CHAPTER IV

DESCRIPTION OF EXPERIMENTAL MANOEUVRE

Materials and methods

Mice

In all experiments with mice, female Swiss mice were used of about 6 weeks of age. They were bred in the Institute.

Rabbits

New Sealand white rabbits (= 2.5 kg) were obtained from the Institutes The rabbits were infected orally with 100,000 embryonated eggs and killed seven days later. The lungs were collected and prepared using the Baermans technique to obtain migrating L3 larvae.

Parasites

Ascaris suum adult worms were collected in a pig abattoir. Females were used to obtain eggs directly from the uteri. Washed eggs were deposited in petri dishes containing 0,5% formaldehyde to let them embryonate and ripen at room temperature. Three weeks later the embryonated eggs were stored in Flashes at +4°C until use.

Ascaris lumbricoides adult worms were obtained from human patients in a large hospital in Hanoi. Collection of eggs, embryonation and storage occurred as mentioned above.

Hatching and collection of larvae

- Wash embryonated eggs three times at 1500 rpm during three minutes in distilled water to remove formaldehyde.
- Resuspend in 6% sodium hypochloride (NaOCl) and incubate in a 37°C waterbath to remove egg coat (check under microscope).
- Wash the decoated egg suspension immediately in distilled water until no chlorine odor is evident (at least 10 washings at 1500 rpm/3 minutes).
- Wash hatching slurry, three times in warm (37°C) Eagle's Minimal Essential Medium with Hank's Salts (HMEM) containing 100 units/ml penicillin and 250 μg/ml streptomycin.
- After the last rinse, eggs were transferred to a Potter- Elvehjem tissue Grinder fitted with a Teflon pestle ground to a 92 mm clearance with its glass steeve. Deshelling is accomplished by 25 strokes of the pestle stirring at approximately 1-2 r/sec driven by rotor.
- Cultures are prepared by placing a louse 0.5 high, absorbent cotton plug into a Pasteur pipette. The pipette's tip has been broken and fine polished so that the tapered protion of the pipette is only 1-2 cm long. After sterilization, the plugged pipette were transferred to sterile 16 mm x 125 mm plastic serene; cap culture tubes and FIG.
- were added to cover the upper surface of the cotton plug. Predetermined larvae were carefully pipetted on to the cotton plug. Too rapid addition of larvae-debris mixtures would force debris through the plug. Loosely capped cultures were placed on 37°C.

- After 48 hours the larvae or media were collected from the bottom of the culture tubes by first slowly moving the cotton-plugge pipette with the aid of a forcep.

In Vitro maintenance

The maintenance of both L2 and L3 larvae of either Ascaris species was carried out as described in detail earlier for Toxocara (de Savignu, 1976, Van Knapen et al., 1983). Since preliminary studies with L2 larvae resulted in a rapid death of the larvae within a week in the medium without serum, the culture fluid of the L2 larvae was enriched with 400 mg glycol-L- histidyl- 1-lysine acetate tetrahydrate (calbiochem) per 20 ml of culture medium (Stromberg et al., 1977), The actual maintenance of the larvae at 37°C and collection of supernatant fluid was carried out for approximately 3 weeks or shorter if the presence of dead larvae became obvious. As soon as the culture medium color turned from red into yellow, the supernatant fluid was collected and fresh medium was added. All supernatants were purled and stored at -20°C until use or futher preparation.

Production of somatic antigens

Somatic antigens were prepared as follows. The isolated larvae were lyophilized. Lyophilized material was pulverised in a matter and suspended in sodium carbonate buffer (0,1 M, pH 9,6) with 0,02 NaN_3 and treated by ultrasonic vibration (20 K Hz). The degree of homogenization was periodically checked by microscopic observation. The suspension was stirred at 4°C for two days, and then centrifuged at 45.000 g for 1 hour. The supernatant fluid was decanted and the protein content determined. The solution was stored at -20°C until use.

Enzyme linked immunosorbent assay (ELISA)

A sandwich ELISA was carried out with different antigens in order to demonstrate antibodies in the mouse sera. Somatic antigens prepared from the L2 and L3/4 larvae were diluted in sodium carbonate buffer (0,1 M, pH 9.6) until the protein content was 5 μg/ml. Coating of the micro-ELISA plates was carried out in 100 ul quantities at 37°C for 1 hour. The ES antigens were coated by overnight incubation at 37°C with undiluted culture fluid until complete evaporation had occurred. Before serial dilutions were added, the coated plate was washed for two times 30 seconds in an automatic washing device (Ruitenberg et al., 1978) with tap water containing 0.05 % Tween 20. Serial dilutions were prepared starting at 1:10 with PBS (0,01 M, pH 7.2) containing 2% bovine serum albumin (BSA) and 0,55% Tween 20. Incubation occurred at 37°C for one hour. After washing (as above), the conjugate was added, optimally diluted in PBS and 0.05% tween 20. Conjugate was a peroxides labeled rabbit - anti - mouse lgG commercially obtained from. Incubation was done for 1 hour at 37°C. After washing again the substrate was added consisting of 80 mg 5- amino salicylic acid (5-amino-2-hydroxyl benzoic acid) in 100 ml of destilled water adjusted to a pH of 6.0.

After incubation for one hour at room temperature, the plate was read with a Multiscan micro-ELISA photometer (t 449 nm) and Polaroid photographs were taken for visual interpretation.

SDS polyacrylamide gel-electrophoresis and immunoblotting

Antigen samples were electrophoretically separated by the method of Laemli (1970). After preparation of the 50 ul antigen samples by boiling for 5 minutes with 10 ul of SDS (10 %), 10 ul of destined water and 2-mercap-toethanol, they were put in top of the stacking gel. An electrophoretic run was

made with 60-100 voltage and a current of 20 MA in the stocking gel and 40 MA in the running gel. The total running time was approximately 3 hours until the bromiumphenolblue dye had reached the bottom. The separated proteins were transferred immediately to nitrocellulose sheets essentially as described by Towbin et al. (1979). It was carried out in a Trans Blot all of Bided, according to the manufacturers prescription. The transfer was made overnight with 0,1 KV and 1OMA. The staining procedure was carried out according to the Peroxides anti-peroxides method described by Glass et al (1981).

Experiment I

The course of migrating larvae of Ascaris suum in experimentally infected mice. In two consecutive experiments 22 and 40 mice respectively were orally infected with 2000 embryonated eggs (by incubation). Animals were killed at days 0, 3, 5, 7 and 14 in the first experiment, and at days 1, 2, 3, 5, 6, 7, 8 and 9 in the second experiment (groups of 5 animals per day). The materials collected for examination on migrating larvae were: total blood in heparine small intestine, liver, lung, heart, spleen, kidney's and brains. Examination on migrating larvae was done in a classical Baermann method after cutting the organ material into small pieces.

Experiment II

Immunisation of mice with somatic and excretory/secretory (ES) antigens of L2 and L3/4 larvae respectively.

Antigen preparation and immunization

An estimation of protein content was made by the Lowry method, with the somatic antigens, 0,1 mg protein per mouse was injected subcutaneously (at two sides) after mixing with Freund Complete Adjuvant (FCA) (vol.vol) at day 0. Freunds Incomplete Adjuvant (FIA) was used for the for the consecutive immunizations at day 14 and 28, Since no protein was detectable in the ES antigen, a 3 times concentrated culture medium (by lyophilisation) was mixed with FCA (vol/vol) for subcutaneous injection at day 0 and with FIA for the consecutive immunizations at days 14 and 28. The treatment schedule was as follow:

- Day 0: 20 animals for each antigen were injected,

  Day 14: 15 animals per antigen were boostered. 5 animals were bled for serum collection.

  Day 28; 10 animals per antigen were boostered, 5 animals were bled for serum collection.

- Day 42: The 40 remaining (immunised) animals and 10 control animals were orally infected with 2000 embryonated Ascarus suum eggs each.

- Day 45: 5 animals of each group, 4 challenge groups and one control group were killed by bleeding. The livers were collected to examin for migrating larvae.

- Day 49: The remaining animals were killed and bled. The lungs were collected for counting of migrating larvae.

Experiment III

Immunization of mice with ES antigen of Ascaris suum L2 or L3/4 larvae, and a combination of ES antigens.

Antigen preparation and immunization

ES antigen (culture fluid) of L2 and L3/4 larvae were lyophilized and concentrated 3 times. Then 0,3 ml. of antigen was thoroughly mixed with FCA (vol/vol) for subcutaneous injection at day 0, and with FIA for booster injections at day 14 and 28. A similar procedure was done with equal volumes of the mixture of both ES antigens. A control group was injected with similar volumes (0,6ml) of FCA and FIA alone at days 0, 14 and 28. A second control group was not pretreated at all but obtained an oral infection with 2000 embryonated A.suum eggs like all animals at day 42. Section of the animals was done at day 45 (5 animals per group) for blood and liver collection, and at day 49 (5 animals per group) for blood and lungs collection.

Experiment IV

Cross immunisation of mice with ES-antigens of Ascaris suum and Ascaris lumbricoides L2 larvae.

Antigen preparation and immunisation:

ES antigen culture fluid of L2 larvae was lyophilised and concentrated 3 times. The preparation with FCA, FIA and subcutaneous injections were similar to those in Experiment III. Groups with 10 mice each consisted of:

1. A.suum/ES antigen immunized, challenged orally with 2000 A.suum eggs.

2. A.suum/ES antigen immunized, challenged orally with 2000 A.lumbricoides eggs.
3. A.lumbricoides/ES antigen immunized challenged orally with 2000 A.lumbricoides eggs.
4. FCA/FIA stimulated, challenged orally with 2000 A.suum eggs.
5. FCA/FIA stimulated, challenged orally with 2000 A.lumbricoides eggs.
6. No immunisation, challenged orally with 2000 A.suum eggs.
7. No immunisation, challenged orally with 2000 A.lumbricoides eggs.

Section was carried out with 5 mice of each group at day 45 for blood and liver collection. The remaining 5 mice per group were killed and bled at day 49 for blood and lung collection.