IN VITRO PRODUCTION AND CHARACTERIZATION OF EXCRETORY/SECRETORY PRODUCTS OF *ONCHOCERCA VOLVULUS*

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Summary :

Onchocerca volvulus excretory/secretory products (ESP) free of host contaminants are often needed for immunologic and biochemical studies. Since prolonged *in vitro* survival of *O. volvulus* in culture requires the presence of human serum, as a supplement, it was necessary to investigate other culture medium supplements in which pure ESP could be generated. Thus heat-inactivated normal rabbit serum, fetal calf serum and the synthetic serum substitute Ultrosor-G were tested for their abilities to sustain *O. volvulus* adult females and nodular microfilariae *in vitro* and their abilities to promote the synthesis and release of ESP. Using [³⁵S]-methionine as the radioactive precursor, *O. volvulus* nodular microfilariae and adult females were shown to actively synthesize and release excretory/secretory proteins with increasing efficiency in human serum, rabbit serum, Ultrosor-G and fetal calf serum. Analysis of the ESP by SDS-PAGE revealed that at least 30 polypeptides in the 7-174 kDa range were continuously released. About 14 of these components were immunogenic to the human host as shown by immunoprecipitation. Prominent antigenic polypeptides were those with relative molecular weights of 13, 16, 33, 68 and 170. Rabbit serum, fetal calf serum and Ultrosor-G could therefore, conveniently replace human serum in cultures of *O. volvulus* nodular microfilariae and adult females.

KEY WORDS : *Onchocerca volvulus*, *in vitro* culture, excretory/secretory products, Ultrosor-G.

MOTS CLÉS : *Onchocerca volvulus*, culture *in vitro*, produits d'excrétion/sécrétion, Ultrosor-G.

Résumé : PRODUCTION ET CARACTÉRISATION *IN VITRO* DES PRODUITS D'EXCRÉTION/SÉCRÉTION D'*ONCHOCERCA VOLVULUS*

Les produits d'excrétion/sécrétion (PSE) d'*Onchocerca volvulus* exempts d'impuretés de l'hôte sont utiles pour les études immunologiques et biochimiques. Cependant la survie prolongée d'*O. volvulus* en culture *in vitro* nécessite la présence de sérum humain comme additif au milieu de culture. Mais les PSE obtenus avec du sérum humain sont souvent contaminés par des composants du sérum. Il est donc indispensable d'évaluer d'autres additifs de milieu de culture pour lesquels des PSE plus purs peuvent être générés. Ainsi, le sérum normal de lapin inactivé à la chaleur, le sérum de veau fœtal, le sérum humain et l'additif synthétique Ultrosor-G ont été testés pour leur capacité à maintenir *in vitro* les femelles adultes et les microfilaries nodulaires d'*O. volvulus* ainsi que pour leur capacité à promouvoir la synthèse et la libération des PSE. En utilisant la [³⁵S]-méthionine comme précurseur radioactif, les deux stades du parasite étudiés ont montré une synthèse et une libération active de PSE avec une efficacité croissante pour les milieux de culture contenant respectivement comme additif le sérum humain, le sérum de lapin, l'additif synthétique Ultrosor-G et le sérum de veau fœtal. L'analyse des PSE par SDS-PAGE a révélé qu'au moins 30 polypeptides de taille allant de 7 à 174 kDa sont continuellement libérés. Par immunoprécipitation, il a été démontré qu'au moins 14 de ces polypeptides sont immunogéniques pour l'homme. Les principaux polypeptides antigéniques ont pour poids moléculaires relatifs : 13, 16, 33, 68 et 170 kDa. Le sérum de lapin, le sérum de veau fœtal et l'Ultrosor-G peuvent ainsi convenablement remplacer le sérum humain pour la maintenance *in vitro* de ces stades d'*O. volvulus* et pour l'obtention de PSE moins contaminés.

INTRODUCTION

Parasitic filariae release into the tissues and body fluids of their hosts excretory-secretory products (ESP) which circulate freely (Ouaissi *et al.*, 1981; Des Moutis *et al.*, 1983; Petralanda *et al.*, 1988; Maizels *et al.*, 1990) or as immune complexes (Paganelli *et al.*, 1980; Steward *et al.*, 1982). The origin of these ESP appears to be diverse, ranging from uterine secretions of gravid adult females to surface-associated components released as a result of the dynamic nature

of the cuticle (Haque & Capron, 1986). Some of these ESP have been found to be biologically active molecules such as enzymes (Harnett *et al.*, 1989) which aid in the migration of these tissue-dwelling parasites through host tissues. By virtue of their direct contact with the host immune system, they may also persistently stimulate the host immune system, causing local inflammatory responses (Maizels *et al.*, 1982; Ottessen, 1984; Philipp *et al.*, 1988). Furthermore, ESP act as the most reliable indicators of current infection or the presence of viable parasites in the host (Selkirk *et al.*, 1986).

O. volvulus has been maintained in culture for two to three weeks in medium supplemented with human serum (Ngu *et al.*, 1981; Engelbrecht & Schulz-Key, 1984; Walter, 1988) with regular changes of the

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medium. Prolonged survival of the parasite *in vitro* is however, required for metabolic studies as well as for the testing of anti-filarial products. Based on the hypothesis that the *in vitro* released products in culture media may correspond to those synthesised *in vivo*, culture supernatants have been considered as a rich source of ESP (Selkirk *et al.*, 1986). The frequent approach to the collection and characterization of ESP is therefore, by the *in vitro* culture of viable parasites for several hours in the presence or not of radiolabelled precursor amino acids.

Several studies have demonstrated that filarial ESP are immunologically important though fewer in number than the corresponding somatic antigens (Maizels *et al.*, 1982; Ottesen, 1984; Selkirk *et al.*, 1986). However, ESP derived from medium supplemented with human serum are not suitable for further work especially for the immunization of experimental animals because of the presence of contaminating human serum proteins. The aim of this study was to investigate conditions for the *in vitro* production of pure ESP.

MATERIALS AND METHODS

PARASITES

Nodular microfilariae (mf) were isolated from fresh nodules by dissection of the latter under sterile conditions. The emergent mf were then purified by centrifugation on percoll (Pharmacia LKB) gradients (Chandrasheker *et al.*, 1984; Titanji *et al.*, 1987). Adult female worms were on the other hand obtained from undamaged fresh nodules treated with collagenase from *Clostridium histolytica* (Boehringer, Mannheim), under aseptic conditions (Engelbrecht & Schulz-Key, 1984). The isolated worms were then sub-cultured for 2-3 hours and the viability ascertained by observation of the movements of the adult parasites with the naked eye and that of the nodular mf under the microscope. After isolation, the viability exceeded 98 % for the nodular mf and was 100 % for the adult female worms.

ANTISERA

Human onchocerciasis patient sera were prepared from blood obtained from consenting nodule donors living in a hyper-endemic area in the SA'A sub-Division in the Sanaga River basin (Cameroon). A pool was made from the sera of 54 patients as a hyper-immune patient serum pool. Normal human serum pool was similarly reconstituted from blood donated by 10 Swedes with no sign of the disease.

IN VITRO CULTURE OF NODULAR MF IN THE PRESENCE OF ULTROSER-G

Nodular mf (1,000-2,000) in a total volume of 1.5 ml, were cultured in medium RPMI 1640 (Gibco) containing 0.16 mg/ml gentamycin and supplemented with the synthetic serum substitute, Ultrosor-G at 1, 2 or 4 %. Medium RPMI 1640 containing the antibiotic only or the same medium supplemented with 10 % human serum were used as controls. The cultures were maintained at room temperature under sterile conditions for as long as 72 hours during which the viability of the parasites was determined as a function of the incubation time.

METABOLIC LABELLING OF PARASITES

All *in vitro* culture of parasites were carried out under sterile conditions. The procedure that follows, represents the final protocol adopted for a typical experiment. The typical experiment consisted of performing simultaneously, all the six assays with one adult female worm per assay, after a series of trials to determine the optimal concentration of each supplement, the incubation time as well as the reaction volume.

In this procedure, the parasites were selectively starved from the precursor amino acid, methionine by maintaining them in methionine-free medium, i.e. EMEM without methionine and glutamine, (Flow laboratories) containing 0.16 mg/ml gentamycin for 3 hours at room temperature. After washing twice in the same medium, the parasites were then re-suspended in 1.5 ml for 6,000-10,000 nodular mf or 2.5 ml per adult female of the labelling medium (methionine-free medium containing 0.16 mg/ml gentamycin, 4 mM glutamine). To each assay was added either of the following medium supplements : 10 % normal human serum (NHS), 10 % heat-inactivated normal rabbit serum (NRS), 10 % fetal calf serum (FCS), or 1 % Ultrosor-G. In another assay, the two parasite stages were selectively starved from methionine, pre-incubated at 42 °C for 20 min (heat-shock treatment) in the labelling medium supplemented with 10 % FCS.

The radioactive precursor, [³⁵S]-methionine (Amersham, SJ 123) was added at 200-250 µCi per assay, and labelling performed for 24 hours either at 37 °C or at room temperature (20-25 °C). During the labelling, aliquots of 100 µl were taken from the adult female cultures following a time course and processed for liquid scintillation counting (see below). At the end of the labelling, the media from the adult female cultures were transferred to new tubes and then centrifuged at 5,000 × g for 10 min at 4 °C to pellet the *in vitro* released microfilariae. The nodular mf cultures were also centrifuged as above. The supernatant in each case constituted the pool of adult female ESP and nodular

mf ESP respectively. To inhibit proteolysis, freshly prepared phenylmethyl sulfonylfluoride (PMSF) was added to each tube to a final concentration of 1 Mm before storage in aliquots at -20°C .

The nodular mf pellet and the adult female worms were washed twice in methionine-free medium and stored frozen at -20°C until required for the extraction of antigens. During the entire labelling period, adult females released eggs some of which developed into microfilariae. These *in vitro* released eggs and nodular mf were separated and purified over percoll gradients (Titanji *et al.*, 1987). Somatic antigens were prepared from the radiolabelled nodular mf and the purified *in vitro* released eggs as previously described (Titanji *et al.*, 1990).

SCINTILLATION COUNTING

The aliquots taken from the adult female cultures at various time points were immediately centrifuged at $10,000 \times g$ for 5 min at 4°C to pellet the mf and the supernatant transferred into clean tubes. These were precipitated with 10 % trichloroacetic acid (TCA) in the presence of 10 % normal human serum or normal rabbit serum as carrier proteins. The precipitates were washed thrice with PBS, then air-dried and redissolved in a scintillation cocktail comprising 0.05 % POPOP, 0.7 % PPO, 35 % triton X100 in scintillation grade toluene for counting using a Packard (2002) liquid scintillation counter.

IMMUNOPRECIPITATION

The frozen radiolabelled ESP were thawed and either used directly or cleared with NHS. In the latter option, the immune complexes were removed by precipitation with 10 % washed pansorbin (*Staphylococcus aureus* stock, Calbiochem). After centrifugation at $3,000 \times g$ for 15 min at 4°C , the supernatant constituted the cleared ESP.

Radioimmunoprecipitation was performed as described (Kessler, 1975) with some modifications (Titanji & Ngu, 1986) using the hyper-immune patient serum pool and the control normal human serum pool. The immune complexes were precipitated with 10 % washed pansorbin, washed thrice with immunoprecipitation buffer (50 mM Tris-HCl pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02 % sodium azide) and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

ELECTROPHORESIS, FLUOROGRAPHY AND AUTORADIOGRAPHY

Electrophoresis of ESP, somatic extracts from nodular mf and *in vitro* released eggs and immune complexes was performed by SDS-PAGE under reducing condi-

tions according to the method of Laemmli (1970) in either 6-20 % gradient or 10 % slab gels. The SDS gels were loaded with samples on the basis of TCA precipitable cpm ($10-15 \times 10^3$ cpm were loaded per well) and Pharmacia low molecular weight standards were applied in parallel lanes. After electrophoresis, the gels were fixed for 1 hour in 10 % TCA, stained with Coomassie brilliant blue R-250 and then destained. Fluorography and drying were performed according to the method of Westermann (1985). Briefly, the fixed gels were rinsed in distilled water and then immersed overnight at room temperature in 20 volumes of absolute acetone containing 20 % diphenyloxazole (PPO). The dehydrated gels were then exposed directly onto either Agfa safety curix RP1 or Hyperfilm- β max (Amersham) films.

RESULTS

EFFECT OF ULTROSER-G ON THE SURVIVAL OF NODULAR MF *IN VITRO*

To investigate the effect of Ultrosor-G on the *in vitro* survival of nodular mf, as a substitute of normal human serum, three concentrations of the product were used. As shown in figure 1, the *in vitro* culture of nodular mf could conveniently be performed in medium containing Ultrosor-G at either 1 or 2 % of this product. The viability of the nodular mf was found to decrease to less than 40 % after 48 hours in medium supplemented with either serum or Ultrosor-G. After the same incubation time the viability was less than 20 % in medium RPMI 1640 only (Fig. 1). It should be noted that the viability of the nodular mf in medium supplemented with 1 % (v/v) Ultrosor-G was found to approach that in NHS in the early periods of the cultures (within 24 hours). Hence, this concentration of the synthetic product and a total incubation period of 24 hours were used in our subsequent experiments and could as well be recommended for prolonged *in vitro* maintenance of parasites in serum-free media.

EFFECT OF INCUBATION TIME ON THE SYNTHESIS AND RELEASE OF ADULT FEMALE ESP

In order to determine the optimal conditions for the *in vitro* synthesis and release of radiolabelled parasite molecules with time, the effect of FCS, NHS, NRS and the serum substitute Ultrosor-G on the synthesis and release of adult female ESP, was investigated as a function of the amount of TCA-precipitable radioactivity. Due to the difficulty in obtaining large numbers of living adult female worms, each assay was performed with one adult female worm only, with the six worms being observed simultaneously. The viability of the

Fig. 1. — Effect of Ultrosor-G on the survival of *O. volvulus* nodular microfilariae. Nodular mf were cultured in medium RPMI 1640 only (RPMI) or this medium supplemented with either 10 % human serum (HS) or Ultrosor-G at 1 % (1 % UTS), 2 % (2 % UTS) and 4 % (4 % UTS). The viability of the worms was assessed over a 72 hours period under sterile conditions and at room temperature.

parasites during the entire period of the experiment was carefully monitored by both visual and microscopic movements of the worms. All the worms were found to be viable at the end of the 24-hour incubation. The results depicted in figure 2 indicate that the amount of TCA-precipitable radioactivity in the adult female cultures containing the sera or Ultrosor-G was influenced by the nature of the medium supplement and the time after addition of the radiolabelled methionine. Although the overall trend was similar in all the assays, the rate of synthesis and hence release of *de novo* synthesized parasite polypeptides appeared to be rapid within the first six hours following their selective starvation from the precursor amino acid. Prolonged *in vitro* culture of the parasites (beyond 6 hours) led to gradual decrease in the amounts of the TCA-precipitable ESP (Fig. 2). With the exception of NHS, all other supplements displayed rather similar rates of ESP.

SDS-PAGE PATTERNS OF THE *IN VITRO* RELEASED PRODUCTS (ESP)

In the preliminary experiments, adult female worms and nodular mf were labelled in medium supplemented with FCS. When the culture supernatant were analyzed by SDS-PAGE and autoradiography, at least 30 polypeptides with molecular weights ranging bet-

ween 7.0 and 174 kDa appeared to be synthesized and released *in vitro* as early as 2 hours after addition of the radioactive methionine (data not shown).

When the nodular mf and adult female *in vitro* released products generated in culture media supplemented with the different sera or Ultrosor-G were similarly analyzed, the polypeptide patterns of the [³⁵S]-methionine labelled ESP from adult females (Fig. 3) and nodular mf (Fig. 4) were as shown. For both parasite stages, the synthesis and release of ESP under these conditions appeared to be most effective in medium containing FCS and least in that containing NHS. Other supplements such as heat-inactivated NRS and Ultrosor-G appeared to be equally effective.

Although the gel patterns of the ESP were similar in both stages, significant quantitative and qualitative differences in the released polypeptides were observed with respect to the medium supplement. Thus adult female worms produced stable high molecular weight polypeptides when cultured in medium supplemented with FCS and not in that containing either NHS, NRS or Ultrosor-G (Fig. 3). Nodular mf on the other hand produced identical polypeptide patterns when cultured in media supplemented with either FCS, NRS or Ultrosor-G (Fig. 4). Furthermore, the detected components were present in the labelled somatic extracts from nodular mf and *in vitro* released eggs (Fig. 4, lanes 7 and 8 respectively).

Fig. 2. — Effect of incubation time on the *in vitro* synthesis and release of [35 S]-methionine labelled proteins of adult female worms. One adult female worm per assay was labelled in methionine-free medium supplemented with either normal human serum (NHS), heat-inactivated normal rabbit serum (NRS), Ultrosor-G (UTS), normal human serum incubated at 37 °C (NHS at 37 °C), fetal calf serum with heat-shock treatment of parasites (HSR) and fetal calf serum (FCS). Aliquots of the culture supernatant from each of the assays were TCA-precipitated and analyzed by liquid scintillation counting.

Fig. 3. — Major *in vitro* released polypeptides of *O. volvulus* adult female worms. One adult female worm per assay was labelled in methionine-free medium supplemented with either NHS (lane 1), NRS (lane 2), Ultrosor-G (lane 3), FCS with heat-shock treatment of the parasites (lane 4), FCS (lane 5) or NHS at 37 °C (lane 6) and aliquots of the culture supernatant analyzed by SDS-PAGE and autoradiography.

Fig. 4. — Major *in vitro* released polypeptides of *O. volvulus* nodular microfilariae. Nodular mf cultures containing 6.000 to 10.000 worms per assay were labelled in methionine-free medium supplemented with either NHS at 37 °C (lane 1), NRS (lane 2), Ultrosor-G (lane 3), FCS after heat shock treatment (lane 4), FCS at 37 °C (lane 5) or FCS (lane 6). Aliquots of the culture supernatant or somatic extracts from labelled nodular mf (lane 7) and *in vitro* released eggs (lane 8) were then analyzed by SDS-PAGE and autoradiography.

Heat-shock treatment of the parasites at 42 °C for 20 min followed by labelling and continued incubation at 37 °C had little or no stimulatory effect on the synthesis and/or shedding of proteins from nodular mf (Fig. 4, lane 4). Under the same conditions adult females were able to release a 63 kDa polypeptide (Fig. 3, lane 4).

REACTIVITY OF THE *IN VITRO* RELEASED PRODUCTS WITH HOST ANTIBODIES

In order to determine whether the secreted proteins were antigenic to the host, the ESP from the two worm stages prepared in media supplemented with the sera or Ultrosor-G were immunoprecipitated with a hyper-immune patient serum pool ($n = 54$) and a control NHS pool ($n = 10$). As shown in figure 5, at least 14 of the secreted polypeptides from nodular mf maintained in medium with either NRS or Ultrosor-G, appeared to react with the patient antibodies. Under similar conditions two of the polypeptides with molecular weights of 7.0 and 174 kDa were found to react

Fig. 5. — Excretory/secretory antigens of *O. volvulus* nodular mf. Nodular mf ESP prepared from parasites labelled in methionine-free medium supplemented with NRS (lane 1) and Ultrosor-G (lane 2) were immunoprecipitated with a hyper-immune patient serum pool ($n = 54$) and the immune complexes analyzed by SDS-PAGE and autoradiography. Nodular mf ESP generated from parasites cultured in medium supplemented with FCS were used to precipitate the normal human control serum pool (lane 3).

with the normal human serum proteins (Fig. 5, lane 3). Similar results were obtained with the ESP from the adult female cultures (Fig. 6).

There was also apparent similarity in the secreted antigens and components with molecular weights of 174, 68, 33, 16 and 13 kDa were prominently secreted by both stages. The 63 kDa antigen from the adult female worms which was secreted/excreted after heat-shock treatment appear to be an adult female stage specific antigen. When the ESP from both stages were preliminarily cleared with NHS before immunoprecipitation, no detectable bands could be seen on the autoradiograms even after prolonged exposure times (data not shown).

DISCUSSION

We have successfully used the synthetic product Ultrosor-G as a serum substitute for the *in vitro* maintenance of *O. volvulus* adult females and nodular mf. The viability of the parasites in medium supplemented with 1 % Ultrosor-G as well as the proteins and antigens released *in vitro* by these life-cycle stages were similar to those obtained by culturing the two life-cycle stages in the presence of serum.

Fig. 6. — Excretory/secretory antigens of *O. volvulus* adult female worms. One adult female worm per assay was labelled in methionine-free medium supplemented with either NHS (lane 1), NRS (lane 2), Ultrosor-G (lane 3), FCS with heat shock treatment (lane 4), NHS at 37 °C (lane 5) or FCS (lane 6). Aliquots of the culture supernatant (ESP) were immunoprecipitated with a hyper-immune patient serum pool and the immune complexes analyzed by SDS-PAGE and autoradiography. Adult female ESP generated from medium supplemented with FCS were used to precipitate the control NHS pool (lane 7).

Several studies have demonstrated the importance of human serum for prolonged *in vitro* maintenance of *O. volvulus* (Engelbrecht & Schulz-Key, 1984; Walter, 1988). However, human serum especially in the endemic areas has become a risky reagent for this and other applications due to the spread of HIV/AIDS and hepatitis infections. The present investigation demonstrates that medium supplements such as fetal calf serum (FCS), heat-inactivated normal rabbit serum (NRS) and Ultrosor-G can also facilitate prolonged *in vitro* survival of this connective tissue-dwelling parasite and moreover, promote the active synthesis and release of parasite products.

The viability of the parasites stages used in this study was carefully monitored by both visual and microscopic movements. Adult parasites were found to be viable throughout the experiments while the viability of nodular mf decreased from more than 95 % to about 75 %. It was therefore expected that only components labelled *de novo* could be detected by the methods described in this study. Following preliminary experiments to determine the optimal conditions, the effect of these supplements on protein synthesis and secretion was monitored as described in materials and methods. Further investigations on the secreted molecules were nevertheless limited to their sizes (Figs. 3 and 4) and antigenicity (Figs. 5 and 6). This notwithstanding, the effect of these supplements on *in vitro* protein synthesis and secretion (Fig. 2) sufficiently reproduces the characterization of the secreted proteins, as six worms were observed simultaneously. Moreover, individual medium supplements have subsequently been used to culture adult worms separately although radiolabelling could not be done in these cases (results not illustrated).

The excretory/secretory products (ESP) derived from parasites cultured in the presence of these supplements were found to be immuno-reactive with patient serum antibodies, thereby indicating that *in vitro* maintenance of these parasite stages in the presence of these supplements did not compromise the antigenicity of the secreted/excreted molecules. This finding also suggests that *in vitro* maintenance of the parasite stages as such is a reliable method of obtaining relatively pure and functionally important ESP suitable for immunization of rabbits or other experimental animals. The resulting antibodies would be expected to be void of contaminating antibodies to human serum components which could be generated if the ESP were prepared from medium supplemented with human serum. Active protein synthesis and release appeared to be observed up to six hours after addition of the radioactive precursor in all the assays (Fig. 2), indicating that a change in the culture medium or the replenishment of the components of the medium became necessary.

The detected ESP were as well detected in the somatic extracts of nodular mf and *in vitro* released eggs (Fig. 4), probably indicating that most of the secreted components could be of somatic origin (Haque & Capron, 1986). Although the reaction between pan-sorbin and the serum proteins could be non-specific (data not shown), about 50 % of the secreted proteins were observed to react with host serum antibodies. Those components which could not be detected by the host serum antibodies may be non-immunogenic in man.

In conclusion, depending on the application intended for parasite ESP Ultrosor-G, heat-inactivated preimmune rabbit serum and fetal calf serum appear to be convenient substitutes of human serum for the *in vitro* maintenance of *O. volvulus* worm stages and for the collection of secreted and/or excreted parasite molecules void of host contaminants.

ACKNOWLEDGEMENTS

This study was supported by grants provided by the International program in the Chemical Sciences (CAM-01 project), Uppsala University, Sweden.

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Reçu le 15 janvier 1997

Accepté le 9 juin 1997