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ACKNOWLEDGMENTS

Funds for this study were provided by U.S. Food and Drug Administration Contract 223-78-3006.

Acrylic Microspheres *In Vivo* V: Immunological Properties of Immobilized Asparaginase in Microparticles

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Received February 2, 1981, from the *Department of Pharmaceutical Biochemistry, Biomedical Center, University of Uppsala, Box 578, 751 23 Uppsala, Sweden.* Accepted for publication September 3, 1981.

Abstract □ L-Asparaginase was immobilized in microparticles of polyacrylamide. Such particles were then injected by intramuscular/subcutaneous, intraperitoneal, or intravenous routes into mice to investigate the immunological consequences of the immobilization. Entrapment of L-asparaginase in microparticles did not prevent the formation of antibodies in intensively treated animals. Intraperitoneal and intravenous injections of particles produced significantly higher antibody levels than soluble L-asparaginase. Antigen administered intramuscularly/subcutaneously in microparticles elicited, however, a weak immune response. Dependent on the route of administration, the particles may thus function as an adjuvant. A modified Arthus reaction in the foot pads of immunized mice indicated that antigenicity decreased when L-asparaginase was immobilized in microparticles. Injection of free L-asparaginase, intramuscularly/subcutaneously (2 × 20 IU) in the preimmunized mice produced no effects on the serum level of L-asparagine, whereas intramuscular/subcutaneous injection of L-asparaginase in microparticles produced a depression of the serum concentration. It is concluded that the intramuscular/subcutaneous injection of L-asparaginase in microparticles is the choice route of administration with respect to duration and the immunological reaction.

Keyphrases □ L-Asparaginase—immunological properties of immobilized L-asparaginase in microparticles □ Microparticles—immunological properties of immobilized L-asparaginase □ Immunological properties—of immobilized L-asparaginase in microparticles

Exogenous enzymes have been used increasingly in biological systems to test their usefulness in treating genetic disorders, *e.g.*, lysosomal storage diseases (1, 2), or for therapeutic purposes, *e.g.*, L-asparaginase to depress circulating L-asparagine in the treatment of acute lymphatic leukemia (3). These enzymes are often used in the immobilized or polymerized form in order to prolong their duration (4–6). However, the desired prolonged effect is seemingly in conflict with the efforts to decrease their immunological properties manifested by the production of antibodies and hypersensitivity reactions, which is enhanced when exposure to the exogenous protein is prolonged. Thus, enzymes in liposomes have been shown to be immunogenic[1]. The liposomes have even, in some instances, been shown to exert adjuvant effects (7). This effect may be due partly to leakage of enzyme molecules out of the liposomes or lysis of the liposomes, but the findings that the adjuvant properties are related to the composition of the liposomes (7, 8) suggest that the adjuvant effects are inherently connected with the liposomes themselves. Likewise, polymethylmethacrylate has been shown to increase the immunogenic properties of simultaneously administered influenza virons (9). The adjuvant effect is strongly correlated to the route of administration, with intravenous or intraperitoneal injections of the immobilized systems generally producing relatively higher antibody titers (7, 10).

The present study was undertaken to investigate the immunological consequences of the utilization of microparticles of polyacrylamide as carrier of exogenous proteins *in vivo*. The polymer itself is not immunogenic (11), but immobilized proteins are partly localized on the surface of the microparticles during the preparation as evidenced by their interaction with cellular surface structures (12) or affinity chromatography material (13). Even if the major portion of the immobilized protein is secluded inside the microparticles, the fraction on the surface should exert immunological properties. The aim was to find the optimal route for the administration of immobilized L-asparaginase.

EXPERIMENTAL

Materials—Aspartate aminotransferase[2] (83 IU/mg) isolated from porcine heart, and malic dehydrogenase[2] (2000 IU/ml) from pigeon breast

[1] In the present paper, the term immunogenic is used to describe the property of a macromolecular system to evoke an immunological response, *e.g.*, antibody production and T-cell stimulation, while the term antigenic is restricted to the property to react with the immunological effectors in a sensitized organism.
[2] Sigma Chemical Co.

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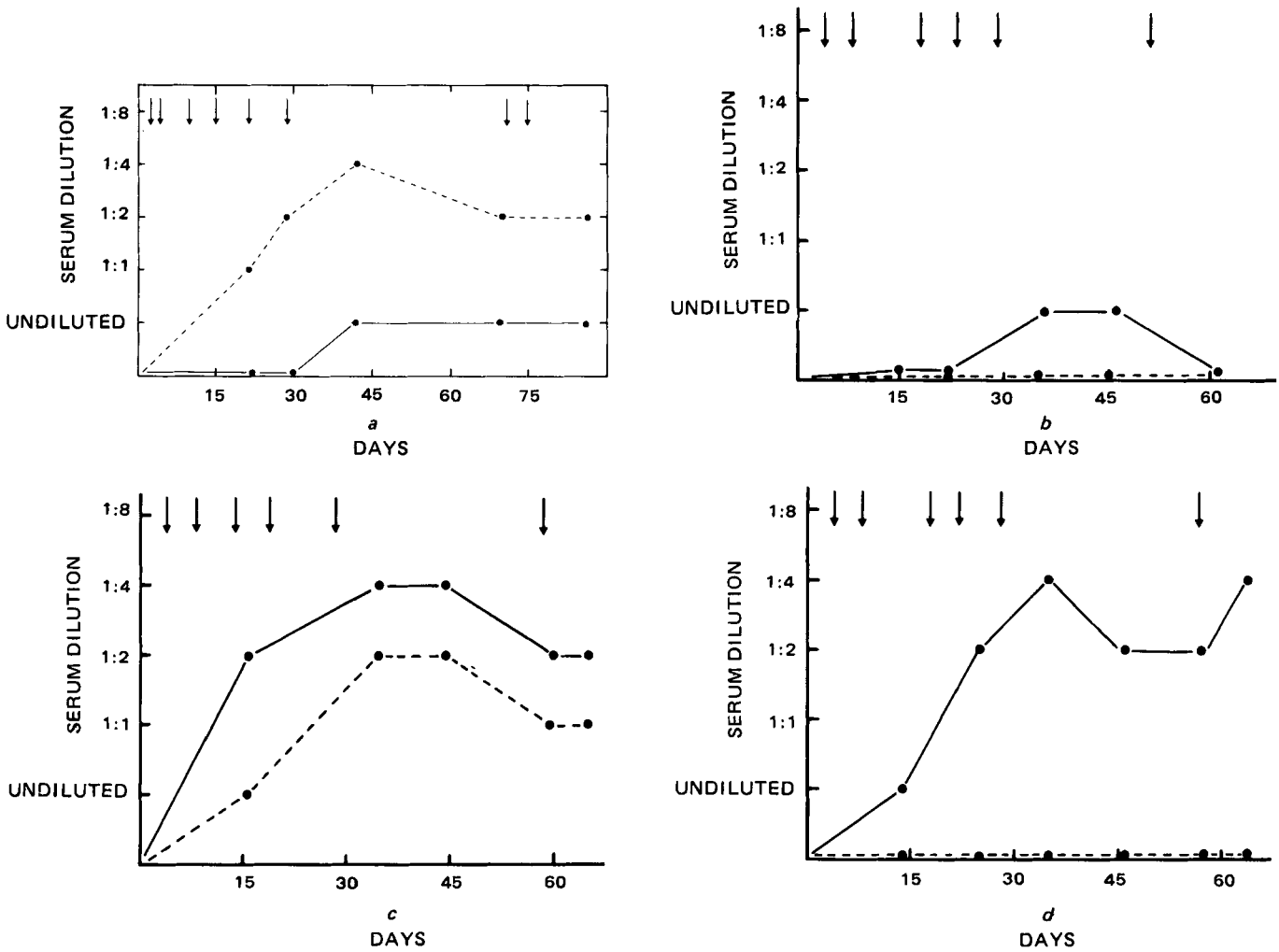

Figure 1—*Antibody response in mice after immunization with* L-*asparaginase in microparticles or in soluble form. 5 IU of* L-*asparaginase was administered on the days indicated by arrows at the top of the graphs. The antibody titer of the serum is measured by Ouchterlony double diffusion as described under* Materials and Methods. *(a)* L-*Asparaginase was given im/sc in microparticles (●—●) or in free form (●- - -●), with Freund's complete/incomplete adjuvant in the back of mice*[10]. *(b)* L-*Asparaginase was given im/sc in microparticles (●—●) or in free form (●- - -●), in 0.1 ml of physiological saline in the back of mice*[9]. *(c)* L-*Asparaginase was given ip in microparticles (●—●) or in free form (●- - -●) in physiological saline in mice*[10]. *(d)* L-*Asparaginase was given iv in microparticles (●—●) or in free form (●- - -●) in physiological saline in mice*[9].

muscle, were used without further purification. Acrylamide[3], *N,N'*-methylenebisacrylamide[3], *N,N',N',N'*-tetramethylethylenediamine[2], tromethamine[2], α-ketoglutaric acid[2], nadide[2] (NADH, reduced form), L-asparagine monohydrate[4], Nessler's reagent[2], and other chemicals were of analytical grade. L-Asparaginase[5] from *Escherichia coli* was used without further purification.

Preparation of Microparticles—Microparticles[6] (TC% = 8–25[7]) with immobilized L-asparaginase were prepared according to a reported method (5) by emulsion polymerization of acrylamide and bisacrylamide with L-asparaginase. In a typical example, the aqueous phase was composed of 5.0 ml of acrylamide (6% w/v) and *N,N'*-methylenebisacrylamide (2% w/v) in 0.005 *M* phosphate buffer, pH 7.4.

The protein was dissolved in the acrylic monomer solution. After the addition of 100 μl of ammonium peroxydisulfate (500 mg/ml in water) to the water phase, it was immediately homogenized with 200 ml of chloroform–toluene (1:4) containing a detergent[8], polyoxyethylene–polyoxypropylene (0.5 g), to produce a w/o emulsion. The polymerization was initiated by adding 1.0 ml of *N,N,N',N'*-tetramethylethylenediamine to the emulsion. The microparticles were isolated by centrifugation, washed several times with buffer, and after the last washing suspended in physiological saline. The mean diameter of the microparticles was ~0.25–0.30 μm.

[3] Eastman Kodak Co.
[4] Merck Co.
[5] Crasnitin was obtained as a gift from Bayer (Sverige) AB, Stockholm.
[6] U.S. pat. 4,061,966.
[7] The nomenclature suggested by Hjertén (1962) (14).
[8] Pluronics F-68, Ugine Kuhlman, Paris, France.

Assay of Native and Immobilized L-Asparaginase—L-Asparaginase activity was determined by direct nesslerization of the ammonia produced by its reaction with L-asparagine (5, 15). The absorbance was determined at 500 nm. Enzyme and substrate blanks were included in all assays. A standard curve was prepared with ammonium sulfate. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1.0 μmole of ammonia/min at 37°.

Determination of L-Asparagine—Assay of serum of L-asparagine was performed according to a reported fluorometric method (16). Blood was taken from the tail vein or the orbital plexus of 4 to 5 mice and centrifuged at 4° for 15 min to obtain serum. The serum samples were pooled to obtain 60 μl of serum and carefully mixed with 150 μl of 5% trichloroacetic acid. After centrifugation at 4° for 10 min, the supernate was extracted three times with diethylether and the L-asparagine was analyzed.

Animals—Male mice[9,10] were used throughout. At the start of the experiments they weighed 20 g.

Immunization Procedures—Groups of 10 mice were injected with L-asparaginase (5 IU in microparticles or in soluble form) by different routes: intramuscular/subcutaneous, intraperitoneal, and intravenous. The enzyme was administered in physiological saline (intramuscular/subcutaneous, intraperitoneal, and intravenous) or in Freund's complete/incomplete adjuvant (intramuscular/subcutaneous). The animals received booster injections after 4, 8, 18, 22, 28, and 52 days. Blood samples were taken before the first injection and after 2, 4, 5, 8, and 10 weeks.

[9] NMRI-mice, Anticimex, Stockholm, Sweden.
[10] C3H-mice, Bomholtsgård, Denmark.

Table I—Arthus Reaction in Preimmunized Mice after Asparaginase Injections

Number of Mice	Immunization Procedure	Provoking Enzyme Preparation[a]	Mean Diameter ± *SD* of Left and Right Foot Pad[b], cm	Probability[c]	Mean Ratio ± *SD* of Foot Pad Thickness	Probability[c]
4	Soluble L-asparaginase im/sc[d]	Soluble Enzyme	0.36 ± 0.02 0.25 ± 0.01	<0.01	1.43 ± 0.17	<0.05
4	Soluble L-asparaginase im/sc[d]	Microparticles	0.37 ± 0.03 0.33 ± 0.01	>0.05	1.14 ± 0.10	
5	Microparticles im/sc[d]	Soluble Enzyme	0.37 ± 0.01 0.26 ± 0.01	<0.01	1.41 ± 0.08	<0.01
4	Microparticles im/sc[d]	Microparticles	0.37 ± 0.01 0.34 ± 0.01	>0.05	1.11 ± 0.06	
5	Soluble L-asparaginase ip	Soluble Enzyme	0.36 ± 0.01 0.26 ± 0.01	<0.01	1.39 ± 0.10	<0.01
5	Soluble L-asparaginase ip	Microparticles	0.33 ± 0.01 0.31 ± 0.01	>0.05	1.06 ± 0.06	
4	Microparticles ip	Soluble Enzyme	0.36 ± 0.01 0.23 ± 0.01	<0.01	1.58 ± 0.14	<0.01
3	Microparticles ip	Microparticles	0.33 ± 0.01 0.30 ± 0.01	>0.05	1.12 ± 0.03	

[a] Mice were challenged by injection into the left hind foot pad of either 10 μl of L-asparaginase (80 IU/ml) or 10 μl of immobilized L-asparaginase in microparticles (80 IU/ml, dry weight 23.1 mg/ml) and into the right food pad of 10 μl of physiological saline or 10 μl of microparticles not containing any enzyme (dry weight 23.1 mg/ml). [b] The thickness of the foot pads was measured 3 hr after provocation. [c] The significance of the difference was calculated by variance analysis. [d] These groups were previously injected with 50 IU L-asparaginase ip in microparticles or in soluble form.

The blood was collected from the tail vein or from the orbital plexus, pooled, and centrifuged at 4° to obtain serum. Each mouse received 35–45 IU of L-asparaginase during the study.

Antibody Determination—The antibody titer was assayed by double immunodiffusion in agar gel as described previously (17). The titer was determined by the highest dilution of serum which produced precipitation with the antigen (5 μg of L-asparaginase in 10 μl of physiological saline was placed in the center well).

Mice, pretreated with immobilized or native L-asparaginase by different routes of administration, were tested for the Arthus reaction (18). The enzyme, 10 μl containing 0.8 IU in soluble form or in microparticles (dry weight 0.23 mg), was injected into the left foot pad of the hind leg. The right foot pad, which served as control, was injected with the same volume, 10 μl, of microparticles not containing any enzyme, or with physiological saline.

Three hours later, and again at 24 and 48 hr, the thickness of both foot pads was measured with a micrometer gauge and the ratio between the two calculated.

Autoradiography—Whole body autoradiography was performed according to the method of Ullberg (19). ^{14}C-Labeled microparticles (4.1 mg; 400,000 dpm) were injected intramuscularly/subcutaneously in some mice with physiological saline in the back. After 2 months the animals were sacrificed with ether and frozen in hexane–solid carbon dioxide (−78°). The mice were embedded in a gel of carboxy-methylcellulose and cut into 20- or 60-μm sagittal sections with a microtome. The sections were cut at −15–20° and freeze-dried. The slices were pressed against photographic films which were exposed for up to 6 months.

RESULTS

Antibody Production in Mice after Treatment with L-Asparaginase in Microparticles—L-Asparaginase was immobilized in microparticles as described previously. The microparticles were injected in mice by different routes and the antibody titers obtained were followed up to ~2.5 months by double immunodiffusion tests. The results are shown in Figs. 1*a*–1*d* and compared with the response obtained after treatment with the same amounts of free, soluble enzyme given under identical conditions.

Immobilization of L-asparaginase in microparticles of polyacrylamide did not prevent the formation of specific antibodies in intensively treated animals. However, it is evident from Fig. 1*a* that the immune response after intramuscular/subcutaneous injection in Freund's adjuvant is weak and significantly weaker than after injection of soluble enzyme. A weak precipitin line was detectable only in undiluted serum. Any antibody production after intramuscular/subcutaneous injection of soluble enzyme in physiological saline is not detectable (Fig. 1*b*), presumably as a consequence of the short half-life of the enzyme.

Figures 1*c* and 1*d* demonstrate the adjuvant effect of the microparticles

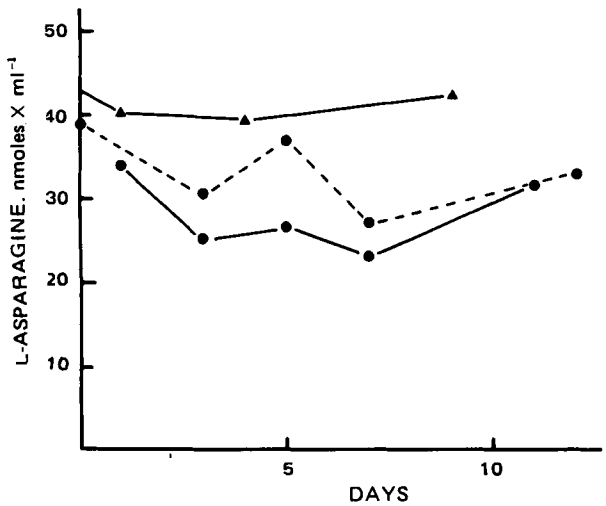

Figure 2—*L-Asparagine concentration in serum after a single ip injection of L-asparaginase (50 IU) in microparticles (●—●) or in free solution (●---●). Each point represents samples drawn from 4–5 mice[10]. The animals were preimmunized with L-asparaginase in microparticles injected im/sc in Freund's adjuvant. The normal L-asparagine concentration (▲—▲) was obtained from a control group given only physiological saline.*

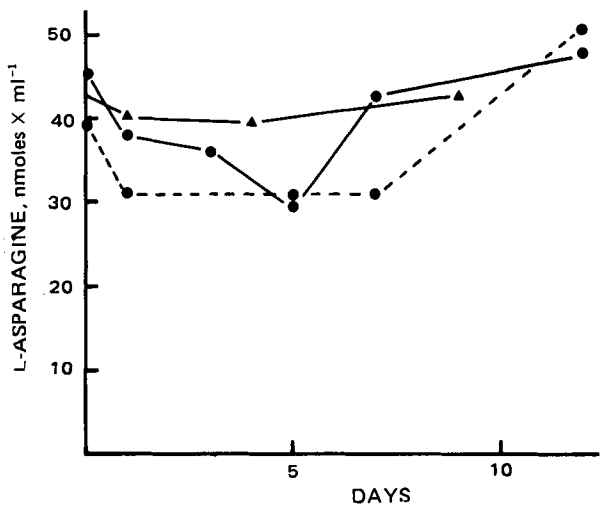

Figure 3—*L-Asparagine concentration in serum after a single ip injection of asparaginase (50 IU) in microparticles (●—●) or in soluble form (●---●). Each point represents serum samples drawn from 4–5 mice[10]. These animals were preimmunized with free L-asparaginase injected im/sc in Freund's adjuvant. The normal L-asparagine concentration (▲—▲) was obtained from a control group given only physiological saline.*

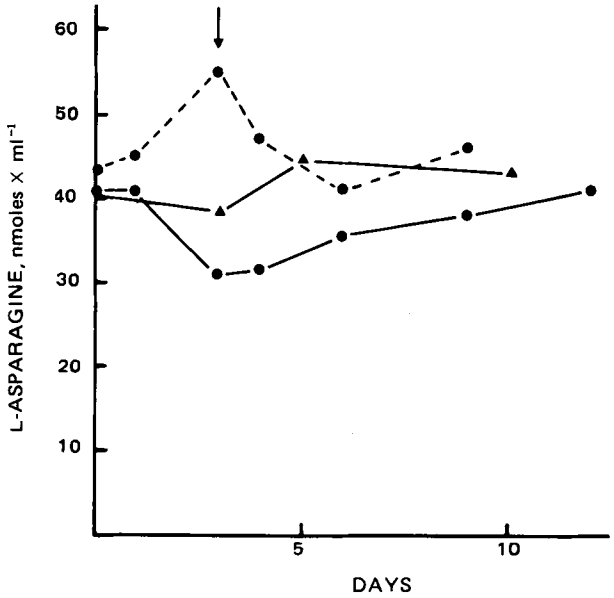

Figure 4—*L-Asparagine concentration in serum. Each point represents pooled samples from 4–5 mice[10] given im/sc 2 × 20 IU of* L*-asparaginase (with an interval of 3 days) in microparticles (●—●) or in free solution (●---●). The animals were preimmunized with* L*-asparaginase in microparticles injected ip. The arrow shows the time for the second injection of 20 IU* L*-asparaginase in microparticles or in free form. The normal* L*-asparagine concentration (▲—▲) was obtained from a control group given only physiological saline.*

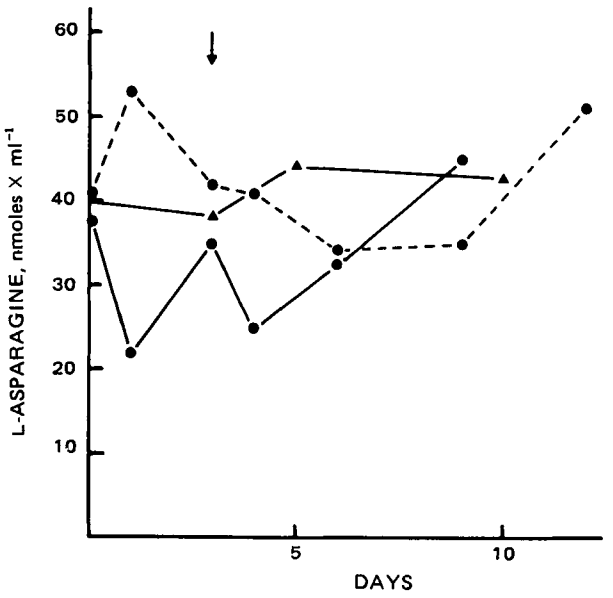

Figure 5—*L-Asparagine concentration in serum. Each point represents pooled serum from 4–5 mice[10] given im/sc 2 × 20 IU of asparaginase (with an interval of 3 days) immobilized in microparticles (●—●) or in free form (●---●). The animals were preimmunized with free* L*-asparaginase given ip. The arrow shows the time for the second injection of 20 IU* L*-asparaginase in microparticles or in free form. The normal* L*-asparagine concentration (▲—▲) was obtained from a control group given only physiological saline.*

administered as intraperitoneal and intravenous injections. After intraperitoneal injection of L-asparaginase in microparticles, a rapid and high production of specific antibodies was seen and in addition, high titers were obtained after intravenous injection. Intravenous injection of native L-asparaginase did not produce any antibodies which could be detected by immunodiffusion, while intraperitoneal injection was effective in producing high amounts of antibodies. The abdomen is drained by the lymphatic vessels, and it is obvious that the strong response is a consequence of the administration of the L-asparaginase close to the lymph nodes.

Studies with a Modified Arthus Test—Under standardized conditions the level of circulating specific antibodies can be estimated in different Arthus tests, in which an antigen–antibody complex mediated hypersensitivity reaction is provoked in different tissues.

Table I presents results from an Arthus reaction test in the foot pads of mice immunized in different standardized ways. The reaction is expressed as the ratio of the thickness of the foot pad injected with the sample (L-asparaginase in soluble or immobilized form) to that of the foot pad injected with empty microparticles or with physiological saline. Mice treated with L-asparaginase intraperitoneally and intramuscularly/subcutaneously were used for the Arthus reaction. Independent of the state of the antigen (immobilized or soluble) with which the mice were immunized, all mice gave an Arthus reaction 3 hr after injection when both native and immobilized L-asparaginase had been used. The swelling at the sites of injection were retracted after 24 hr. L-Asparaginase in the microparticles gave the same or smaller response (thickness of the left foot pads) than the same dose of soluble enzyme. However, the reaction was partly unspecific, in the sense that the microparticles themselves produced an increase in the size of the foot pads, which was significantly larger than that produced by physiological saline. In fact, the response given by the enzyme in microparticles was statistically not significantly larger than that given by the particles themselves. Consequently, the response measured as the ratio of the thickness of the two foot pads was significantly lower with the enzyme administered in microparticles than with soluble enzyme. This means that the antigenicity of the enzyme is decreased by immobilization in microparticles.

L-Asparaginase Effect in Preimmunized Mice—The capacity of differently immunized mice to neutralize and inhibit the effect of L-asparaginase administered in different ways has also been studied. Figures 2–5 show the results obtained from mice in which the serum concentration of L-asparagine was followed as a measure of the L-asparaginase activity and duration. Normally, intramuscular injection of 2 × 2.5 IU of L-asparaginase in microparticles or 5–10 IU injected intravenously depresses the L-asparagine to almost 0 values for 5–7 days, but in the preimmunized animals, considerably higher doses have to be used for any effects on the L-asparagine. Even 50 IU of the enzyme produced only a small and transient effect. Figure 2 illustrates the effects obtained after intraperitoneal injection of 50 IU of L-asparaginase in microparticles or in native form into mice previously treated with free L-asparaginase by the intramuscular/subcutaneous route. Only small effects on the serum level were obtained and no significant differences could be detected between free and immobilized enzyme. Similar results were obtained when the same amount of enzyme in microparticles or in soluble form was injected intraperitoneally into mice pretreated with microparticles given by the intramuscular/subcutaneous route (Fig. 3). A 10–20% reduction of the serum concentration of L-asparagine was achieved the day after injection, but the level was soon normalized.

The results shown in Fig. 4 illustrate the effect of enzyme in microparticles and native enzyme given intramuscularly/subcutaneously in two doses (2 × 20 IU, with an interval of three days) into mice previously treated with immobilized L-asparaginase in microparticles by the intraperitoneal route. Injection of free enzyme did not produce any depression of the serum level of L-asparagine, while some reduction of the serum concentration was achieved when the particles were given intramuscularly/subcutaneously.

Figure 5 shows the changes seen after intramuscular/subcutaneous injection of 2 × 20 IU of L-asparaginase in free or immobilized forms into mice previously treated with native L-asparaginase intraperitoneally. In this case, injection of immobilized enzyme produced lower levels the day after injection, but they were normalized again after 3 days. The same procedure was repeated after the second injection; low values (25–30 nmoles) were found the day after injection, which were normalized again after 2–3 days. Intramuscular injection of L-asparaginase in free solution did not affect the serum concentration of L-asparagine at all.

Whole Body Autoradiography after Intramuscular Subcutaneous Injection of Microparticles in Mice—Figure 6 shows the distribution of C^{14}-labeled microparticles 2 months after intramuscular/subcutaneous injection. The particles were encapsulated at the injection site, and no radioactivity could be detected in the liver or spleen.

DISCUSSION

The microparticle is a macroporous bead in which the immobilized macromolecules are partly entrapped, partly fixed in the polymer bundles forming the network (20). Consequently, a fraction of the immobilized molecules, and their antigenic determinants, will be exposed on the

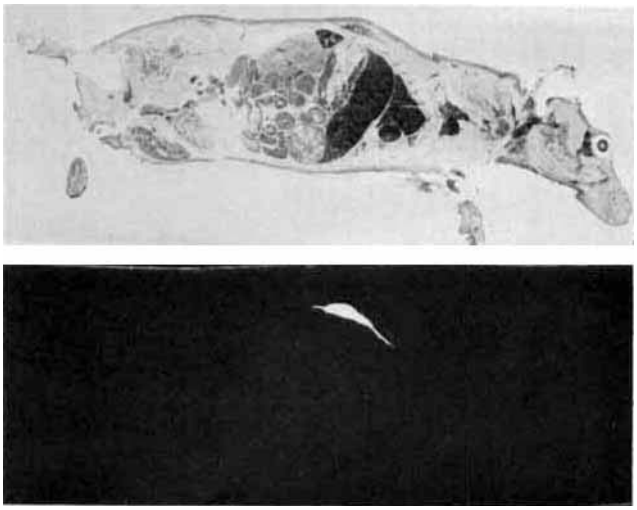

Figure 6—*Whole body autoradiogram of a mouse 2 months after im/sc injection of C^{14}-labeled microparticles (400,000 dpm in 4.1 mg). Bottom: Corresponding tissue section 20 μm.*

surface of the microparticles, and this fraction will be larger, the smaller the beads are. The polyacrylamide forming the microparticle is not immunogenic itself. Therefore, it is to be expected that the immunological response provoked by the immobilized proteins will be determined by the immunogenic properties of the exposed macromolecules and, providing the microparticle has no adjuvant effect, will be related to the size of the particles and will be smaller than that exerted by the native macromolecule administered under the same conditions. As shown, this hypothesis holds true when L-asparaginase is injected intramuscularly with Freunds adjuvant. As a result, soluble enzyme slowly diffuses out from the oil depot with a strong immunological response, which is amplified by the adjuvant. On the other hand, the microparticles stay at the site of injection, as shown by the autoradiogram, and are not reached by the immunocompetent cells to any significant degree. Consequently, there is a low immunological response. When the microparticles are given in saline, the response is quantitatively the same, while the soluble L-asparaginase is rapidly distributed and degraded with poor reaction as a result. The reaction in the two strains of mice used is the same in this respect.

After intraperitoneal administration of L-asparaginase in microparticles, high antibody production could be seen both in the diffusion test and in the modified Arthus reaction, which was stronger than with soluble enzyme. This is a surprising result, as it is known that the particles are rapidly cleared from the abdomen and the circulation and taken up by cells belonging to the reticuloendothelial system in bone marrow, liver, and spleen (21), and thereby are prevented from a primary reaction with the immune system. However, it is apparent that the rapid uptake in the macrophages triggers a secondary response mediated *via* some lymphocyte activating factor (LAF as found in ref. 22). In this sense the microparticles may function as an adjuvant, considerably improving the immunological response.

The distribution of the microparticles after intravenous injection is the same as after intraperitoneal injection, and the antibody production is consequently the same. However, the immune response after intravenous injection of free L-asparaginase is significantly impaired and no antibodies could be detected in the double immunodiffusion tests. The $t_{1/2}$ of the enzyme is short and the direct effect on the lymph system is not obtained in the same way as after intraperitoneal administration.

The modified Arthus test employed gives information not only about the antibody production in the mice, but also, in this modification, about the antigenicity of the L-asparaginase in soluble and in immobilized form. The antibody-mediated reaction on injection of different samples in the foot pads is estimated from the degree of the edema produced, when the sample is injected in one foot and the appropriate control in the other. It can be seen in Table 1 that the injections of microparticles with L-asparaginase produce a much smaller reaction than native enzyme, despite the fact that the unspecific reaction from the microparticles themselves is stronger than from physiological saline. Thus, it can be concluded that the antigenicity is decreased as a consequence of the immobilization in the microparticles.

Earlier studies have shown that the optimal way of administration of immobilized L-asparaginase is the intramuscular/subcutaneous route, when the duration of the effect on the L-asparagine level *in vivo* is considered. The present results also indicate that the intramuscular injection is the preferred way of administration as far as the immunological response is concerned, giving the smallest antibody production. In addition, the effect of L-asparaginase given in microparticles as intramuscular/subcutaneous injection in immunized animals is more pronounced than of intraperitoneal injection. Thus, 50 IU of L-asparaginase given intraperitoneally gave a slight decrease in the L-asparagine level, while an intramuscular/subcutaneous 2 × 20 IU dose slightly decreased the level for 5–7 days.

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ACKNOWLEDGMENTS

This work was supported by the Swedish Board for Technical Development (project no 78-3614) and the I.F. Foundation for Pharmaceutical Research, Stockholm.

The technical assistance of Miss Siv Larsson and Mrs. Linnéa Wallsten was appreciated.