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Isolation and Characterization of Excretory/Secretory Products from *In Vitro* Developmental Stages of *Echinococcus granulosus*

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Abstract

Excretory/secretory (E/S) products of *Echinococcus granulosus* were prepared from presegmentation stages (PS1, PS2, PS3 and PS4) and one segmented (S5) stage which corresponded to 1-, 5-, 12-, 26- and 43-day old *in vitro* cultured parasites, respectively. Cultures were initiated from protoscolices collected from hydatid cysts of indigenous Jordanian ewes. The E/S products were characterized using SDS-PAGE with silver, Coomassie-blue and PAS staining as well as immunoblotting with sera from hydatid patients and immunized mice. Younger cultured stages yielded small quantities of E/S materials, compared to the banding (PS4) and segmented (S5) stages. The E/S products from various stages of cultured parasites were heterogeneous and contained 15–28 protein bands and 3–6 PAS^+ bands. A total of 13 protein bands were shared among the E/S products of various cultured stages, 11 of which were also shared with crude sheep hydatid fluid (CSHF), tegumental and somatic antigens. The E/S products of PS1 – S5 stages and CSHF shared 3 PAS^+ bands. All the immunoreactive components of E/S products except for a band of <14.4 KDa, were detected by sera of unilocular hydatid Jordanian patients and were also reactive to sera of patients infected with *E. multilocularis*.

Key words: *Echinococcus granulosus*, excretory/secretory products, immunoblotting, antigen

Introduction

Unilocular hydatidosis is caused by the metacestode stage of *Echinococcus granulosus*. The disease is endemic in many parts of the world including Australia, the Far East, China, Eastern Europe and the Middle East, mainly Turkey, Lebanon and Jordan (Mattosian *et al.*, 1977; Schwabe, 1986; Al-Yaman *et al.*, 1988; Craig *et al.*, 1991).

Crude hydatid cyst fluid is currently used for the serodiagnosis of human hydatidosis (Schantz and Kagan, 1980; Di Felice *et al.*, 1986; Rickard and Lightowlers, 1986; Shepherd and McManus, 1987; Maddison *et al.*, 1989). Several important and specific antigenic components were found in the crude hydatid fluid, particularly those having a molecular weight (Mr) of 16, 12 and 8 kilodaltons (KDa) (Shepherd and McManus, 1987; Maddison *et al.*, 1989). However, the crude hydatid fluid is heterogeneous and predominantly of host origin (Di Felice *et al.*, 1986; Shepherd and McManus, 1987). Recently, the excretory/secretory (E/S) antigens of parasites have been investigated for specific and sensitive immunodiagnosis. For example, studies on E/S antigens were carried out on various parasite infections: *Nematospiroides dubius* in mice (Adams *et al.*, 1987); *Strongyloids stercoralis* in humans (Brindley *et al.*, 1988); larval stages of *Ascaris suum* in rabbits, mice and rats (Kennedy and Qureshi, 1986), *Taenia saginata* cysticerci in cattle (Harrison *et al.*, 1989; Joshua *et al.*, 1989); *E. multilocularis* (Schuppert *et al.*, 1989) as well as *E. granulosus* in humans (Craig and Nelson, 1984) and in dogs (Gasser *et al.*, 1989).

The nature of E/S materials produced by the rostellar glands of *E. granulosus* was found to be a protein or a polypeptide containing a large quantity of cysteine with the possibility of a lipid component being present (Smyth, 1964; Thompson *et al.*, 1979). No studies on the nature of E/S products from *in vitro* or *in vivo* stages have been attempted.

The purpose of this study is to characterize the E/S products from cultured developmental stages of *E. granulosus* and to determine the immunogenic fractions using sera from infected humans and

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mice injected with E/S or somatic antigens.

Materials and Methods

Parasite culture and collection of E/S products: Unilocular hydatid cysts were isolated from the liver of ewes indigeneous to Jordan. The dissection of cysts and the culturing of *E. granusolus* protoscolices (psc's) in CMRL-1066 (Flow labs, Irvine, Scotland, U.K.) were performed as described by Smyth (1967, 1985). The E/S material was prepared from presegmentation (PS) and segmentation (S) stages following the system of the *in vitro* development described by Smyth (1979) and Hijjawi *et al.* (1992). The E/S products were collected as described by Craig and Nelson (1984). The E/S from the following stages were collected: PS1, PS2, PS3, PS4 and S5 which correspond to 1-, 5-, 12-, 26- and 43-day old cultures, respectively. Following the development of each stage, the parasites were harvested and washed 3 times with Hank's balanced salt solution (HBSS, pH 7.2) and incubated for 5-days in RPMI-1640 medium free from foetal bovine serum (Gibco, Paistey, U.K.). For each stage the conditioned medium was collected daily and collections on days 2–5 were pooled together and centrifuged immediately at 11,000×g for 30 min at 4°C, then concentrated using YM-10 Amicon membrane (Amicon Canada Ltd., Ontario, Canada). The Molecular weight (Mr) cut-off point was 10 KDa. The protein content of the collected E/S was determined by the method of Bradford (1976).

Preparation of other antigens: Crude sheep hydatid fluid (CSHF) was prepared as described by Al-Yaman *et al.* (1988). The protoscolex tegumental antigen (TA) was prepared from sheep psc's which were cultured for 5 days in a diphasic medium. These parasites were rinsed 3 times in HBSS then washed for 10 min in HBSS with 20 mM Tris-HCl (HBSS-T). One ml of the packed volume of psc's was added to 3 ml of HBSS-T and immersed in a container of liquid nitrogen (–196°C) for 1 hr in an ice bath. Then the preparation was centrifuged at 3,000×g for 30 min at 4°C. The somatic antigen (SA) was prepared from the PS2 and PS4 stages of cultured *E. granulosus*. At the end of each stage, the parasites were removed and washed 3 times with HBSS, subjected to ultrasonication for 5 min (10×30 sec pulses) and centrifuged at 11,000×g for 30 min at 4°C. The supernatant was concentrated 15 fold using a 10 KDa cut-off point YM-Amicon membranes. *Moniezia expansa* adult antigen (MAA) was prepared as described by Conrath and Coupe (1978). *Cysticercus tenuicollis* cyst fluid antigen (CTFA) was collected from cysts obtained from infected sheep. The fluid was centrifuged at 11,000×g for 30 min at 4°C and dialysed in 3 changes of PBS (pH 7.2) for 12 hr.

Characterization of E/S Products: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Laemmli (1970). Analysis of the E/S products and other antigenic preparations of *E. granulosus* including CSHF, TA and SA was carried out on 15.5% SDS-PAGE. Tested samples contained 1 mg/ml of protein except for the E/S products collected from the S5 stage in which 0.7 mg/ml samples were used. Onto each well 15 μl of each sample was applied. Following electrophoresis, both silver and Coomassie-blue stains were used for fraction analysis of each stage. The PAS stain was used for the detection of carbohydrate-containing components. Immunoreactive fractions of E/S antigens collected from various developmental stages were characterized by the immunoblot technique (Towbin *et al.*, 1979) using sera from Jordanian patients infected with unilocular hydatidosis as well as sera of patients infected with *E. multilocularis* and *Fasciola hepatica* (obtained from Bernhard Nocht Institute for Tropical Medicine, Hamburg, West Germany through the courtesy of Dr. F. Al-Yaman). Sera from non-infected new born humans used as controls. Immunoblotting was also performed using polyclonal IgG antibodies prepared from BALB/c mice immunized with either the E/S of SA products prepared from the PS4 stage. For immunization, each mouse was injected intraperitoneally with 50 μg of the antigen. The inoculum was prepared in 0.5 ml HBSS mixed (1:1) with complete Freund's adjuvant (CFA; Sigma, St. Louis, U.S.A.). Two boosters were given at 2 and 4 weeks after the first inoculum using the same regimen except that incomplete FA was used instead of CFA. Mice were bled through the retroorbital route on days 7, 14, 28 and killed on the 35th day after initial inoculation. On that day responding animals showed

high IgG antibody titers (yielded and optical density of >1.00 using the enzyme linked immunosorbent assay-ELISA).

Results

Components of E/S products of cultured parasites:

Figure 1 compares the amounts of E/S proteins produced by 5 *in vitro* developmental stages of *E. granulosus*. The production rate increased slowly during the first 12 days of culture, but a steady and sharp increase in E/S production was seen in the PS4 and S5 stages (Fig. 1). Apparently, the production of E/S materials by the segmented worms (S5) exceeded 40 folds that of the evaginated psc's (PS1).

Silver and Coomassie-blue stains showed common and unique fractions in the E/S preparations made from the various *in vitro* developmental stages. Since silver staining showed a greater number of bands which appeared more refined and intense, all information presented here pertain to fractions revealed by this technique (Fig. 2A). As many as 24, 10, 25, 8 and 20 major and 4, 8, 3, 7, and 4 minor protein bands were fractionated in the E/S products of PS1, PS2, PS3, PS4 and S5 stages, respectively. Only bands whose Mr was less than 97.4 KDa are considered here. Of these, 13 fractions were common to all stages. These were of Mr 29.5, 30.5, 31, 33, 35, 38–40, 45, 50, 53–70, 75, 77 KDa and additional two bands whose Mr was lower than 14.4 KDa (Fig. 2A). All of these fractions were detected in CSHF, TA and SA preparations except for two bands of Mr 35 and <14.4 KDa. Moreover, bands of Mr 24.5, 75 and 77 KDa appeared faint in SA and TA, while they were major in CSHF and E/S collected from PS1, PS3 and S5 stages. Four bands (Mr 42, 16 and <14.4 (a and b) KDa) appeared in preparations from most cultured parasites but not SA and TA preparations. The E/S products of PS1, PS3 and S5 stages contained a larger number of protein bands in lower molecular weight (Mr <28 KDa). In contrast, the E/S products of PS4 showed only two bands in this category. Both bands were lower than Mr 14.4 KDa (Fig. 2A). The E/S materials collected from PS2 showed several distinct extra bands (Mr 16, 22.5 and 23.5 KDa), which appeared much sharper in the E/S preparations made from PS1, PS3 and S5 stages (Fig. 2A).

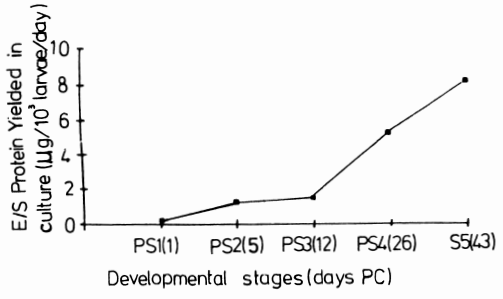

Fig. 1 Amount of excretory/secretory (E/S) products yielded by various developmental stages of *E. granulosus* grown *in vitro* at various days post culturing (PC). Viability and evagination rates of each stage ranged between 80–90% and 20–30%, respectively.

The PAS stain revealed 3 shared carbohydrate-complexed bands (Mr <14.4, 53 and 63–69 KDa) with varying intensities in all E/S preparations. Several extra bands with Mr >97.4 KDa have been revealed (Fig. 2B). Additional bands (Mr 45, 47 and 50 KDa) were detected only in the E/S products collected from PS1, PS3 and PS4 stages.

Immunoreactivity of E/S products:

Figure 3 shows immunoreactivity of various E/S antigenic preparations against pooled sera from Jordanian patients with confirmed hydatidosis. One immunoreactive component (Mr <14.4 KDa) was obvious in all E/S antigenic preparations. A 16–17 immunoreactive fraction was present in E/S products from PS1, PS2 and PS3 stages but not the other two stages (Fig. 3B). The PS1 stage contained an additional immunoreactive component of Mr 22 KDa. All of these immunoreactive components were also found in CSHF (Fig. 3A, B). A 27–28 KDa component was identified in E/S products from PS1 and PS3 stages but not in CSHF. None of the above mentioned immunoreactive fractions was detected using sera of patients infected with *E. multilocularis, F. hepatica* or newborn human serum. A non-specific fraction of 38–40 KDa was detected in the E/S preparations of several *in vitro* stages as well as in CSHF using sera of patients infected with *E. granulosus, E. multilocularis* or *F. heptica*. Additional 9 immunoreactive bands with Mr >43 KDa were identified in the E/S products of PS1 and PS3 stages using pooled sera of confirmed hydatid pa-

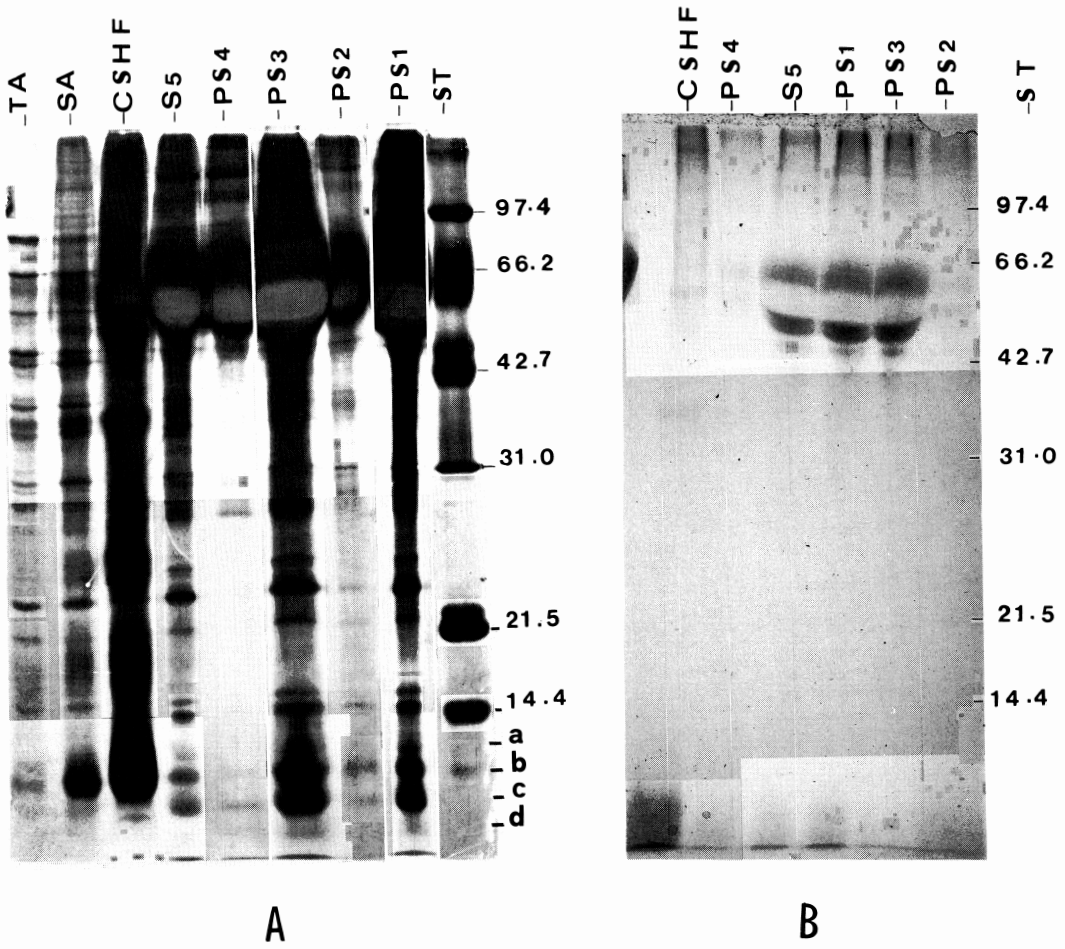

Fig. 2 SDS-PAGE of various excretory/secretory (E/S) products, crude sheep hydatid fluid (CSHF), tegumental antigen (TA) and somatic antigen (SA) of *E. granulosus* from Jordanian sheep. The E/S products were prepared from 5 *in vitro* developing stages (PS1, PS2, PS3, PS4 and S5) which corresponded to 1-, 5-, 12-, 26- and 43-day old larvae in culture, respectively. All antigens were reduced prior to SDS-PAGE. ST represents the molecular weight standards in kilodaltons. A: Silver staining, B: PAS staining.

tients. All of these bands were also detected in E/S products of PS4 except for 3 bands of Mr 43, 45, and 50 KDa. Furthermore, all of these bands mentioned above were crossreactive to pooled sera of patients infected with *E. multilocularis* (Fig. 3A, B).

The E/S products of PS4 stage, CSHF, TA and SA of *E. granulosus* as well as MAA and CTFA preparations were blotted with sera of mice immunized with E/S products of PS4 stage. The sera of mice injected with PBS were used as controls. With the exception of 45 KDa band, all other 14 immunoreactive components which reacted with immunized mice sera were also detected in CSHF (Table 1). None of the other antigenic preparations shared any immunoreactivity except for one fraction (Mr 53 KDa) which appeared faint in the CTFA preparation.

Discussion

The E/S products of *E. granulosus* larvae cultured *in vitro* were heterogeneous and composed of

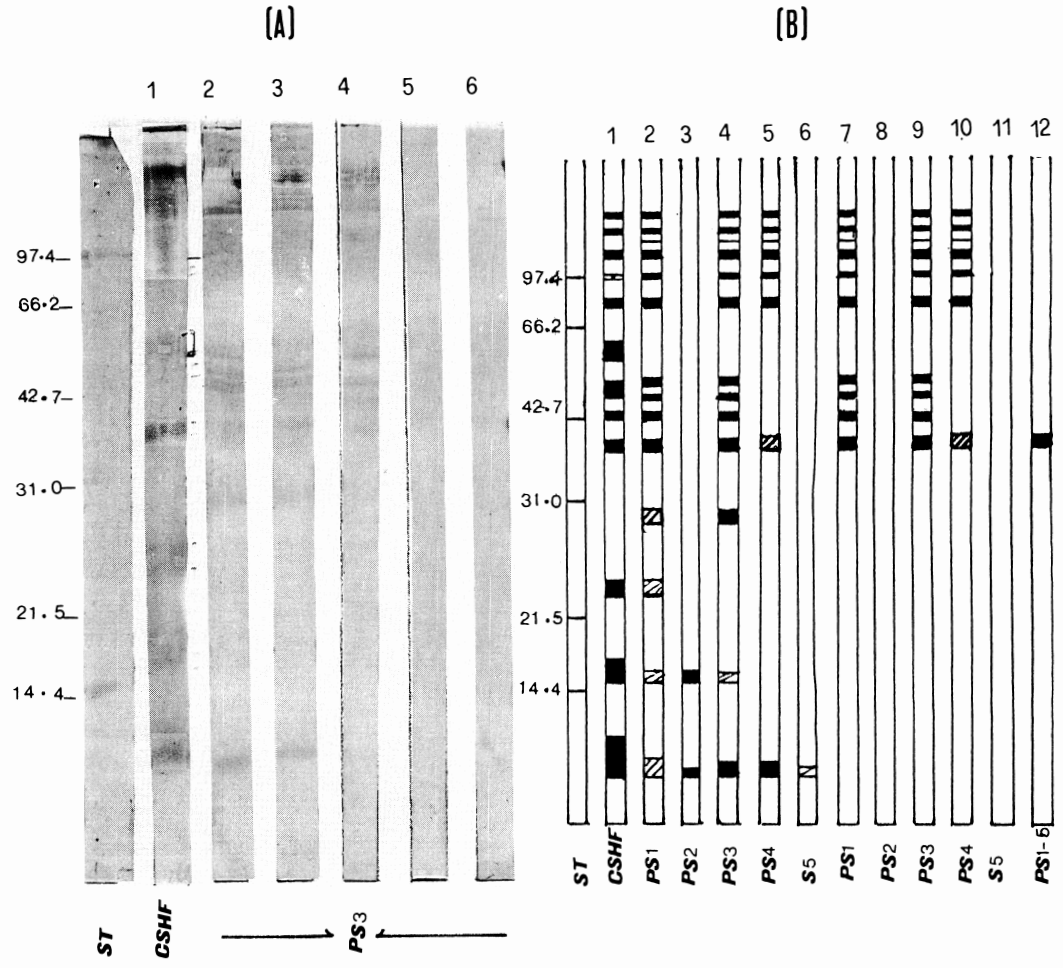

Fig. 3 Immunoreactive components of crude sheep hydatid fluid (CSHF) and excretory/secretory (E/S) antigens from 12-day old (PS3-stage) *in vitro* cultured larvae of *E. granulosus*. In A, CSHF (lane 1) or PS3 (lanes 2, 3) fractions were treated with pooled sera from 5 Jordanian hydatid patients. For crossreactivity and control, fractionated PS3 was reacted with sera of patients infected with *E. multilocularis* (lane 4), *F. hepatica* (lane 5) or normal newborn human serum (lane 6). B shows a diagramatic representation of immunoreactive bands identified in CSHF and various *in vitro* developmental stages of *E. granulosus* (PS1, PS2, PS3, PS4, S5) using pooled sera of Jordanian hydatid patients (lanes 1–6), and *E. multilocularis* patients (lanes 7–11). Lane 12 shows the reaction with normal newborn human serum. ST represents the molecular weight standards in kilodalton.

many proteins and carbohydrate-conjugated proteins. Older stages yielded greater quantities of E/S products reflecting greater larval activity and tissue differentiation. *T. saginata* showed similar pattern of E/S product yield (Joshua *et al.*, 1988).

Electrophoretic characterization of E/S antigenic preparations revealed 13 protein bands shared among all E/S preparations. Additional bands were seen in the E/S products of PS1, PS3 and S5 stages, many of which were of lower molecular weight. Intra-stage variation have been reported for viable *T. saginata* metacestode stages (Joshua *et al.*, 1989). Among 5 stages, the E/S products of PS3 stage showed the largest number of bands while those of PS4 showed the fewest. This may be attributed to the high metabolic activity of *E. granulosus* at the PS3 stage

Table 1 Immunoreactive bands of *E. granulosus* antigen and other parasite antigens identified by sera of mice immunized with E/S products of PS4

Band M_r (kDa)	Reactivity of immunized mouse serum against						Reactivity of PBS injected mouse serum against PS4
	PS4	CSHF	TA	SA	MAA	CTFA	
>97.4 x	+	+	–	–	–	–	–
>97.4 y	+	+	–	+	–	–	–
>97.4 z	+	+	–	+	–	–	–
97.4	+	+	–	+	–	–	–
94	+	+	+	–	–	–	–
77	+	+	–	+	–	–	–
70	+	+	+	+	–	–	–
66	+	+	–	–	–	–	–
60	+	+	+	–	–	–	–
53	+	+	–	+	–	+F	–
50–48	+	+	+	–	–	–	–
45	+	–	–	–	–	–	–
40–38	+	+	+	+	–	–	–
31.0	+F	+	+	+	–	–	–
29.5	+F	+	–	–	–	–	–

(+) Present
(–) Absent
(F) Faint
Immunoblot technique was used to detect immunoreactive components in E/S isolated from 26-day old larvae of *E. granulosus* (PS4), crude sheep hydatid fluid (CSHF), tegumental (TA), somatic (SA) preparations as well as *Moniezia* adult antigen (MAA) and *C. tenuicollis* fluid antigen (CTFA) by sera from mice immunized with E/S. All antigens were reduced prior to electrophoresis.

which is reversed in the following PS4 stage, however. This last stage is characterized by the disappearance of calcarious corpuscles (cc) when banding and constriction occurs at one point to initiate the process of proglotidization of the worm. It has been suggested that the cc play an important role in the metabolism of early strobilar differentiation stages (Smyth and McManus, 1989). Thus, cc disappearance is indictive of increased metabolism. It may be that most of the metabolites during PS4 stage are quickly reutilized by the developing worms.

A sharp decline in E/S products of low molecular weight (Mr <28 KDa) appears evident in PS2 and S5 larvae which correspond to the appearance of excretory canals (PS2 stage) and segmentation (S5 stage). It is worthwhile mentioning that all bands (Mr <14.4, 22.5, 27, 35, 43, 76 and 97.4) KDa) identified in E/S materials isolated from evaginated psc's (Gasser *et al.*, 1989) were found in E/S products of PS1 in the present study. As expected, all E/S antigenic components were also found in CSHF which contains many other extra fractions. Ten components were shared among all E/S antigenic preparations, CSHF, TA and SA. This cross-reactivity may result partly from the shedding phenomenon or from the secretory nature of rostellar region. Although the contribution of surface components to E/S preparations is clearly defined in trematodes and nematodes, there is no such evidence in cestodes (Lightowlers and Rickard, 1988). Gasser *et al.* (1989) identified 7 major components in the E/S products prepared from *E. granulosus* psc and most of them are shared with the SA prepared from the same stage.

Most of the E/S bands which are shared with CSHF are expected to be of parasite origin. Components of Mr 53–70, 75 and 77 KDa (Fig. 2A) were of massive quantity in all E/S preparations. Less quantities of these components were detected in CSHF, TA and SA. Within these bands, 3 components of Mr 53, 56 and 67 KDa were distinctly recognizable in CSHF, TA and SA preparations. It is established

that Mr 67 KDa component in CSHF is of host origin, probably serum albumin (Rickard and Lightowlers, 1986; Shepherd and McManus, 1987). This component appears to be the major one in the E/S preparations as well. The albumin molecules from foetal bovine serum added in culture medium may be assimilated into the parasite or attached to the tegument during culturing. But, Kennedy and Qureshi (1986) reported that the 67 KDa fraction found in culturing medium of *A. suum* larvae was of two origins: one of parasite origin and the other from serum albumin assimilated into the parasites from the culture fluid.

Some of the major E/S products of cultured *E. granulosus* larvae are glycoproteins in nature as revealed by PAS and silver staining. As many as 3–6 PAS-positive components appeared in various E/S preparations from these larvae. Some of the most important antigens in E/S preparations from *Schistosoma* spp. and *T. saginata* (Lightowlers and Rickard, 1988; Joshua *et al.*, 1989) are glycoprotein in nature.

Using sera of confirmed Jordanian hydatid patients, a species-specific component of Mr <14.4 KDa was identified in all E/S preparations. Another specific one (Mr 16–17 KDa) was also identified in E/S products of PS1, PS2 and PS3 stages. These two components were also detectable in CSHF in the present study and elsewhere (Shepherd and McManus, 1987). These workers suggested that these two antigens were of E/S origin. The present study provides evidence that these two antigens are excretory/secretory and specific to *E. granulosus*. Recently, an additional 8 KDa *Echinococcus*-specific antigen has been reported (Maddison *et al.*, 1989).

Various immunoreactive components found in the E/S products of the PS1 stage against human sera have also been detected by Gasser *et al.* (1989) using sera of infected dogs. The 38–40 KDa component of the present E/S preparations is cross-reactive with antigens from CSHF and was reactive with sera of patients infected with *E. granulosus*, *E. multilocularis* and *F. hepatica*. This is the same fraction which has been reported to be cross-reactive with a wide variety of cestodes and nematode helminths (Shepherd and McManus, 1987).

The sera of mice immunized with E/S preparations reacted with most of the E/S products (Mr >28–30 KDa) from PS4 and were also immunoreactive to human sera. Neither 16–17 KDa nor <14.4 KDa component was immunoreactive to the immunized mouse serum. Similarly, Gasser *et al.* (1989) did not detect a 16–17 KDa band upon using dog serum. Several extra bands were detected by immunized mouse serum but not by infected human serum (Fig. 3, Table 1). Extra bands are expected to occur due to host difference and also to varied ways of exposure to the antigen, i.e., injection vs infection (Mitchell, 1987).

The cross-reactivity between some E/S products and CSHF, TA and SA preparations (Table 1) is expected since the E/S products would accumulate in CSHF and also since most of them originate from the SA of the parasite or shedding of its tegument (Lightowlers and Rickard, 1988).

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