strate the existence of both antibody and CMI responses to *A. americanum*[2], *R. sanguineus*[2], *R. appendiculatus*[13], *Ixodes holocyclus*[13] and *D. variabilis*[14], but only CMI to *D. andersoni*[15] and antibody mediated immunity to *B. microplus*[16].

The observation that young guinea-pigs are better responders to tick infestation than older mature animals suggests age-dependent depression of immune responsiveness. A similar phenomenon has been described in other vertebrates associated with the depression of specific subpopulations of T-lymphocytes (CMI)[17]. Resistance to *A. americanum* involves both cell and antibody-mediated reactions[2-4,6], therefore, the importance of antibody in this observed age-dependent decrease in immune responsiveness should not be overlooked. The finding of an age-dependent immunity in guinea-pigs to ticks is critical to our studies aimed at elucidating the hosts immune response to ectoparasites; where high titered antibodies are needed as probes for antigens, and where large populations of highly specific lymphocytes are used for in vitro assays. These findings support our initial prejudice that young guinea-pigs are immunologically more reactive to tick feeding than older mature individuals, and presents another variable that must be considered in evaluating host resistance to ticks.

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Monoclonal antibodies to L-asparaginase

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Summary. Five hybridomas secreting monoclonal antibody to *E. coli* L-asparaginase were isolated. These monoclonal antibodies were classified into 3 different subclasses; Ig G_1 (1 clone), Ig G_2 (2 clones) and Ig G_3 (2 clones). One of them possessed anti-L-asparaginase neutralizing activity. Four antibodies examined demonstrated a linear Langmuir binding plot and binding affinities, with equilibrium dissociation constant (K_d) ranging between 2.5×10^{-9} M and 6.3×10^{-10} M. The monoclonal antibodies should be useful probes for investigation of the enzyme activity.

There are several reports of the preparation and exploitation of documented monospecific antibodies against enzymes such as phenylalanine hydroxylase[1], glucose-6-phosphate dehydrogenase[2], acetylcholinesterase[3], urokinase[4] and phosphofructokinase[5].

This paper describes the isolation and characterization of monoclonal antibodies against *E. coli* L-asparaginase. This is the first report describing the preparation of monoclonal antibody to L-asparaginase.

Materials and methods. Enzyme assay: Enzyme solution (0.1 ml) is added to sodium borate buffer (0.1 M, pH 8.5) to give a volume of 1.5 ml. The reaction is started by the addition of 0.5 ml of L-asparagine monohydrate (0.04 M) and allowed to proceed for 30 min at 37 °C. The incubation is stopped by adding 0.5 ml of 15% trichloroacetic acid, and the mixture if centrifuged. The supernatant is transferred quantitatively to 10-ml graduated test tubes, and diluted to 9 ml with water. 1 ml of Nessler's reagent is then added, and the mixture is allowed to stand for 15 min at room temperature. Ammonia is estimated by determining the optical density at 500 nm. Enzyme and substrate blanks are incubated in all assays[6].

Immunization protocol: Five male BALB/c mice, 2 months of age, received i.p. injections of L-asparaginase (500 units, Kyowa Hakko Kogyo Co.) with complete Freund's adjuvant on days 0, 10, 20, and 30 and i.v. injections of 300 units of enzyme on day 40. The mice were killed 3 days later for somatic cell hybridization.

Cell fusion protocol: Lymphocyte suspensions from spleens of immunized mice were prepared by gentle teasing, and the red blood cells were removed by NH_4Cl (0.83%). In each fusion experiment, spleen cells (1×10^8 cells) were mixed with mouse myeloma cells (P3 × 63Ag8, 1×10^7 cells) and co-pelleted with 30% of polyethyleneglycol (PEG 1000) for a total of 8 min at room temperature. Pellets were washed with Dulbecco's modified Eagle's medium (DMEM) to remove PEG. The pellets containing fused cells were gently resuspended in a small volume of DMEM-HAT medium (10% fetal calf serum, penicillin, streptomycin, nonessential amino acids, glucose, glutamine,

Antibody	Subclass	Binding activity (K_d)	Neutralizing activity
84	Ig G_1 (k)	1.8×10^{-9} M	–
116	Ig G_{2a} (k)	8.3×10^{-10} M	–
119	Ig G_3 (k)	2.5×10^{-9} M	–
280	Ig G_{2a} (k)	6.3×10^{-10} M	–
105	Ig G_3 (k)	+	+

100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine) and distributed into 96-well plates. After 2 weeks of incubation, hybridoma culture supernatants were tested for anti-L-asparaginase activity and positive cultures were cloned in HAT medium by limiting dilution into microtiter wells containing 10^5 normal mouse macrophages as a feeder layer. After the monoclonal cultures had become established, they could be gradually weaned off the HAT medium and stably propagated in DMEM plus fetal calf serum.

Screening of monoclonal antibody to L-asparaginase: Hybridoma culture supernatants were screened for anti-L-asparaginase activity by a solid phase immunoadsorption assay. Culture supernatants (0.1 ml) were preincubated with 50 µl of a 20% formalinized *S. aureus* suspension in 0.1 M Tris-HCl pH 8.2 for 15 min at 4 °C. The mixture was centrifuged for 3 min at 2000 × g, washed once, and resuspended in 0.2 ml of the same buffer. L-asparaginase (3 units) was then incubated with the treated *S. aureus* suspension for 1 h at 4 °C. The suspension was centrifuged, and both the supernatant and the resuspended pellet fractions were assayed for L-asparaginase activity. In the presence of specific antibody, asparaginase activity was removed from the supernatant fraction, and if the antibody was not neutralizing, most of that activity could be detected in the pellet fraction. In the absence of specific antibody, more than 90% of the initial L-asparaginase activity was recovered in the supernatant fraction.

Purification of monoclonal antibodies: The positive hybridomas were used to produce ascites fluid after i.p. injection of 1×10^7 cells into mice that had been primed by injection of 0.2 ml of pristane at least 3 days previously. Ascites fluid was collected with a syringe 20 days after hybridoma injection, centrifuged to remove cells, and stored at −70 °C. Homogeneous monoclonal antibody was obtained after chromatography on protein A-sepharose at 4 °C. Samples were loaded onto the column in 50 mM Tris-HCl, pH 8.6, 150 mM KCl and the monoclonal Ig Gs were eluted in 50 mM Na acetate, pH 4.0, 150 mM KCl, concentrated by ammonium sulfate precipitation and finally suspended by dialysis into 50 mM KPO_4, pH 7.2, 150 mM NaCl.

Class of monoclonal antibodies: To determine the class of the monoclonal antibody, Ouchterlony analysis was performed using anticlass, anti-κ and anti-λ rabbit anti-mouse antisera.

Langmuir plots of monoclonal antibodies: After preincubation of L-asparginase with various Ig G species, a suspension of formalinized *S. aureus* (20%) was added to a final concentration of 10% and further incubated for 20 min at 4 °C. The immune complexes were then sedimented at 2000 × g for 5 min, and the supernatant and pellet fractions were assayed for L-asparaginase activity. Control incubations containing no Ig G were processed identically.

Results. Screening of positive hybridomas: After hybridization, 10 out of 348 wells were found positive for anti-L-asparaginase activity using the solid phase binding assay. Out of these 10 clones, 5 clones, 5 clones showing high anti-L-asparaginase antibody activity and giving stable lines were selected, expanded in suspension culture and grown as ascites in mice for mass production of antibodies.

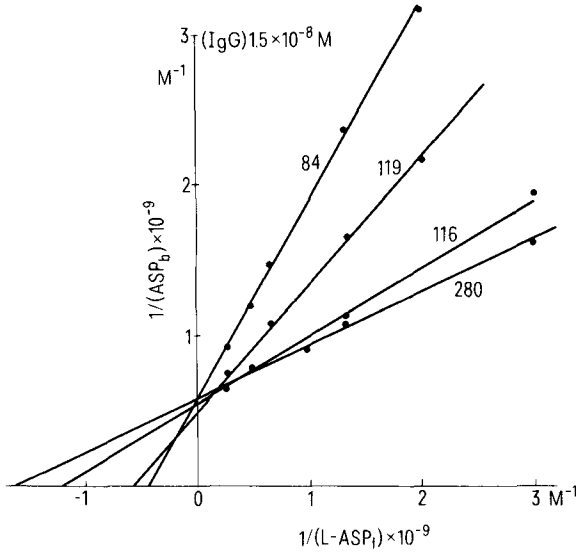
Properties of the monoclonal antibodies to L-asparaginase: Physical properties of the monoclonal antibodies are summarized in the table. These monoclonal antibodies are classified into 3 different subclasses Ig G_1 (1 clone), Ig G_{2a} (2 clones) and Ig G_3 (2 clones) with κ-chains.

Kinetic properties of monoclonal antibodies on L-asparaginase: Langmuir plots are linear indicating that each antibody recognises a single class of binding site with respect to L-asparaginase (fig.).

Discussion. The molecular weight of L-asparaginase from *E. coli* A-1-3 (Kyowa) is approximately 141,000 as determined by sedimentation equilibrium. The enzyme is composed of 4 subunits (mol. wt 34,100). An apparent K_m for L-asparagine is 1.9×10^{-5} M and an isoelectric point is approximately 4.75[7].

In this paper, we have described our establishment of 5 stable murine monoclones which produce homogenous Ig G molecules that specifically recognize L-asparaginase, as assessed by a sensitive binding assay. One of 5 monoclonal antibodies exhibits inhibitory activity against L-asparaginase. All of the 4 antibodies with which quantitative binding studies have been performed to date generate the theoretically predicted linear Langmuir plots and demonstrated dissociation constants (K_d) ranging between 2.5×10^{-9} and 6.3×10^{-10} M. These binding antibodies are homogeneous and possess a single affinity for a single antigenic determinant on the L-asparaginase molecule. The catalytic activity of L-asparaginase is unaffected by preincubation with each of the binding antibodies, this suggesting that none of the monoclonal antibodies interacts with the catalytic sites of the enzyme protein.

These antibodies provide sensitive tools in the analysis of enzyme structure, genetics and related fields.



Langmuir plots of anti-L-asparaginase antibodies. L-ASP_b, antibody bound L-asparaginase; L-ASP_f, free L-asparaginase; Ig G, 1.5×10^{-8} M.

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