PN-ABC-892

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 7/1/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. C7-162
semi-annual PR - mid 9/87 - 3/88

Attachment

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PN-ABC-892

ISN: 61845

US-ISRAEL CDR PROGRAM PROJECT C7-162

Grant No. DPE-5544-G-SS-7019-00

Towards an antigranuloma vaccine in schistosomiasis mansoni: molecular cloning and characterization of a putatively relevant antigen

1st Annual Progress Report

DECEMBER, 1988

FEB 24 1989

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Activity items reported herewith are listed according to the original items of the proposed work-plan, Phase A. In the 1st semi-annual progress report we have given a rather detailed account on these items. We have incorporated these details in the present annual report which provides updated results. We have also addressed problems and relevant problem-solving activities.

Phase A: FURTHER CHARACTERISATION OF MEG

A.1. Suitable antigens and antibody reagents

A.1.1. Rabbit antisera

The glycoprotein fraction of soluble egg antigen (SEA-gly) of S. mansoni was separated by lectin-affinity chromatography on con-A sepharose as previously described [1]. For immunization of rabbits 100 ug SEA-gly were incorporated with Freund's complete adjuvant and injected intramuscularely. Booster doses of 50 ug SEA-gly incorporated with Freund's incomplete adjuvant were administered twice at 3 weeks intervals. Rabbit serum anti-SEA-gly (R anti SEA-gly) was collected and stored at -20°C. SEA-gly were run electrophoretically on agarose gel against R anti SEA-gly for line immunoelectrophoresis as previously described by Kroll [2]. ^{125}I-labelled MEG, purified by ion exchange chromotography, was added as a tracer to identify MEG-containing precipitin lines as previously described [1]. Two precipitin lines were obtained one of which contained the bulk of radioactivity indicating that it was rich in MEG. This precipitin line was excised from the agarose, washed and injected to a

rabbit as previously described by Kroll [2], for producing rabbit anti-MEG (R anti MEG).

Following production of monoclonal antibodies (Mab) to MEG (see below) we have used an alternative method for producing R anti MEG. This procedure involved affinity purification of MEG on CNBr-Sepharose to which Mab KCSmeg1 (see below) was bound as previously described [3]. MEG was eluted from the affinity column by glycine buffer pH 2.8 and neutralized by 1M K_2HPO_4. In order to avoid loss of material during dialysis of the product we avoided dialysis and instead adsorbed the neutralized eluate onto nitrocellulose paper (NCP). This was done by introducing a square piece (0.5x0.5 cm) of NCP to an Eppendorf tube with 800 ul of eluate containing up to 50 ug MEG. About 95% adsorbance to the NCP was achieved following overnight shaking of the NCP with antigen solution. For immunization the NCP was washed, dried, powdered, suspended in saline and injected subcutaneously to a rabbit. A total of 100 ug MEG was injected each time in 5 divided doses. This was repeated at 3 weekly intervals. For quality control of affinity purified MEG, material eluted from the NCP was run by SDS Polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemli [4]. This was done on a 16x17 cm slab gel containing 10% acrylamide. The electrophoretic run was carried out overnight with a constant voltage of 40 V. Molecular weight markers (MW-SDS 200 Kit and MW-SDS 70 Kit, Sigma) were run in parallel. Following completion of the run the gel was stained with Coomassie Blue. One major band was exhibited (Fig. 1) at Mr

67-70 Kd which is similar to the molecular weight of MEG (1). In some of the products a second band, a minor one, of a molecular weight of about 60 Kd was seen. Whether this minor band is MEG with a lesser degree of glycosilation or whether it is a different antigen altogether is presently unknown. The specificity of rabbit anti-MEG was tested by Western blotting. To do this, SEA components were initially resolved by SDS-PAGE according to the method of Laemli [4] as desdribed briefly above. The resolved SEA components were electroblotted onto NCP (Membrane Filters, BA85 Schleicher and Schull, West Germany) according to the method of Tobin et al. [5] and of Burnette [6].

Following electroblotting the NCP was treated as follows for immunodetection of antigens reactive with R anti-MEG. The NCP was first treated for blocking non specific sites by incubation in "blocking solution" (PBS pH 7.2, Milk powder 5%, Triton X-100 0.1% and NP-40 0.5%) for 2 hr at 37°C. After washing in "blocking solution" the NCP was reacted for 2 h at room temperature with R anti MEG diluted 1:500 in "blocking solution". Subsequently the NCP was washed and reacted with affinity purified goat anti rabbit alkaline phosphatase-conjugated antibody, light chain specific (Bioyeda Rehovot, Israel), and a color reaction induced by reaction with the substrate (naphtyl phosphate disodium mixed with Fast red TR (Sigma), in Tris 50 mM pH 9.0). After completion of color development the NCP was treated with a fixative (H_2O: Acetic acid: methanol at 5:1:5 ratio), washed with distilled water and dried. Two bands were exhibited at the same locations

as described above for purified MEG (Fig. 2). Rabbit anti-MEG was to be used for identification of ^{35}S-labeled MEG in SEA from cultured eggs, and of MEG-related translation products.

1.1.2. Monoclonal antibodies

Initial immunization experiments were carried out for inducing antibody response to MEG in Balb/c mice. To do this mice received 3 repeated intraperitoneal injections of 5000 live S. mansoni ova at 7 days intervals. Serum was collected before injection of eggs was started, and subsequently just before each injection of eggs. Sera from each collection were pooled, and antibody activity towards MEG determined by Farr-type radioimmunoassay employing ^{125}I-labeled MEG as previously described [1]. Various concentrations of test sera were employed along with normal mouse serum (NMS) negative control, and chronic-infection serum (CIS) as a positive control. Maximal binding with CIS was considered 100% anti-MEG antibody activity, and binding with NMS was considered 0% binding. After a single injection of 5000 S. mansoni eggs no antibody response was observed. Two injections induced 62.5% of the maximal antibody activity and 3 injections induced 77.5% of the maximal antibody activity. Immunization of splenocyte donors for hybridoma production was subsequently done by 3 injections of S. mansoni ova. The immunized mice were rested for at least one month (after the last injection of ova) and 4 days before fusion the mice received an intravenous injection of 20 µg SEA-gly. Mab were

prepared according to established protocols [7,8]. Myeloma cells (line x63-Ag8.653 [9]) were fused with spenocytes at a ratio of 5:1, by employing polyethyleneglycol (M.W. 3600). Screening of culture fluids was carried out by ELISA [10]. So far 9 stable hybridomas were obtained which secrete antibodies, 5 of which are reactive with MEG. Two have been initially subcloned (KCSmeg1 and KCSmeg2) and Mab produced as culture supernatants and ascitic fluids. Of the 5 hybridomas secreting anti MEG Mab 4 were found to be directed to periodate sensitive epitopes (see below) and one gave inconsistent results. This clone (21-7) was therefore recloned in search of Mab reactive with the peptide moiety of MEG, such Mab being considered important for detecting MEG-related polypeptides among cell-free translation products.

A.1.3. Characterization of polyclonal and monoclonal antibodies

This item was not presented as a separate item in the workplan. We included this study as part of our problem solving activity since it was obvious that only antibodies reactive with MEG-related polypeptides are suitable for identifying relevant products of a cell-free translation system or of relevant products of a DNA expression library. We therefore tested the periodate sensitivity of the epitopes reactive with the corresponding Mab and with R anti MEG. To do this SEA was coated onto a microtiter plate and then treated with various concentrations of periodate as previously described [11]. Periodate treatment was followed by ELISA. Epitopes which are

destroyed even by a mild periodate treatment are considered to be of a carbohydrate nature. Table 1 presents a summary of the results of these tests. Two separate rabbit anti-MEG sera were tested. The results indicate that at least 77% of R anti-MEG activity is directed to carbohydrate (periodate sensitive) epitopes of MEG. This puts in question the suitability of R anti-MEG for effective detection of MEG-related polypeptides in a cell free translation system, or among products of an expression library. Monoclonal antibodies to MEG (KCSmeg 1, KCSmeg 2, KCSmeg 3, and KCSmeg 4) are directed against periodate sensitive epitopes. A representative results of a periodate sensitivity test with Mab KCSmeg1 are presented in Table 1. One hybridoma (21-7) which was reactive with purified MEG and exhibited variable periodate sensitivity was subcloned and periodate sensitivity tested in the 5 MEG-reactive subclones obtained. All the subclones were active with periodate resistant epitopes. However, they were all reactive with a variety of antigens unrelated to MEG such as: BSA, ovalbimin, Phosphorylise B, -galactosidase, Myosin and carbonic anhydrase. These Mab were therefore considered unsuitable as specific detectors of MEG-related polypeptides. Preparation of Mab to deglycosilated MEG (see below) is considered in order to obtain Mab reactive with MEG-peptide epitopes.

A.1.4. Further Characterization of MEG Structure

This item was not included in the original work plan. However, since production of antibodies directed primarely to peptide epitopes of MEG appears to be a problem (see above), we decided to subject MEG to amino acid microsequencing so that oligopeptides can be chemically constructed and, in turn, employed for immunization. Recent studies have indicated the usefulness of peptide antibodies as a new tool to identify gene products, to assay or isolate pure proteins, to study protein domains and to immunolocalize proteins [12]. In addition, partial elucidation of the peptide sequence of MEG should enable us to construct a corresponding oligonucleotide probe which may help identify the corresponding gene.

Preliminary Amino acid sequencing of highly purified MEG was carried out by the Edman degradation reaction [13]. Applied Biosystem Model 470 Sequencer was employed. The heavy glycosilation of MEG hindered definite sequence identification due to very large "carry over" effect combined with unindentifiable glycosilated residues. Sequence assignement beyond residue #10 was not possible. Table 2 presents the results of two sequencing cycles.

The absence of methionine from the amino-terminal end of MEG suggests post-translational cleavage of the polypeptide. Residues #11-20 were provided, not as a sequence but rather as definite

evidence of presence, as follows: Val; Asn/Thr/Ser; Glu; Gly; Vol; Leu; Asp; Asn/Thr/Ser; Lys.

Deglycosilation of MEG is considered essential before further analysis of A.A sequence can be accomplished. Deglycosilated MEG can also be used to prepare peptide-specific Mab, and also to elucidate the immunopathological relevance of MEG polypeptide moiety.

A.2. Synthesis of MEG by S. mansoni eggs cultured in vitro

Attempts to harvest S. mansoni ova from livers of infected mice [14] were unsuccessful as yields were repeatedly too small. Harvesting of sterile ova from intestines of infected mice was therefore attempted. Mice were infected by subcutaneous injection of 350 cercariae and sacrified 9 weeks later. Procedures previously employed for harvesting S. mansoni ova from the intestines were applied [15,16]. In order to maintain sterility of the ova, harvesting was carried out by employing sterile homogenizer, filtration devices, labware and solutions. In addition, all solutions employed were supplemented with streptomycine and penicilline. Sterile ova were reproducibly obtained (up to 5×10^4/mouse) and sterility was maintained through 48 hours in culture (as demonstrated by inoculation of aliquotes of cultured egg suspension onto proteose-peptone agar plates).

S. mansoni ova were maintained in Minimal Essential Medium-Eagles (MEM) supplemented with oxalacetic acid, insuline, glutamine and antibiotics. Methionine-free MEM was employed in

experiments on protein synthesis. In these experiments ^{35}S-methionine was added and the eggs underwent incubation at 37°C in an atmosphere of 7% CO_2 in air. Ova were separated from culture fluids and TCA precipitable radioactivity in the culture supernatants and in solubilized ova was determined. Fig. 3 demonstrates the kinetics of ^{35}S-methionine incorporation into total proteins of S. mansoni ova. A sigmoid incorporation curve is exhibited with a lag period of about 1 hour and with maximal incorporation being reached at about 4 hours. By contrast secretion of ^{35}S-labeled, TCA precipitable, constituents into the culture medium (Fig. 4) lags about two hours behind total protein synthesis. Furthermore, secretion continues to take place after 6 hours of incubation. Separate experiments show that when eggs are cultured in the presence of ^{35}S methionine for 24 hours, then washed thoroughly and incubated for another 24 hours in the absence of labeled methionine, a considerable amount of secreted ^{35}S-methionine-labeled, constituents can be identified in the supernantant medium (Table 3). The ongoing secretion of labeled constituents after arrest of external ^{35}S-mentionine suggests the presence of preformed (perhaps precursor) molecules. SEA was prepared from ova which incorporated ^{35}S-methionine in culture. Such metabolically labeled SEA was analyzed by SDS-PAGE [4] and autoradiographed. Multiple bands with a wide range of molecular weights were revealed (Fig. 5). Metabolically labeled SEA underwent immunoprecipitation with R anti MEG (or NRS as a negative control), and complexes were removed by protein A-

spherose beads. Specific precipitation of labeled constituents by R anti MEG was observed by radioactivity counts. Subsequently, immunoprecipitates were run on SDS-PAGE. Following autoradiography (amplyfied by fluorography) (Fig. 5) 4 major bands reactive with R anti MEG (Mr = 90, 70, 48 and 42.5kd), and 3 minor ones (Mr = 135, 66 and 37.5kd) were exhibited, the one at Mr = 70kd corresponding to MEG. These results indicate that MEG is actively synthesized in cultured S. mansoni ova. Furthermore since R anti MEG regularly detects a single major band in SEA (Fig. 2), we assume that the additional bands detected in SEA from eggs cultured in vitro with ^{35}S-methionine represent newly synthesized MEG components, presumably MEG precursors and cleavage pproducts. The finding that the N-terminal amino acid of MEG is not methionine (Table 2), suggests that post-translational modifications of MEG polypeptide indeed occur. Such modifications are in line with the secretory nature of MEG.

A.3. The immunopathological significance of the polypeptide moiety of MEG

Stockpiling of sufficient amounts of MEG for this study presented a problem since much of the harvested eggs were consumed for in vitro egg cultures, for producing RNA (see below) and for screening and characterizing Mab. It is anticipated that more MEG will be consumed for preparing enough RNA to enable us prepare a cDNA library, and also for preparing deglycosilated

MEG, to be used for purposes described above. Deglycosilated MEG may also be used in order to establish the suspected importance of MEG polypeptide for inducing/eliciting delayed type hypersensitivity and immunopathology. This approach seems to us complementary to testing whether proteolytic digestion of MEG abolishes its immunopathologic activity, as originally proposed.

A.4. The abundance and translatability of MEG-specific message

Initial attempts for harvesting RNA from S. mansoni ova were carried out by the Guanidinium Isothiocianate/CsCl method [17]. Eggs were found to be resistant to disruption by homogenization with Polytron or With Dounce glass homogenizer. Disruption of eggs by sonication or by mortar and pestle (under liquid nitrogen) provided very low yields of RNA, and RNA degradation was marked. Ova deep-frozen in liquid nitrogen were employed in these trials. In subsequent experiments RNA was harvested from fresh ova. Guanidinium isothiocyanate was added and ova disrupted by a ground glass/glass homogenizer and RNA purification proceeded by the method mentioned above [17]. RNA yield were relatively small, amounting to only between 15.2 and 32.5 ug total RNA from 200.000 to 500.000 S. mansoni ova. For the purpose of preparing a cDNA library we will have to stockpile sufficient amounts of RNA. Since eggs will be used for both production of RNA and MEG, stockpiling of sufficient amounts of both these materials simultaneously will be required. This presents a problem. However, since microsequencing as compared to RNA

production requires less material (S. mansoni ova) we decided to focus initially on microsequencing, so that production of peptide antibodies and corresponding oligonucleotides can follow without unnecessary delay. Production of RNA at the initial stage was confined for in vitro translation purposes. In this regard it should be reemphasized that since α anti MEG is directed primarely to carbohydrate moieties (Table 1) its suitability to identify MEG polypeptides is considered limited.

A representative preparation of total RNA from S. mansoni ova was run by agarose gel electrophoresis (Fig. 6). The presence of relatively undergraded mRNA is suggested. The pattern obtained also suggested that there is a nick in the large rRNA unit to produce 2 small RNA species which comigrate with the RNA from the small subunit as previously described for RNA from adult worms [18]. This RNA was subjected to translation in an in vitro cell free translation system from rabbit reticulocytes (New England Nuclear). Two concentrations of each RNA product (between 1 and 4 ug) were taken for in vitro translation reaction. RNA was diluted in H_2O, mixed with Mg acetate, K acetate, "translation cocktail", reticulocyte lysate and ^{35}S-methionine, according to the manufacturers instruction. Samples were taken for radioactivity count at 0 time (before onset of incubation) and after 60 minutes incubation. Negative (no RNA) and positive (yeast RNA) controls were included. Table 4 summarizes the results of a representative test. Products of cell free translation of RNA from S. mansoni ova and of a control reaction

without RNA were subjected to SDS-PAGE. Molecular weight markers were run in parallel. The gel was subsequently stained with coomassie blue, dried and subjected to autoradiography. Immunoprecipitation of translation products with R anti MEG consistently gave between x2 to x3 the radioactivity of controls immunoprecipitated with NRS. This however is not considered a sufficiently specific immunoprecipitation. Translation products immunoprecipitated with R anti MEG or NRS were also analyzed by SDS-PAGE along with total SEA into which ^{35}S-methionine was incorporated. Fig. 7 constitutes a combined presentation of the results of these experiments. Small molecular weight constituents that migrate differentially from controls without RNA or from NRS-immunoprecipitation control were tentatively identified. Such small constituents are compatible with non glycasilated MEG-specific constituents. Constituents of higher molecular weight were also tentatively identified and may represent precursor molecules. Further analysis is required in order to establish these tentative results. The use of a more efficient antibody reagent for detecting MEG polypeptide (e.g. peptide antibodies) is highly required for these studies.

B. SCIENTIFIC COLLABORATION (corresponds to proposal items)

A. Work to be done in Kenya

A.1. Collection of human sera and schistosome egg (S. haematobium)

S. haematobium eggs were collected in Kwale District, the

Coast Province, from the urine of infected school children. The schools were Tserezani where 400 individuals were examined (of which 20% had visible haematuria and 50% had S. haematobium eggs in urine); Burani, in which 368 children were examined (16% had visible haematuria and 48% had eggs in the urine); and Mihirini where 300 children were examined of which 3% had visible hematuria and 10% had S. haematobium ova in the urine). Subsequently, Tserezani and Burani were chosen for urine collection. Pooled urine (4x20 liters) was processed by filtration as previously described [19] and washed with saline containing 100 units/ml of penicillin/streptomycin. About 3.2×10^{6} ova were collected. Some were used to immunize balb/c mice (see below) and the rest stored at -70°C for preparing antigens.

About 20 samples, 5 ml each, of sera from S. haematobium cases were collected.

A.2. Production of rabbit antisera to S. haematobium egg antigens

Soluble egg antigen of S. haematobium (SEA) was prepared and the glycoprotein fraction separated by lectin affinity chromatography as previously described [1]. Immunization of rabbits with these antigens is underway. We have also started attempts to isolate S. haematobium MEG (MEG_{1}) by methods previously described for S. mansoni MEG [1].

A.3. In vitro synthesis of S. haematobium egg glycoproteins

This item has not started yet and is pending the harvesting

of S. haematobium ova under sterile conditions. Harvesting and maintaining sterile S. haematobium ova under conditions existing in field laboratories in Kenya is highly unlikely. We will therefore attempt organizing a days' egg-collection expedition and shipment by air to the central laboratory in Nairobi for further processing and culture.

Once sufficient amount of MEG_H is available and methods for deglycosilation and preparation of peptide antibodies worked out for S. mansoni MEG, we will apply these methodologies for MEG_H.

Data obtained on culture of S. mansoni ova should be helpful for culturing S. haematobium eggs. Identification of newly synthesized MEG_H components in cultured ova will be done by immunoprecipitation and is dependent on successful preparation of specific antibody reagents.

A.4. Preparation and characterization of S. haematobium egg RNA

This has not been started yet. Methods for efficient harvesting of RNA from S. mansoni ova will have to be worked out in detail before we can attempt harvesting S. haematobium egg RNA.

A.5. Preparation of monoclonal antibodies to S. haematobium egg antigens

As mentioned above (8.A.1) S. haematobium eggs have been collected. In addition 20 balb/c mice were immunized by repeated intraperitoneal injections of 5000 ova. Only several mice have

survived this immunization and are now ready for boosting in order to use their splenocytes for producing hybridomas. Boosting will be done by intravenous injection of SEA-glyco or MEG_{11}. MEG_{11} is preferred so that high proportion of anti-MEG antibodies can be obtained.

We also consider postponing this part of the work until deglycosilated MEG_{11} is available, hoping that immunization (boosting) with this antigen may increase the chance of obtaining antibodies to the peptide moiety of MEG_{11}. Preparations for applying the hybridomas technology at KEMRI have started and training of a senior technician for this purpose has been completed (see B.1. below).

B. transfer of technology

B.1. By training Kenyan scientists

Mr. Jim Mwaniki Kagai of the Kenya Medical Research Institute spent two and a half months (8.1.87-28.1.88) in our centre for training on hybridoma technology, which included the following subjects:

- Immunization procedures
- Fusion procedures
- Culture and selection of hybridomas
- Screening of culture supernatants and ascitic fluids by ELISA
- Subcloning by limiting dilution
- Cryopreservation of myeloma and hybridoma cells
- Production of ascitic fluids

- Concentration of monoclonal antibodies (Mab)
- Characterization of Mab for Ig isotypes by ELISA
- Characterization of corresponding antigens by western blotting and immunodetection
- Determination of the carbohydrate or polypeptide nature of corresponding antigens
- Record keeping
- Organization of, and supplies for a hybridoma facility
- Logistics and problem-solving in the operation of a hybridoma facility.

During his stay with us Mr. Kagai worked in our hybridoma facility and performed independently all the necessary steps on a routine basis. He has compiled the acquired information in a neatly organized handbook, and in addition received photocopies of relevant information. He received materials and cells necessary for setting up a hybridoma facility at KEMRI. Subsequently a myeloma cell culture has been established in KEMRI, but difficulties encountered in maintaining the CO_2 incubator in a good operating order have resulted in loss of myeloma cells. Also, establishing a satisfactory supply of culture media, FCS, etc. is still a problem.

A summary with special emphasis on emerging problems and suggestions for their solving

A. Activities in Israel

A.1. Antibodies and antigens: Although rabbit ansera and Mab to

MEG have been already prepared, a major problem in this part of the work was encountered. Namely, that suitable Mab to MEG peptide epitopes are not yet available and that the activity of R anti MEG is largely (about 70%) directed to carbohydrate moiety of MEG. This is only a minimum estimate of the proportion of antibodies to carbohydrate epitopes. Suitable antibodies to peptide epitopes of MEG are essential for identifying cell free translation products and for immunoscreening of expression libraries. Microsequencing of MEG amino acids has been started aiming at constructing corresponding peptides for inducing corresponding peptide antibodies. The highly glycasilated nature of MEG presents a problem in obtaining a clear sequence information. To overcome this problem we intend to prepare deglycosilated MEG. In this context we also intend to improve procedures for MEG purification. We have recently acquired HPLC equipment and preliminary experiments for MEG purification (from SEA-gly) by HPLC are underway.

A.2. Synthesis of MEG by S. mansoni eggs in vitro: Harvesting of eggs from livers of infected mice, as originally proposed, was attempted but discontinued since the number of eggs obtained was insufficient, and fungal contamination prevailed. We developed a procedure for harvesting sterile eggs from the intestines of infected mice. Demonstration of in vitro incorporation of ^{35}S-methionine into MEG was accomplished. This in itself strongly indicates that the corresponding mRNA is present in ova harvested

from the intestines (the very same egg preparation is used to prepare RNA). Precursor molecules of MEG were tentatively identified in these experiments.

The quantity of S. mansoni ova is a limiting factor in our studies since they are required for several purposes as follows:

a. Production of MEG to be used for: Preparation of peptide antibodies for immunoprecipitation and immunoscreening; deglycosilation; microsequencing; elucidation of the immunopathological activity of MEG polypeptide.

b. Production of RNA to be used for: In vitro translation; construction of a cDNA library; Northern blotting

Since production in MEG for the purposes listed above seems more important at this stage we decided to use S. mansoni eggs primarely for MEG production and, in parallel, for stockpiling just enough RNA to enable definite identification of MEG polypeptides among cell-free translation products.

A.3. The immunopathological significance of the polypeptide moiety of MEG: This study has not been yet accomplished. Once deglycasilation is accomplished we will study in parallel the effect of proteolysis and deglycasilation on the immunopathological activity of MEG.

A.4. The abundance and translatability of MEG specific message

Harvesting of RNA from S. mansoni ova and its in vitro translation have been accomplished. The yield of RNA will have to

be improved so that sufficient amount can be available for constructing a cDNA library. Tentative identification of MEG-related translation products has been accomplished. However, definite evidence is still required and this will depend on the availability of suitable antibodies (see above).

If compatible oligonucleotides can be prepared on the basis of A-A sequence we will attempt their use as primers for polymerase chain reaction in order to obtain corresponding DNA sequences.

B. Activities in Kenya

Collection of S. haematobium eggs has been accomplished and rabbit antisera to S. haematobium antigens have been prepared.

In view of the results we obtained with S. mansoni MEG, it appears that preparation of polyclonal or monoclonal antibodies directed to MEG_{11} polypeptide may present a problem. We anticipate that at this stage preparation of MEG_{11}, its deglycasilation, and microsequencing can contribute the most to the understanding of the molecular differences between MEG of the two schistosomes. Also, peptide antibodies to MEG_{11} can be prepared. Collection of S. haematobium eggs, and production SEA and of glycoproteins thereof in Kenya, followed by purification of MEG_{11} in Israel is considered.

Establishing a functional hybridoma unit at KEMRI may require an extended visit of the P.I. for troubleshooting and problem-solving. A short visit during the 2nd year is planned.

Attemps will be made to obtain funds to make it possible to extend this visit for the following purposes

- Help establish a hybridoma facility
- Help carry out experiments on *in vitro* culture of *S. haematobium* eggs
- Help preliminary fractionation of SEAr

Other activities in Kenya will have to await corresponding developments of our work with *S. mansoni*.

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Table 1. Periodate sensitivity of epitopes corresponding to rabbit anti-MEG (R anti MEG)

Antibody	Antigen	ELISA O.D. with periodate (Pi) treated antigens at various PI concentrations (mM)				
		0	0.1	1	5	10
R anti MEG$_1$	SEA	1.095	1.061	0.779	0.394	0.340
R anti MEG$_2$	SEA	1.470	1.374	0.861	0.230	0.342
R anti BSA (negative control)	BSA	1.931	1.876	1.909	1.914	1.945
Mab KCSmeg^1 (positive control)	SEA	0.721	0	0	0	0

Two lots of R anti MEG were tested. BSA/anti BSA was included as a negative (pi insensitive) control and Mab KCSmeg^1/SEA as a positive (Pi sensitive) control.

Table 2. Amino acid sequence information from MEG

Residue #	A.A.	Comments
1	Leu/gly	lower probability (LP)-ser.
2	Val	
3	Asn*	
4	Ser*	
5	Pro	
6	Thr*	LP-Pro.
7	glu	
8	gly	
9	Pro	
10	Thr*	LP-Pro.

* Lower yields of phenylthiohydantoin point to glycosilation.

Table 3. Incorporation of ^{35}S-methionine into *S. mansoni* ova: Continuous (A) and interrupted (B) incubation with ^{35}S-methionine for 24 hours

	A	B
Incubation	24 h w ^{35}S-Met (2 mci)	24 h w ^{35}S-Met. then 24 h w/o ^{35}S-Met
No. of eggs	2.5×10^5	2.5×10^5
Total incorporation of ^{35}S-Met into ova	22×10^6 cmp	19.7×10^6 cpm
Total ^{35}S-Met in SEA alone	6.5×10^6 cpm	5.0×10^6 cpm
TCA-precipitable radioactivity from SEA	3.5×10^6 cpm (57%)[a]	3.1×10^6 cpm (62%)[a]
Radioactivity in Sup. 24 h. after interrupted ^{35}S-Met	not applicable	10.4×10^6 cpm
Radioactivity precipitated from Sup. by TCA	7.3×10^5	2.6×10^5 (2.5%)[b]

S. mansoni ova were incubated for 24 h in the presence of (w) ^{35}S-methionine (^{35}S-Met). An aliquote of eggs was then washed thoroughly from extraneous ^{35}S-Met and incubation continued without (w/o) ^{35}S-Met.

a TCA precipitable radioactivity from SEA represents precipitable secretable and non-secretable SEA constituents.

b This low value (2.5%) of TCA precipitable material represents secreted constituents, most likely glycoproteins which do not precipitate well with TCA. Similar TCA precipitation was previously obtained with purified MEG (1).

Table 4. *In vitro* translation of total RNA from *S. mansoni* ova

Total RNA	RNA quantity (ug)	^{45}S-methionine in TCA insoluble material (cpm)	
		at 0 time	at 60 minutes
no RNA (negative control)	-	15,631	23,987
Yeast RNA (positive control)	2	17,323	178,855
S. mansoni egg RNA product 1	1	16,396	57,170
	2	14,197	76.310
S. mansoni egg RNA product 2	1	14,210	83,411
	2	15,856	103,701

S. mansoni egg RNA was translated in a cell free translation system from rabbit reticulocytes. Two RNA products are presented, 1 and 2 ug of each were subjected to translation. A reaction mixture with no RNA served as negative control and yeast RNA served as a positive control. Samples were taken for radioactivity counts at 0 time and after 60 minutes incubation.

LIST OF FIGURES

Fig. 1: SDS-PAGE of purified MEG.

MEG (5 ug) was run by SDS-PAGE on a 10% polyacrylamide slab gel under reducing conditions. Mr. of MEG is 70kd.

Fig. 2: Western blot analysis of SEA with immunodetection of bands reactive with R anti MEG.

SEA was separated electrophoretically by SDS-PAGE then electroblotted onto NCP.

Lane 1: Stained with Amido-Black.

Lane 2: Underwent immunodetection with R anti MEG.

Lane 3: Underwent immunodetection with normal rabbit serum.

Fig. 3: Incorporation of ^{35}S-methionine into total constituents of S. mansoni ova.

Fresh S. mansoni ova (2.15×10^5) in 5 ml medium were incubated for 15 minutes without ^{35}S methionine. ^{35}S-methionine (215 uci) was then added and the ova incubated at 37°C while rocking in 5% CO_2 atmosphere. Aliquotes of 900 ul were taken at 0, 1, 2, 3 and 4 hours and subjected to

centrifugation, precipitation by TCA followed by methanol, solubilization of precipitates with soluene, and radioactivity measurement.

Fig. 4: Incorporation of ^{35}S methionine into constituents of S. mansoni ova: Identification of labeled polypeptides secreted from ova cultured in vitro.

Fresh ova (1×10^5) in 2 ml medium were incubated for 15 minutes without ^{35}S-methionine. ^{35}S-metionine (100 uci) was then added and the ova incubated at 37°C while rocking in 5% CO_2 atmosphere. Aliquotes of 250 ul were taken at 1, 2, 3, 4 and 6 hours. The supernatants were collected and polypeptides adsorbed onto NCP. Aliquotes from a control culture without ova were collected in parallel. The results represent net cpm (after subtracting control value of adsorption onto NCP).

Fig. 5: Identification and characterization of newly synthesized MEG constituents among SEA constituents.

SEA was harvested from ova maintained *in vitro* with ^{35}S -methionine, and subjected to SDS-PAGE, with or without immunoprecipitation analysis with R anti MEG.

Lane 1: ^{35}S-SEA - 48,000 cpm

Lane 2: ^{35}S-SEA - 21,000 cpm

Lanes 3,4: MEG constituents immunoprecipitated by R anti MEG (1,500 cpm).

Autoradiography/Fluorography involved exposure for 1 week at -70°C (lanes 1, 2, 3) or 2 1/2 weeks (lane 4). Analysis of control immunoprecipitates (with NRS) did not exhibit any bands (not shown).

Fig. 6: Agarose gel electrophoresis of RNA from *S. mansoni* ova.

RNA was harvested from fresh ova by the Guanidinium isothiocyanate method, exhibiting a 260 nm/280 nm O.D. ratio of 1.769. RNA from *S. mansoni* (left lane) and control (*E. coli*) RNA (right lane) were run on 1.5% agarose under non-denaturing conditions.

Fig. 7: Identification of MEG constituents among products of cell free translation of RNA from S. mansoni ova.

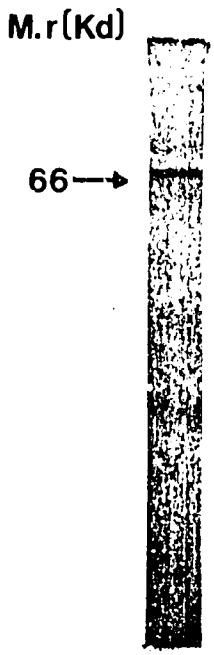
Lane 1: Control - no external RNA added.

Lane 2: Total translation products from S. mansoni RNA.

Lanes 3,5: Translation products immunoprecipitated with control antiserum (NRS).

Lanes 4,6: Translation products immunoprecipitated with R anti MEG.

Figure 1.



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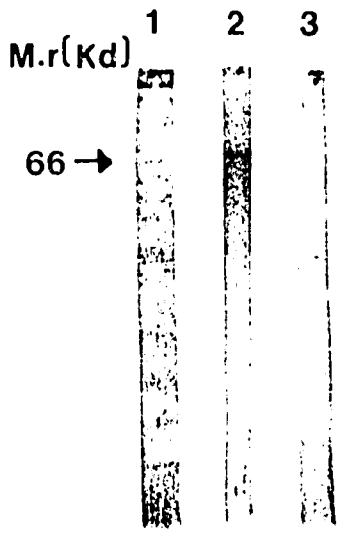


Figure 3.

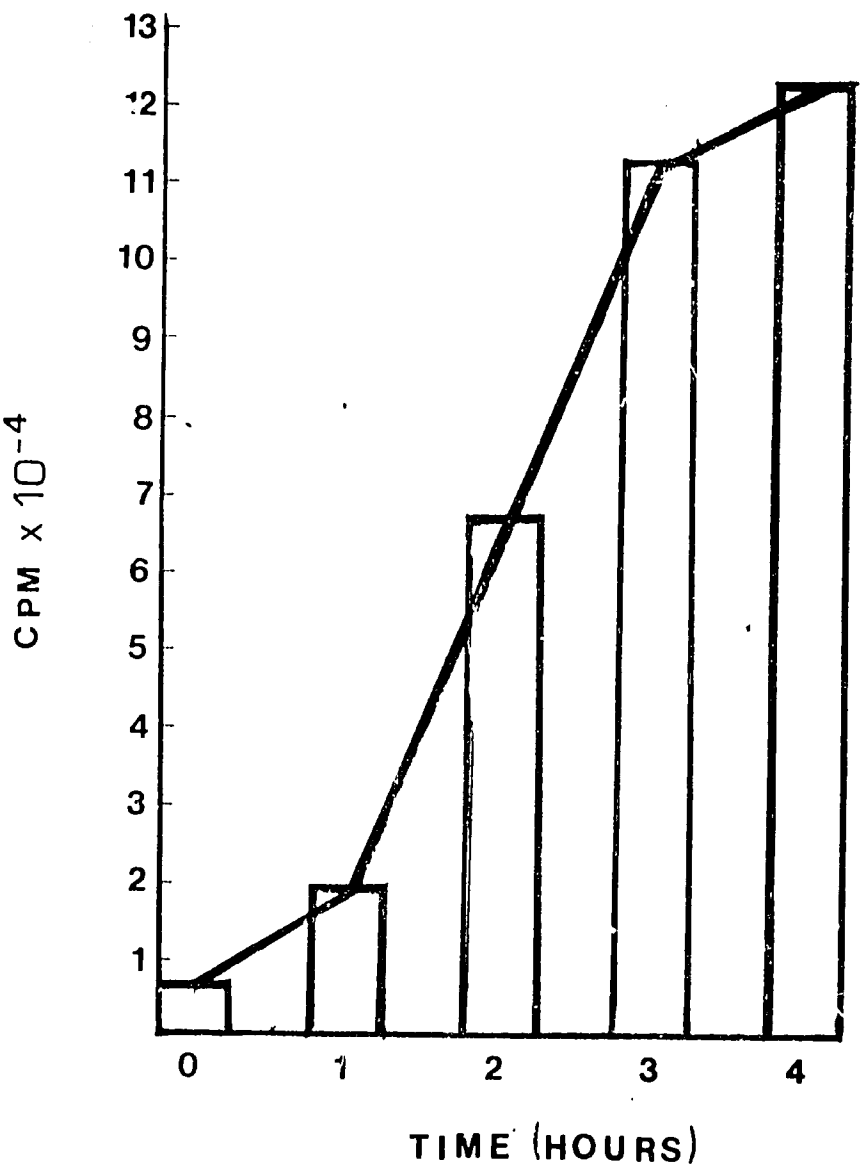
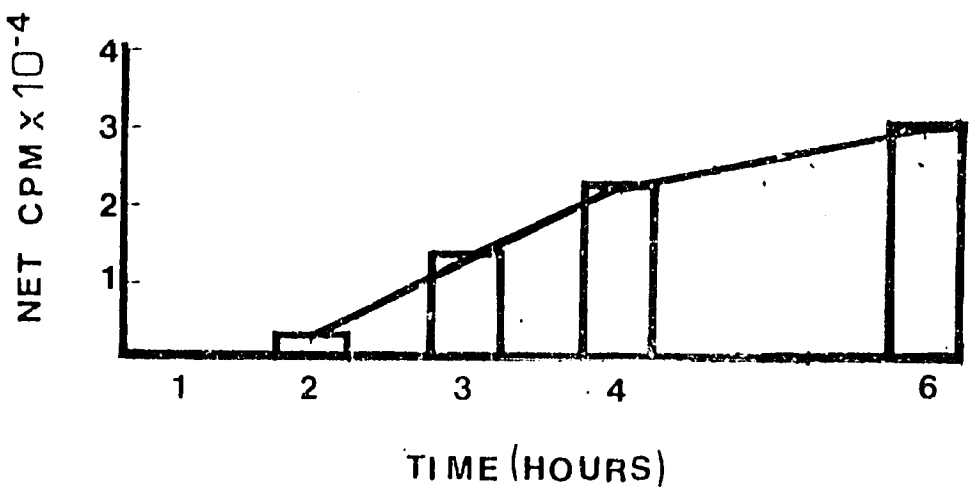


Figure 4.



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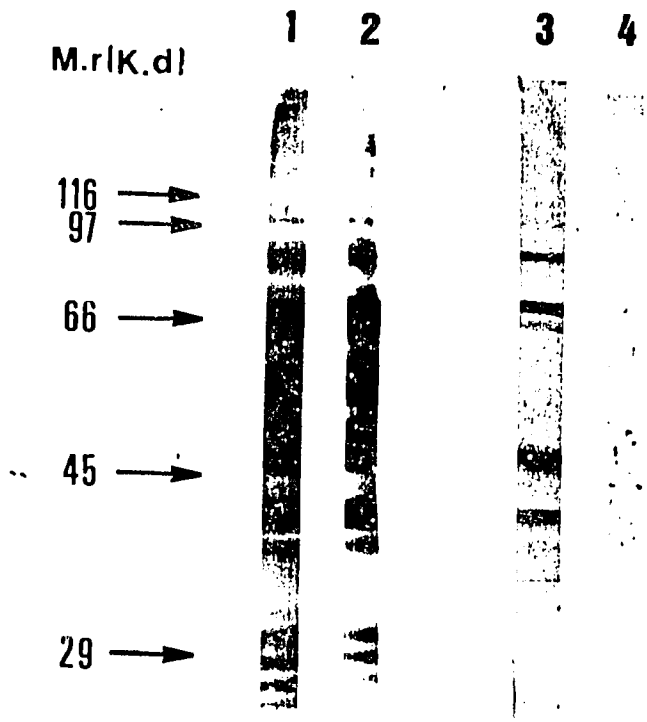


Figure 6.

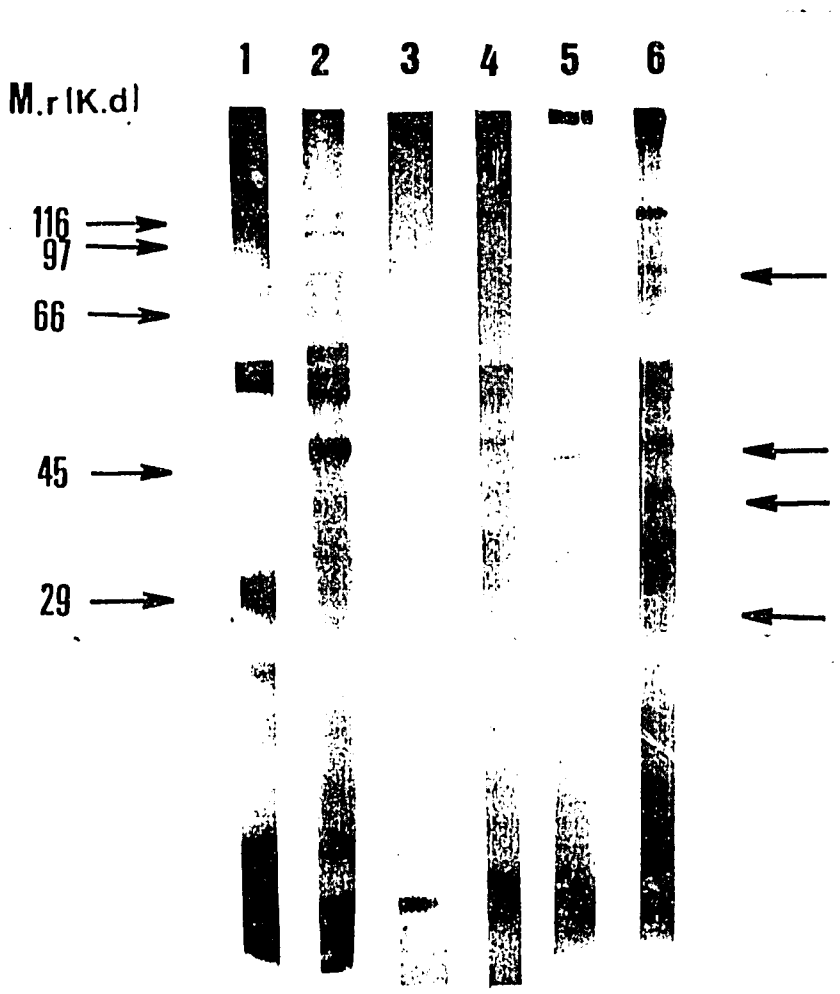
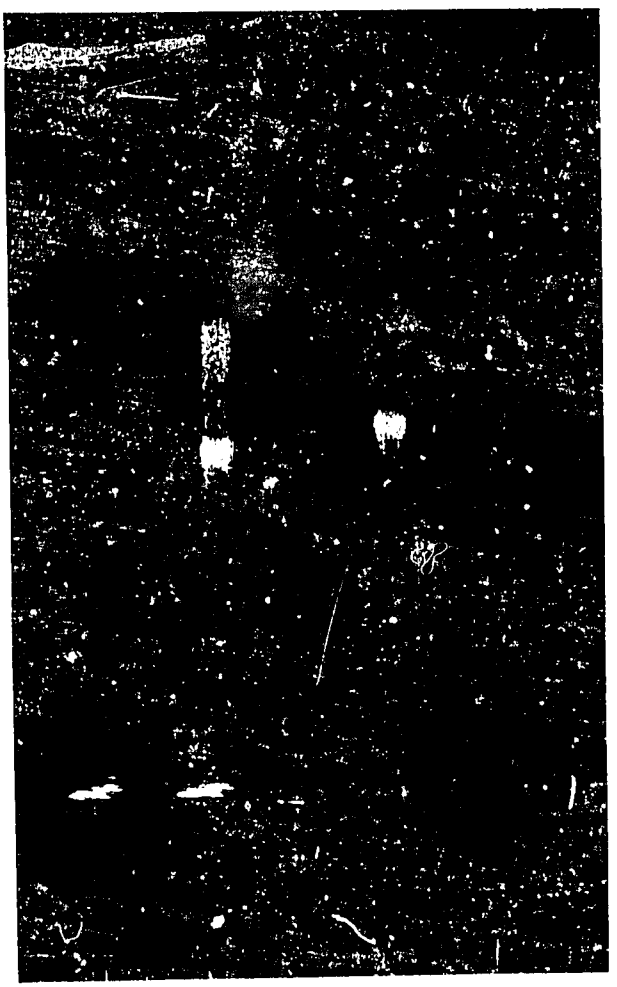


Figure 7.



US-ISRAEL CDR PROGRAM PROJECT 87-162

Grant No. DPE-5544-G-SS-7017-00

"Towards an antigranuloma vaccine in schistosomiasis mansoni: molecular cloning and characterization of a putatively relevant antigen"

Principal Investigator: Dr. Joseph Hamburger

1st Semi Annual Progress Report

The effective start of the proposed project was mid-September 1987. The report covers activities through March 1988.

The activities, during the period covered, centered on preparation of polyclonal and monoclonal antibodies to the major egg glycoprotein (MEG), and on standardizing culture conditions which will allow protein synthesis by *S. mansoni* ova maintained in short-term *in vitro* culture.

Rec'd in SCI JUN 2 0 1988

A. PREPARATION OF ANTIBODY REAGENTS

A.1. Polyclonal antibodies to MEG

The glycoprotein fraction of soluble egg antigen of *S. mansoni* (SEA-gly) was separated by lectin-affinity chromatography on con-A sepharose as previously described (1). For immunization of rabbits 100 ug SEA-gly were incorporated with Freund's complete adjuvant and injected intramuscularly. Booster doses of 50 ug SEA-gly incorporated with Freund's incomplete adjuvant were administered twice at 3 weeks intervals. Rabbit anti-SEA-gly (R & SEA-gly) was collected and stored at -20°C. SEA-gly were run electrophoretically on agarose gel against R & SEA-gly for line immunoelectrophoresis as previously described by Kroll (2). ^{125}I-labelled MEG, purified by ion exchange chromatography, was added as a tracer to identify MEG-containing precipitin lines as previously described (1). Two precipitin lines were obtained one of which contained the bulk of radioactivity indicating that it was rich in MEG. This precipitin line was excised from the agarose, washed and injected to a rabbit as previously described by Kroll (2) for producing rabbit anti-MEG (R & MEG).

Following production of monoclonal antibodies (Mab) to MEG (see below) we have used an alternative method for production of R & MEG. This procedure involved affinity purification of MEG on CNBr-Sepharose to which Mab KCsmegi was bound as previously described (3). MEG was eluted from the affinity column by glycine buffer pH 2.8 and neutralized by IM K_2HPO_4.

In order to avoid loss of material during dialysis of the product we avoided dialysis and adsorbed the neutralized eluate onto nitrocellulose paper (NCP). This was done by introducing a square piece (0.5x0.5 cm) of NCP to an Eppendorf tube with 300 ul of eluate containing up to 50 ug MEG. Following overnight shaking, the NCP was washed, dried, powdered, suspended in saline and injected subcutaneously to a rabbit. A total of 100 ug MEG was injected each time in 5 divided doses. This was repeated at 3 weekly intervals. For quality control of affinity purified MEG, material eluted from the NCP was run by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE), a single major band with Mr of about 70 kd (equivalent to that of MEG) was exhibited.

Polyclonal antisera are to be used for immunoprecipitation experiments for identification of ^{35}S-labeled MEG in SEA from cultured eggs, and in cell-free translation products.

A.2. Monoclonal antibodies

Initial immunization experiments were carried out for inducing antibody response to MEG in Balb/c mice. When three repeated weekly intraperitoneal injections of live *S. mansoni* ova were administreted for immunization a substantial antibody response, as determined by a radioimmunoassay (1), was induced. The mimunized mice were rested for at least one month, and 4 days before fusion the mice received an intravenous injection of 20 ug SEA-gly. Mab were prepared according to established protocols (4,5). Myeloma cell line x63-Ag8.653 (6) were fused with

splenocytes at a ratio of 5:1, by employing polyethyleneglycol (M.W. 3600). Screening of culture fluids was carried out by ELISA (7). So far 9 stable hybridomas were obtained which secrete antibodies. 5 of which are reactive with MEG. Two have been subcloned and Mab KCSmeg1 and KCSmeg2 produced as culture supernatants and ascitic fluids.

Affinity purification of MEG was carried and with KCSmeg1 (see above).

3. MAINTENANCE OF S. MANSONI OVA IN CULTURE AND ANALYSIS OF PROTEIN SYNTHESIS

Attempts to harvest S. mansoni ova from livers of infected mice (8) were unsuccessful as yields were repeatedly too small. Harvesting of sterile ova from intestines of infected mice was therefore attempted. Mice were infected by subcutaneous injection of 300 cercariae and sacrified 9 weeks later. Procedures previously eployed for harvesting S. mansoni ova from the intestines were applied (9,10). In order to maintain sterility of the ova, harvesting was carried out by employing sterile homogenizer, filtration devices, labware and solutions. In addition, all solutions employed were supplemented with streptomycine and penicilline. Sterile ova were reproducibly obtained (up to 5×10^4/mouse) and sterility was maintained through 48 hours in culture (as demonstrated by inoculation of aliquotes of cultured egg suspension onto protease-peptone agar plates).

S. mansoni ova were maintained in Minimal Essential Medium-

Eagles (MEM) supplemented with oxalacetic acid, insulin, glutamine and antibiotics. Methionine-free MEM was employed in experiments on protein synthesis. In this case ^{35}S-methionine was added and the eggs underwent incubation at 37°C in an atmosphere of 7% CO_2 in air. Aliquotes of cultured egg suspensions were collected from the culture at various time intervals. Ova were separated from culture fluids and TCA-precipitable radioactivity in supernatants and in solubilized ova was determined. Incorporated ^{35}S-methionine was detected in both. Maximal incorporation of ^{35}S-methionine into ova was observed after hours in culture. SEA was prepared from ova which incorporated ^{35}S-methionine in culture. Such metabolically labeled SEA was separated by SDS-PAGE (11). Autoradiography revealed multiple bands within a wide range of molecular weights. Metabolically labeled SEA was reacted with R & MEG (prepared by the method of Kroll - see above). Immune complexes were precipitated by Protein A-Sepharose then washed thoroughly suspended in sample buffer, boiled and run on SDS-PAGE. Following autoradiography 2 major bands were exhibited, one of which at Mr corresponding to that of MEG. These results suggest that MEG is actively synthetized in cultured S. mansoni ova.

Further immunoprecipitation analyses with rabbit anti-immunoaffinity purified MEG are underway.

C. THE IMMUNOPATHOLOGICAL SIGNIFICANCE OF THE PROTEIN MOIETY OF MEG

Stockpiling of affinity purified MEG is underway for studies on the effect of proteolysis on the delayed foot pad swelling-eliciting activity of MEG.

D. THE ABUNDANCE AND TRANSLATABILITY OF MEG-SPECIFIC MESSAGE

Initial attempts for harvesting RNA from S. mansoni ova were carried out by the Guanidinium Isothiocianate/CsCl method (12). Eggs were found to be resistant to disruption by hemogenization with Polytron or Downce glass homogenizer. Disruption of eggs by sonication or by mortar and pestle (under liquid nitrogen) provided very low yields of RNA, and RNA degradation was marked. Ova deep-frozen in liquid nitrogen were employed in these trials. Further attempts to harvest RNA from S. mansoni ova are underway. The use of freshly prepared ova, and employment of other means of disruption are considered.

E. TRAINING AND COLLABORATION

Mr. Jim Mwaniki Kagai of the Kenya Medical Research Institute spent 2 1/2 months (8.11.87-28.1.88) in our centre for training on hybridoma technology, which included the following subjects:

- Immunization procedures
- Fusion procedures

- Culture and selection of hybridomas
- Screening of culture supernatants and ascitic fluids by ELISA
- Subcloning by limiting dilution
- Cryopreservation of myeloma and hybridoma cells
- Production of ascitic fluids
- Concentration of monoclonal antibodies (Mab)
- Characterization of Mab for Ig isotypes by ELISA
- Characterization of corresponding antigens by western blotting and immunodetection
- Determination of the carbohydrate or polypeptide nature of corresponding antigens
- Record keeping
- Organization of, and supplies for a hybridoma facility
- Logistics and problem solving in the operation of a hybridoma facility.

During his stay with us Mr Kagai worked in our hybridoma facility and performed independently all the necessary steps on a routine basis. He has compiled the acquired information in a neatly organized handbook, and in addition received photocopies of relevant information. He received materials and cells necessary for setting up a hybridoma facility in the collaborating institution. Subsequently a myeloma cell culture has been established in KEMRI. Preparations for harvesting ova of <u>Schistosoma haematobium</u> from urine of infected school children are in advanced stage. Collection of ova, immunization of mice and fusion experiments are expected to start during the 2nd half

of the 1st year.

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