PN-ABM-429

US-ISRAEL CDR PROGRAM PROJECT C7-162

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

Towards an antigranuloma vaccine in Schistosomiasis mansoni: molemolecular cloning and characterization of a putatively relevant antigen

2nd Annual Progress Report

December 1989

Rec'd in SCI FEB 12 1990

This report summarized the research activity during one whole year since December 1988, from which (late) the last annual report was dated.

The present year's research was dedicated to the preparation of a cDNA library and to attemps directed at deglycosilation of MEG for two main purposes, namely: (a) to enable induction of antibodies to peptide epitopes of MEG, and (b) to obtain partial amino acid sequences of MEG. In the original proposal and work plan deglycosilation of MEG was not included. However initial results previously reported have indicated that deglycosilation of MEG is an essential step before induction of antipeptide antibodies and amino-acid sequence analysis can be accomplished. Because of the revised approach the present report is not itemized according to the original items of the proposed work plan.

A. PREPARATION OF A cDNA LIBRARY

A.1. Preparation of RNA from S. mansoni ova

All glassware was treated with DEPC-H_2O and sterilized. All solutions for RNA production were prepared with DEPC-H_2O. Successful production of RNA by the Guanidinium Isothiocianate (GIT)/CsCl method [1] was accomplished by the following adaptations dictated by the resistance of the egg to disruption by Polytron homogenizer or Dounce glass homogenizer. A representative harvest by the procedure finally chosen is

herewith described: total of $1.5x10^5$ ova/0.9ml PBS were taken for the RNA harvest. (1) Three ml of GIT buffer was added to each egg aliquote when frozen. After thawing another ml of GIT buffer was added. (2) The eggs were homogenized for 15 minutes in a Ten Broek (glass-glass) homogenizer (GIT buffer stands for 4 M GIT, 25 mM sodium acetate, pH 6, 0.83% final -mercaptoethanol) (a sonication step at this stage was found useful fur further shearing of DNA). (3) The homogenate was transfered to a Corex tube and centrifuged for 20 min. at 10,000 rpm at room temperature. (4) The supernatant was transfered to a polycarbonate tube (ultraclear-Beckman, pretreated with 1M KOH) over CsCl. (5.7M CsCl, 25mM sodium acetate, pH 6) and ultracentrifuged for 21h in a SW41 rotor at 32,000 rpm and at room temperature. 5) The supernatant was carefully removed and the bottom of the tube (containing the RNA pellet) was cut, put on ice and the RNA dissolved in 300 ul 0.3M acetate buffer pH6 in 3 aliquotes of 100ul each. (6) The dissolved RNA was transferred to an eppendorf tube and alcohol precipitated. (7) The RNA pellet was washed twice with 80% Ethanol, dried and reconstituted with 40ul ddw. The total amount of RNA from $1.5x10^6$ eggs was 243ug. Fig. 1 presents the results of agarose gel electrophoresis exhibiting undergraded RNA. It should be recalled that the large subunit of rRNA undergoes nicking that results in formation of 2 small RNA species which comigrate with the RNA from the small subunit [2]. Bands present in the mRNA

zone suggest the presence of defined mRNA species (the identity of which is yet unknown).

Poly A^+ RNA was isolated from total RNA by oligo dT. A total of about 300ug RNA at a time underwent the procedure of isolating poly A^+ RNA. Oligo (dT) cellulose type 7 (Pharmacia) was used. An oligo (dt) cellulose column was prepared to a packed volume of 0.5ml wet matrix, and equilibrated in 0.5M KCl in 1xTE. The total RNA sample was made to 0.5M KCl by adding the appropriate amount of 4M KCl. The total RNA sample was cycled several times onto the column to allow hybridization of poly $A(^+)$ mRNA, and flow through fractions of 1ml were subsequently collected by washing the column with equilibration buffer. TE in DEPC-H_2O was then aded in 10 aliquotes of 1ml each and eluate fractions of 1ml collected. OD_{260} of each fraction was determined.

Fractions containing poly $A(^+)$ mRNA were pooled and concentrated by extraction with secondary butyl alcohol or by lyophilization. NaAc and ethanol were added to the concentrated poly $A(^+)$ pool to precipitate the RNA. The average amount of poly $A(^+)$ RNA produced from 300u total RNA was 3.2ug.

A.2. <u>Construction of a cDNA library</u>

Several attempts were made to produce a cDNA library in phage gt11. A small library was eventually constructed by the method based on the procedure described by Gubler and Hoffman

[3] which describe classical oligo (dT) primed first strand Synthesis coupled with the use of RNAase H - DNA polymerase I mediated second strand synthesis.

Poly A($^+$) mRNA (7ug) was dried up and first strand DNA was synthesized by adding to the dry DNA 38ul of a mixture containing 50mM Tris HCl pH 8.3, 10mM $MgCl_2$ 10mM Dithiotriatrol (DTT), 1.5 mM of each dATP dGTP and dTTP, 0.5mM of dCTP, and 4mM of Na Pyrophosphate. Subsequently 4.7ul of 1mg/ml oligo DT and 3.5 ul of reverse transcriptase (3000 u/ml) were added. This was termed the Main reaction (MR) Mix. A 6.5ul aliquote was removed from the main reaction and 1ul of ^{32}P dcTP 800 ci/mM was added. This was termed the Radioactive reaction (RR) mix. Both MR and RR mix were incubated in parallel for 45 minutes at 43°C and the reactions were terminated by adding EDTA (17 mM final). An aliquote of 1.5ul of the RR was diluted 1:10 in TE and 5ul aliquotes of the diluted RR was used to determine total vs. acid precipitable counts. Incorporation ratio in the RR was 0.046. In the MR we had 6ug RNA in about 40ul. Estimated amount of dCTP (an estimated quarter of the total amount of nucleotides) was 1.5x10^{-}6 gr, therefore the estimated amount (by molarity) of dcTP available for replacement is:

$$\frac{\frac{\text{Amount of dCTP (gr.)}}{\text{M.W. of dcTP}}}{\text{volume (l)}} = \frac{\frac{1.5\times10^{-6}}{280}}{40\times10^{-6}} \doteq 130 \text{ uM dcTP}$$

the amount of cold dcTP in the reaction was 500uM therefore the calculated incorporation is:

$$\frac{\text{incorporation ratio (in RR) x total dcTP input (uM)}}{\text{estimated dcTP available for replacement (uM)}} = 0.1769$$

or <u>17.7% incorporation to 1st strand DNA</u>

In order to follow through the steps of extraction of 1st stand DNA we added an amount of readioactive material (about 35,000 cpm) from RR to MR and this was followed by extraction in phenol and back extraction in phenol and in chloroform and subsequently by adding Na Acetate and alcohol precipitation.

The 1st strand DNA was dried and then disolved in a 100ul second strand reaction mixture containing 20mM Tris pH 7.6, 5mM $MgCl_2$ 5mM Mg Cl_2, 10mM $(NH_4)_2$ SO_4, 100mM KCl, 5ug BSA, 40uM each of dATP dGPT and dTTP, 40uM of dCTP, 0.15mM of -NAD. Two aliquotes of 5ul were taken for radioactivity counts. The estimated recovery of 1st strand after extraction was 60%. Considering that we started 1st strand synthesis (in MR) with 6ug RNA and obtained 17.7% incorporation it can be calculated that we obtained 1.062 ug 1st strand cDNA. About 40% were lost during extractions so that about 0.637ug were left. For 2nd strand reaction to 130ug mixture as above was added 0.64ul RNAase ("Enzo" 2000 u/ml) and 6.4ul DNA polymerase I (Boehringer - 50u/ul). This was termed second

strand main reaction (2nd MR). From it 10 ul were taken and 1ul ^{35}P-labeled dcTP was added to make 11 ul of second strand radioactive reaction (2nd RR). The 2nd MR and 2nd RR were incubated in parallel for 1h at 12°C and then 1h at 22°C. EDTA was added to final 20mM to stop the reaction. A 1ml aliquote of 2nd RR was diluted in TE and radioactivity counts of total to acid precipitable exhibited 3.5% incorporation (One ul from the 2nd RR was added to the 2nd MR for monitoring purposes during extraction).

If 1st strand was 0.637 ug and estimating C to be 1/4 of the nucleotides then the amount of C in the 1st strand DNA available for copying was $1.5^{9}x20^{-7}$ gr or $5.687x10^{-10}$ moles. In the 2nd MR we had 40uMC x 120ul or $4.8x10^{-9}$ Mol C. If incorporation ratio was 0.035 (3.5%, see above) then 2nd strand synthesis was:

$$\frac{0.035\ (4.8 \times 10^{-9})}{5.687 \times 10^{-7}} = 0.0717$$

or about 70% 2nd strand.

About $4x10^{4}$ cpm of ss(cDNA) and ds(cDNA) underwent agarose gel electrophoresis in 1.4% agarose. Alkaline buffer 30mM NaOH, 1mM EDTA) was employed for the run and DNA samples were loaded with sample buffer (50mM NaOH, 1mM EDTA, 2.5% Ficoll and 0.025% Bromo cresol green) ØX 174 Hae cut was used as molecular weight marker. The gel was run at 100V for 3h, then packed in a cellophane bag and underwent autoradio-

graphy. Fig. 2 presents the results of this electrophoresis showing the synthesis of both ss and ds DNA with ss MW ranging between about 250 bp and 1350 bp, while dsDNA appeared to be somewhat longer suggesting that some hairpin loop might have been synthesized. The cDNA underwent methylation to protect it from EcoRI cleavage at the stage which follows the addition of EcoRI linkers (see below). For methylation, the dried ds(cDNA) was dissolved in 20ul TE. A small amount of 2nd RR was added to the reaction mixture in order to increase the radioactive counts for the purpose of subsequent monitoring. S Adenyl-mentionine to 1mM final and 40u/2ul of EcoRI methylase (New England Biolabs) were added. The methylation reaction took place at 37°C for 10 min. The cDNA was then recovered by alcohol precipitation and finally resuspended in TE.

A filling reaction was carried out by mixing 10ul of cDNA in TE, 2ul 10xT_4 pol bufer [4], 5ul H_2O, 1ml 2mM each of dATP, dTTP, dGTP and dCTP cold,and 1ul of ^{32}P dCTP. An aliquote of 0.5ul from the above mixiture was removed to monitor acid precipitable counts before the reaction. The count was 348 cpm. One microliter (3u) of T_4 polymerase (New England Biolabs) was added and the reaction took place at 37°C for 15 min. EDTA (1ul of 500mM) was added and 0.5ul of post-reaction sample was taken for determining acid precipitable counts after the reaction (948 cpm). The DNA was extracted once in phenol chlorophorm and twice in chloroform then back

extracted, alcohol precipitated, rinsed in EtOH, dried and suspended in 10ul TE for continued processing.

Next step was the addition of EcoRI linkers. Kinasing of EcoRI linkers was carried out as described by Maniatis et al [4] by employing ^{32}P- γ -ATP (Amersham 3000 ci/mM) and polynucleotide kinase (New England Biolabs 12u/ul). An aliquote of the linkers underwent a ligation assay. Ligation was done using T_4 ligase (Boehringer 0.94u/ul) and an aliquote thereof restricted with EcoRI as described by Maniatis et al. [4]. Electrophoresis of kinased linkers, kinased and ligated linkers and of kinased, ligated and EcoRI-restricted linkers was carried out in 8% polyacrylamide gel as described by Maniatis et al. [4]. Fig. 3 presents the autoradiograph of this run exhibiting the ladder-like appearance of the various "Mers" of ligated linkers. This run also demonstrates that the ligated linkers can be cut. Once ligation and restriction of the linkers was demonstrated, another aliquote of kinased linkers was employed in a ligation reaction with ds(cDNA). For this purpose ds(cDNA) in 10ul TE was mixed with an excess of the kinased linkers (pretreated by warming to 65°C for 5 min then leaving at room temperature for 15 min.) in a volume of 14ul, 5ul 3x ligase buffer, 1.5ul of 20mM ATP and 1.6ul T_4 ligase. The mixture was incubated at 12°C for 25h. After ligation, EDTA (1.2ul of 0.5M) was added to the reaction tube, an aliquote of 1ul was taken for electrophoretic analysis and the remaining ligation

reaction mixed with NaAc, alcohol precipitated and dried. EcoRI digestion then followed in order to generate cohesive EcoRI cloning ends. For this purpose the dried ds(cDNA) to which linkers were ligated was dissolved in 200ul TE and mixed with 22ul 10x EcoRI buffer and 20ul EcoRI (BRL 10u/ul). Digestion took place overnight at 37°C. EDTA (4ul of 0.5M) was then added and the DNA precipitated in alcohol and dried. It was subsequently resuspended in 10ul TE and 0.5u aliquote taken for analysis by PAGE as described above. Fig. 4 shows the autoradiograph of this run (only the lane containing the cDNA + linkers is presented).

The vector phage λgt 11 was employed for cloning. It was received as EcoRI pre-digested gt 11 arms of which the 5' termini have been dephosphorilated with calf intestinal alkaline phosphatase to minimize self ligation.

A ligation reaction ("first ligation") was set with an estimated tenfold excess of vector to cDNA. A control ligation ("second ligation") reaction was set without cDNA. The "first ligation" reaction included 1ul 6X ligation buffer, 0.5ul DNA (roughly 150 ng), 3ul cDNA, 1ul 6mM ATP and 0.5ul T_4 ligase. The "second ligation" contained the same materials except for cDNA which was replaced by TE for volume. Total volume of the ligation was 6ul. Packaging was carried out using a kit provided by Stratagene according to the manufacturers instructions. Following packaging, SM buffer (0.1M NaCl, 0.05M Tris-Hcl pH 8.5, 10mM $MgSO_4$) and a

drop of chloroform were added. The final volume of the library was about 600ul. For titration, volumes of 0.1ml, 1.0 ul and 10ul of the packaging reaction were mixed with SM and underwent plating on Y1088 cells according to Huynh et al [5]. IPTG and x-gal were included in the top agarose for white/blue plaques differentiation. When 0.1ul packaging reaction was used for titration 78% white colonies were detected with the "first reaction"(Vector + Insert) and 65% white colonies were detected in the "second reaction" (no insert). In a second titration the percentages were 81 and 62 respectively. These figures suggest that 10-20% of the white colonies represent recombinant phage, the total size of the library was about $0.5x10^5$.

Screening of the library by antibodies awaits the preparation of suitable antibodies against peptide epitopes of MEG. On the basis of the relative abundance of MEG, which is a major glycoprotein in SEA and comprises about 5% of the total SEA constituents we assume that although the egg cDNA library is small, clones relevant to MEG should be identified providing suitable antibodies are prepared.

B. DEGLYCOSILATION AND OTHER MANIPULATIONS OF MEG

Deglycosilation of MEG was found to be necessary for two main reasons. Firstly, anti- MEG antibodies produced by us (both rabbit policlonals and murine monoclonals) were directed to

resistant epitopes of MEG was observed). Secondly, clear-cut amino acid microsequencing was not possible because of the high degree of glycosilation. Deglycosilation was therefore attempted by chemical and ezymatic approaches which are expected to leave the polypeptide backbone intact.

B.1. Attempts at deglycosilation of MEG by trifluoromethyl sulfonic acid (TFMS)

TFMS has been said to cleave O-glycosyl bonds [5]. Two alternative procedures were employed with equal success. Nanogram quantities of ^{125}-labelled MEG to mg quantities of Fetuin (which was used as control) underwent TFMS treatment. The first procedure was according to Weiss et al. [6]. Fetuin (1.5mg) was weighed into a V-vial (Wheaton) and dissolved in 200ul TFMS: Anisole (4:1) at 0°C. N_2 was bubbled in (all steps took place under a fume hood) and the vial closed tightly. The reaction vial was put on ice for 3h and the reaction terminated by adding 0.6ml of pyridine:meter (3:4) at 0°C. After 30min on ice the reaction mixture and 0.2ml of water wash were dialized against 0.01M phosphate buffer pH 7.4. Alternatively, desalting on Sephadex G-25 took place in which case water wash was not included. When ^{125}I-MEG was treated by TFMS, the volumes of the reaction components was reduced ten-fold. The second procedure was according to Edge et al. [5]. The following is a description of the procedure as carried out with 1.5 mg Fetuin. Fetuin in a V-vial was

dissolved in 200 ul TFMS:Anisole. N_2 was bubbled in for 30 sec and the reaction proceeded for 3 h at 0°C. Extraction then followed by adding 400ul diethyl ether cooled to -40°C, and 400ul of ice cold 50% aqueous pyridine. The resulting precipitate was redissolved, the ether phase removed and the extraction repeated. Dialysis was then carried out. During both procedures the recovery ranged between 45 and 60%. The products were run by PAGE and 10% polyacrylamide gel according to Laemli [7]. Fig. 5 presents the results of the electrophoretic analysis of Fetuin before and after TFMS treatment. About 30% reduction in molecular mass was observed. Fig. 6 demonstrates the results of PAGE analysis of ^{125}I-SEA glycoprotein fraction before and after deglycosilation with TFMS. Fig. 7 demonstrates the results of PAGE analysis of ^{125}I-MEG. TFMS has an effect on ^{125}I-labeled SEA-gly. Thus, small molecular species can be seen in TFMS treated SEA-gly but not in untreated SEA gly. ^{125}I-MEG on the other hand was not affected by TFMS. We assume that MEG is unaffected by TFMS due to its structural qualities and not because TFMS reaction was not effective.

B.2. <u>Attempts at deglycosilation of MEG by Endoglycosidase H (Endo H).</u>

Endo H (Endo-β-N-acetylglycosaminidase H) will cleave an N-linked oligosaccharide from a glycoprotein leaving an N-acetylglucosamine residue attached to the polypeptide. The

cleavage occurs between the residues of the N,N'-diacetylchitobiose ($GlcNAc_c$ 1-4GlcNAc) within the core sequence, R,Man 1 -- 3 (R_2 Man 1 -- 6) Man 1 -- 4 GlcNac - Asn. Endo H cleaves the oligosaccharide if R_2 is a hydrogen, a mannose (Man) or a series of Man residues. The susceptibility of oligosaccharide structures to hydrolysis by Endo H is summarized in [8]. The reaction was carried out as described by the manufacturer (Boehringer) with ^{125}I-MEG and with Fetuin. The effect of Endo H cleavage on Fetuin and on MEG was tested by running the products in PAGE. No effect was observed in both cases, it was therefore concluded that the enzyme was inactive. Radioimmunoassay by the method of Farr as described by us for MEG analysis [9] was carried out using a monoclonal antibody (Mab) Kcsmeg2 which is active with carbohydrate epitopes of MEG. No cleavage activity was observed since both treated and untreated MEG were equally reactive with the Mab (results not shown).

B.2. Attempts at deglyconsilation of MEG by Glycopeptidase F (PNGase F)

PNGase F (Glycopeptide-N-Glycosidase; N-glycanase) will cleave an intact oligosaccharide from a native or denatured glycoprotein. The cleavage occurs between the innermost residue of N,N'-diacetylchitobiose and the asparagine to which the oligosaccharide is linked. It readily cleaves most high mannose oligosaccharides, hybrid oligosaccharides and

high mannose oligosaccharides, hybrid oligosaccharides and biantennary, triantennary or tetra antennary complex oligossacharides. Its substrate specificity has been described in details [8].

Two sets of reaction conditions were used in our study. The first one described by the manufacturer (Boehringer), and the other by Haraguchi et al. [10]. None of these conditions succeeded to deglycosilate Fetuin nor ^{125}I-MEG or even N-glycopeptide- galactosidase-labeled (which is recommended by Boehringer for measuring PNGase activity). Several repeats as well as testing three separate batches of PNGase did not succeed to deglycosilate MEG and the above-mentioned controls. In each case analysis of the post-deglycosilation material by SDS PAGE did not reveal any change in molecular mass, nor did IRA with anti-carbohydrate epitopes of MEG show any binding differences (Details of the negative experiments are not provided here). It thus became apparent that the PNGase batches used by us were inactive. This was eventually confirmed by the manufacturer. Further experiments with a new batch of PNGase are under way.

The deglycosilation by PNGase is considered by us a key step for obtaining MEG polypeptide suitable for inducing antipeptide antibodies and for obtaining undisturbed amino-acid sequence of MEG.

B.3. Partial deglycosilation of MEG by sodium metaperiodate ($NaIO_4$)

This was done on the basis of the work we have previously described on the treatment of MEG by $NaIO_4$ [11]. ^{125}I-MEG was oxidized by mixing ^{125}I-MEG exhibiting desired radioactive counts with $NaIO_4$ (5mM final) in a final volume of 100ul 50mM acetate buffer pH 4.5. Fetuin and BSA (both at 90ug/100ul) were treated as controls. The reaction took place at 4°C for 2h in the dark. It was stopped by adding 1ul glycerol (130mM final). The samples were then desalted on Sephadex G-25 column (in Pasteur pipettes) and analyzed by SDS-PAGE, and by antibody binding. Fig. 8 shows the results of $NaIO_4$ treatment of ^{125}I-MEG for 2-24 h. Upon PAGE a progressive reduction in molecular mass can be observed down to about half the original molecular mass (about 50 kd). The question of the variable molecular weights observed for MEG (50 kd and about 70 kd) is discussed below. Fig.9 shows that periodate treatment of MEG reduces its capacity to bind to chronic infection (20 weeks) mouse-serum (IMS_{20}). However about 30% of the binding activity remains intact suggesting that IMS_{20} includes antibodies to periodate resistant epitopes (presumably peptide epitopes) of MEG. This finding is of importance since if we fail to produce artificially antibodies to MEG polypeptide we may have to compromize and use IMS for immunoscreening of our cDNA library.

Preparative $NaIO_4$ treatment of MEG was carried out under similar conditions. This was done in order to obtain partially deglycosilated MEG for immunization in an attempt to obtain a higher proportion of antipeptide antibodies.
To 50ug/4.4ml MEG were added 500ul of 50mM $NaIO_4$ in 500mM Na Acetate pH 4.5. The mixture was incubated overnight (16h) at 4^oC in the dark. The reaction was stopped by adding 50ul glycerol and desalting took place on a Sephadex G-25 column with 120ml bed volume against water. The column was calibrated with ^{125}I-MEG, the relevant fractions lyophilyzed and run on SDS-PAGE. A control gel lane was stained with silver stain and the corresponding band cut out from the main gel slab and used to immunize mice.

B.4 Mapping of MEG fragments

The molecular mass of MEG as originally described was about 70 kd [9]. In a later study we described a carbohydrate rich fragment (CRF) which cross reacts serologically with MEG but does not induce granulomatous hypersensitivity nor elicit delayed foot-pad swelling [13]. The analog of MEG from the Puerto Rican strain of S. mansoni is MSA_1 [11,14,15], the molecular mass of which has been estimated to be about 50kd. We have observed that very old batches of MEG or of SEA from which the MEG M.W. was originally about 70kd yielded a 50kd MEG. We assume therefore that MEG (70kd) undergoes cleavage in vitro at conditions yet unknown to give rise to a molecule

of 50kd similar to MSA_1, and to a breakdown product, possibly CRF. This assumption has yet to be confirmed experimentally but so far there are some supporting evidence from studies on *in vitro* egg culture (see below). We have started a study on MEG fragments with regard to size, serological activity and immunopathological activity. Fragmentation by Cyanogen Bromide [16], Formic Acid [17], N-cholorosuccinimide/urea [18] and hydroxylamine [19] are underway. Fragments will be used for inducing gramolomatous hypersensitivity.

C. *IN VITRO* CULTURE OF *S. MANSONI* OVA AND METABOLIC LABELING

In vitro

Culture of MEG was carried out in the presence of ^{35}S-Methionine as described in the 1st report. SDS PAGE analysis of the products was carried out. When ^{35}S-labeled (metabolically labeled) SEA was analyzed a major band was observed with molecular mass of about 70 kd. Only a minor band corresponding to 50kd was seen in metabolically labeled MEG, as can be seen in Fig. 10. Stored SEA usually did not contain a major 70kd band suggesting that this band undergoes breakdown during storage. This is in line with the abovementioned assumption that "70kd MEG" gives rise to a "50kd MEG" (plus small fragments). Culture supernatant (taken after eggs were maintained in it for 24h with ^{35}S-methionine) was analyzed by SDS-PAGE. For concentrating the constituents in the supernatant and washing out the excess radioactivity and culture materials, the culture supernatant

underwent adsorbtion onto NCP. This was carried out by incubating an NCP piece measuring 0.5 x 0.5cm in an eppendorf tube containing culture supernatant for 24h while shaking. The NCP piece was then rinsed in 0.01M phosphate buffer then mixed with SDS-PAGE sample buffer, boiled and the supernatant after boiling applied onto the gel. A 60% of the adsorbed radioactivity was recovered in the sample buffer after boiling the NCP. It was seen (Fig. 10) that the major band in the ^{35}S-labeled culture supernatant was a 50kd molecule. This finding tentatively suggests that upon secretion the 70kd molecule becomes a 50kd molecule. Freshly prepared SEA gives rise to a major 70kd band in the glycoprotein fraction as seen in Fig. 11. A 50kd molecule was also present. This is in line with the assumption that 70kd MEG is present in freshly prepared SEA (from freshly harversted eggs) and that it disintegrates upon storage to give rise to a 50kd molecule. Studies are underway to add evidence to support this assumption.

An experiment was carried out in order to demonstrate <u>in vitro</u> incorporation of M^3-Mannose into egg constituents. Ova were harvested under sterile conditions and 450000 ova (exhibiting 38% mature eggs and 31% movement or hatching) were cultured in 8ml medium DMEM minus glucose, and supplemented with 100ug/ml streptomycin and 100u/ml penicillin, 292ug/ml L-glutamine 0.2u/ml insulin and 250ug/ml oxalacetic aid. Mannose (D 2 ^3H, New England Nuclear. Specific activity 30 ci/mMol, 1mci/ml). For <u>in vitro</u> culture 5ml of supplemented medium plus 1ml ^3H-Man + eggs were incubated in a 2ml tissue culture flask at 37°C in a 5% CO_2

atmosphere. Following 48h incubation egg viability was 23% and sterility was proven by innoculating supernantant on proteose peptone agar plates.

SEA and culture supernatant were harvested, aliquotes adsorbed onto NCP and the radioactivity counted. Radioactivity (^{3}H-Mannose) in SEA was only 2.5 times higher than background radioactivity, while the supernatant exhibited NCP-bound radioactivity about one hundred times higher than the background. SDS-PAGE analysis of the culture supernatant exhibited aggregated material at the top of the gel, plus a 50kd constituent and a 45kd constituent. No 70kd constituents were observed (Fig. 12). These results were now repeated. They seem to indicate that ^{3}H-Mannose is incorporated mainly to substances extruded from the eggs of which a 50kd MEG may be one. The presence of a gramuloma inducing activity in culture supernatants was demonstrated in the mid seventies [20], since MEG is a proven immunopathologically active egg antigen it can be concluded that it was present in the culture sup. The finding that the putative ^{3}H-labeled MEG in the culture fluid is a 50kd molecule again suggests that upon secretion the native 70kd molecule of MEG is cleaved.

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LIST OF FIGURES

Fig. 1: Agarose gel electrophoresis of RNA from S. mansoni ova

RNA was harvested from ova by the Guanidinium isothiocyanate method and exhibited a 260nm/280nm O.D. ratio of 1.9. RNA from S. mansoni was run on a 1.5% agarose gel under non-denaturing conditions.

Fig. 2. Agarose gel electrophoresis of ss(cDNA) and ds(cDNA).

About $4x10^4$ cpm of ss(cDNA) and ds(cDNA) underwent electrophoresis in 1.4% agarose/alkaline buffer (see text). The gel was run at 100V for 3h and then underwent autoradiography.

Fig. 3: Polyacrylamide gel electrophoresis of EcoRI linkers

Linkers (lane 1), kinased and ligat ed linkers (lane 2), and kinased ligated and EcoRI-restricted linkers (lane 3) were run on 8% polyacrylamide gel with TBE buffer at 150V, and autoradiographed.

Fig. 4: Polyacrylamine gel electrophoresis of cDNA with attached EcoRI liners

Conditions of the run were as described in Figure 3.

Fig. 5: Polyacrylamide gel electrophoresis of Deglycosilated Fetuin

TFMS treated Fetuin (lane 1) and untreated Fetuin (lane 2) were analyzed by SDS-PAGE on 10% gel.

Fig. 6: Polyacrylamide gel electrophoresis of TFMS treated SEA-gly. TFMS treated SEA-gly (lane 1) and untreated control SEA-gly (lane 2) were analyzed by SDS-PAGE on 10% gel.

Fig. 7: Polycrylamide gel electrophoresis of TFMS treated MEG TFMS treated MEG (lane 1) and untreated control SEA-gly (lane 2) were analyzed by SDS-PAGE on 10% gel.

Fig. 8: Polyacrylamide gel electrophoresis of $NaIo_4$ treated MEG ^{125}I-MEG was treated with 5mM $NaIO_4$ for 2h (lane 2), 4h (lane 3), 8h (lane 4), 16h (lane 5) and 24h (lane 6), and analyzed by SDS-PAGE on 10% gel. Untreated ^{125}I-labelled MEG (lane 1) was run as a control.

Fig. 9: Antibody binding courves of $NaIO_4$ treated and untreated ^{125}I-labelled MEG. Analysis of antibody binding was carried out by the ammonium sulfate method of Farr. Binding was tested with serum collected 8 weeks after infection (IMS_8) and 20 weeks after infection (IMS_{20}). The results are presented as net % binding (after subtraction of binding with normal mouse serum).

Fig. 10: Comparative analysis of freshly synthesized SEA and freshly secreted egg constituents. These are combined results of 2 separate analyses by SDS-PAGE on 10% gels. Eggs were cultured *in vitro* in the presence of ^{35}S-Methionine the

supernatant separated and eggs hemogenized and ultracentrifuged to produce SEA. The fresh products were analyzed.
Lane 1- SEA. Lane 2- Culture supernatant.

Fig. 11: Analysis by SDS-PAGE of SEA-gly prepared from fresh SEA metabolically labeled with ^{35}S-Methionine

SEA was prepared from egs cultured *in vitro* in the presence of ^{35}S-Methionine. The glycoprotein fraction was separated by affinity chromatography on Con-A-Sepharose 4B and analyzed.

Fig. 12: Analysis by SDS-PAGE of in vitro secreted egg constituents metabolically labeled with ^{3}H-mannose

Figure 1.

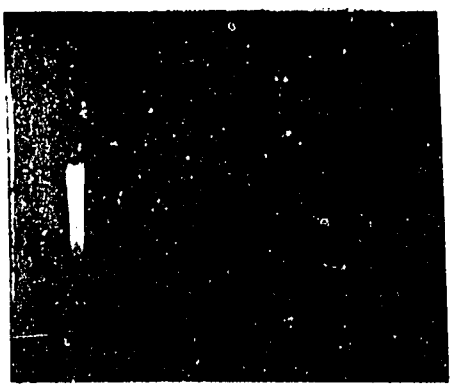

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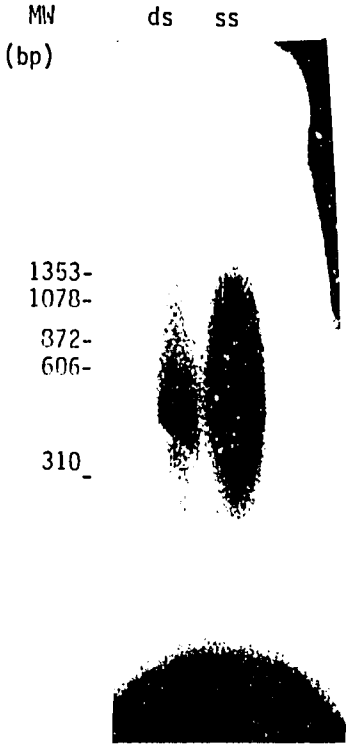
Figure 2.



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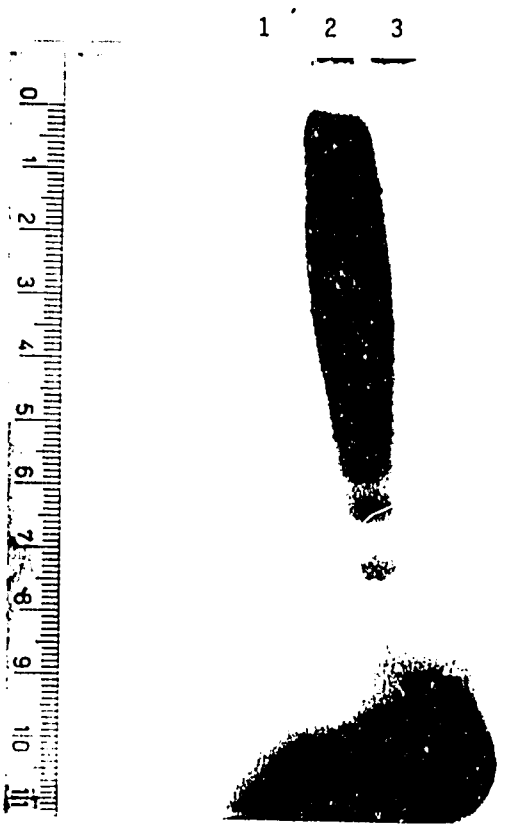

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Figure 4.

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Figure 5.

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Figure 6.

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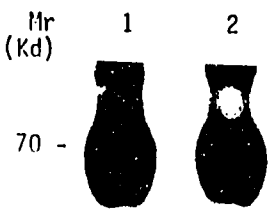
Figure 7.



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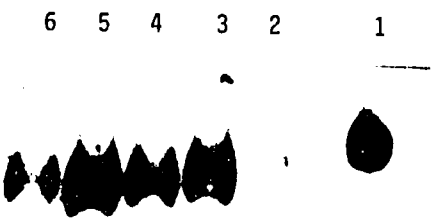

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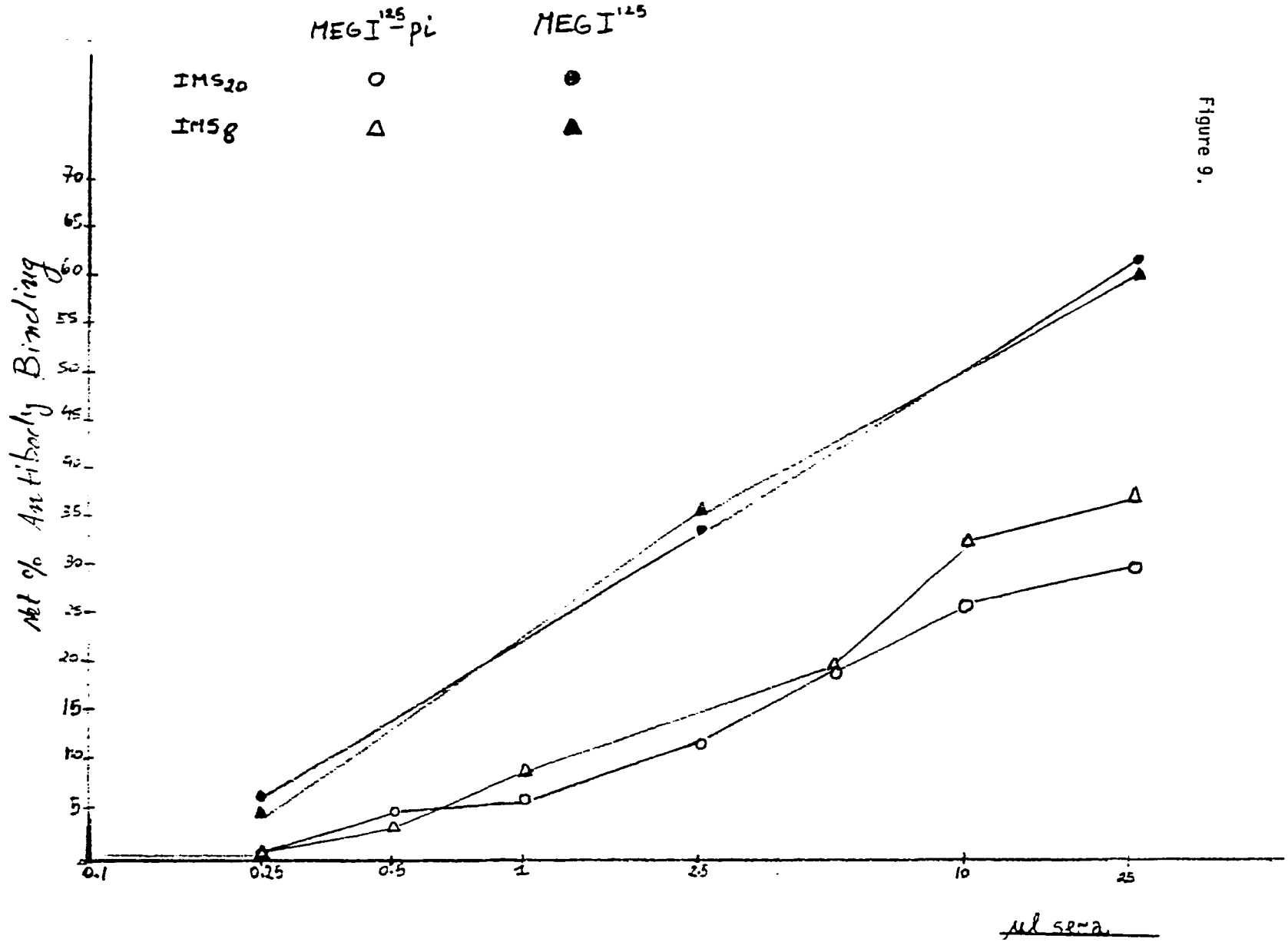

Figure 9.

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Figure 10.

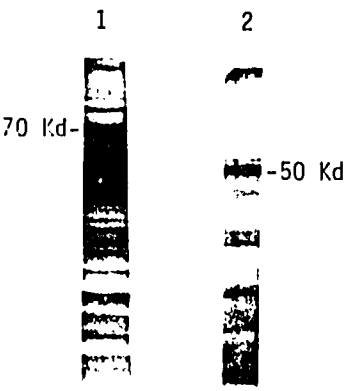

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Figure 11.

-70 Kd

-50 Kd

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Figure 12.

Aggregates ?

- 50 Kd
- 45 Kd

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