

## **Antigen stripping from the nematode epicuticle using the cationic detergent cetyltrimethylammonium bromide (CTAB)**

D.I. PRITCHARD,\* C.R. CRAWFORD,\* I.R. DUCE & J.M. BEHNKE\*

\*MRC Experimental Parasitology Research Group, Department of Zoology, University of Nottingham, Nottingham NG7 2RD, UK

Accepted for publication 25 April 1985

**Summary** The cuticular antigens of adult *Nematospiroides dubius* were selectively removed using the cationic detergent cetyltrimethylammonium bromide (CTAB). Nonionic, zwitterionic or anionic detergents were ineffective in comparison. The biochemical profile of the antigens removed by detergent was identical to that of surface antigens removed by homogenization, with the added advantage that detergent-stripped antigens lacked many of the background antigens (excretory/secretory—ES and somatic) seen in homogenates. In addition, the detergent was shown to act in a non-invasive manner as electron micrographs failed to reveal any gross damage to the nematode outer cuticle. The observed selective release of significant quantities of relatively clean nematode surface antigen by CTAB in a non-invasive or destructive manner provides the impetus for definitive studies on the relevance of surface antigens (in the absence of ES or somatic antigens) to the overall immunogenicity of this and other parasites.

**Keywords:** nematode, epicuticle, antigen, cetyltrimethylammonium bromide

### **Introduction**

The outermost surface of the nematode cuticle consists of a polyanionic glycocalyx overlying a trilamellate layer; together, they form the epicuticle complex (Lee 1977, Lumsden 1975, Inglis 1983). The net surface charge of the epicuticle is negative, and it has been shown to bind polyvalent cationic complexes such as cationized ferritin and ruthenium red (Himmelhoch & Zuckerman 1983, Murrell & Graham 1983).

Correspondence: Dr D.I. Pritchard, MRC Experimental Parasitology Research Group, Department of Zoology, University of Nottingham, Nottingham NG7 2RD, UK.

It is also apparent that the nematode epicuticle expresses molecules which are highly immunogenic to the parasitized host (Philipp, Parkhouse & Ogilvie 1980), and that these surface antigens can be shed into the external milieu (Vetter & Klaver-Wesseling 1978, Smith *et al.* 1981). These observations suggest that surface antigens may be relevant to the generation and expression of immunity, and recent data, albeit indirect and utilizing protective monoclonal antibodies specific for surface antigens, supports this possibility (Ortega-Pierres, Mackenzie & Parkhouse 1984). However, there are as yet no examples of successful vaccination against helminth infections using purified preparations of antigens identified by surface-labelling methods (Philipp & Rumjaneck 1984).

Therefore it was decided to attempt to accelerate the relatively slow turnover of surface-expressed molecules in a controlled manner using a technique of selective antigen-stripping from the parasite surface by detergents. Detergents have been widely used to solubilize nematode surface antigens (reviewed by Philipp & Rumjaneck 1984), but apart from a few unpublished reports do not seem to have been used in a controlled manner. In the present study, advantage was taken of the polyanionic surface charge of the nematode, and the fact that cationic detergents exist and have been successfully used to remove surface-determinants from viruses (Bachmayer 1975, Laver & Webster 1976). The selective removal of surface antigens was demonstrated, and the potential of the technique to provide sufficient relatively pure immunogenic cuticular material for immunization studies discussed.

## Materials and methods

### NEMATODE RECOVERY AND ANTISERA

*Nematospiroides dubius* adults were harvested from CFLP mice as described previously (Pritchard *et al.* 1983). Immune sera (IMS) were raised in CFLP mice using a multiple infection schedule (Behnke & Parish 1979). Normal mouse sera (NMS) were obtained from uninfected controls.

### SURFACE-LABELLING, IMMUNOPRECIPITATION AND SDS-PAGE

Parasite antigens were analysed biochemically following the methods of Philipp *et al.* (1980) and Pritchard *et al.* (1984). Briefly, freshly recovered parasite material was washed extensively before labelling with  $I^{125}$  using Iodogen (Fraker & Speck 1978). Labelled nematodes were homogenized in the presence of protease inhibitors and centrifuged for 30 min at 11 000 g (4°C) to obtain a source of solubilized surface-labelled material (\*SAg).

Otherwise, labelled nematodes were washed extensively in Eagle's MEM containing 50 µg penicillin/ml, streptomycin/ml 50 µg and fungizone (2.5 µg/ml) until the washings were 'cold'. The washed, labelled worms were then cultured in the same medium at 37°C for up to 28 h. At regular time intervals, aliquots of the culture supernatant were aseptically removed for analysis by TCA precipitation (10 µl + 50 µl 10% TCA + 50 µl Fetal Calf Serum (FCS) and immunoprecipitation (50 µl + 50 µl IMS or NMS).

## DETERGENTS

Detergents were incorporated into the Eagle's MEM in an attempt to selectively strip surface antigens. The selection of detergents was determined by their surfactant properties (Helenius & Simons 1975), and the following were used: Triton X-100, nonionic; sodium deoxycholate (NaDOC), anionic; CTAB, cationic and Empigen BB (zwitterionic). The release in the presence of detergent was compared with that of medium alone.

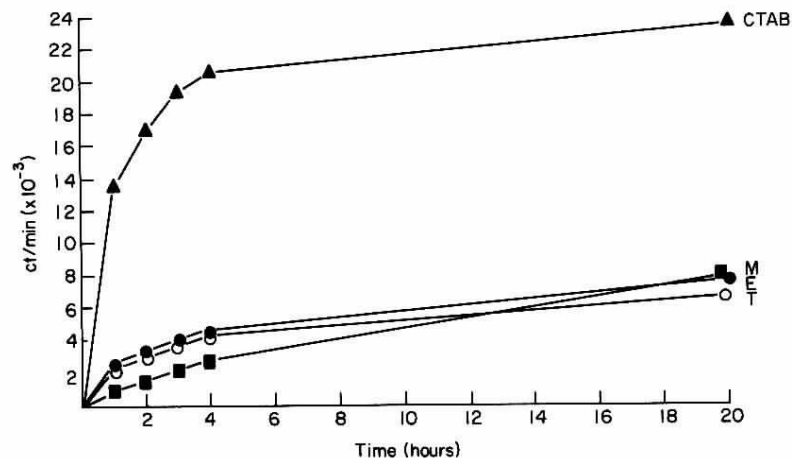
## ELECTRON MICROSCOPY

Adult *N. dubius* were transferred to 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for 2 h, and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h (at room temperature). Following dehydration, the worms were transferred to propylene oxide before being infiltrated and embedded in Spurr resin. For light microscopy autoradiography (LMARG) 0.5  $\mu$ m sections were coated by dipping in a 1:1 mixture of Ilford K2 Nuclear Emulsion and 0.1% glycerol at 47°C. LMARGs were developed in D19 developer, fixed in 30% sodium thiosulphate, and examined and photographed unstained under Nomarski optics on a Zeiss Photomicroscope II. Unlabelled material and LMARGs exposed to light showed no evidence of positive or negative chemography.

## RESULTS

*Antigen stripping by detergents during culture in vitro*

The cationic detergent CTAB selectively stripped appreciably more TCA-precipitable  $^{125}$ I-labelled material from the surface of adult *N. dubius* when compared with Triton X-



**Figure 1.** The removal of  $^{125}$ I-surface-labelled (Iodogen) protein from adult *N. dubius* using detergents. Adult *N. dubius* were surface-labelled by the Iodogen method before incubation at 37°C in Eagle's MEM containing Empigen BB (E), Triton X-100 (T) or cetyltrimethylammonium bromide (CTAB), or in medium alone (M). This graph indicates the time course of removal of soluble surface-labelled protein (TCA precipitable) into each medium over a period of 20 h. The ct/min expressed at 20 h are the cumulative counts released over the whole incubation period.

**Table 1.** The removal of surface antigens from adult *N. dubius* using detergents. Adult *N. dubius* were surface-labelled ( $^{125}\text{I}$ ) by the Iodogen method before incubation at 37°C in various detergents. This figure compares the ability of each detergent to release TCA precipitable and immunogenic material from the parasites' surface.

| Conditions of incubation | Incubation time (h) | ct/min in 10 $\mu\text{l}$ culture supernatant (TCA precipitable) | % of original c.p.m. in total supernatant (i.e. % ct/min available released) |             | ct/min precipitated from 50 $\mu\text{l}$ of culture supernatant |     |
|--------------------------|---------------------|---|--|-------------|--|-----|
|                          |                     |   |  |             | NMS  | IMS |
| Eagle's MEM              | 1                   | 978   | 2.8%   | 281         | 480  |     |
| + 1% Triton $\times$ 100 | 1                   | 2375  | 6.8%   | 168         | 1646   |     |
| + 1% Empigen BB          | 1                   | 2477  | 7.1%   | 96          | 2269   |     |
| + 0.25% CTAB             | 1                   | 13494   | 39.0%  | 609         | 16172†   |     |
| Eagle's MEM              | 20*                 | 7850 (5064)   | 22.0%  | 2978 (2382) | 2962 (491)   |     |
| + 1% Triton $\times$ 100 | 20                  | 6639 (2416)   | 19.0%  | 410 (88)    | 5131 (2443)  |     |
| + 1% Empigen BB          | 20                  | 7717 (3136)   | 22.0%  | 427 (74)    | 5435 (1492)  |     |
| + 0.25% CTAB             | 20                  | 23499 (2932)  | 67.0%  | 4936 (3703) | 22677 (1588)   |     |

\* The ct/min expressed at 20 h are the cumulative counts released over the whole incubation period (see Figure 1). The actual counts precipitable by TCA or sera to 20 h are shown in brackets.

† This figure represents 24% of the total TCA precipitable radioactivity. However, this % can be increased dramatically by titration to optimal proportions.

**Table 2.** The effect of an anionic detergent (NaDOC) on the release of  $I^{125}$ -labelled protein from adult *N. dubius* in culture compared with the cationic detergent CTAB

| Conditions of incubation | Time of incubation (h) | ct/min in 10 $\mu$ l supernatant (TCA precipitable) | % of original ct/min in total supernatant |
|--------------------------|------------------------|---|---|
| MEM (Eagle's)            | 1                      | 550   | 4.9%                                      |
| +0.025% CTAB             | 1                      | 4216  | 37.9%                                     |
| +0.25% CTAB              | 1                      | 4932  | 44.2%                                     |
| +0.1 NaDOC               | 1                      | 521   | 4.7%                                      |
| +1% NaDOC                | 1                      | 1610  | 14.4%                                     |
| MEM (Eagle's)            | 20                     | 2696 (1269)   | 24.0%                                     |
| +0.025% CTAB             | 20                     | 6681 (350)  | 60.0%                                     |
| +0.25% CTAB              | 20                     | 8485 (968)  | 76.1%                                     |
| +0.1 NaDOC               | 20                     | 2976 (1121)   | 26.6%                                     |
| +1% NaDOC                | 20                     | 4521 (1395)   | 40.4%                                     |

100, Empigen BB and medium alone (Figure 1). Release was seen to occur very rapidly over the initial 4 h of culture, during which time the nematodes were still motile and appeared morphologically intact.

By 20 h of culture, 67% of the total available  $I^{125}$ -labelled material had been released compared with 19–22% released in medium by the other detergents used in these experiments (Table 1).

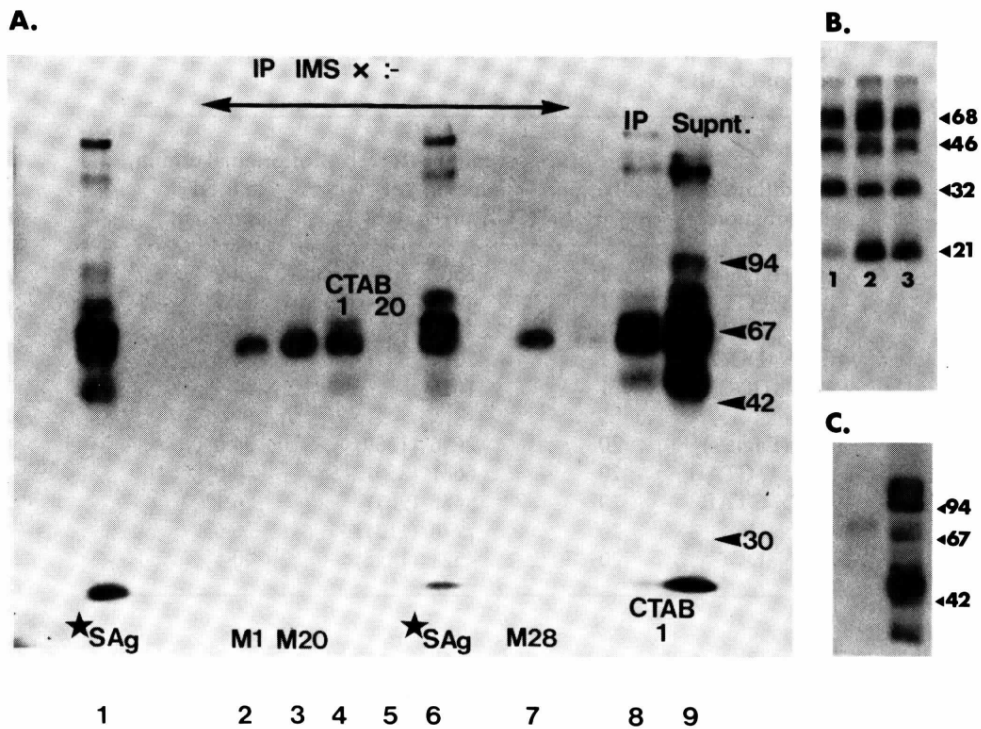
Precipitation of the same supernatants with IMS revealed that the pattern of release of immunogenic surface-labelled material was identical to the release of TCA-precipitable material (therefore graph not shown). Although immunogenic material was gradually released in medium alone over the 20 h incubation period, this process was rapidly accelerated by CTAB but not by the other detergents. The quantity of labelled material precipitated by normal mouse serum (NMS) was negligible in comparison (Table 1).

When CTAB (cationic) was compared directly with NaDOC (anionic), the cationic detergent was far superior in releasing appreciable quantities of surface-labelled material into the culture supernatants (Table 2). Even concentrations as low as 0.025% CTAB were more effective than 1% NaDOC, which might be surprising considering the more stringent mode of action of the latter detergent (Helenius & Simons 1975).

It was also demonstrated that surface-labelled material was effectively released from larval (d6L4) *N. dubius*, adult *Necator americanus* and the infective muscle larvae of *T. spiralis* (data not shown).

#### *SDS-page analysis of the CTAB-stripped antigens (Figure 2)*

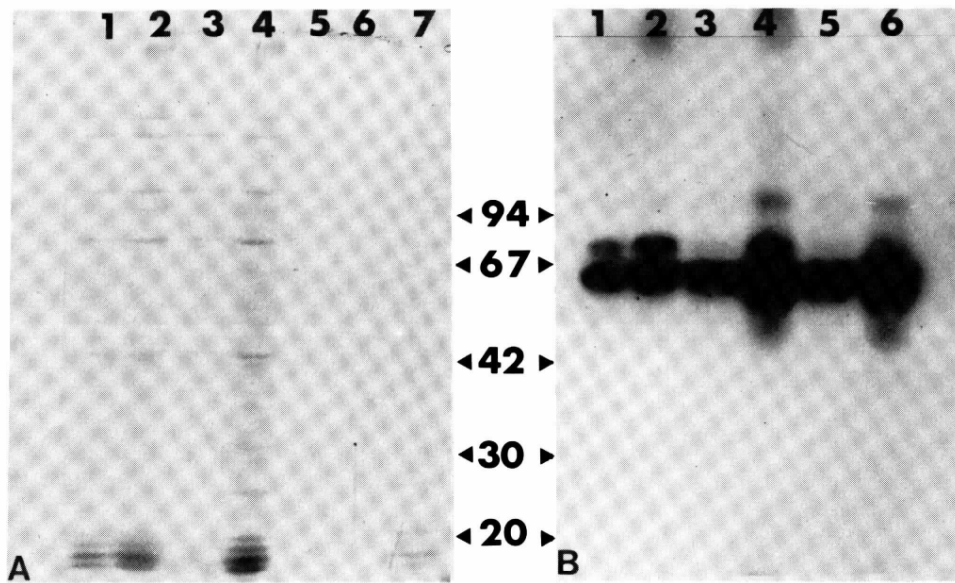
Immunoprecipitates manufactured between IMS/NMS and the supernatants of CTAB cultures and supernatants of control (medium) cultures were analysed. The resulting profiles on autoradiography were compared with those of CTAB supernatants alone



**Figure 2.** SDS-PAGE analysis of CTAB-stripped antigens. *A.* *N. dubius* Immunoprecipitates (IP) manufactured between immune mouse sera (IMS) and the supernates of control (medium, M) cultures and CTAB cultures were analysed by SDS-PAGE, and the  $^{125}$ I-labelled antigens precipitated compared with those released by the homogenization of labelled adult worms (\*SAg—lane 1), and those released by CTAB into the culture supernatant after 1 h (CTAB 1 supernatant—lane 9). For full explanation see text; *B.* *N. americanus*, immunoprecipitates manufactured between 'immune' hamster sera (long-term 100 L3 infection of 117 days) and CTAB-stripped antigens (lane 1) were compared on SDS-PAGE with the CTAB culture supernatant after 1 h (lane 2) and those labelled surface antigens released by homogenization (lane 3); *C.* *T. spiralis*, The molecular weight of the labelled surface antigens released by CTAB during a 1 h culture of infective muscle larvae of *T. spiralis*.

(stripped antigen alone after 1 h incubation—lane 9), radiolabelled whole surface antigen released by homogenization (\*SAg—lane 1) and an immunoprecipitate (IP) manufactured between immune mouse serum (IMS) and \*SAg (lane 6).

It can be seen (Figure 2) that the major surface antigens of adult *N. dubius* (c.f. \*SAg, lanes 1 and 6) were released into the supernatant of cultures within 1 h in the presence of CTAB (Supernatant—lane 9) and that these antigens were immunoprecipitable (IP) by IMS (lane 8). The antigens present in the CTAB supernatant after 1 h were comparable in molecular size to those released from labelled adults by homogenization (\*SAg). The major immunogenic moiety which has been detected on the adult surface resolves at 65 000 on SDS-PAGE (Pritchard *et al.* 1984) and it can be seen from Figure 2 that this antigen was released into medium alone during the course of incubation (immunoprecipi-

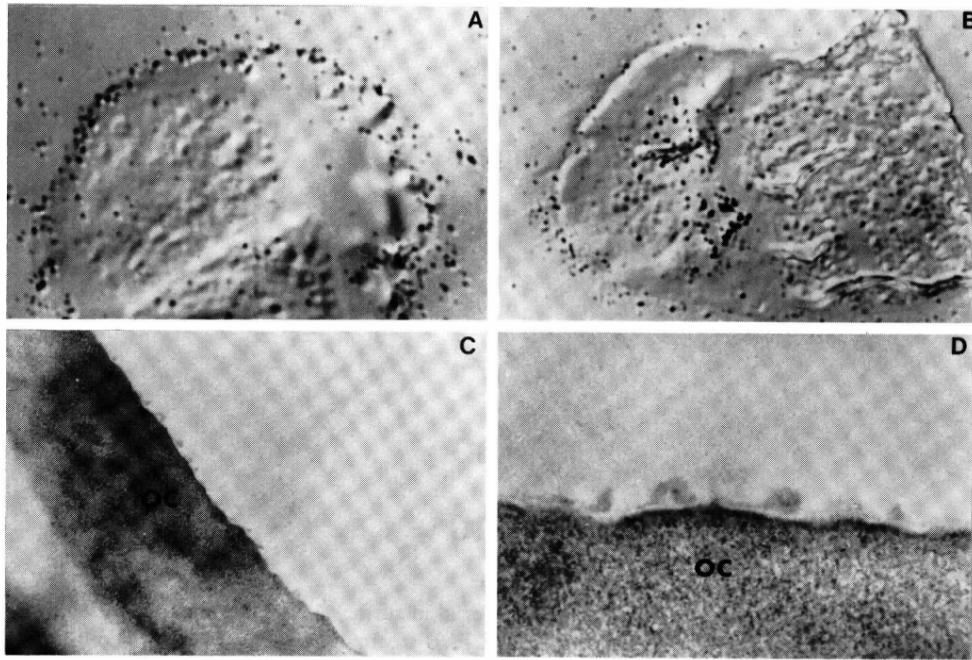


**Figure 3.** The enrichment of CTAB-stripped material in surface antigens compared with antigen obtained by homogenization before and after CTAB treatment (where  $x = 35 \times 10^3$  ct/min) A, Coomassie brilliant blue; B, autoradiograph of A. Lanes 1 and 2,  $x$  and  $3x$  ct/min (TCA precipitable) recovered by the homogenization of labelled adult *N. dubius* following incubation of the worms for 1 h in CTAB, lanes 3 and 4,  $x$  and  $3x$  ct/min precipitable recovered by the homogenization of labelled *N. dubius*; lanes 5 and 6,  $x$  and  $3x$  ct/min (TCA precipitable) stripped from the surface of intact labelled *N. dubius* by CTAB (1 h); lane 7, Cold adult homogenate.

tates M1, M20, M28—lanes 2, 3, 7) and that an antigen of identical molecular weight was released by CTAB within 1 h (immunoprecipitate CTAB 1—lane 4) but that little else was released by hour 20 of the incubation (CTAB 20—lane 5). It would therefore appear that CTAB strips the majority of the immunogenic surface-labelled material of *N. dubius* within 1 h of incubation. It is also pertinent that the major immunogenic band (65 000) released into culture is also prominent in immunoprecipitates manufactured between IMS and \*SAg.

Finally here, the major surface-labelled proteins of *Necator americanus* and *Trichinella spiralis* (see inset—Figure 2) were similarly removed by CTAB after 1 h, indicating that CTAB is indeed a useful agent for the rapid removal of surface-labelled material from the nematode cuticle.

However, it does not suffice that a method should be rapid without being clean. Therefore, it was decided to compare the protein content of CTAB-stripped material with that of \*SAg released by homogenization. Labelled preparations of each type were equalized in terms of ct/min precipitable by TCA before analysis by SDS-PAGE, and the resultant gel stained with Coomassie brilliant blue. If CTAB is preferentially stripping surface antigen, then the track containing this preparation should contain significantly less protein than that track containing the homogenization-prepared \*SAg, despite the fact that identical ct/min were loaded onto the gel.



**Figure 4.** A and B, Light microscopy autoradiographs of transverse sections of adult *N. dubius* maintained in: A, medium alone and B, medium + CTAB (1 h). Nomarski optics. Comparative silver grain count: A,  $21.94 \pm 5.87/100 \mu\text{M}$ ; B,  $7.86 \pm 2.26/100 \mu\text{M}$  (results = mean + 1 sd.  $n = 13$  different transverse sections. A vs B,  $P = < 0.001$ , Student's *t*-test). C, D. Electron micrographs of the surface (outer cuticle, OC) of adult *N. dubius* following incubation in CTAB for 1 h. A,  $\times 370$ ; B,  $\times 310$ ; C,  $\times 33\,250$ ; D,  $\times 127\,980$ .

It can be seen from Figure 3 that this was indeed the case, as material from worms homogenized to release their surface antigens both before (lanes 3, 4) and after (lanes 1, 2) CTAB treatment shows the characteristic profile on SDS-PAGE of adult worm homogenate (Pritchard *et al.* 1983) whereas the CTAB preparation contained none of these proteins despite having an equivalent quantity of radiolabelled surface antigen (in ct/min). The autoradiograph corresponding to these lanes is also shown to demonstrate this point. It is also interesting to note here that the CTAB-stripped preparation was devoid of labelled material in the 'ES-fraction' of adult *N. dubius* antigens. The ES antigens of *N. dubius* are known to resolve between molecular weight 14 000 and 20 000 under the conditions imposed (12–15% running gel—Pritchard *et al.* 1983) and can be seen to present as four distinct bands in the homogenized preparations (lanes 1–4, cold AH lane 7).

#### *Electron microscopy*

Electron microscopical analysis was conducted on parasitic worms in the presence or absence of CTAB. Gross analysis (Figure 4A) revealed that labelling was largely restricted to the surface of the worm and that significant quantities of label were removed



by CTAB (Figure 4B—c.f. Figure 1). At the EM level, CTAB was shown to cause disruption of the outermost layer (glycocalyx?) of the outer cortex (Figures 4C, D) without appearing to cause significant disruption of the cortical layers themselves.

## Discussion

There is little doubt that parasite surface antigens generate an immune response (Philipp *et al.* 1980) and that the resulting immune response can be effective against parasites *in vitro* and *in vivo*. For example, the infectivity of *T. spiralis* newborn larvae to mice is significantly reduced when worms are pre-incubated with an IgG, monoclonal antibody directed against a surface antigen of molecular weight 64 000 (Ortega-Pierres *et al.* 1984). In addition, monoclonal antibodies directed against surface determinants of *S. mansoni* schistosomula have been obtained by several workers (Grzych *et al.* 1982; Tavares *et al.* 1983) and shown to mediate *in vitro* killing of larvae and passive protection against *in vivo* challenge. Despite these elegant studies, there are as yet no examples of successful vaccination against helminth infections using purified preparations of antigens identified by surface-labelling methods (Philip & Rumjanek 1984). The limiting factor in the quest to assess directly the biological significance of surface antigens has been the lack of sufficient purifiable material for bioassay. In the present study, surface antigens have been selectively harvested from a number of species of parasitic nematode using a cationic detergent cetyltrimethylammonium bromide. Approximately 10 times as much surface-labelled material was released by incubation in the presence of CTAB compared with medium alone or anionic, zwitterionic or nonionic detergents, suggesting that a charge interaction is occurring between CTAB and the polyanionic 'glycocalyx' of the parasite surface (Inglis 1984).

The surface-labelled material released by CTAB retained its antigenicity, as shown by immunoprecipitation with postinfection sera, and the released material appeared biochemically identical on SDS-PAGE to those antigens released by the conventional homogenization techniques. CTAB-stripped material was further shown to lack the background proteins released by homogenization, in particular the protein bands usually associated with ES-products (Pritchard *et al.* 1983).

Significantly, worms incubated in CTAB were visually viable during the first 4 h of incubation (during which time the majority of releasable material appeared in culture supernatants) although infections following incubation will verify viability absolutely. For example, preliminary data using *T. spiralis* has revealed that infective larvae incubated for 1 h in CTAB retain both infectivity and immunogenicity. In the same context, electron microscopical analysis of adult *N. dubius* failed to reveal any gross damage to the nematode surface following incubation for 1 h in CTAB despite the removal of labelled material (as assessed by a decrease in the number of silver grains on autoradiography). The most noticeable effect of CTAB was a disruption of the outermost surface ('glycocalyx') of the outer cuticle. It is possible that the formations seen at the outer cuticle following CTAB-treatment represent the aggregated clusters described above.

In conclusion, treatment of some species of live nematodes with a cationic detergent releases significant quantities of relatively clean surface antigens in a non-invasive or grossly destructive manner. This observation provides the impetus for definitive studies

on the absolute relevance of surface antigens (c.f. ES, somatic antigens) to the overall immunogenicity of the parasite—in terms of antibody binding, antibody-mediated cellular cytotoxicity and immunization.

### Acknowledgements

The authors would like to acknowledge the intellectual input of Professors Derek Wakelin and Chris Arme to this work, and the photographic expertise of Mr Brian Case. The work was supported by a grant from the Medical Research Council. CC was supported by a post-doctoral assistantship from the MRC.

### References

- BACHMAYER H. (1975) Selective solubilisation of haemagglutinin and neuroaminidase from influenza viruses. *Intervirology* **5**, 260
- BEHNKE J.M. & PARISH H.A. (1979) Expulsion of *Nematospiroides dubius* from the intestine of mice treated with immune serum. *Parasite Immunology* **1**, 13
- FRAKER P.J. & SPECK J.C. (1978) Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1, 3, 4, b-tetrachloro-3a, 6a-diphenyl glycoluril. *Biochemical and Biophysical Research Communications* **80**, 849
- GRZYCH J.M., CAPRON M., BAZIN H. & CAPRON A. (1982) *In vitro* and *in vivo* effector functions of rat IgG2a monoclonal anti-*S. mansoni* antibodies. *Journal of Immunology* **129**, 2326
- HELENIUS A. & SIMONS K. (1975) Solubilisation of membranes by detergents. *Biochimica et Biophysica Acta* **415**, 29
- HIMMELHOCH S. & ZUCKERMAN B.M. (1983) *Caenorhabditis elegans*: Characters of negatively charged groups on the cuticle and intestine. *Experimental Parasitology* **55**, 299
- INGLIS W.G. (1983) The design of the nematode body wall: the ontogeny of the cuticle. *Australian Journal of Zoology* **31**, 705
- LAVER W.G. & WEBSTER R.G. (1976) Preparation and immunogenicity of a purified influenza virus haemagglutinin and neuraminidase subunit vaccine. *Postgraduate Medical Journal* **52**, 373
- LEE D.L. (1977) The nematode epidermis and collagenous cuticle, its formation and ecdysis. *Symposium of the Zoological Society of London* **39**, 145
- LUMSDEN R.D. (1975) Surface ultrastructure and cytochemistry of parasitic helminths. *Experimental Parasitology* **37**, 267
- MURRELL K.D. & GRAHAM C.E. (1983) *Strongyloides ratti* and *Trichinella spiralis*: Net charge of epicuticle. *Experimental Parasitology* **55**, 331
- ORTEGA-PIERRES G., MACKENZIE C.D. & PARKHOUSE R.M.E. (1984) Protection against *Trichinella spiralis* induced by a monoclonal antibody that promotes killing of newborn larvae by granulocytes. *Parasite Immunology* **6**, 275
- PHILIPP M., PARKHOUSE R.M.E. & OGILVIE B.M. (1980) Changing proteins on the surface of a parasitic nematode. *Nature* **287**, 538
- PHILIPP M. & RUMJANECK F.D. (1984) Antigenic and dynamic properties of helminth surface structures. *Molecular and Biochemical Parasitology* **10**, 245
- PRITCHARD D.I., MAIZELS R.M., BEHNKE J.M. & APPLEBY P. (1984) Stage-specific antigens of *Nematospiroides dubius*. *Immunology* **52**, 325.
- PRITCHARD D.I., WILLIAMS D.J.L., BEHNKE J.M. & LEE T.D.G. (1983) The role of IgG<sub>1</sub> hypergammaglobulinaemia in immunity to the gastrointestinal nematode *Nematospiroides*

- dubius*. The immunochemical purification, antigen specificity and *in vivo* anti-parasite effect of IgG<sub>1</sub> from immune serum. *Immunology* **49**, 353
- SMITH H.V., QUINN R., KUSEL J.R. & GIRDWOOD R.W.A. (1981) The effect of temperature and antimetabolites on antibody binding to the outer surface of second stage *Toxocaria canis* larvae. *Molecular and Biochemical Parasitology* **4**, 183
- TAVARES C.A.P., DE ROSSI R., PAYARES G., SIMPSON A.J.G., McLAREN D.J. & SMITHERS S.R. (1983) A monoclonal antibody raised against adult *Schistosoma mansoni* which recognizes a surface antigen on Schistosomula. *Zeitschrift fuer un Parasitenkunde* **70**, 189
- VETTER J.C.M. & KLAVER-WESSELING J.C.H. (1978) IgG antibody binding to the outer surface of infective larvae of *Ancylostoma caninum*. *Zeitschrift fuer Parasitenkunde* **58**, 91