Fasciola hepatica: surface and internal tegumental changes induced by treatment *in vitro* with the sulphoxide metabolite of albendazole ('Valbazen')

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SUMMARY

A morphological study has been carried out to determine the effect of the active sulphoxide metabolite of the benzimidazole anthelmintic, albendazole (ABZ-SO) on the adult liver fluke, *Fasciola hepatica*. Whole flukes were treated with ABZ-SO for 12 and 24 h at a concentration of 10 μg/ml. The changes in response to drug treatment were examined by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and tubulin immunocytochemistry (ICC). No surface changes were apparent following 12 h ABZ-SO treatment, but localized blebbing was observed after 24 h, which became more extensive towards the posterior region of both surfaces. TEM of sections from the posterior midbody region revealed that ABZ-SO caused the accumulation of secretory bodies in the tegumental cells and in their cytoplasmic connections and, after 24 h, just above the basal plasma membrane. Localized blebbing of the apical membrane also occurred. The morphology of the Golgi complexes within the tegumental cells began to change after 12 h treatment with ABZ-SO and, by 24 h, few complexes were observed. A distinct increase in tubulin immunoreactivity occurred after 12 h treatment, but this decreased after 24 h. The results obtained are consistent with those expected for microtubule inhibition. They are discussed in relation to the action of established microtubule inhibitors, as well as the benzimidazole derivative, triclabendazole.

Key words: *Fasciola hepatica*, liver fluke, tegument, albendazole sulphoxide, SEM, TEM, ICC.

INTRODUCTION

Albendazole (ABZ) is one of the most important drugs in use today to treat parasitic infections of humans and livestock. It is marketed in 3 forms: as the pro-drug netobimin ('Hapadex'), as albendazole ('Valbazen', 'Proftril') and as the metabolite albendazole sulphoxide ('Rycoben', 'Bental'). ABZ probably has the broadest spectrum of activity of any member of the benzimidazole group of drugs. Thus, it is extensively used in human and veterinary medicine against nematodes (Coles, 1994; Torgerson, 1995; Albonico, Crompton & Savioli, 1999; Ottesen, Ismail & Horton, 1999; Horton, 2000); cestodes, especially hydatid disease (Arundel, 1986; Horton, 1997; Reuter *et al*. 2000); trematodes (Dubey *et al*. 1978; Pungpark, Bunnag & Harinasuta, 1984; Boray, 1986) and protozoa (Katiyar *et al*. 1994; Gardner & Hall, 2001). Apart from triclabendazole (TCBZ), which has high specificity for *Fasciola hepatica*, ABZ is the only common benzimidazole that is recommended for the treatment of fasciolosis in the USA. Higher dose rates are required in fasciolosis than are used against nematodes and cestodes (McKellar & Scott, 1990; Fairweather & Boray, 1999). At these higher dosages, ABZ has good efficacy against adult but not juvenile fluke (Knight & Colglazier, 1977; Johns & Dickeson, 1979; Van Schalkwyk *et al*. 1979; Theodorides & Freeman, 1980; Malone *et al*. 1982).

The phenomenon of TCBZ resistance is a growing problem in the control of *F. hepatica* (Mitchell, Maris & Bonniwell, 1998; Thomas, Coles & Duffus, 2000; Moll *et al*. 2000; Gaasenbeek *et al*. 2001). Recently, it has been shown that ABZ is active against adult TCBZ-resistant flukes, and so may have use as an alternative to TCBZ therapy (Coles & Stafford, 2001). Since the major target for both drugs is likely to be tubulin, this suggests that the two drugs may bind at different sites on the tubulin molecule. However, other possibilities exist and have been discussed by Robinson *et al*. (2002). Nevertheless, this finding indicates that it would be worthwhile to investigate further the differential sensitivity of the fluke to TCBZ and ABZ. Several studies have been carried out with TCBZ (reviewed by Fairweather & Boray, 1999), but relatively few with ABZ. The latter drug is known to cause stunting of flukes, as well as severe damage to the reproductive system and reduction in egg production (Lang *et al*. 1980; Masaba, 1981).

The aim of the current study was to examine the effects of ABZ against adult flukes *in vitro*. The tissue of choice was the tegument because it serves a number of important metabolic roles in the fluke (Fairweather, Threadgold & Hanna, 1999) and is one of

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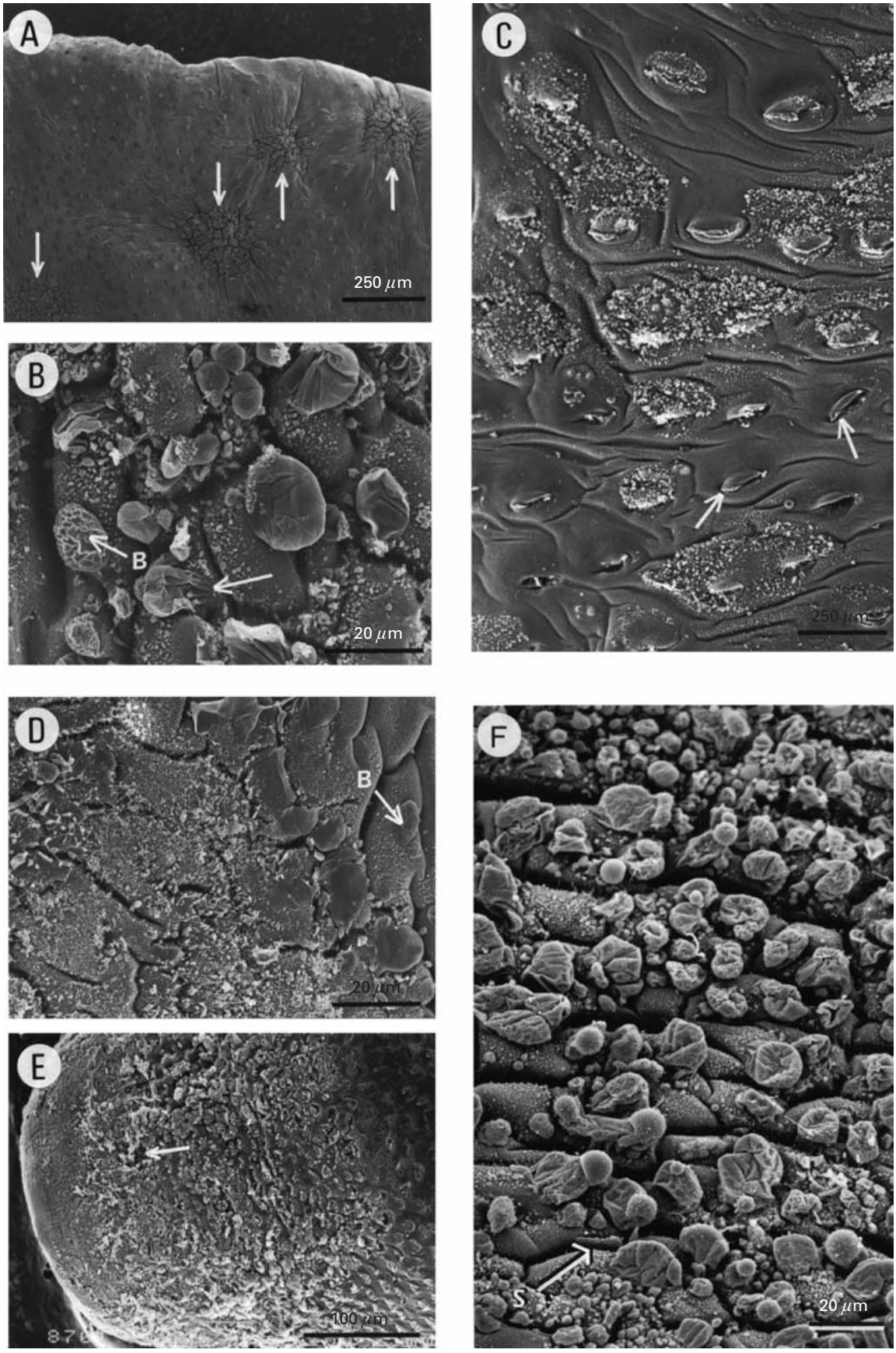

Fig. 1. For legend see opposite page.

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the principal sites of drug entry into the fluke, therefore rendering it a potential drug target. Both scanning and transmission electron microscopy (SEM/TEM) were employed to investigate morphological changes induced by ABZ. Immunocytochemistry using a polyclonal antibody against tubulin was also carried out to investigate changes to what is a likely molecular target for ABZ action. All three techniques have been used in previous studies to elucidate the effect of drug action on the liver fluke (reviewed by Fairweather, 1997). Albendazole sulphoxide (ABZ-SO) was used for this study, since it is the most abundant metabolite found in plasma and tissues of the host (Hennessy *et al*. 1989; Fetterer, Rew & Knight, 1982; Alvarez *et al*. 2001) and it is considered to be the most pharmacologically active form of the drug (Lubega & Prichard, 1990).

MATERIALS AND METHODS

An experimental infection of *Fasciola hepatica* was maintained in male albino (Wistar) rats. Adult flukes (at least 12 weeks old) were removed from the bile ducts of the rats under sterile conditions in a laminar flow cabinet. They were subsequently washed in several changes of warm (37 °C), sterile NCTC 135 culture medium containing antibiotics (penicillin, 50 i.u./ml; streptomycin, 50 μg/ml). (NCTC 135 and antibiotics were obtained from Flow Laboratories, Irvine, Scotland, UK). Whole flukes were transferred to fresh culture medium containing albendazole sulphoxide at a concentration of 10 μg/ml. This concentration is comparable to maximum blood levels of the sulphoxide metabolite *in vivo*: 3·9 μg/ml in sheep following a therapeutic (oral) dose of 20 mg/kg (Fetterer, Rew & Knight, 1982) and 3·2 μg/ml in sheep following a therapeutic (oral) dose of 10 mg/kg (Marriner & Bogan, 1980); and to the level in bile: 6·2 μg/ml in sheep following an intra-ruminal dose of 4·5 mg/kg (Hennessy *et al*. 1989). The concentration was also chosen on the basis of concentrations used *in vitro* with experiments involving *Echinococcus* spp. (Perez-Serrano *et al*. 1994; Ingold *et al*. 1999; Urrea-Paris *et al*. 2000). A stock solution was initially prepared by dissolving 0·01 g of the drug in 1·0 ml dimethyl sulphoxide (Me_2SO) and added to the culture medium to give a final solvent concentration of 0·1% (v/v). Whole flukes were incubated for 12 and 24 h. Controls were prepared for each individual experiment by incubating whole flukes in NCTC 135 containing 0·1% (v/v) Me_2SO for the appropriate time-periods. The ABZ-SO was a generous gift from Mr Nigel Walshe, Pfizer Ltd, Sandwich, Kent, UK.

Tissue preparation for SEM

Flukes were removed from the incubation medium, lightly flat-fixed for 1 h in a 3 : 1 mixture of 4% (w/v) aqueous glutaraldehyde and 1% aqueous osmium tetroxide. The flukes were subsequently free-fixed in fresh fixative for a further 4 h at room temperature. They were washed in several changes of deionized water and dehydrated in an ascending series of acetone concentrations. After critical-point drying, the flukes were mounted on aluminium stubs, coated with gold and viewed in a JEOL 6400 scanning electron microscope operating at 10 keV.

Tissue preparation for TEM

The specimens were lightly flat-fixed for 1 h at room temperature in 4% (w/v) glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·4) and 3% (w/v) sucrose. The worms were divided into 4 regions: oral cone (including ventral sucker); anterior midbody; posterior midbody and tail. The tail region from each fluke was discarded and the posterior midbody region was further divided into transverse sections approximately 1 mm wide. The specimens were subsequently free-fixed overnight at room temperature (25 °C) in 4% (w/v) glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·4) and 3% sucrose (w/v), washed overnight in 0·1 M sodium cacodylate buffer (pH 7·4) and 3% sucrose (w/v), post-fixed in 1% aqueous osmium tetroxide for 1 h, washed in 0·1 M sodium cacodylate buffer (pH 7·4), dehydrated through an ascending series of ethanol concentrations and infiltrated and embedded in Agar 100 resin (Polaron Equipment Ltd, Watford, UK). Ultra-thin sections, 60–70 nm in thickness were cut on a Reichert Ultracut ultramicrotome, mounted on bare 200 mesh copper grids and double-stained with alcoholic

Fig. 1. Scanning electron micrographs (SEMs) of adult *Fasciola hepatica* following 24 h incubation in ABZ-SO (10 μg/ml). (A) SEM of the dorsal midbody lateral margin showing localized areas of severe blebbing (arrows). (B) High-power SEM of an area of localized blebbing from the lateral margin of the dorsal midbody region. Numerous blebs (B) are present on the tegumental surface, some of which appear to have burst, giving a raisin-like appearance. These are seen to arise from the tegumental surface (arrow). (C) SEM of the dorsal posterior midbody region. Blebbing is associated with the spines. The tegumental covering is beginning to come away from some spines (arrows). (D) SEM of the dorsal tail region. Large blebs (B) are present on the surface as well as very small blebs, which carpet the surface. (E) SEM of the extreme dorsal tail region, showing the extensive damage induced by the drug. Spine loss becomes evident as the posterior of the fluke is approached. In a few areas, loss of tegument also occurs (arrow) which exposes the basal lamina. (F) SEM of the ventral tail region, showing severe blebbing of the tegumental surface. Small spherical blebs cover the surface. Most of the larger blebs have burst. The spines (S) are barely visible, due to the swelling of the tegument surrounding them.

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uranyl acetate (15 min) and aqueous lead citrate (10 min). The sections were viewed in a JEOL 100-CX transmission electron microscope operating at 100 keV.

Tissue preparation for tubulin immunocytochemistry

Specimens were fixed in absolute methanol for 3 h at room temperature (25 °C) and embedded in JB4 resin. Sections, 4 μm in thickness, were cut on a pyramitome, mounted on a multiwell slide and washed in 0·1 M phosphate-buffered saline (PBS), pH 7·2. The tissues were then incubated overnight in a polyclonal anti-tubulin antiserum (1 : 50) at room temperature (25 °C). The antibody was raised in rabbit against tubulin purified from chick embryo (Sigma-Aldrich Co. Ltd, Poole, Dorset, UK). Western blots previously performed in our laboratory with fluke homogenates showed that the antiserum bound exclusively to a polypeptide with a molecular mass of approximately 54 kDa, indicating that it was reactive with fluke tubulin. Following a further wash in PBS, the sections were incubated in an FITC-conjugated swine anti-rabbit secondary antibody (1 : 50) (Dako Ltd, High Wycombe, UK). The sections were washed in PBS, mounted in anti-fade solution (90% glycerol, 10% PBS (pH 7·2), 2·5% (w/v) diazabicyclo) and observed using a Leitz Epifluorescent microscope. Sections of untreated flukes were prepared for each of the time periods. Experimental controls were also set up, with omission of the primary antibody.

RESULTS

Scanning electron microscopy (*SEM*)

Following incubation for 12 h in albendazole sulphoxide (ABZ-SO), no changes were observed on the dorsal or ventral surfaces. However, after 24 h treatment, isolated circular areas of pronounced blebbing occurred along the lateral margins in the dorsal midbody region (Fig. 1A). Small blebs were observed on the surface of the tegument, which appeared swollen. Larger blebs were also present, while a number had advanced beyond this stage and had burst, giving them a shrivelled 'raisin-like' appearance (Fig. 1B). In other areas of the dorsal posterior midbody, blebbing was observed around the spines and some of them were beginning to lose the tegumental lining over their tips. The blebs in this region were mostly small in size (Fig. 1C) In the dorsal tail region, a fine carpet of blebs was observed on the tegumental surface (Fig. 1D). The blebbing became more pronounced as the extremity of the fluke was approached. Spine loss was evident in the tail region and, in some areas, a loss of tegument was also observed. In isolated areas, the basal lamina was exposed (Fig. 1E). The extent of blebbing observed in the ventral tail region was also severe. Many of the blebs present had burst and the spines were virtually invisible due to the swollen tegument (Fig. 1F). No other disruption to the ventral surface was noticed. The surface architecture of the control specimens appeared normal.

Transmission electron microscopy (*TEM*)

Sections from the posterior midbody were examined. After 12 h treatment with ABZ-SO, few ultrastructural changes were detectable within the tegumental syncytium, although it did appear quite vacuolated (Fig. 2A). Accumulations of T1 secretory bodies were observed in localized regions close to the basal plasma membrane (Fig. 2B) and in the cytoplasmic connections running between the subtegumental muscle blocks (Fig. 2C). The tegumental cells generally appeared active, with Golgi complexes present (Fig. 2D). The latter adopted a normal morphology on the whole, although some were slightly diffuse in appearance (Fig. 2E).

Following 24 h treatment, the apical region of the tegument appeared vacuolated, due to the presence of 'open' bodies (Rogan & Threadgold, 1984). These are indicative of a release of secretory bodies at the apical surface. Very few secretory bodies were observed in this region. Mitochondria were present in their normal chain-like arrangement throughout the syncytium, but were significantly swollen and rounded with distinct cristae. Autophagic vacuoles containing these organelles were also present in the syncytium (Fig. 3A). In some areas, there were signs of blebbing (Fig. 3B). Swelling of the basal infolds was not a general feature of the 24 h incubation, although quite severe swelling of the mucopolysaccharide masses surrounding the infolds was present in some locations (Fig. 3C). The subtegumental muscle blocks beneath these areas remained normal. Distinct accumulations of secretory bodies occurred at the base of the syncytium close to the basal plasma membrane and in the cytoplasmic connections (Fig. 4A). In the tegumental cells themselves, accumulations of secretory bodies were also observed. Few Golgi complexes were present within the cells and the GER cisternae had a normal covering of ribosomes on their membranes (Fig. 4B). The tegumental ultrastructure of the control specimens was normal.

Tubulin immunocytochemistry (*ICC*)

Control specimens. Immunofluorescence in the tegumental syncytium was confined to the apical region (Fig. 5A). A distinct band of immunofluorescence below the apical plasma membrane followed the contours of the tegumental folds. At higher magnification, it appeared to be composed of discrete rod-like structures. A thread-like pattern of staining was observed in the tegumental cell bodies and in their cytoplasmic connections with the syncytium (Fig. 5B).

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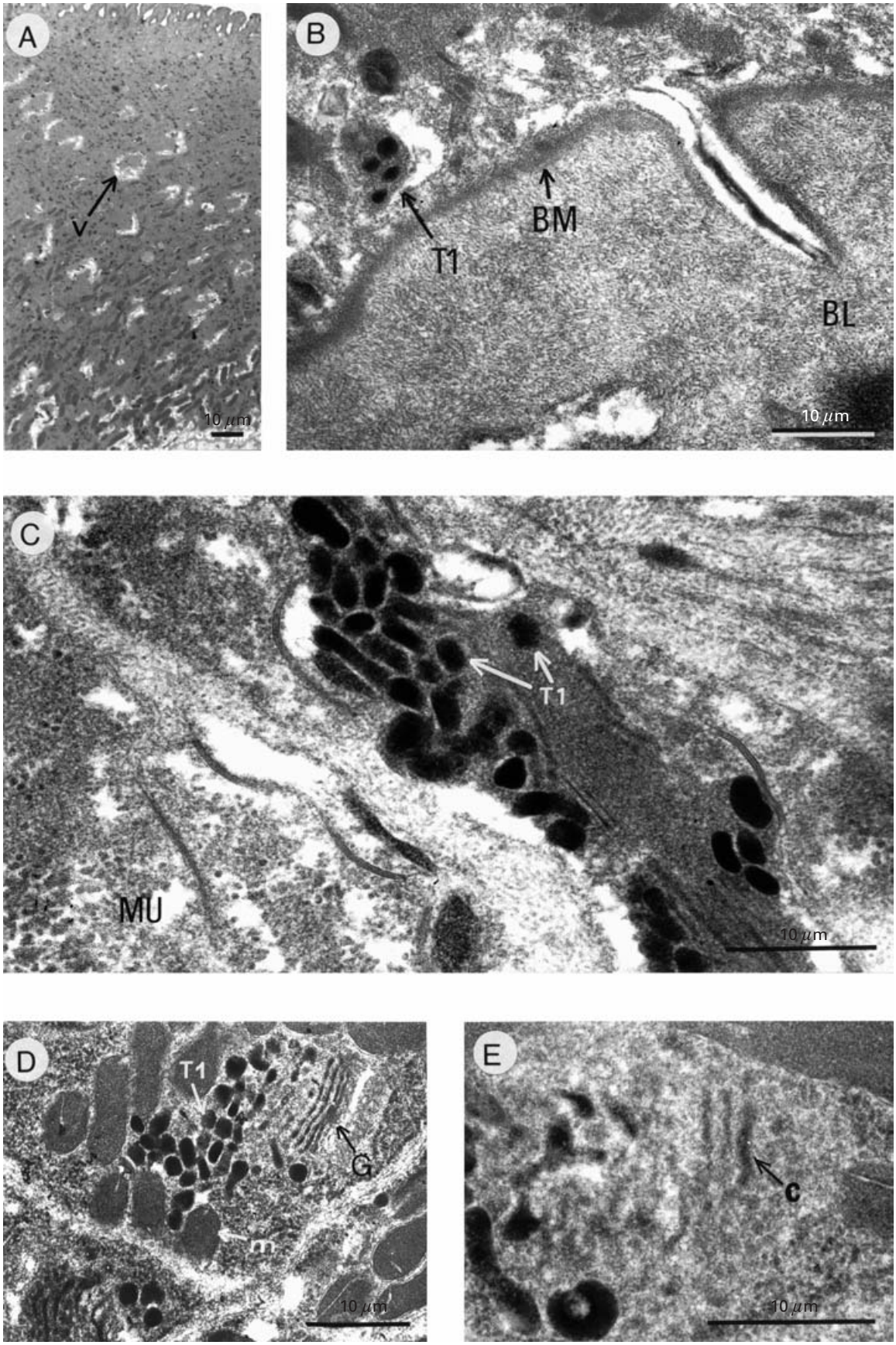

Fig. 2. Transmission electron micrographs (TEMs) of adult *Fasciola hepatica* following incubation in ABZ-SO for 12 h (10 μg/ml). (A) TEM of the tegumental syncytium, showing the presence of vacuoles (V). (B) TEM showing an accumulation of T1 secretory bodies close to the basal plasma membrane (BM). BL, basal lamina. (C) TEM showing a 'pool' of T1 secretions within a cytoplasmic extension. MU, muscle. (D) TEM showing part of a T1 tegumental cell body, containing an accumulation of T1 secretory bodies. Golgi complexes (G) and mitochondria (m) are present. (E) TEM of an abnormal Golgi complex in a T1 tegumental cell body. It is diffuse, consisting of only 3 distinct cisternae (c).

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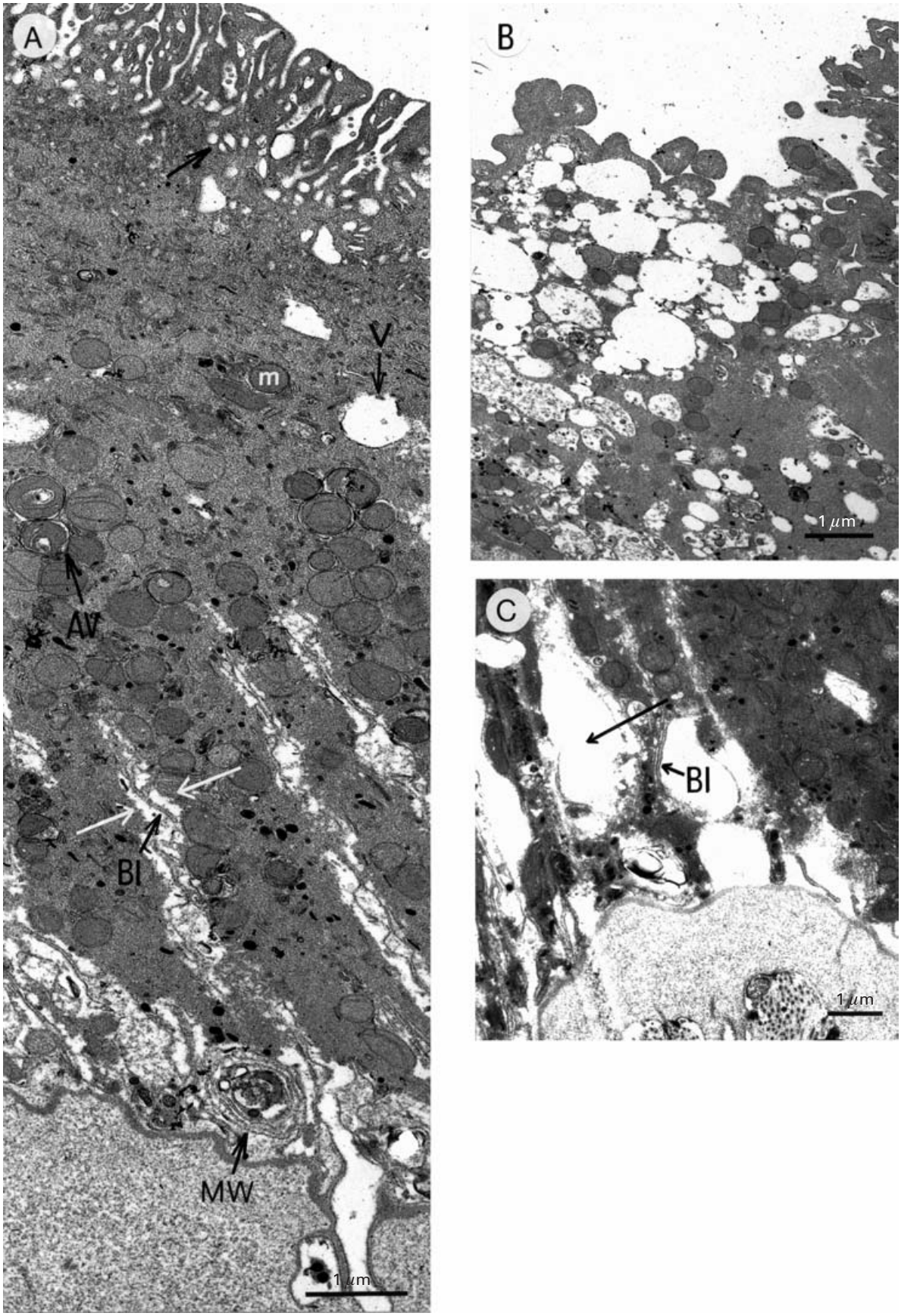

Fig. 3. Transmission electron micrographs (TEMs) of adult *Fasciola hepatica* following 24 h incubation in ABZ-SO (10 μg/ml). (A) TEM of the tegumental syncytium. 'Open bodies' (black arrow) are present at the apex. The mitochondria (m) are swollen and rounded in appearance, with defined cristae. They are distributed throughout the syncytium. Certain of the mitochondria are present in autophagic vacuoles (AV). Other vacuoles are also present in the syncytium (V).

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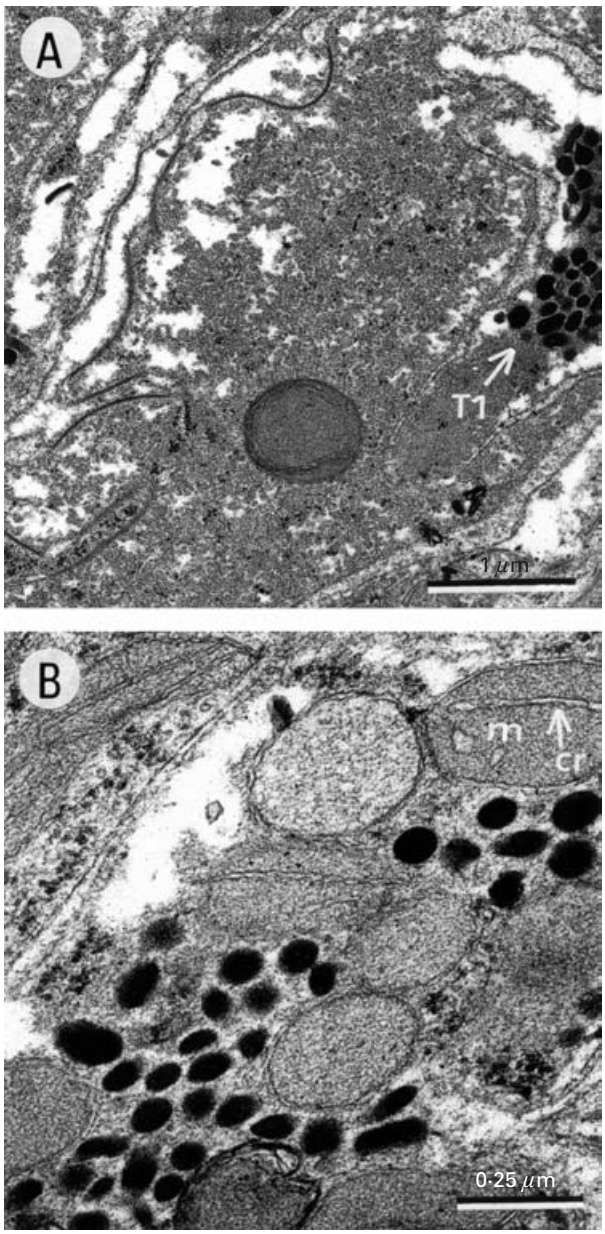

Fig. 4. Transmission electron micrographs (TEMs) of adult *Fasciola hepatica* following 24 h incubation in ABZ-SO (10 μg/ml). (A) TEM showing a 'pool' of secretions in a cytoplasmic connection between the tegumental cell body and syncytium. T1 secretory bodies (T1) are present. (B) TEM showing an accumulation of T1 bodies in a tegumental cell. The mitochondria (m) have distinct cristae (cr).

Treated specimens. Following 12 h incubation of flukes, there were significant changes in the pattern of immunofluorescence observed as compared to control flukes (Fig. 5C). The level of staining throughout the syncytium was markedly increased. The fluorescence was concentrated in discrete areas scattered uniformly throughout the syncytial layer, giving the whole tegument a 'speckled' appearance. In addition, the areas of fluorescence were more intense as compared to those of control flukes. The apical band observed in control specimens was present, although it was more difficult to define, because of the over-riding effect of the fluorescence throughout the syncytium. The pattern and intensity of staining within the tegumental cells and the cytoplasmic connections were similar to those of the untreated specimens (Fig. 5D).

After 24 h, the distribution of immunoreactivity within the tegument was significantly different as compared to that of control and 12 h-treated sections. The level of immunostaining in the apical band was less intense, and little fluorescence was observed throughout the syncytium itself (Fig. 5E). However, a thread-like pattern of immunostaining was observed in the tegumental cells and their connections, and was similar to that of the control and 12 h-treated flukes (Fig. 5F). In the control sections where the specific anti-tubulin antibody was omitted, no immunofluorescence was observed.

DISCUSSION

An analysis of the response by the parasite to anthelmintic drugs can provide insight into the action of such compounds. Such an approach has previously been used with *Fasciola* to study the action of TCBZ and drugs from chemical groupings other than the benzimidazoles (see review by Fairweather, 1997). In this investigation, the benzimidazole metabolite ABZ-SO was used. The results of the present work demonstrate that treatment with ABZ-SO leads to surface alterations in the fluke, particularly in the posterior region; disrupts secretory processes within the tegument; and causes changes in the intensity and distribution of tubulin immunostaining in the tegumental syncytium. These results will be discussed in turn and compared with the actions of other benzimidazoles, particularly TCBZ-SX, and established microtubule inhibitors.

The SEM observations demonstrate tegumental swelling that gave rise to the formation of blebs around spines and in localized areas of the dorsal midbody region. This blebbing was more pronounced in the dorsal tail region. Damage to the posterior region of the fluke has also been observed following treatment with triclabendazole sulphoxide (TCBZ-SX) and mebendazole (Verheyen *et al*. 1982; Stitt & Fairweather, 1993*a*; Meaney *et al*. 2002). It has been

The basal infolds (BI) are normal, although the mucopolysaccharide masses associated with them appear disrupted (white arrows). MW, membranous whorl. (B) TEM of the tegumental apex, showing localized blebbing. The cytoplasm of the blebs is highly vacuolated. (C) TEM of the base of the tegument, showing swelling of the mucopolysaccharide masses (arrow) surrounding the basal infolds (BI).

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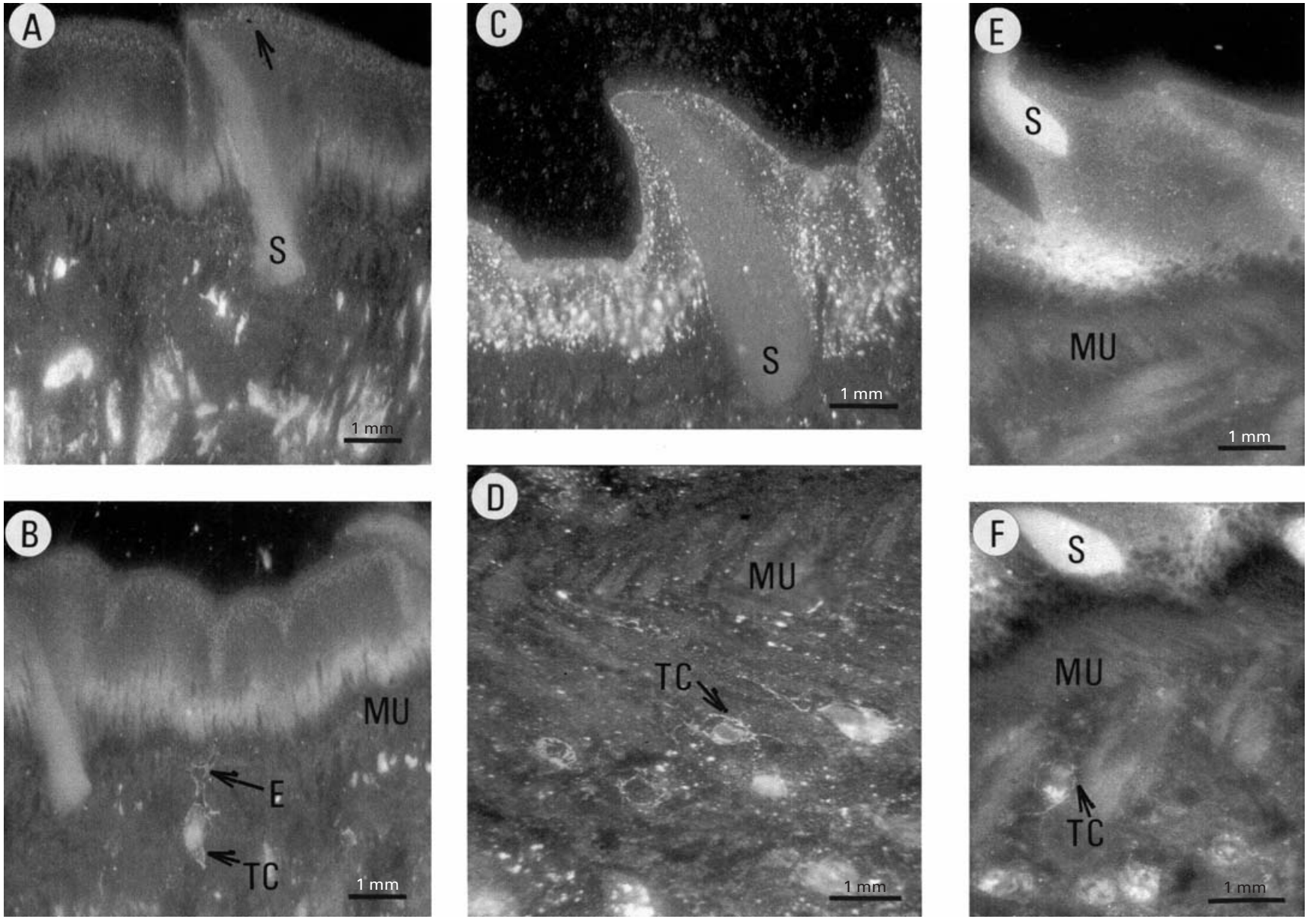

Fig. 5. For legend see opposite page.

seen, too, with other fasciolicides, but is not a general rule for all such compounds (see review by Fairweather & Boray, 1999). The surface changes were far less severe than those observed following incubation in TCBZ-SX. Some spine loss and limited loss of tegument occurred in the tail region of the fluke but was not as widespread as that seen with TCBZ-SX. The blebbing was remininscent of the early stages of TCBZ-SX treatment, but far more localized in appearance. Blebbing in response to drug action has been observed in other helminth parasites and is believed to represent an attempt by the parasite to replace damaged surface membrane. In *Schistosoma mansoni*, at least, the phenomenon is thought to be a calcium-dependent process (Bricker *et al*. 1983).

The TEM studies showed that transport of the secretions responsible for maintenance of the apical tegumental membrane was blocked by ABZ-SO treatment. The 12 h incubation was characterized by a build-up of secretory bodies in the cytoplasmic connections, as well as at their site of synthesis in the tegumental cells. This was still observed after 24 h, with distinct accumulations also occurring at the base of the syncytium. Another marked feature of the 24 h treatment was a disruption in the distribution of secretory bodies throughout the syncytium which, again, is indicative of a block in transport. Within the tegumental cells, some of the Golgi complexes were beginning to adopt an abnormal morphology after 12 h and these organelles were scarcely present in the cells by 24 h. This block by ABZ-SO of secretory body transport and disruption of the Golgi complexes are features typical of microtubule inhibition in secretory cells (Allan, 1995; Hirokawa, Noda & Okada, 1998; Lippincott-Schwartz, 1998). Reduction of Golgi activity and inhibition of secretory vesicle movement are also typical of TSBZ-SX activity against *F. hepatica* (Stitt & Fairweather, 1994). However, the necrosis of tegumental cells following TCBZ-SX treatment was not observed in the present study. Furthermore, similar morphological changes have been observed in the tegument of cestodes treated with both ABZ (Casado *et al*. 1996; Schmidt, 1998; Urrea-Paris *et al*. 2000) and mebendazole (Borgers *et al*. 1975*b*; Verheyen *et al*. 1976; Verheyen, 1982). They have also been described in the intestinal cells of nematodes following incubation with mebendazole (Borgers & De Nollin, 1975; Borgers *et al*. 1975*a*; Atkinson, Newsam & Gull, 1980). A physical loss of microtubules in response to benzimidazole treatment has been observed in studies involving cestodes, nematodes and protozoans (Borgers *et al*. 1975*a*, *b*; Atkinson *et al*. 1980; Laclette *et al*. 1981; Verheyen, 1982; Zintz & Frank, 1982; Sangster, Prichard & Lacey, 1985; Franz, Zahner & Benten, 1990; Oxberry, Thompson & Reynoldson, 1994).

All of the apparent effects of microtubule inhibition in the tegument of *F. hepatica* observed in the present study have been described in previous studies on *F. hepatica* and other trematode and cestode parasites using established microtubule inhibitors (Bogitsh & Carter, 1977; Etges & Bogitsh, 1985; Stitt & Fairweather, 1993*b*; Stoitsova & Gorchilova, 1997; Schmidt, 1998). For example, treatment of flukes with colchicine was seen to block transport within the tegumental cells and transport from the cells to the base of the syncytium, although no inhibition of their synthesis occurred (Stitt & Fairweather, 1993*b*). In a separate study involving colchicine, the number of T2 bodies within the tegument was also reduced (Stoitsova & Gorchilova, 1997). With tubulozole-C, accumulations of secretory bodies occurred, particularly at the base of the tegument. Tubulozole-C elicited more severe effects by causing disruption to the apical membrane and eventual sloughing of the syncytium. In contrast to the action of colchicine, synthesis of the secretions within the tegumental cells appeared to be affected by tubulozole-C (Stitt & Fairweather, 1993*b*). The results obtained with tubulozole-C resemble those obtained for TCBZ-SX on the tegument of the fluke (Stitt & Fairweather, 1994).

As well as altering the distribution of secretions throughout the tegument, 24 h incubation of specimens with ABZ-SO also significantly affected the morphology of the mitochondria within the syncytium, in that they appeared swollen and adopted a rounded appearance. This is in contrast to TCBZ-SX treatment, following which a reduction in both the number and size of the syncytial mitochondria was observed (Stitt & Fairweather, 1994). The induction by ABZ-SO of their autophagy seen in the present

Fig. 5. Tubulin immunofluorescence images of *Fasciola hepatica* tegument. (A) Untreated specimen. An intense band of immunostaining is present just beneath the apical plasma membrane (arrow). Note the absence of staining in the spines (S). (B) Untreated specimen. A thread-like pattern of immunostaining is evident in the tegumental cells (TC) and in their cytoplasmic extensions (E) to the syncytium. No immunoreactivity is present in the muscle layers (MU). (C) Tegumental syncytium following 12 h ABZ-SO incubation (10 μg/ml). An intense pattern of immunostaining occurs throughout the syncytium, giving it a 'speckled' appearance. S, spine. (D) Subtegumental region of the fluke following 12 h ABZ-SO incubation (10 μg/ml). The immunoreactivity in the tegumental cells (TC) appears thread-like. The muscle layers (MU) remain unstained. (E) Specimen treated for 24 h with ABZ-SO (10 μg/ml). There is a low level of immunoreactivity throughout the syncytium and the apical band of immunoreactivity is less distinct. The muscle layers (MU) are unstained. (F) Distribution of tubulin immunostaining in the subtegumental region following 24 h ABZ-SO incubation (10 μg/ml). Staining within the tegumental cells adopts a thread-like pattern. No immunofluorescence is associated with the spines (S) or the muscle blocks (MU).

study has also been seen with the microtubule inhibitor, colchicine (Stoitsova & Gorchilova, 1997), but not with TCBZ-SX (Stitt & Fairweather, 1994).

However, the swelling of the mitochondria may also be indicative of an effect on the metabolism of the fluke. In addition, other morphological changes that could not be directly linked to microtubule inhibition were observed in the tegument, including a swelling of the basal infolds and the presence of autophagic vacuoles in the syncytium. Similar changes, together with the presence of greater numbers of lipid bodies and decreased glycogen levels have been described in other studies with both ABZ and ABZ-SO (Perez-Serrano *et al*. 1994; Casado *et al*. 1996; Magambo, 1996; Ingold *et al*. 1999; Urrea-Paris *et al*. 2000; see also De Nollin *et al*. 1974; Borgers & De Nollin, 1975). There is some evidence for the disruption of energy metabolism in *F. hepatica* by mebendazole (for data, see Fairweather & Boray, 1999) and it has been suggested that ABZ (together with other classical benzimidazoles and TCBZ) is capable of uncoupling oxidative phosphorylation in isolated mammalian mitochondria (McCracken & Stillwell, 1991; Carr, McCracken & Stillwell, 1993). However, these studies were not carried out with *F. hepatica*, or any other helminth. Moreover, many authors regard the 'metabolic' changes as being secondary to (and even as a consequence of) the disorganization of the presumed primary target, namely, microtubules. That is, the changes may simply represent a state of stress in a parasite that stems from the disruption of such fundamental cell organelles.

The tegument of flukes treated for 12 h with ABZ-SO showed a dramatic increase in the level of diffuse tubulin immunostaining in the syncytium. A similar phenomenon has been observed in *Giardia duodenalis* trophozoites following ABZ treatment (Oxberry, Reynoldson & Thompson, 2000). The authors have suggested that the increase in fluorescence was due to a rupturing of the cell membrane, thus permitting more antibody to penetrate the cell. However, this cannot be the case in the present study as sections, as opposed to intact cells, were used. An alternative explanation given was that of an inherent conformational change having occurred in the tubulin molecule as a result of drug binding, allowing an overall increase in antibody binding. Differential tubulin immunostaining was also seen in isolated cells from the germinal layer of hydatid cysts and from the African Green monkey kidney in response to colchicine and a number of benzimidazoles (including mebendazole and parbendazole) (Havercroft, Quinlan & Gull, 1981; Rubino *et al*. 1983). In these cases it was attributed to the inhibition of microtubule polymerization, leading to a change in polymer/dimer equilibrium of the microtubule, thus increasing the concentration of tubulin subunits in the syncytium for interaction with the antibody. The latter explanations imply that there are epitopes present on the tubulin molecule(s) that vary in their accessibility to antibody. This accessibility will depend on the state of polymerization of the α and β subunits. As a polyclonal antibody, presumably specific for several epitopes, was used in this study, this effect would be possible. The possibility remains that if accelerated transport of secretory bodies was indeed occurring this may result in a greater number of microtubules being required to facilitate the transport as a response by the fluke to compensate for microtubule disruption.

Following 24 h treatment, the level of fluorescence throughout the syncytium was greatly reduced. A decrease in the intensity of immunostaining was also observed in the protoscolices of *Echinococcus granulosus* following treatment with ABZ, ABZ-SO or a combination of the two (Perez-Serrano *et al*. 1995). Furthermore, in a separate study on *F. hepatica*, incubation with TCBZ-SX led to a total loss of tubulin immunoreactivity in the tegumental syncytium (but not in the tegumental cells or in their connections to the syncytium) in TCBZ-susceptible, but not -resistant, flukes (Robinson *et al*. 2002). The decrease in immunoreactivity seen with ABZ-SO and TCBZ-SX probably explains the block in transport of secretions also observed with both drugs. The difference in the immunostaining patterns observed with ABZ-SO and TCBZ-SO remain to be resolved.

As explained previously, there are similarities in the tegumental response to ABZ-SO and TCBZ-SX. However, the effects of TCBZ-SX seem to be more severe (Stitt & Fairweather, 1993*a*, 1994; Robinson *et al*. 2002). It appears that ABZ elicits similar effects to that of colchicine, which is what would be expected, since benzimidazoles are believed to bind at the colchicine-binding site on β-tubulin (Russell & Lacey, 1995). Certainly, benzimidazoles can compete with colchicine for binding to tubulin, and this is one of the standard assays used to assess tubulin binding activity. It has been suggested that TCBZ binds at an alternative site on the molecule (Stitt & Fairweather, 1993*b*). This fits with data that ABZ is effective against TCBZ-resistant strains (Coles & Stafford, 2001). Even though both drugs target microtubules, the fact that they may bind at separate sites may mean that they could both be used in drug rotation schemes and in synergistic combinations. Because different groups of amino acids on the tubulin molecule would be involved in their binding, resistance to both drugs probably develops independently. Thus, ABZ presents itself as a drug that may become increasingly important in fluke chemotherapy. However, additional studies need to be carried out on ABZ to confirm its precise mode of action, both at the morphological and molecular level. In relation to the potential use of ABZ against TCBZ-resistant flukes, the present study has highlighted a number of differences in the morphological profile of flukes treated with the two drugs. These need to be explored further

in order to explain the differential activities of the two drugs.

Since ABZ-SO is generally considered to be the more active of the ABZ metabolites, it is assumed that the effects observed following treatment with ABZ-SO are representative of ABZ action within the fluke. It has recently been shown that *F. hepatica* is capable of metabolizing ABZ to ABZ-SO, rather than simply relying on its host for this transformation (Solana, Rodriguez & Lanusse, 2001). Solana's study confirmed a previous observation that the concentration of sulphoxide in the fluke was greater following incubation in ABZ than ABZ-SO (Fetterer & Rew, 1984). Moreover, the uptake of the more lipophilic parent albendazole is greater than that of the more hydrophobic sulphoxide metabolite (Alvarez *et al*. 2001). The combined data raise intriguing questions concerning the relative uptake of ABZ and its metabolites by the fluke *in vivo*, the route of drug entry into the fluke (trans-tegumental or oral ingestion) and which is the active form of the drug (ABZ itself or its sulphoxide metabolite). The sulphone metabolite is not considered to possess any biological activity. A relatively greater activity of ABZ (and other parent benzimidazoles) has been demonstrated in a number of assays, including inhibition of benzimidazole binding to nematode tubulin and inhibition of egg hatching and larval development in nematodes (Lacey *et al*. 1987; Lacey, 1990; Lubega & Prichard, 1991). It is known that ABZ binds with higher affinity to tubulin (Lacey *et al*. 1987; Lubega & Prichard, 1991); therefore future work into the action of the parent molecule would prove of great value. Such studies are in progress.

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