ASCARIS SUUM: **PARTIAL FRACTIONATION OF METABOLIC ANTIGENS FROM** IN VITRO **CULTURED LARVAE.**

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Ascaris suum *metabolic antigens were obtained from second and early third stage larvae cultured* in vitro *in supplemented Eagle's minimum essential medium. Metabolic antigens harvested after 12 and 16 days from* in vitro *cultures were eluted through Bio-Gel Al.5.*

Three main elution peaks were identified, dialysed, lyophilized and injected into mice with 4% sodium alginate. Peak II from elution of two preparations of metabolic antigens protected mice against a challenge infection of 10,000 A. suum *embryonated eggs.*

INTRODUCTION

Ascaris suum larval metabolic fluids have been shown to be good protective antigens when injected into mice, prior to an infection (13, 14, 15). Because of the complexity of the medium in which the larvae have to be grown (23) there might be a diminution of the immune response due to the presence of competing non-protective immunogens.

It is well known that the simultaneous exposure of an animal to several antigens decreases the immune response to some of the antigens in the mixture (1, 2, 11, 12). It is, therefore, desirable to separate a complex antigenic mixture into its components. One of the preferred methods with the least biological damage to the antigens is fractionation on the basis of molecular size differences using filtration through a column of agarose gels. These are more rigid than many other gels and have low compressibility so that a good flow rate and reproducible separation might be insured (18).

The present paper concerns attempts to isolate the *A. suum* metabolic larval intigens from the culture medium using exclusion chromatography.

MATERIALS AND METHODS

Animals. One hundred and ten albino Swiss mice averaging 22.2 ± 1.5 gm in weight were divided into 11 groups of 10 animals each.

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Preparation of Ascaris suum eggs. Eggs were embryonated following the method previously described by Guerrero and Silverman (14).

Preparation of antigens. Larval metabolic antigens of *A. suum* were collected from *in vitro* cultures (23). Embryonated eggs were deshelled using the method of Haskins and Weinstein (16), hatched by the method of Jaskoski and Colucci (20), and cultured in sterile bottles in an atmosphere of CO_2 at 37°C for 12 and 16 days. Eagle's minimum essential medium (MEM) plus 10% antibiotic mixture (both from Grand Island Biological Co; 3175 Staley Road, Grand Island, N.Y. 14072) and 10% pig serum was used in the culture medium.

On day 12 and 16 the cultures were terminated and the number of dead and live larvae assessed and expressed as a percentage of the total. The total number of larvae in culture in the batch that was maintained *in vitro* for 12 days was 36,000, of which 70% were in the early third stage, showing 35% viability (i.e. activily moving). The larval batch that was cultured for 16 days had a total of 34,000 larvae, 90% of which were in the early or midthird stage according to the criteria of Douvres and Tromba (7); this culture showed a 32% viability.

Once the concentration was ascertained the larvae in the culture medium were sedimented at 1600 g for 10 minutes in a refrigerated centrifuge. Immediately after centrifugation the supernate medium was applied to the gel in the chromatography column.

Column chromatography. Bio-Gel A1.5, 100-200 mesh, was obtained fully swollen in Tris EDTA buffer 0.001M with 0.02% sodium azide from Bio-Rad Laboratoires (Richmond, California). It was handled as described by Hjertén (17). A Pharmacia chromatography column, K2.5/45cms. was used after washing several times with deionized distilled and 70% ethanol.

After drying, a gel bed of 42 cm. in height was poured into the column utilizing the device described by Flodin (9). This gel bed was subsequently washed with 500 ml of Tris HCl 0.1M at a 7.4pH to eliminate the sodium azide. The same buffer was used as an eluent. All the chromatography experiments were carried on at 4°C. The approximate separation range of Bio-Gel A1.5 for proteins is between ten thousand and 1.5 million molecular weight (6). A fraction collector (Gilson Medical Electronics, Middleton, Wisconsin) was used to collect fractions every 3 ml of elution. Separation of the original compound was determined by scanning the fraction through a Beckman, DB Spectrophotometer at 260 nm (for nucleid acids) and 280 nm (for proteinaceous materials) (21). Samples to be separated were applied following the method described by Determan (6), and were all in a 10 ml volume.

The efficiency of the gel filtration technique and the baseline of elution was first assessed by eluting 10 ml of MEM plus 10% pig serum and 10% antibiotic mixture (supplement), then the 36,000 larval equivalents (LE) of 12 days *A. suum* metabolic larval antigens in 10 ml MEM and supplement, and the 34.000 LE of 16 day *A. suum* metabolic larval antigens in 10 ml MEM and supplement were eluted. Once the fractions that formed an elution peak were identified, they were pooled, dialyzed against demineralized H_2O, lyophilized and stored at 4°C until used. Three major peaks (I, II, and III) were identified at 260 and 280 nm.

Estimation of protein concentration. The concentration of proteins in each antigenic fraction and in the medium used as control was estimated using the method by Lowry *et al.* (24).

Adjuvant and immunizing schedule. Four percent sodium alginate (Algivant Colab, Laboratoires, Inc., Chicago Heights, Illinois) was used in doses of 0.5 ml per animal. Injections were administred intraperitoneally on days 0 and 14.

Challenge inoculation procedures. All animals except the uninoculated control group, were challenged with dose of 10.000 embryonated *A. suum* eggs. The animal were inoculated *per os* on day 21 (i.e. 7 days after the second immunizing injection).

Lung scoring technique. The mice were killed on day 28 (i.e. 7 days after inoculation) by chloroforming, and the degree of infection was assessed by scoring visible

petechiae in the lungs. This method was devised by Brown and Chen (5), and was modified by Lehnert (22). It employs an arbitrary scale according to the number and size of hemorrhages on the lungs, grading them from 0 to 5. A lung score of zero represents completely normal lungs and a lung score of five represents complete hepatization of both lungs or death of the infected animals as a consequence of the challenge infection.

Statistical analysis. The lung scores were analyzed as completely Randomized Design with Bayes' least significant difference at approximately 5% probality (8).

Experimental design. Mice in groups 1 to 3 received two injections of adjuvant and 1800 LE of each *A. suum* metabolic antigen fraction from larvae cultured *in vitro* for 12 days and eluted through Bio-Gel Al.5 Mice in group 1 were injected with fraction I, and each mouse received 0.742 mg of protein per injection; mice in group 2 were injected with fraction II, and each mouse received 1.061 mg per injection; group 3 received two injections of fraction III and protein concentration of each injection per mouse was 0.445 mg.

Mice in groups 4 to 6 received two injections of adjuvant and 1700 LE of each *A. suum* metabolic antigen fraction from larvae cultured for 16 days *in vitro* and eluted through Bio-Gel Al.5. Mice in group 4 were injected with fraction I, and each animal received 0.870 mg of protein per injection; mice in group 5 were injected with fraction II, and each mouse received 1.485 mg of protein per injection; group 6 received two injections of fraction III and the protein concentration of each injection per mouse was 0.615 mg.

Mice in groups 7, 8, and 9 controls were inoculated with the products of elution of the medium and supplements through a Bio-Gel Al.5 column. Mice in group 7 were injected with fraction I and each mouse received 0.933 mg protein per injection; mice in group 8 were injected with fraction II, and each mouse received 0.976 mg of protein per injection; group 9 was injected with fraction III, and each mouse got 0.403 mg of protein per injection.

Mice in group 10 were inoculated twice with 8,000 embryonated *A. suum* eggs 21 and 7 days before challenge and were considered infection immunity control. Mice in group 11 were injected twice with saline and were kept uninoculated as a normal background control.

RESULTS

The Bio-Gel Al.5 fractionation patterns of medium plus supplement, and of the larval metabolic antigens in a complex medium are presented in Figures 1 and 2. Three major fractions are identified as indicated by their peak OD values at 260 and 280 nm and they were present in the complex medium, and metabolic antigens.

The concentration of proteins in each eluted fraction after concentration and reconstitution to 10 ml of volume is presented in Table I. The results indicate that most of the protein is eluted in fraction II.

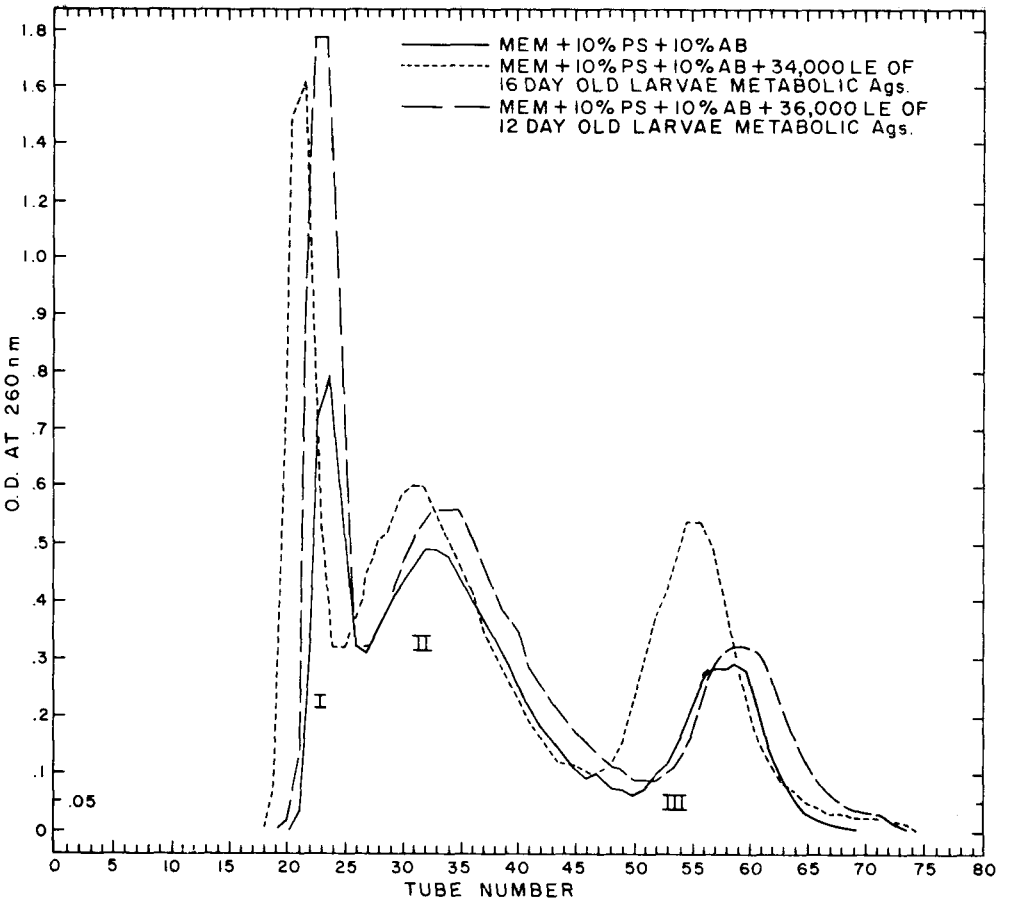
The results of the studies of immunogenicity of the fractionated *A. suum* larval metabolic antigens are presented as mean lung scores in Table II. Fraction II from elution of *A. suum* metabolic antigens from larvae cultured for 12 days *in vitro* conferred the best protection of all experimental groups that were immunized with injections of antigen.

Fibrosis of the liver was absent in animals from groups 1, 2, 3, 4, 5, 6, 7, 8, 9, and 11, and present in 4 animals from group 10.

DISCUSSION

The results obtained in this experiment show that fraction II from the elution of *A. suum* metabolic larval antigens through Bio-Gel Al.5. is biologically active and induces a good degree of immunity. There seems to be no marked difference in biological activity between fraction II from metabolites of larvae cultured *in vitro* for 12 or 16 days. This results confirm those found by Guerrero (13) using Sephadex G-200 as the gel for exclusion chromatography.

There is no significant difference (at the 5% level) between the mean lung scores of groups 2 vs. 5. There is a significant difference (at the 5% level) between the mean lung scores of groups 2 vs. 1, 3, 4, 6, 7, 8, 9, 10 and 11. In this particular experiment the best protection against the migration of *A. suum* larvae was conferred by previous infections.



Graph 1. Optical density readings at 260 nm following elution of three different samples through a Bio-Gel A1.5 column.

TABLE I

Total Concentration of Protein in 10 ml of Each Concentrated Antigens Fraction After Elution Through a Bio-Gel A1.5 Column.

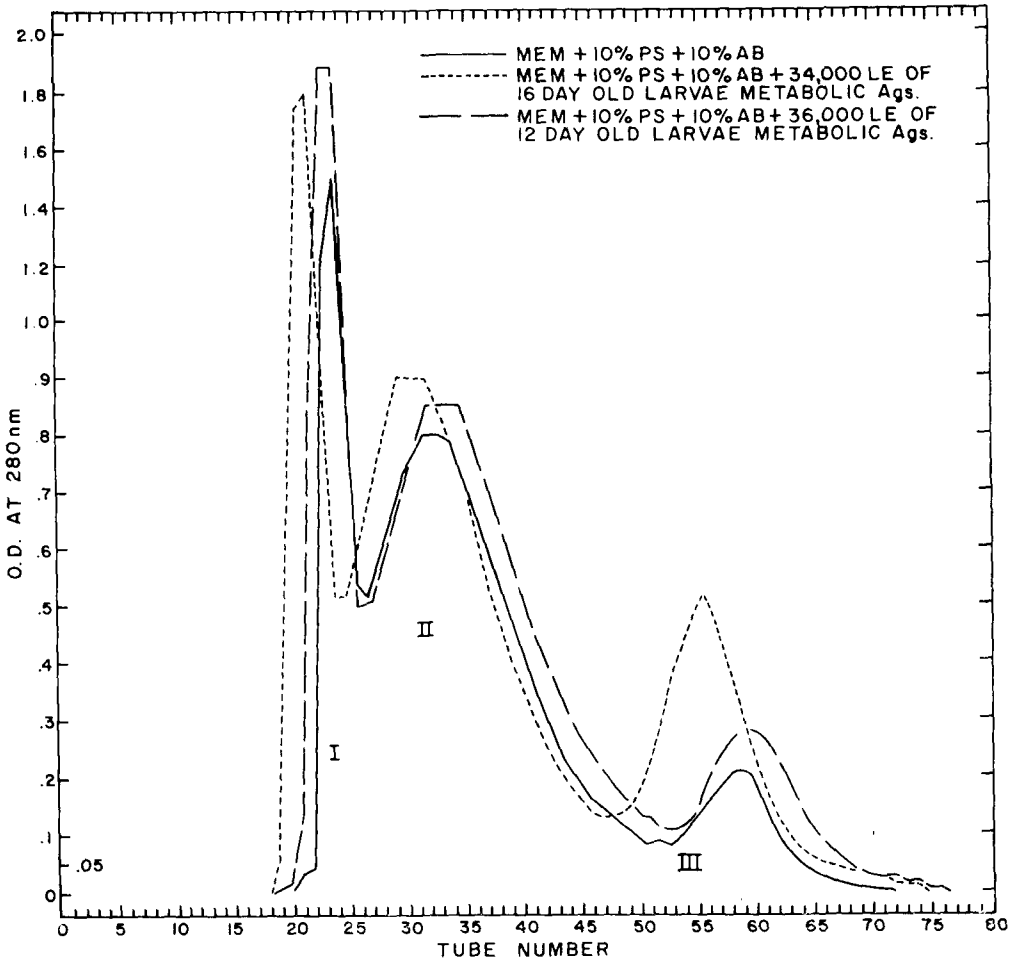
	Complex medium and 12 day metabolic larval antigens	Complex medium and 16 day metabolic larval antigens	Complex medium
Fraction I	14.85 mg	17.40 mg	18.67 mg
Fraction II	21.22 mg	29.70 mg	19.52 mg
Fraction III	8.91 mg	12.30 mg	8.06 mg

TABLE II

Lung Lesion Scores on Day 7 Following Inoculation of Mice with Embryonated *Ascaris suum* Eggs

Group Nº	Treatment	Mean Lung Scores *
1	Fraction I (Bio-Gel Al.5m) of 3,600 LE of metabolic antigens from 12 day old larvae and adjuvant	2.5
2	Fraction II (Bio-Gel Al.5m) of 3,600 LE of metabolic antigens from 12 day old larvae and adjuvant	1.4
3	Fraction III (Bio-Gel Al.5m) of 3,600 LE of metabolic antigens from 12 day old larvae and adjuvant	3.0
4	Fraction I (Bio-Gel Al.5m) of 3,400 LE of metabolic antigens from 16 day old larvae and adjuvant	2.4
5	Fraction II (Bio-Gel Al.5m) of 3,400 LE of metabolic antigens from 16 day old larvae and adjuvant	1.5
6	Fraction III (Bio-Gel Al.5m) of 3,400 LE of metabolic antigens from 16 day old larvae and adjuvant	2.1
7	Fraction I (Bio-Gel Al.5m) of MEM plus supplement and adjuvant (control)	3.1
8	Fraction II (Bio-Gel Al.5m) of MEM plus supplement and adjuvant (control)	3.4
9	Fraction III (Bio-Gel Al.5m) of MEM plus supplement and adjuvant (control)	2.7
10	Infection immunity control	0.3
11	Uninoculated (background) normal control	0.3

* Bayes least significant difference .51



Graph 2. Optical density readings at 280 nm following elution of three different samples through a Bio-Gel A1.5 column.

Bio-Gel Al.5 has been found to be satisfactory for the separation of biologically active macromolecules obtained from the metabolites of *A. suum* larvae cultured *in vitro*. The exclusion chromatography profile using this type of gel were completely reproducible, and the results obtained suggest that the molecular weights of the metabolic products correspond to materials with a molecular weight higher than 10,000 (minimum fractionation range from Bio-Gel Al.5) (3). According to Boyd (4) antigens must have more than a certain minimal degree of complexity and molecular weight; this minimal molecular weight in synthetic polypeptide antigens is as low as between 4 and 5 thousand (29). Hogarth-Scott (19) found that the allergic activity resided in the second peak in helminth allergens after filtration of *Ascaris* body fluid through Sephadex G-75, and that peak was found to be formed by molecules of more than 50,000 in MW. Oliver-Gonzales and Gadea (26) found 3 main peaks after filtration of the coelomic fluid of *A. suum* adults, through a Sephadex G-200 column.

Oliver - Gonzales *et al.* (27) utilized the first peak of this elution in the diagnosis of visceral larvae migrans. Utilizing an indirect hemagglutination test, they were able to relate positive serologic results with previous contact of the patients not only with the homologous antigen, but with the antigens from migrating larvae. They claim to have found the same type of antigens in third and fourth stage *A. suum* larvae. Unfortunately, these authors did not describe the way they collected these larval antigens or if they were used in protection trials. Ozerol and Silverman (28), found that the biologically active fractions of metabolites from third and fourth stage *Haemonchus contortus* larvae is the one formed by the heavy molecules that are eluted in the first peak after filtration through Sephadex G-200. They were able to separate further this active fraction into 3 more of different molecular weights but they did not test them to find the one responsible for the induction of resistance to the infection.

The doses of antigen given to the experimental animals were between 0.403 mg to 1.485 mg of protein per mouse. According to Mitchison (25) these should be considered actively immunizing doses in mice. Using BSA injected in mice, he found that low doses (1-10 μg) induced low zone paralysis, and that higher doses of the order of 100 μg to 1 mg immunize, while still higher doses (5-50mg) paralyse.

The culture system used in this experiment is suitable for the maintenance and development of *A. suum* larvae. Eagle's Minimum Essential Medium supplemented with 10% pig serum and 10% antibiotic mixture provided a good nutrient medium in which larvae grew and developed to a point where functional metabolic antigens were released. The viability of larvae at the end of the *in vitro* culture period, expresses the percentage of active larvae in the culture on the last day of incubation, but does not represent the number of developing larvae in the system during the period of culture. Because the culture medium is not sufficient to make larvae reach adulthood, when larvae reach the third stage most of them die due to lack of proper nutrients or perhaps stimulus. Levine and Silverman (23)) found that a protein was especially necessary for the development of the late second and early third stage of *A. suum* larvae; Douvres and Tromba (7) also found that the highest yields of advanced stages of *A. suum* were obtained when the medium was supplemented with swine kidney cells, calf or swine serum.

A disadvantage of the culture system utilized in this experiment is that the medium was not completely defined, and that the larvae needed the presence of 10% pig serum to survive and develop. Until *in vitro* cultures and metabolic antigen production is carried on in a completely defined medium, free of macromolecular supplements, the characterization of *A. suum* larval functional antigens will be a problem. An alternative way to solve this problem could be the elimination of these supplements by immunological or chemical methods.

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SUMÁRIO

Os antígenos metabólicos de Ascaris suum *foram obtidos de larvas de segundo e de terceiro estágio (precoce) cultivadas* in vitro *em meio mínimo essencial de Eeagle com suplemento proteico. Os antígenos metabólicos coletados depois de 12 a 16 dias das culturas* in vitro *foram eluidos em Bio-Gel Al 5.*

Três picos de eluição foram identificados, dializados, liofilizados e injetados em camundongos, com alginato de sódio a 4%. O pico II da eluição de duas preparações dos antígenos metabólicos protegeram os camundongos da infecção com 10.000 ovos infectivos de A. suum.

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