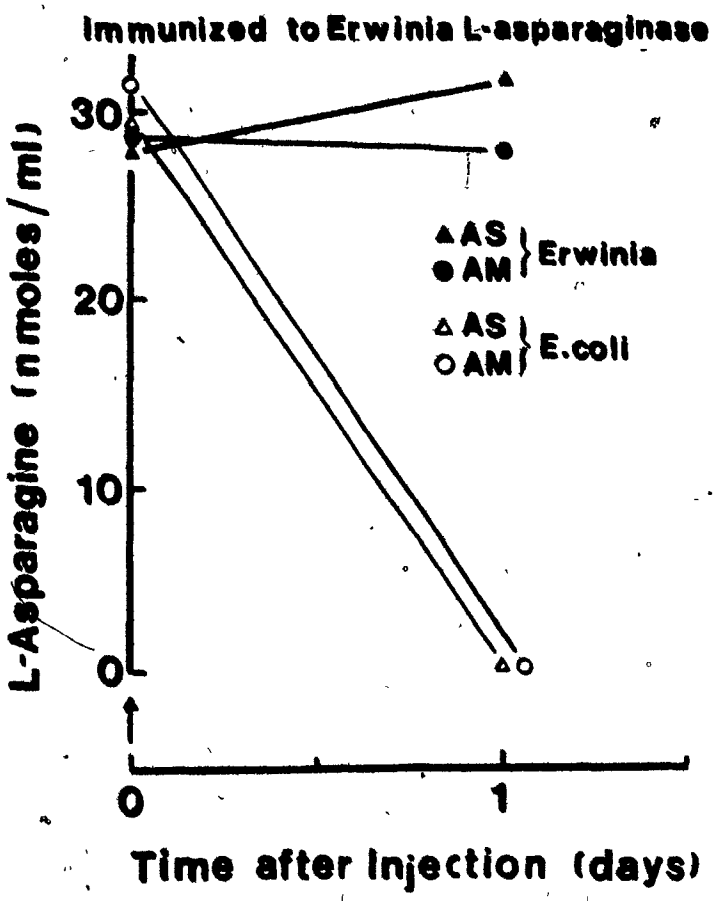


FIGURE 8

Plasma L-asparagine levels in mice immunized to Erwinia carotovora L-asparaginase after one of the following i.p. injections:

Each ▲ = the mean of 2 immunized mice injected with Erwinia carotovora L-asparaginase solution (AS) and sacrificed at the indicated time interval.

Each ● = the mean of 2 immunized mice injected with Erwinia carotovora L-asparaginase microcapsules (AM) and sacrificed at the indicated time interval.

Each △ = the mean of 2 immunized mice injected with E. coli AS and sacrificed at the indicated time interval.

Each ○ = the mean of 2 immunized mice injected with E. coli AM and sacrificed at the indicated time interval.

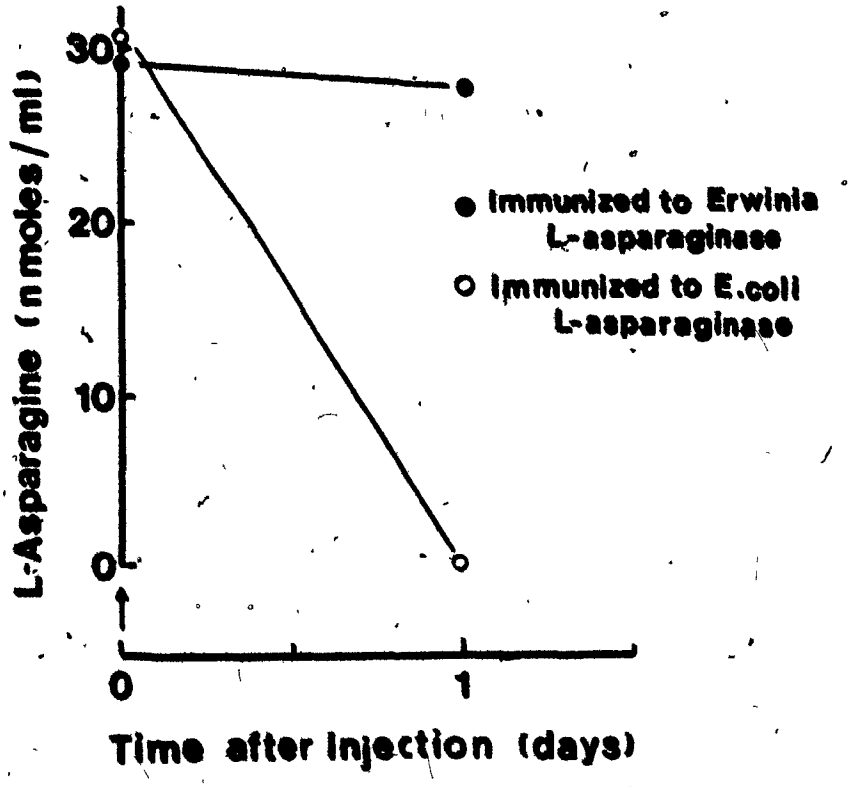


FIGURE 9

Plasma L-asparagine levels in mice immunized to E. coli L-asparaginase or Erwinia carotovora L-asparaginase after i.p. injection of Erwinia carotovora L-asparaginase microcapsules previously exposed to E. coli L-asparaginase solution.

Each ● or ○ = the mean of 2 immunized mice sacrificed at the indicated time interval.

of Erwinia L-asparaginase microcapsules exposed to E. coli L-asparaginase solution was not able to lower the plasma L-asparagine concentration to zero on day 1 after injection (Figure 9). Control experiments with non-immunized mice showed that i.p. injection of this type of microcapsules reduced the plasma L-asparagine concentration to zero on day 1 after injection.

Preliminary studies with microcapsules containing both E. coli and Erwinia L-asparaginase (prepared as described in Materials and Methods) showed that i.p. injection of these specially prepared microcapsules was able to lower the plasma L-asparagine concentration to zero on day 1 in mice immunized to E. coli L-asparaginase or to Erwinia L-asparaginase (Figure 10). I.p. injection of this type of microcapsules into control, non-immunized mice also lowered the plasma L-asparagine level to zero on day 1 after injection.

Histological studies

Similar cellular reactions to i.p. injected microcapsules were seen on day 1:

(a) in non-immunized mice receiving an injection of one of the following- control microcapsules, E. coli L-asparaginase microcapsules, Erwinia L-asparaginase microcapsules, or Erwinia L-asparaginase microcapsules that had been exposed to E. coli L-asparaginase solution; and

(b) in (E. coli) immunized mice receiving an injection of either control microcapsules or Erwinia L-asparaginase microcapsules.

In all of these cases, the cell infiltrate observed around the microcapsules was typical of a foreign-body reaction, consisting primarily of neutrophils on day 1. This host response was probably non-specific in nature and was mounted

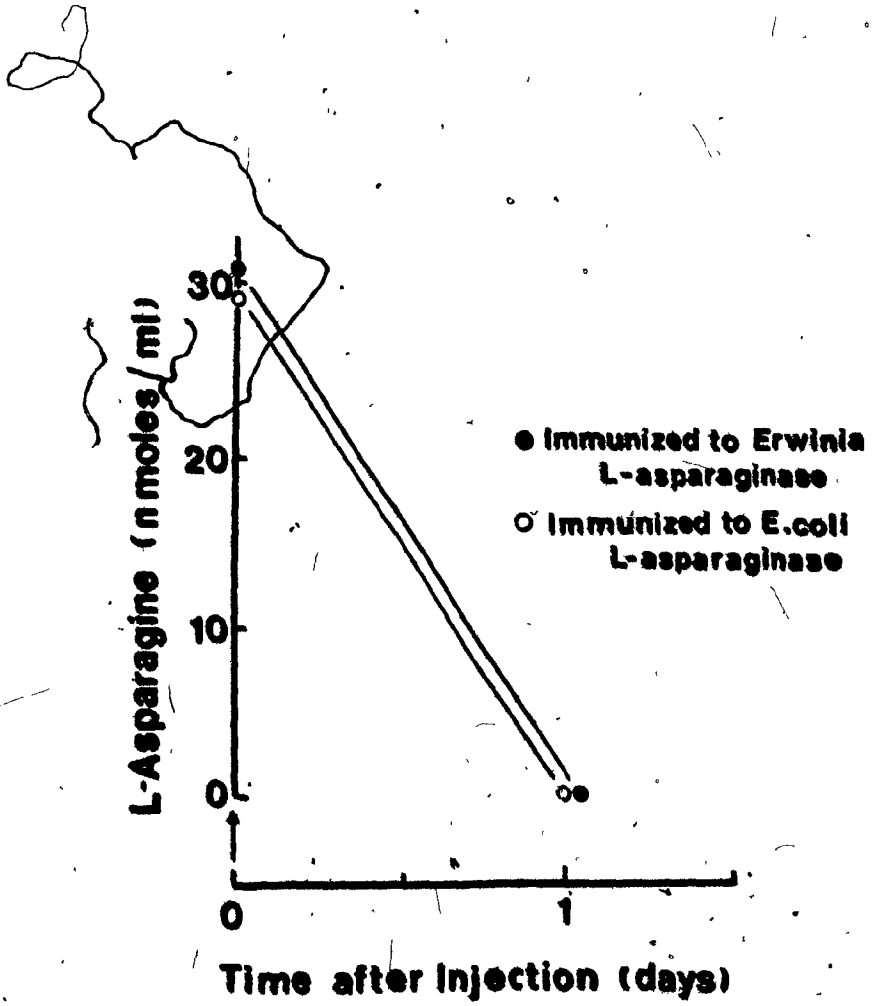


FIGURE 10

Plasma L-asparagine levels in mice immunized to E. coli L-asparaginase or Erwinia carotovora L-asparaginase after i.p. injection of microcapsules containing both E. coli and Erwinia carotovora L-asparaginase.

Each ● or ○ = the mean of 2 immunized mice sacrificed at the indicated time interval.

against the microcapsules themselves, regardless of their contents.

In contrast, a different cellular response was seen on day 1 in (E. coli) immunized mice injected i.p. with E. coli L-asparaginase microcapsules or Erwinia L-asparaginase microcapsules that had been exposed to E. coli L-asparaginase solution. There was an increase in density of cells around the microcapsules and in addition to neutrophils, there was a large infiltration of lymphocytes in these specimens. This infiltration of lymphocytes was specific in that it was only seen in animals which had been immunized against the specific type of enzyme present within or on the surface of L-asparaginase microcapsules.

DISCUSSION

Results obtained in these studies demonstrate that there is marked inhibition of the catalytic activity of L-asparaginase in free solution after incubation with anti-L-asparaginase serum (Table 1). This inhibition, however, was incomplete, even under conditions of antibody excess. The inability of antiserum to completely inhibit the catalytic activity of L-asparaginase is in accord with other findings on the effect of antibodies on enzymes that act on low molecular weight substrates (10). Reports by several other investigators (11 - 14) also indicate that inhibition of L-asparaginase activity by antibody is incomplete, the maximum inhibition reported ranging from 45 - 53%. According to the immunization schedule described in these studies, immunization of mice over a period of one month also resulted in the production of antisera which inhibited 45%. However, we have found that more potent antisera which inhibited 70% could be obtained with a more prolonged immunization schedule over a period of one year. This probably represents an increased avidity of antibody molecules produced after prolonged immunization. This finding is not peculiar to production of anti-L-asparaginase antibodies since others have found that the inhibiting power of antisera produced against the enzymes ribonuclease (15) and tyrosinase (16) also increase in the course of prolonged immunization.

In marked contrast to results obtained with L-asparaginase solution, there was no inhibition of the catalytic activity of L-aspara-

ginase microcapsules incubated with anti-L-asparaginase serum (Table 2). This observation indicates that in vitro:

(a) there is no leakage of L-asparaginase from the microcapsules; and

(b) the presence of humoral antibodies in the surrounding medium does not affect the catalytic action of L-asparaginase inside the microcapsules.

These findings are related to the protection afforded the enzyme by the microcapsule membrane which is selectively impermeable to macromolecules. Thus, in vitro, neither is there leakage of L-asparaginase from nor entry of antibodies into the microcapsule.

The maximum L-asparaginase activity found in the blood of immunized mice after i.p. injection of L-asparaginase solution was only 7.5% of the maximum blood L-asparaginase activity observed in similarly treated, non-immunized mice (Figure 2). This may be attributed to the presence of catalytically active antigen-antibody complexes that had not yet been eliminated from the circulation. The zero plasma L-asparagine concentration found in samples taken 1 and 2 hours after injection of L-asparaginase solution (Figure 4) is probably a result of the continued in vitro action of these catalytically active immune complexes in the blood and does not reflect the actual in vivo plasma L-asparagine concentration at the time of bleeding.

The observation that the L-asparaginase activity remaining in the "body", immediately after i.p. injection of L-asparaginase solution, was lower in immunized than in non-immunized mice (Figure 3) is most likely a consequence of inhibition of the native enzyme by humoral

antibodies present in the "body" homogenate. On the other hand, after i.p. injection of L-asparaginase microcapsules, the L-asparaginase activity remaining in the "body" of immunized mice was as high as that assayed in non-immunized mice up to 2 days post injection (Figure 3). This finding suggests that the presence of circulating antibodies also does not inhibit the catalytic activity of L-asparaginase entrapped within microcapsules in vivo, up to this time.

Measurement of plasma L-asparagine levels after i.p. injection of L-asparaginase solution into immunized mice (Figure 4), show that the native enzyme was incapable of suppressing the plasma L-asparagine to any significant extent in these mice. This finding provides the physiological basis for explaining the loss of therapeutic activity of L-asparaginase solution in 6C3HED tumor-bearing, immunized mice, observed in earlier experiments (5). The inability of L-asparaginase solution to suppress the plasma L-asparagine level in immunized mice is directly related to the inhibition of catalytic activity of the native enzyme by humoral antibodies (Table 1) and the rapid clearance of injected enzyme from the circulation (Figure 2). Moreover, since i.p. injection of 10 times the usual dose of L-asparaginase solution was unable to lower the plasma L-asparagine concentration to zero in the present studies (Figure 5), the shortened half-life of the enzyme in the circulation is probably the more important influence. Many other studies (11 - 14, 17 - 19) have also related the loss of antitumor activity of L-asparaginase solution in immunized hosts to the presence of humoral antibodies which both inhibit the catalytic activity of injected enzyme

and cause a dramatically accelerated removal of the enzyme from the circulation.

The finding that i.p. injection of L-asparaginase microcapsules into immunized mice also was unable to suppress the plasma L-asparagine level significantly provides the physiological basis to explain the loss of antitumor activity of L-asparaginase microcapsules in the treatment of tumor-bearing, immunized mice, observed in experiments described earlier (5). However, since humoral antibodies do not inhibit the catalytic activity of L-asparaginase microcapsules in vitro (Table 2) and the activity of L-asparaginase microcapsules remained high in vivo for up to 2 days post injection (Figure 3), the plasma L-asparagine results obtained with L-asparaginase microcapsules were somewhat enigmatic. This is particularly so in the light of extensive supporting data concerning the absence of any significant leakage of microcapsule contents in vitro and in vivo in non-immunized animals, as reviewed in detail in Chapter III. Further studies were therefore carried out to investigate possible mechanisms that may explain the inability of microencapsulated L-asparaginase to deplete the plasma L-asparagine content in mice immunized to the encapsulated enzyme.

There are at least 4 possible mechanisms that may account for the latter finding. The likelihood of each of these as the probable explanation will be examined in turn in the discussion following.

(a) The possibility existed that circulating anti-L-asparaginase antibodies in immunized mice may inhibit the catalytic activity

of L-asparaginase microcapsules. However, the in vitro results presented in Table 2 showing that there is no inhibition, do not support this possbility. Furthermore, in the in vivo situation there was no decrease in the L-asparaginase activity remaining in the "body" of immunized, as compared to non-immunized, mice for up to 2 days after i.p. injection of microencapsulated L-asparaginase (Figure 3).

(b) Repeated injections of L-asparaginase during the immunization schedule may result in increased levels of L-asparagine synthetase activity in host cells, particularly those of the liver and pancreas which have been implicated as possible regulatory sites for the homeostasis of plasma L-asparagine levels (20, 21). It is possible that these host cells respond to L-asparagine depletion by increasing production of large quantities of endogenous L-asparagine at a rate sufficient to effectively counteract L-asparaginase therapy. However, the following 4 lines of evidence seem to suggest that this was probably not a crucial factor in the present study:

(1) It was not possible to lower the plasma L-asparagine concentration to zero even after injection of microencapsulated L-asparaginase at 10 times a dose which is already in excess of that required to effectively suppress the plasma L-asparagine concentration (Figure 5).

(2) There have been several clinical reports (22 - 24) that indicate that plasma L-asparagine could successfully be depleted

with Erwinia carotovora L-asparaginase, which is immunologically distinct from E. coli L-asparaginase (25, 26), in leukemic patients immunized to the E. coli enzyme.

(3) Studies by Goldberg et al (19) have also demonstrated that tumor-bearing mice, previously immunized to E. coli L-asparaginase, received similar benefit from the therapeutic activity of Erwinia carotovora L-asparaginase as did non-immunized, tumor-bearing mice.

(4) In the present study, Erwinia L-asparaginase solution was able to maintain the plasma L-asparagine at zero concentration (Figure 7) in mice immunized to the E. coli enzyme. That these latter results were as a consequence of the immunologically distinct nature of the enzyme and not a property peculiar to it, was ruled out by the results of reciprocal experiments presented in Figure 8. In these experiments with mice immunized against the Erwinia enzyme, it was found that injection of Erwinia L-asparaginase in the solution or microencapsulated form was unable to lower the plasma L-asparagine to zero concentration but E. coli L-asparaginase solution could (Figure 8). Therefore, these data indicate that the inability to deplete the plasma L-asparagine to zero concentration was probably not due to an insurmountable production of L-asparagine by cells in the immunized host.

(c) A third possibility to be considered is that in order to effectively deplete the plasma L-asparagine concentration to zero in immunized

mice, particularly under compromising conditions of increased endogenous L-asparagine production, it may be essential that L-asparaginase exert its action intracellularly. However, diffusional restrictions imposed upon the enzyme entrapped within the confines of the microcapsule membrane do not allow for such an effect. Thus, the rate of depletion of plasma L-asparagine by microencapsulated L-asparaginase under these circumstances may not be efficient enough to overcome the host's compensatory mechanisms for maintaining a normal plasma L-asparagine level. Initially, this hypothesis appears to be in accord with the experimental results obtained in the present studies. It adequately explains why in mice immunized to E. coli L-asparaginase, Erwinia L-asparaginase in solution can effectively suppress the plasma L-asparagine concentration, while E. coli L-asparaginase entrapped within microcapsules cannot (Figure 7). It also explains why in mice immunized to Erwinia L-asparaginase, E. coli L-asparaginase in solution is effective, while Erwinia L-asparaginase entrapped within microcapsules is not (Figure 8). However, the following 2 lines of evidence do not support this hypothesis:

(1) Erwinia L-asparaginase microcapsules are effective in lowering the plasma L-asparagine concentration to zero in mice immunized to E. coli L-asparaginase (Figure 7), and

(2) E. coli L-asparaginase microcapsules are effective in mice immunized to Erwinia L-asparaginase (Figure 8).

Thus, the diffusional limitations inherent in the microencapsulated

form of the enzyme also do not seem to be a crucial factor in the present studies.

(d) Finally, the fourth possibility that will be considered is that L-asparaginase antigenic determinants may be present on the surface of L-asparaginase microcapsules, thus conferring certain surface properties to the microcapsules. In the immunized animal, recognition of these surface antigens as being identical to ones to which it had been exposed, may elicit a host response that in some way renders the catalytic action of L-asparaginase microcapsules ineffective in vivo. Two ways by which this may be brought about are:

(1) A decreased permeability of the microcapsules. This may be a significant factor in the immunized host because in addition to a foreign-body reaction to the microcapsules, which results in an infiltration of inflammatory cells and the deposition of proteins, such as fibrin, onto the surface of the microcapsules (4), there is also the added presence of humoral antibodies and a large number of immunocompetent cells around the microcapsule surface.

(2) Damage to the microcapsule membrane as a result of immune phenomena. This may result in entry of humoral antibodies into the microcapsules and leakage of microcapsule contents into the circulation.

Either of these 2 effects would interfere with the ability of L-asparaginase microcapsules to lower the plasma L-asparagine level effectively, particularly under conditions of increased L-asparagine synthesis. In the remaining discussion, I would first like to consider the likelihood of the presence of antigenic determinants on the surface of L-asparaginase

microcapsules prepared by the interfacial polymerization technique. This will be followed by a discussion of the possible ways in which a host response to the presence of surface antigens may explain the loss of in vivo action of L-asparaginase microcapsules in the immunized host.

Presence of antigenic determinants on the surface of L-asparaginase microcapsules

Since L-asparaginase is chemically a polyamine containing free amino groups, it may react chemically with sebacoyl chloride during the process of interfacial polymerization, forming part of the membrane structure. In fact, Chang (1) has prepared artificial cell membranes consisting entirely of cross-linked protein and sebacoyl chloride. Also, hemoglobin has been shown (1, 27) to be incorporated in the structure of nylon microcapsules prepared by the standard method. It is thus possible that L-asparaginase may be cross-linked, together with hemoglobin, to form a structural component of the membrane. Another possibility is that L-asparaginase molecules may be physically adsorbed onto the surface of L-asparaginase microcapsules during their preparation and washing. In effect, therefore, L-asparaginase molecules on the surface of the microcapsules may present themselves to the host's immune system as "intrinsic" antigens (component of the membrane structure) and as "extrinsic" antigens (adsorbed to the membrane surface). The proposal here is somewhat analogous to the presence of surface antigens on target cells involved in various cell-mediated immune responses.

That the immunized host recognizes and responds to the presence of homologous antigens associated with the microcapsules is supported indirectly by the following histological observations. As described in the present study, the cellular infiltrate seen in immunized mice injected with microcapsules bearing homologous antigens is distinctly different to that seen in non-immunized mice or mice immunized to antigens which do not cross-react with those present on the microcapsule surface. The cellular infiltrate in the former case consisted of neutrophils, lymphocytes and other lymphoid cells, a histologic picture not unlike that seen at sites of cell-mediated immune reactions. The presence of a large number of circulating "sensitized"lymphocytes in immunized mice as early as one day after injection of L-asparaginase microcapsules is presumably attributable to the booster dose of the immunizing antigen that was given 10 days prior to the injection of L-asparaginase microcapsules in these studies.

More direct evidence of an immunological recognition by the host of the presence of immunizing antigens on the surface of L-asparaginase microcapsules stems from the following demonstration. I.p. injection of E. coli L-asparaginase microcapsules into mice immunized against E. coli L-asparaginase elicited an intense, accelerated production of humoral anti-L-asparaginase antibodies, similar to the secondary antibody response observed after injection of a booster dose of L-asparaginase solution ("memory phenomenon").

It was only because of a lack of time that other immunologic studies, for example using the technique of immunofluorescence, were not performed to confirm the presence of L-asparaginase antigenic

determinants on the surface of L-asparaginase microcapsules. However, from the foregoing, it may be surmised that the presence of antigenic determinants is a distinct possibility. Recognition of these surface antigenic determinants may result in immunological events which together with inflammatory reactions to the microcapsules themselves, may affect the in vivo action of L-asparaginase microcapsules in at least two ways, as will be discussed in more detail below.

Decreased permeability of L-asparaginase microcapsules

According to this hypothesis, there is a decrease in the permeability of injected microcapsules as a result of immunological and inflammatory cellular infiltrate, protein deposition, etc, around the surface of injected microcapsules. This decreased permeability may be sufficient to reduce the catalytic efficiency of the microencapsulated enzyme. Thus, particularly under conditions of increased L-asparagine production, the rate of removal of L-asparagine from the peritoneal fluid may not be rapid enough to overcome the compromising effect of an increased synthesis of L-asparagine into the host's systemic circulation.

The increased cellular density observed histologically around microcapsules recovered from mice immunized to the specific antigens present within or on the surface of L-asparaginase microcapsules lends support to this theory. Another line of evidence which adds strong support to this hypothesis, instead of a damage-leakage hypothesis, is that L-asparaginase levels remaining in the "body" of immunized mice are as high as the levels assayed in non-immunized mice for up to 2 days post injection (Figure 3). Perhaps it should

be mentioned here that the ability to assay the L-asparaginase activity remaining in the "body" of immunized mice injected with L-asparaginase microcapsules does not conflict with the idea of a decreased microcapsule permeability since homogenizing of the tissues prior to assay would disperse any cells and protein film surrounding the microcapsule.

Three major attempts were made to alter the surface properties of L-asparaginase microcapsules to test the likelihood of the decreased permeability hypothesis. Firstly, it was reasoned that perhaps microencapsulation of E. coli L-asparaginase by the alternate technique of interfacial coacervation might decrease the probability of L-asparaginase being present on the surface of the microcapsule membrane. Collodion microcapsules containing E. coli L-asparaginase might not, therefore, elicit a immunologic response in hosts immunized to E. coli L-asparaginase and, as a result, might be expected to function efficiently in vivo. In this regard, Poznansky and Chang (28) had already shown that catalase microencapsulated within collodion microcapsules was effective in reducing injected perborate levels in mice immunized to catalase. However, the results presented in Figure 6 indicate that i.p. injection of collodion L-asparaginase microcapsules did not suppress the plasma L-asparagine concentration significantly. This finding does not necessarily conflict with a decreased permeability theory, however. One possible explanation is that the antigenic determinant sites of the L-asparaginase molecule, unlike the catalase molecule, may be exposed externally on the

surface of collodion L-asparaginase microcapsules so that the host can react to their presence.

Secondly, the finding that Erwinia L-asparaginase microcapsules could effectively lower the plasma L-asparagine level to zero in mice immunized to E. coli L-asparaginase (Figure 7) led to the idea that, if one were to adsorb E. coli L-asparaginase onto the surface of Erwinia L-asparaginase microcapsules, perhaps the Erwinia L-asparaginase microcapsules with these particular surface properties would no longer be effective in lowering the plasma L-asparagine to zero in these mice. Although histological studies showed that mice immunized to E. coli L-asparaginase did react to these microcapsules in a manner qualitatively similar to E. coli L-asparaginase microcapsules, Erwinia L-asparaginase microcapsules exposed to E. coli L-asparaginase solution were still found to be capable of lowering the plasma L-asparagine concentration to zero in mice immunized to E. coli L-asparaginase (Figure 9). On the other hand, injection of these microcapsules into mice immunized to Erwinia L-asparaginase, did not lower the plasma L-asparagine concentration to zero (Figure 9). Again, these preliminary data do not necessarily rule out a decreased permeability concept. One possible explanation for the results obtained in these experiments is that exposure of Erwinia microcapsules to L-asparaginase solution may not result in adequate masking of the surface antigenic properties of Erwinia L-asparaginase microcapsules.

Another kind of experiment was carried out in which both

E. coli and Erwinia L-asparaginase were microencapsulated at the same time and in concentrations equivalent to those used in the separate microencapsulation of each enzyme. In this way, both E. coli and Erwinia L-asparaginase antigenic determinants would presumably be present on the surface of this type of microcapsules. It was reasoned that injection of this type of microcapsules into animals immunized to E. coli or Erwinia L-asparaginase should elicit an immunological response to them similar to that mounted to microcapsules containing the corresponding type of enzyme antigen. If the permeability of these microcapsules was to decrease as a result, then plasma L-asparagine levels should not be lowered to zero in either group of immunized animals. However, plasma L-asparagine levels decreased to zero after i.p. injection of these microcapsules into both groups of immunized mice (Figure 10). These results cannot be explained by a decreased permeability hypothesis.

Immunologically-induced damage to L-asparaginase microcapsules

Another mechanism that attempts to explain the loss of in vivo activity of L-asparaginase microcapsules in mice immunized to the microencapsulated enzyme is that of "immunologically-induced damage" to the microcapsules. It is proposed here that specifically sensitized lymphocytes recognize the presence of homologous (intrinsic and/or extrinsic) antigens on the surface of the microcapsules and thus cause activation of a series of immunological events. These events in turn can cause damage to the microcapsule membrane, particularly at points where cross-linked protein molecules form part of the structure.

The result is that certain areas of the membrane become porous, allowing the entry of humoral antibodies into the microcapsules and release of enzyme into the surrounding medium where it may be bound to circulating humoral antibodies and removed as immune complexes. It should be pointed out here that this hypothesis of possible leakage of L-asparaginase microcapsules after i.p. injection into immunized hosts does not apply to non-immunized hosts. As discussed in Chapter III, microcapsules do not leak significantly in vitro or in vivo in non-immunized mice.

The immune mechanisms that may be involved in causing damage to the microcapsule membrane are not known, but perhaps one can speculate that they are similar to those that have been implicated in causing immunologic injury to tissues carrying membrane-associated antigens (29 - 32). The latter phenomenon involves complex interactions of various cell types, such as neutrophils, lymphocytes, and macrophages, and soluble factors, such as lymphocytic factors, complement and humoral antibodies. Such immune phenomena are very complex in nature and their immunopathologic mechanisms of action are still not yet precisely defined even for biological cells. However, the histologic picture observed around microcapsules recovered from mice immunized to the surface antigens of these microcapsules is similar to that seen in cell-mediated immune reactions involved in tissue injury. The added presence of a high anti-L-asparaginase antibody titre may also be an important factor. Another factor that may be involved is the

release of potent hydrolytic enzymes, such as cathepsins and other lysosomal enzymes, as a result of neutrophilic degranulation at sites of antigen-antibody interaction. It is postulated that the proteinaceous areas of the microcapsule surface may be particularly susceptible to attack by these potent proteolytic enzymes causing leakage.

According to this damage-leakage hypothesis, the results obtained using microcapsules with different surface properties may be interpreted as follows:

Antigenic determinants may be present on the surface of collodion L-asparaginase microcapsules. Host recognition of these immunizing antigens led to immunologic damage of the microcapsule membrane, with subsequent inactivation and removal of enzyme leaking out from the microcapsules. As a result, collodion L-asparaginase microcapsules were not effective in suppressing the plasma L-asparagine level (Figure 6).

In experiments with Erwinia L-asparaginase microcapsules which had been exposed to E. coli L-asparaginase solution, the host immunized to E. coli L-asparaginase recognizes the presence of (extrinsic) E. coli antigens on the microcapsule surface. Subsequent activation of immune events in the host led to immunologic damage of the microcapsule membrane. This caused release of an immunologically different (Erwinia) L-asparaginase into the circulation. As a result, this type of microcapsules effectively lowered the plasma L-asparagine level in mice immunized to E. coli L-asparaginase (Figure 9). On the other hand, injection of this type of microcapsules

into mice immunized to Erwinia L-asparaginase evoked immune mechanisms but, this time, because of host recognition of (extrinsic and intrinsic) Erwinia antigens on the microcapsule surface. However, damage to the microcapsule membrane with subsequent release of Erwinia L-asparaginase cannot lower plasma L-asparagine because of the presence of circulating anti-Erwinia L-asparaginase antibodies in these mice (Figure 9,.

The results obtained with microcapsules containing both E. coli and Erwinia enzymes can easily be reconciled according to a damage-leakage hypothesis. Immunologic recognition of the presence of (extrinisic and intrinsic) surface antigens by hosts previously immunized against either one of the entrapped enzymes would result in immunologic damage to the microcapsule membrane. Subsequent leakage of physiologically active Erwinia L-asparaginase into the circulation of mice immunized to E. coli L-asparaginase would lower the plasma L-asparagine in these mice (Figure 10). Similarly, leakage of physiologically active E. coli L-asparaginase into the circulation of mice immunized to Erwinia L-asparaginase would also lower the plasma L-asparagine in these mice (Figure 10).

It is important to note that a damage-leakage mechanism, though attractive, cannot explain the findings that "body" L-asparaginase levels were just as high in immunized as in non-immunized mice for up to 2 days post inection (Figure 3). If there was significant leakage of microcapsule contents during the first 2 days post injection, one would expect the "body" L-asparaginase levels to be lower in

immunized than in non-immunized mice.

In short, either a "decreased permeability" or a "damage-leakage" hypothesis can explain the plasma L-asparagine results obtained in mice immunized to E. coli L-asparaginase after i.p. injection of E. coli L-asparaginase microcapsules (Figures 4 & 5), and in mice immunized to Erwinia L-asparaginase after i.p. injection of Erwinia L-asparaginase microcapsules (Figure 8). Unfortunately, the results of subsequent studies using collodion L-asparaginase microcapsules (Figure 6) and Erwinia L-asparaginase microcapsules that had been exposed to E. coli L-asparaginase solution (Figure 9), were not definitive enough to exclude the likelihood of either of the 2 proposed mechanisms. Although the results obtained with microcapsules containing both E. coli and Erwinia L-asparaginase (Figure 10) can best be explained by a damage-leakage mechanism, nevertheless, one cannot overlook data showing that "body" L-asparaginase levels remained as high in immunized as in non-immunized mice, at least up to 2 days post injection (Figure 3). The latter findings can only be interpreted according to a decreased permeability mechanism. Thus, from the data presently available, one cannot as yet define the exact detailed mechanism(s) whereby L-asparaginase microcapsules are rendered physiologically ineffective in mice immunized to the microencapsulated enzyme. However, these studies do suggest that antigenic properties associated with the surface of the microcapsules and the host's ability to recognize and respond to their presence, play a prominent role in any such mechanism.

APPENDIX 1

Preparation of collodion L-asparaginase microcapsules

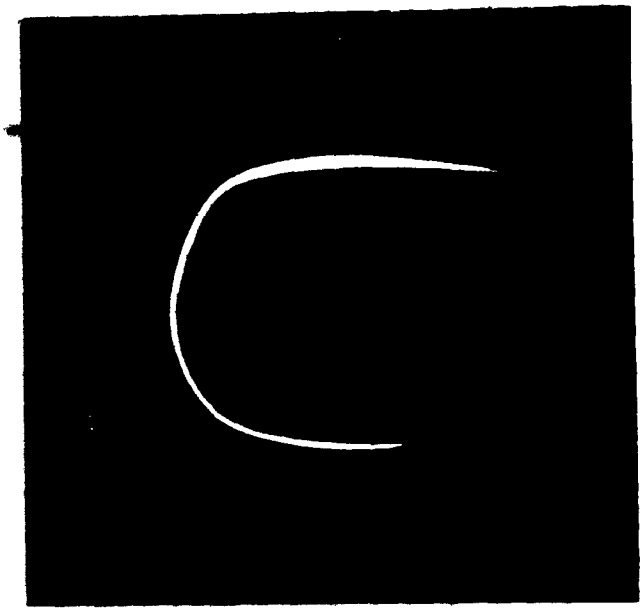
10 gm% hemoglobin solution (hemoglobin substrate, Worthington Biochemical Corporation, Freehold, New Jersey) was buffered by the addition of tris (hydroxymethyl) aminomethane base (final concentration 0.08 M), then filtered through Whatman No.42 filter paper. Collodion (cellulose nitrate) solution was prepared by evaporating collodion U.S.P. (E.R. Squibb & Sons, New York, New York) to 20% of its original weight and made up to its original volume with ether. To a 150 ml glass beaker surrounded by ice the following were added: 4.2 ml tris-buffered hemoglobin solution containing 500 IU L-asparaginase and 42 ml of water-saturated ether containing 1% (v/v) Span 85 (Atlas Chemical Industries, Brantford, Ontario). The mixture was immediately emulsified for 5 seconds, using a "Jumbo" magnetic stirrer (Fisher Scientific Co., Montreal, Quebec) with a speed setting of 7. Without stopping the stirring, 42 ml of collodion solution were added and the reaction mixture stirred for one minute more at the same speed. The beaker was then covered and allowed to stand unstirred at 4°C for 45 minutes to allow the cellulose ester to gradually precipitate at the interface of each microdroplet. The supernatant was removed and 50 ml of n-butyl benzoate (Eastman Kodak Co., Rochester, New York) containing 1% (v/v) Span 85 were added and the mixture stirred for 30 seconds at speed 5. The suspension was then left to stand uncovered and unstirred at 4°C for 30 minutes to allow the ether to evaporate and the outer surface of the microcapsules to set. The supernatant was removed and 42 ml of 50% Tween 20 solution (Atlas Chemical Industries) added. The microcapsules were dispersed in the aqueous medium by stirring the suspension with the Jumbo stirrer at speed 10 for 30 seconds. The speed was decreased to

5, 50 ml of water added and stirring continued for another 30 seconds. The suspension was then further diluted with 200 ml water and allowed to sediment. The supernatant was removed and the microcapsules washed repeatedly with 1% Tween 20 to get rid of fragile microcapsules and any residual n-butyl benzoate. The microcapsules were then washed with saline to remove the Tween 20 and resuspended in saline to produce a 50% microcapsule suspension.

APPENDIX 2

Demonstration of the presence of anti-L-asparaginase antibodies by the Ouchterlony technique

In the present study, the Ouchterlony double diffusion technique in two dimensions was carried out on slides coated with agarose. The centre well of each slide was filled with the test serum and the peripheral wells were filled with different concentrations of L-asparaginase solution (AS). The reactants in the separate wells move toward each other and a immuno-specific, precipitin line forms in the agarose medium when antigen and its homologous antibody combine in optimal concentrations. The figure below shows typically the precipitin lines that develop when anti-L-asparaginase serum, collected from animals that had been immunized as described, was placed in the centre well.



S = test serum

1 = 12.5 µg/ml AS

2 = 25 µg/ml AS

3 = 50 µg/ml AS

4 = 100 µg/ml AS

5 = 150 µg/ml AS

6 = 200 µg/ml AS

APPENDIX 3

Appearance of typical patterns of hemagglutination

The appearance of typical patterns of hemagglutination is shown in the figure below.

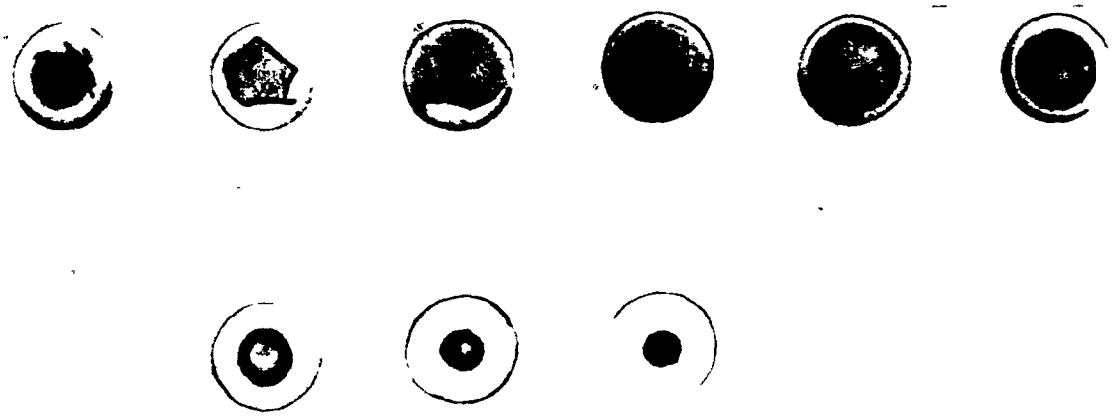


Figure 1. Appearance of patterns of hemagglutination. Top row—left to right ++++, +++, ++, +, ±, reactions. Bottom row—left to right ±, −, −.

(taken from Stavitsky, A.B., (9)).

In a strong reaction, the cells form a compact granular agglutinate or a diffuse "carpet" of agglutinated cells covering the bottom of the tube. Weaker positive reactions are characterized by a mat of cells with folded or ragged edges. A negative reaction consists of a discrete button or small ring of cells in the centre of the bottom of the tube. In a doubling dilution series, a gradation between the appearances of positive and negative reactions usually occur over 2 - 3 tubes. The endpoint chosen in these studies was the last tube showing an even carpet of cells with a slight ring at the edge.

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CHAPTER V

GENERAL SUMMARY AND CLAIMS TO ORIGINAL RESEARCH

GENERAL SUMMARY

* indicates claims to original work with L-asparaginase microcapsules in particular.

*1. There was no detectable L-asparaginase activity in the blood of mice injected i.p. with L-asparaginase microcapsules (AM) for up to 16 days post injection. In marked contrast, after i.p. injection of L-asparaginase solution (AS), L-asparaginase activity quickly appeared in the blood with the highest concentration occurring after 4 hours, and was cleared from the circulation with a half-life of 4.4 hours.

*2. It took 2.5 to 3 days for "body" L-asparaginase levels to decline to 50% of its original activity after i.p. injection of AM, whereas "body" L-asparaginase levels fell with a half-life of 2 hours after i.p. injection of AS. Thus, these results showed that AM retained catalytic activity for a significantly longer period of time than AS in vivo.

*3. Plasma L-asparagine was maintained at zero concentration for 8 days after i.p. injection of (nylon) AM as compared to only 3 days after i.p. injection of AS.

4. It is concluded from (1) and (3) that AM can effectively suppress the plasma L-asparagine level without enzyme leaking out from the microcapsules into the blood.

5. The greater ability of AM to maintain plasma L-asparagine at zero concentration may be explained by the increased stability of microencapsulated over the solution form of the enzyme, and the protective function of the microcapsule membrane in preventing the rapid removal and inactivation of the foreign enzyme by the body's defense mechanisms.

*6. Although 20% of the catalytic activity of injected AM was still retained after day 8 post injection, plasma L-asparagine levels rose back towards normal on day 9.

*7. Histological studies of microcapsules recovered at various times from the peritoneal cavity showed that a foreign-body reaction was mounted by the host to the i.p. injected AM. It is proposed that the progressive fibrosis observed around the microcapsules results in a decreased permeability of the microcapsules to substrate and provides a morphological basis to account for the findings in (6).

*8. Subsequent experimental data show that increasing the in vitro stability of AM, by cross-linking the enzyme with glutaraldehyde after microencapsulation, did not further increase the ability of i.p. injected AM to maintain a zero plasma L-asparagine concentration. This finding is consistent with the hypothesis of decreased permeability of the microcapsules in vivo, as proposed

in (7). This result is also the first in vivo demonstration of the physiological action of cross-linked AM and provides further support that a leakage mechanism is not a sine qua non to explain the mode of action of i.p. injected microencapsulated enzymes.

*9. There was no increase in liver L-asparaginase activity after injection of AM, in contrast to injection of AS. Preliminary data suggest rather that a prolonged depletion of L-asparagine and/or prolonged increase in L-aspartate and ammonia levels in the plasma, brought about by the sustained action of AM, cause a decrease in (endogenous) liver L-asparaginase activity.

*10. It was demonstrated that i.p. injection of AM, like AS, was capable of inducing complete regression of established 6C3HED lymphosarcoma borne in non-immunized, syngeneic hosts.

11. Subsequent, more detailed studies were performed to compare and analyze the tumor inhibitory effectiveness of various doses of free and microencapsulated forms of L-asparaginase in causing the regression of established 6C3HED lymphosarcoma implants in non-immunized mice.

*12. Although the initial rate of tumor regression was more rapid in mice treated with AS than in those treated with AM, 3 days after either type of treatment, tumors were no longer visible in all mice treated with AS or AM at all doses given.

*13. A dose of 0.500 IU/gm body weight of either AS or AM was found to be curative. Six days after treatment with AS or AM at this dosage, mice were sacrificed and post-mortem examination revealed no evidence of lymphosarcoma at the site of tumor implantation or at other sites in the body. There was no reappearance of the tumor after treatment for up to 100 days after tumor implantation and all animals were tumor-free at this time.

*14. However, some tumors reappeared after treatment with doses of 0.125 IU/gm or 0.250 IU/gm body weight of either AS or AM. Tumors reappeared more quickly in animals that received the lower dose of 0.125 IU/gm of AS or AM than in those given the higher dose of 0.250 IU/gm of AS or AM.

*15. For a given subcurative dose, tumors reappeared more quickly in those mice treated with AS than in those treated with AM.

*16. All animals treated with 0.125 IU/gm AS were dead by day 35 after tumor implantation, whereas 27.3% of those treated with the equivalent dose of AM were alive and tumor-free up to 100 days after tumor implantation.

*17. 75% of mice treated with 0.250 IU/gm AS were alive and tumor-free 100 days after tumor implantation, as compared to 91% for those treated with AM after the same period.

18. It is concluded from these results (15 - 17) that microencapsulated L-asparaginase at subcurative doses is more effective than the equivalent dose of AS in the treatment of established 6C3HED lymphosarcoma implants in non-immunized mice. These differences in therapeutic potency correlate well with the physiological properties and in vivo effects (2, 3, 5) of these two enzyme preparations in non-immunized mice.

19. Experiments were performed that confirmed the proposal that the LDH virus decreases the clearance rate of enzyme from the circulation of infected hosts, and thus is an important contributing factor to the therapeutic activity of L-asparaginase. In our case, a 3-fold increase in the half-life of L-asparaginase in the blood of tumor-bearing mice injected with AS was observed.

*20. No L-asparaginase activity was detected in the blood of tumor-bearing mice after i.p. injection of AM. It is noted that since the mode of action of i.p. injected AM does not depend on L-asparaginase activity in the blood, the LDH virus probably does not play an important role in the case of microencapsulated L-asparaginase therapy.

*21. Further studies in mice which had previously been immunized to L-asparaginase indicated that both AM and AS were incapable of inducing regression of the 6C3HED lymphosarcoma borne in these hosts. The median survival times for tumor-bearing, immunized mice treated

with 0.250 IU/gm or 0.500 IU/gm AS or AM were similar to those mice given saline or microcapsules containing no L-asparaginase, as controls.

22. It is concluded from these results that both AM and AS, even at a dose level which is curative in non-immunized mice bearing equivalent tumor loads, are therapeutically ineffective when administered to hosts previously immunized to L-asparaginase.

23. Studies were then carried out with immunized mice in an attempt to explain the physiological basis underlying the loss of therapeutic potency of the enzyme preparations in these mice.

*24. It was found that more potent antisera could be obtained by prolonged immunization over a period of 1 year instead of 1 month. This probably represents an increased avidity of antibody molecules produced after prolonged immunization.

*25. Incubation of hyperimmune anti-L-asparaginase serum with AM did not inhibit the catalytic activity of AM, in marked contrast to similarly treated AS which was inhibited 70%.

*26. In vitro, the L-asparagine content of plasma was effectively depleted to zero by AM in spite of the presence of anti-L-asparaginase antibodies in the surrounding medium.

27. The above results with AM (25, 26) suggest that in vitro there is no leakage of L-asparaginase from the microcapsules and the presence of specific humoral antibodies in the surrounding medium does not affect the catalytic activity of L-asparaginase inside the microcapsules which continue to act on plasma L-asparagine diffusing in.

28. After i.p. injection of AS into immunized mice, very low L-asparaginase activity appeared in the blood, which was very rapidly removed from the circulation.

*29. No L-asparaginase activity appeared in the blood after i.p. injection of AM into immunized mice.

*30. Whereas "body" L-asparaginase levels were lower in immunized than in non-immunized mice after injection of AS, there was no significant difference in the "body" L-asparaginase levels in immunized and in non-immunized mice after injection of L-asparaginase microcapsules for up to 2 days post injection.

31. The plasma L-asparagine concentration in immunized mice could not be lowered significantly after injection of AS. This is related to the results obtained in (28).

*32. Despite the findings in (30) that "body" L-asparaginase levels remained high for up to 2 days after i.p. injection of AM, into

immunized mice, the plasma L-asparagine could not be lowered significantly after injection of AM.

33. The results in (31) and (32) provide the physiological basis to explain the loss of therapeutic activity of AS and AM respectively in tumor-bearing, immunized mice.

*34. The plasma L-asparagine level could not be suppressed significantly even after i.p. injection of AS or AM at 10 times the usual dose of 0.5 IU/gm body weight.

35. With the aid of Erwinia carotovora L-asparaginase, further studies were carried out to investigate possible mechanisms that may explain the inability of AM to deplete the plasma L-asparagine content in mice immunized to the microencapsulated enzyme.

*36. Whereas in mice immunized to E. coli L-asparaginase, injection of E. coli AS or AM could not suppress the plasma L-asparagine significantly, the injection of equivalent doses of Erwinia AS or AM was effective.

*37. Reciprocal experiments in mice immunized to Erwinia L-asparaginase showed that injection of equivalent doses of E. coli AS or E. coli AM could lower the plasma L-asparagine to zero.

*38. However, injection of Erwinia AS or AM could not lower the plasma L-asparagine to zero in mice immunized to Erwinia L-asparaginase.

39. Other studies were also carried out using L-asparaginase microcapsules with different surface properties.

*40. Injection of collodion E. coli AM also was unable to lower the plasma L-asparagine concentration to zero in immunized to E. coli L-asparaginase.

*41. Injection of Erwinia AM previously exposed to E. coli AS decreased the plasma L-asparagine to zero in mice immunized to E. coli L-asparaginase.

*42. However, injection of Erwinia AM previously exposed to E. coli AS did not lower the plasma L-asparagine to zero in mice immunized to Erwinia L-asparaginase.

*43. Injection of microcapsules containing both E. coli and Erwinia L-asparaginase lowered the plasma L-asparagine to zero in mice immunized to E. coli L-asparaginase.

*44. Injection of microcapsules containing both E. coli and Erwinia L-asparaginase also lowered the plasma L-asparagine to zero in mice immunized to Erwinia L-asparaginase.

*45. Histological studies showed that similar cellular reactions occurred in response to i.p. injected microcapsules on day 1:

(a) in non-immunized mice receiving an injection of one of the following-control microcapsules, E. coli AM, Erwinia AM, or Erwinia AM exposed to E. coli L-asparaginase solution; and

(b) in (E. coli) immunized mice receiving an injection of either control microcapsules or Erwinia AM.

In all of these cases, the cell infiltrate observed around the microcapsules was typical of a foreign-body reaction, consisting primarily of neutrophils on day 1.

*46. In contrast, a different cellular response was seen on day 1 in (E. coli) immunized mice injected i.p. with either E. coli AM or Erwinia AM exposed to E. coli AS. There was an increase in density of cells around the microcapsules and in addition to neutrophils, there was a large infiltration of lymphocytes in these specimens. This lymphocytic infiltrate is suggestive of a host immune response to these microcapsules.

47. The results above (45, 46) indicate that the predominantly lymphocytic infiltrate observed in (46) was specific in that it was only seen in animals which had been immunized against the specific type of enzyme present within or on the surface of AM.

*48. I.p. injection of E. coli AM into mice immunized against E. coli L-asparaginase elicited an intense, accelerated production of

humoral anti-L-asparaginase antibodies, similar to the secondary antibody response observed after injection of a booster dose of AS ("memory phenomenon"). This immune response suggests that the host recognized the presence of immunizing antigenic determinants associated with the surface of the microcapsules.

49. Various possible mechanisms that may explain the inability of AM to lower the plasma L-asparagine concentration in mice specifically immunized to the microencapsulated enzyme are discussed in the light of the present data.