Restricted sets of parasite antigens from the surface of different stages and sexes of the nematode parasite *Nippostrongylus brasiliensis*

R. M. MAIZELS, MUNIRA MEGHJI & BRIDGET M. OGILVIE* *Division of Parasitology, National Institute for Medical Research, Mill Hill, London*

Summary. Surface molecules of parasitic stages of the nematode *Nippostrongylus brasiliensis* can be readily iodinated by the chloramine T technique, and assessed for antigenic reactivity with humoral antibody from infected animals. Free-living infective larvae are less amenable to analysis by this, or similar methods, but within 18 hr of larvae entering the host, new macromolecular surface antigens can be detected. The parasites change their surface antigens twice more in the course of their maturation to the adult stage. Surface antigens are stage-specific: lung larvae (L3), intestinal larvae (L4) and gut-living adults each possess characteristic sets of cuticular molecules. Single stage infections result in antibody reactive only to the antigens from the homologous stage. The adult surface appears to bear the greatest number of antigens, one of which is found only on the male worm. The composition of these antigens does not differ grossly between adult worms from a naive or immune host, or worms established after the adaptation of a 'trickle' (multiple low dose) infection. There appears to be an interesting contrast between the rapidity and extent of changes in surface antigens in the early phases of infection, and the stability of adult antigens analysed at different points in the host immune response.

* Present address: The Wellcome Trust, 1 Park Square West, London NW1 4LJ.
    Correspondence: Dr R. M. Maizels, National Institute for Medical Research, Mill Hill, London NW7 1AA.

INTRODUCTION

In the investigation of the immune response to parasitic organisms, the definition of discrete parasite antigens has become an essential step towards understanding and manipulating the interaction between the parasite and its host. The importance of the surface of helminth parasites in immune recognition (Soulsby, 1963; Ogilvie, Philipp, Jungery, Maizels, Worms & Parkhouse, 1980) has been emphasized by the fact that anti-surface antibody will mediate leucocyte adherence and cell-mediated killing of both nematodes (Mackenzie, Preston & Ogilvie, 1978) and trematodes (Butterworth, Sturrock, Houba, Mahmoud, Sher & Rees, 1975). Recently, techniques have been developed which permit the selective radiolabelling of nematode surface proteins, using *Trichinella spiralis* as a model (Philipp, Parkhouse & Ogilvie, 1980; Parkhouse, Philipp & Ogilvie, 1981). These studies produced a striking result; the few protein components which are to be found on the cuticle of *T. spiralis* are strictly specific for a single stage of the parasite life cycle (Philipp, Taylor, Parkhouse & Ogilvie, 1981), as predicted from cell adherence studies (Mackenzie *et al.*, 1978).

Nippostrongylus brasiliensis is widely used in model systems for nematode infections, and the course of its infection in the rat and the immune response to it is well documented (Yokogawa, 1922; Mulligan, Urquhart, Jennings & Neilson, 1965; Jarrett, Jarrett & Urquhart, 1968; Ogilvie & Jones, 1971; Keller & Keist,

1972; Ogilvie & Love, 1974). Further definition has been hampered by the lack of knowledge of the critical antigens and the need to resort to crude somatic extracts. With the surface labelling techniques of Philipp *et al.* (1980) we can now identify cuticular molecules recognized by the humoral arm of the host's immune system (Maizels, Philipp & Ogilvie, 1982).

Nippostrongylus brasiliensis enters the host percutaneously as a third stage larva (L3), migrates to the lungs where it moults to an L4 stage before travelling through the trachea, oesophagus and stomach to the small intestine. There the larva undergoes a final moult to the adult stage, this taking place in the rat on the fourth or fifth day of infection (Yokogawa, 1922). In an experimental infection of $1–5 \times 10^3$ larvae/rat, most of the adult worms are expelled from the gut between day 12 and day 16 post-infection, and nearly all are eliminated by day 20 (Jarrett *et al.*, 1968). Once the parasites are cleared, the animal retains a powerful degree of immunological memory, transferable by immune cells (Kelly & Dineen, 1972; Ogilvie & Love, 1974; Nawa & Miller, 1978), and serum (Ogilvie & Jones, 1968; Miller, 1980). This memory manifests itself by a more rapid expulsion in subsequent infections commencing around day 7 of reinfection (Ogilvie & Love, 1974).

Evidence from experiments in which worms are transplanted between hosts differing in immune status have shown that the host response is predominantly against the adult stage of the parasite (Ogilvie, 1965; Jones & Ogilvie, 1971). Such techniques have also demonstrated a difference between a single infection of say, 2000 L3/rat, and an infection more closely approaching natural conditions, that is 50 L3/rat per day. In the latter 'trickle' infections, the parasite adapts to its host and a persistent infection of relatively non-immunogenic worms results (Jenkins & Phillipson, 1972; Jenkins, 1974).

The characterization of antigens from the surface of this parasite should facilitate the analysis of its reaction with the host at a finer level. In particular we are seeking to identify key protective antigens which lead to worm expulsion under standard experimental conditions, although these may well include secreted molecules not represented on the parasite surface. In carrying out such an analysis by the surface labelling techniques of Philipp *et al.* (1980), we shall also approach the question of whether these authors' finding of a remarkable developmental restriction and sequence of *T. spiralis* surface antigens is a generalized feature of nematode parasites.

MATERIALS AND METHODS

Parasites and hosts

N. brasiliensis was maintained in outbred Sprague-Dawley (SD) rats and the life cycle completed as described previously (Ogilvie, 1965). Worms for experimental analysis, recovered by Baermann isolation, were exhaustively washed (10 times) before use. SD rats and CBA/Ca mice were obtained from the SPF colony at NIMR. Rats were routinely infected with 5000 larvae raised by culture of egg-containing faeces. 'Trickle' infections (Jenkins & Phillipson, 1972) consisted of twenty injections of fifty L3 spread over a 4 week period. In transfer experiments, 2000 viable adults recovered from the gut of 8-day infected rats were injected into the duodenum of a naive rat under anaesthesia. Immunization with irradiated larvae employed 5000 L3 (irradiated with 150 krad from a ^{60}Co source) per injection.

Radiolabelling of surface proteins

The procedure of Parkhouse *et al.* (1981) was adopted. Briefly, worms in 100 μl–1 ml of phosphate-buffered saline (PBS) were incubated with 0·2–1 mCi Na ^{125}I (Amersham International IMS 30) and 50 μg/ml chloramine T for 5 min at room temperature. Excess iodine was consumed by the addition of 10–50 μl of a saturated tyrosine solution, and 10 ml of 10mM Tris buffer pH 8·3. containing protease inhibitors were added. Protease inhibitors were: L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK, Sigma 50 μg/ml), N-α-p-tosyl-L-lysine chloromethyl ketone HCl (TLCK, Sigma 25 μg/ml), and phenylmethylsulphonyl fluoride (PMSF, Sigma, 174 μg/ml). Worms were washed repeatedly in this buffer, by sedimentation at 1 g for adults, and gentle centrifugation (0·5–3 min at 100–400 g) for the larvae at different stages.

Labelling with the Bolton-Hunter reagent (Bolton & Hunter, 1973) was carried out as described by Parkhouse and co-workers. Worms were incubated for 10 min in 0·1 M borate buffer pH 8·3 with 0·1–0·2 mCi of ^{125}I Bolton-Hunter reagent (Amersham International IMS 861) before the addition of glycine to a final level of 0·2 M, and washed as described above.

Solubilization of radiolabelled proteins

Surface labelled proteins were solubilized by the procedure described by Parkhouse *et al.* (1981). To 0·5 ml or 1·0 ml of labelled worms in 10 mM Tris buffer, was added deoxycholate (Merck) solution to a final concentration of 1%. A manual glass homogenizer was

used to disrupt the worm suspension before centrifugation (10,000 g, 30 min, 4°) to recover the soluble components.

The fractions not soluble in detergent-containing buffers were subjected to a harsh extraction procedure. Material pelleted at 10,000 g for 30 min was resuspended in 10% SDS, 2% 2ME for 30 min at 100° with occasional vortex agitation. A further centrifugation (10,000 g, 30 min 4°) yielded a second soluble fraction; material still insoluble was not subjected to further analysis.

Release of radiolabelled protein in vitro

Radiolabelled worms were incubated in PBS or 10 mM Tris buffers, with or without detergent, for up to 24 hr at 37°. Supernatants from worm cultures were centrifuged (10,000 g 30 min 4°) before storage, and cultured worms washed three–four times in fresh buffer before homogenization as described above.

Immunoprecipitation analysis

Serial bleeds from the tail vein of rats and mice provided serum samples at different time points in experimental infection. Immunoprecipitation was then carried out: to 100 μl of 10 mM Tris buffer pH 8·3 was added 2–5 μl of ^{125}I-surface labelled antigen (10^4–10^5 c.p.m.) and a 5 μl sample of test serum. Incubation for 16 hr at 4° or 3 hr at 37° was followed by the addition of 50 μl of rabbit anti-rat immunoglobulin serum (a gift from R. de Rossi), and further incubation for 1–3 hr at 37°. The precipitated antigen–antibody complexes were washed three times in cold PBS, transferred into fresh tubes, and radioactivity in the washed precipitate measured.

Polyacrylamide gel electrophoresis

SDS-PAGE was carried out on a slab gel modification of Laemmli's original procedure (Laemmli, 1970). Samples for analysis were denatured in 6 mM Tris buffer containing 10 mM EDTA 2% SDS, 10% glycerol, for 5 min at 100° with 2% mercaptoethanol when reducing conditions were desired. Gels (8 cm × 8 cm) of 4·5%, 7·5% or 15% acrylamide were electrophoresed on a Pharmacia GE-4 apparatus following the manufacturer's instructions. Fixed, dried gels were autoradiographed on flashed Fuji film exposed at −70°, using where necessary Ilford fast tungstate intensifying screens. Standard molecular weight markers used were: α-lactalbumin (mol. wt 14,400), soybean trypsin inhibitor (mol. wt 20,100), carbonic anhydrase (mol. wt 30,000), ovalbumin (mol. wt 43,000), bovine serum albumin. (mol. wt 67,000) and phosphorylase b (mol. wt 94,000), purchased from Pharmacia. These marker proteins were used to calibrate a slope from which the apparent molecular weights of labelled parasite molecules could be estimated. It should be appreciated that molecular weight estimates of 200,000 or above obtained from extrapolation from this slope are extremely inaccurate and can only serve as a relative guide.

RESULTS

Surface labelling of infective larvae

The L3 (infective larvae) of *N. brasiliensis* represent the free-living stage which infects the rat and thereby comes into contact with the host immune system. Although these larvae proved amenable to radiolabelling by the chloramine T technique, the results do not show the reproducibility characteristic of most other nematode species, or of other stages of this species. Figure 1 presents the PAGE profiles from three such preparations showing some typical gel profiles. In fifteen experiments, bands of 28,000, 41,000 and 65,000 mol. wt were most frequently represented, and a 16,000 component was ubiquitous. This variation was not related to the age of larval culture (which varied between 6 and 21 days), nor to the use of conventional charcoal cultures or axenic filter paper culture conditions. Furthermore, the routine use of selected protease inhibitors in all worm extractions reduced the probability of different degrees of degradation occurring in these experiments.

Another contrast between *N. brasiliensis* L3 and most other nematodes studied with these techniques is that detergent-mediated extraction is relatively inefficient. Typically, only 33% of the surface labelled ^{125}I can be solubilized from *N. brasiliensis* L3 in either DOC or Triton X-100, and this yield is reduced by half if no detergent at all is used. Incubation in detergent for 24 hr failed to increase solubilization. Attempts to solubilize the remaining radioactivity with SDS and 2-mercaptoethanol with or without 8 M urea, did not produce any discrete bands other than those identified previously.

This pattern of variable radiolabelling of L3 molecules is accompanied by results, included in Table 1, which demonstrate the weak and inconsistent reactivity of this material with immune serum antibody. From some preparations, the 16,000 and 41,000 mol. wt bands can be specifically immunoprecipitated (Fig.
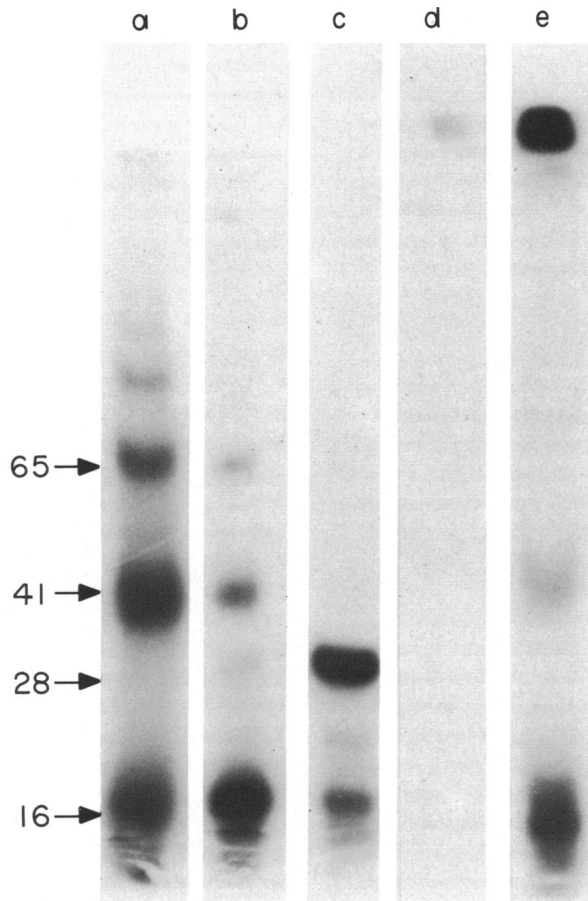
R. M. Maizels, Munira Meghji & Bridget M. Ogilvie

Figure 1. Surface of macromolecules of free-living infective larvae of *N. brasiliensis* analysed by SDS-PAGE. Molecular weight analysis of chloramine-T-mediated surface radioiodinated molecules from infective larvae of *N. brasiliensis*. (a, b and c) Three different preparations run on 7·5% SDS-PAGE; the apparent molecular weights of the major bands, indicated by each arrow, were estimated by the relative positions of marker proteins at 14,400, 20,100, 30,000, 43,000, 67,000 and 94,000. The patterns of molecules immunoprecipitated from surface labelled preparations following incubation with normal rat serum (d) or day 7 secondary immune rat serum (e) are also shown.

1). However, using a cell adherence assay to measure anti-surface antibody, Mackenzie and co-workers (Mackenzie, Jungery, Taylor & Ogilivie, 1980) have shown that the L3 surface is consistently antigenic, even with sera taken shortly (day 8) after primary infection. Two other labelling procedures were therefore used in an effort to identify any surface antigens not possessing the exposed tyrosine residue(s) necessary for chloramine T-mediated labelling. The Bolton and Hunter reagent (Bolton & Hunter, 1973) yielded a very low level of activity, although the 16,000 and 65,000 bands could be detected. Sodium borotritide (Gahmberg & Hakomori, 1973) labelling following neuraminidase and glucose oxidase treatment was not successful in detecting glycosylated macromolecular surface components. One explanation of these results may be that the L3 antigen is an insoluble constituent of the cuticular surface and our techniques are failing to isolate it; alternatively, in the particular instance of *N. brasiliensis* L3, the surface molecules most relevant in immune recognition may be of relatively low molecular weight (for example, glycolipids).

Antigens from larval parasitic stages

Two phases in the development of parasitic larvae may

Table 1. Immunoprecipitation of ^{125}I surface-labelled antigens from four stages in the life cycle of *N. brasiliensis* by serum from normal and infected rats

Source of antigen	Normal rat serum (%)	Day 7, 2° immune serum (%)	Day 94, 2° immune serum (%)
In vitro L3	5.2 ± 4.5	10.8 ± 10.4	6.7 ± 5.3
Lung L3/L4	3.1 ± 2.4	23.7 ± 4.0	23.2 ± 1.7
Gut L4	5.7 ± 1.7	17.8 ± 2.4	ND
Adult	4.3 ± 2.1	25.7 ± 8.3	21.8 ± 4.2

Results presented summarize data from at least three experiments with each stage carried out as detailed in Materials and Methods, excluding results from the experiments presented in Table 2 and Figs 7 and 8. Data are calculated as percent of TCA-precipitable input c.p.m. per assay tube.

readily be recovered from the host. Baermannization of the lung between 24–48 hr yields parasitic L3, and from 48 hr onwards, fourth stage larvae are found in increasing numbers in the gut. Surface labelling of lung larvae, harvested soon after their arrival at 26 hr, gives a clear and reproducible PAGE pattern, as shown in Fig. 2a. Interestingly, four of these major bands, 16,000, 28,000, 41,000 and 65,000 are of similar molecular weight to those often associated with the infective larvae described above. However, there are two additional bands, a minor one of 35,000 and a major band with an apparent molecular weight of 180,000. Furthermore, if analysis is carried out under non-reducing conditions a major 220,000 mol. wt component is detected (not shown).

When lung worms are harvested 20 hr later (at 46 hr) they are, in our host-parasite combination, in the course of the third moult. However, the profile of surface labelled proteins corresponds exactly to that of the newly arrived larvae (Fig. 2). We conclude therefore that lung larvae are still L3, which, once they have entered their tissue-dwelling phase, acquire fresh surface components (predominantly those of 180,000 and 220,000 mol. wt) compared with their free-living predecessors.

In complete contrast to these lung stage worms, gut L4 possess a new series of surface molecules (Fig. 2) whether examined at 46 hr or 76 hr. Analysis on longer polyacrylamide gels reveals two components of approximately 300,000 mol. wt, 20–30,000 apart. Other bands are seen at 75, 100 and 110,000, but none is in common with the earlier larval stage. This complete renewal is thus the second change in surface

composition which the parasite is able to undergo within 48 hr of entering the host.

These major surface components are all antigenic in that they are immunoprecipitated by serum from infected animals (Table 1, Fig. 2). It is particularly interesting that antigens that are evidently so transient in their expression (e.g. the lung larval 180,000 antigen) stimulate high and persistent levels of antibody.

Adult *N. brasiliensis* surface antigen

The greatest number of labelled surface molecules is found in analysis of the adult worms. Figure 3a shows the seven major components revealed by chloramine T labelling (at 70,000, 75,000, 90,000, 115,000, 120,000 and approximately 250,000 and 300,000 mol. wt), two of which (115,000 and 120,000) are susceptible to degradation by 2 ME (Fig. 3b). In addition, less prominent bands of 20,000, 45,000 and 55,000 mol. wt may be detected. A 150,000 mol. wt component is particularly heavily labelled by the Bolton-Hunter reagent, and the differences between the two techniques emphasize the quantitative bias which may be introduced by the use of a single labelling procedure.

N. brasiliensis adults possess a substantial quantity of haemoglobin within the fluid-filled matrix layer of the cuticle (Lee, 1969). In none of the preparations of surface labelled adult worms has radiolabel been detected in the region of 17,000 mol. wt, the reported molecular weight of *N. brasiliensis* haemoglobulin subunits (Sharp & Lee, 1981). This would imply that if the label does penetrate the outermost membrane of
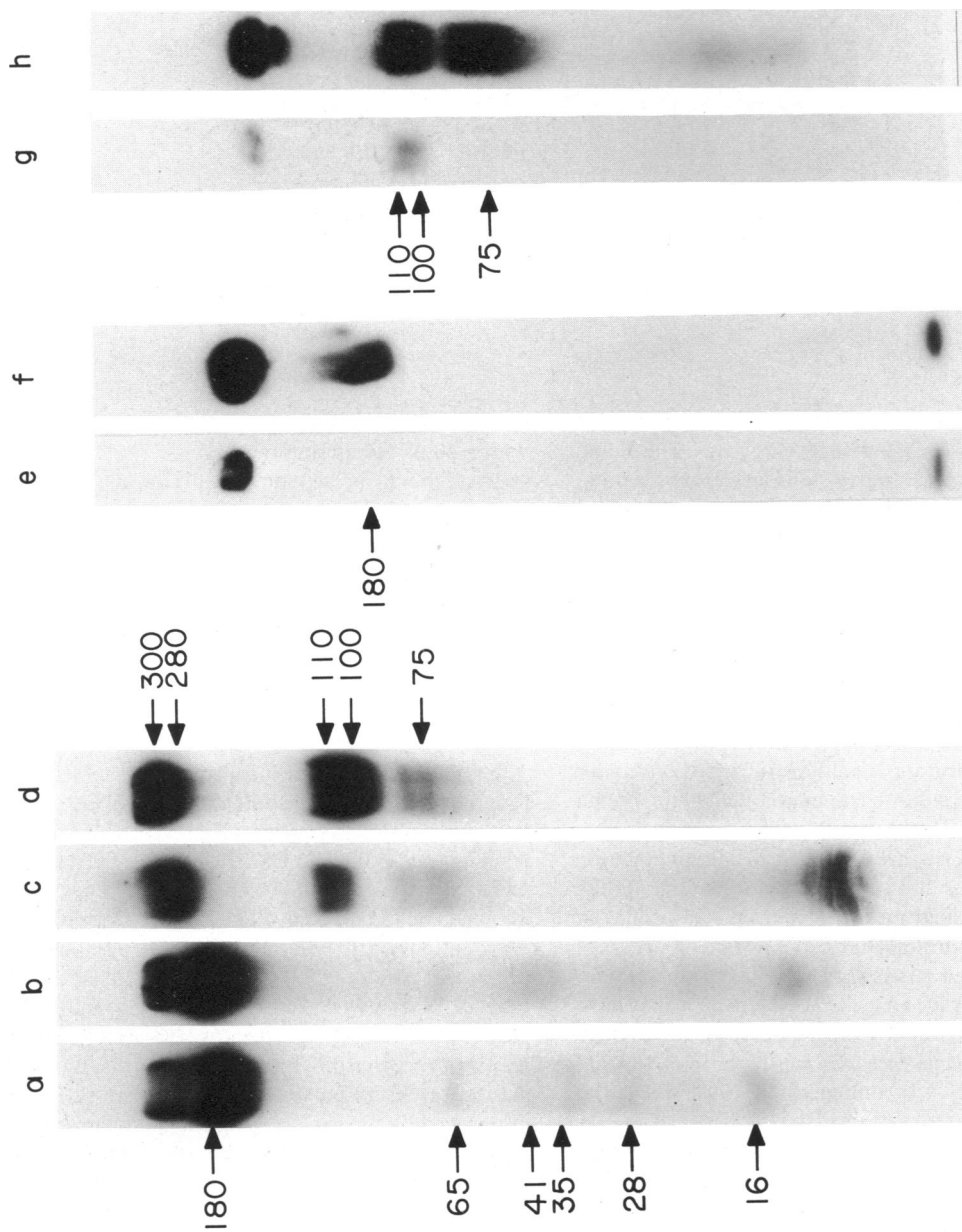
Figure 2. Profile of surface-labelled antigens from parasitic stages of *N. brasiliensis* larvae analysed by SDS-PAGE. Chloramine T-mediated surface radioiodination from four phases of parasitic larvae were analysed. Lung larvae at 26 hr (a) or 46 hr (b) post-infection are compared with intestinal larvae also analysed at 46 hr (c), or at 70 hr (d). Molecular weights, indicated with arrows, were calculated from reference markers. The antigenic nature of these surface molecules was analysed by immunoprecipitation, comparing normal (e) or immune (f) rat serum from binding lung larval antigen, and with the same sera for intestinal larval antigen (g and h).
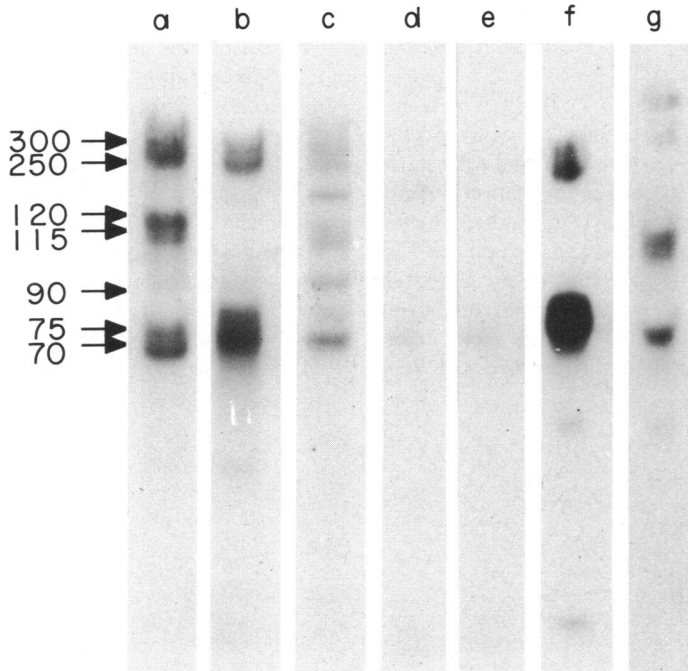
Figure 3. Surface components of adult *N. brasiliensis* worms. SDS-PAGE analysis of adult worm surface labelled antigens, run under non-reducing (a), or reducing (b) conditions. A comparison of surface-extracted molecules may be made with those released overnight in culture (c). Immunoprecipitated antigens bound by normal rat serum (d), rat anti-*T. spiralis* serum (e), or serum from a rat immune to *N. brasiliensis* (f) are all analysed under reducing conditions, or, in the case of homologous immune serum, under non-reducing conditions (g).

the cuticle in this instance, it certainly goes no deeper than the boundary of the cortex, about 150 nm below the surface (Lee, 1969).

The surface labelled adult preparations are very different from those of infective larvae in several respects. Most of the radioactivity (typically 75%) can be recovered from adult worms in DOC, and indeed, a PBS extraction is equally efficient (data not shown). The solubilized material has a higher content of TCA-precipitable material (60·9% *v* 45·5% for L3) and acts as a very sensitive antigen by immunoprecipitation (Table 1). There is some similarity between the adult profile and that of the gut L4, with both stages bearing molecules of 75,000 and 300,000 mol. wt. Experiments described below, however, indicate that these are probably not antigenically related and that *N. brasiliensis* undergoes another complete antigenic change at the time of its final moult.

The finding that adult surface molecules are soluble in detergent-free PBS may be reflected in the fact that these molecules are released into culture medium following labelling (Fig. 3c). This indicates that antigenic preparations made in this way from unlabelled worms should include these molecules (Wilson, 1967; Denham, 1969; Burt & Ogilvie, 1975; Poulain, Pery & Luffau, 1976b; Day, Howard, Prowse, Chapman & Mitchell, 1979; Petit, Pery & Luffau, 1980), although the PBS extract certainly contains many more besides (Ambler & Orr, 1972; Murray, Robinson, Grierson & Crawford, 1979). Wilson (1967) and Petit *et al.* (1980) reported an allergenic 12–15,000 mol. wt component in the secretions of adult worms, but this antigen does not appear to be labelled on the surface before its release.

The surface labelled molecules are antigenic to the infected host. Figure 3d, 3e and 3f compares the material bound by serum from normal rats (d), and rats infected with *T. spiralis* (e) and *N. brasiliensis* (f). Analysed under reducing conditions, and compared with Fig. 3(b) it is apparent that the 70,000 and 75,000 bands in particular, as well as the 250,000 and 300,000 components, are specifically precipitated only by

homologously infected serum. In the absence of 2 ME the 115,000 and 120,000 bands are also detected (Fig. 3g).

Similar immunoprecipitation patterns are seen if immune serum is added to labelled worms before extraction, and worms are incubated and washed free of unbound antibody before solubilization. Precipitation of the antibody will yield only those surface molecules accessible to antibody binding on the living worm. The results of this experiment, showing that the antigens described above are immunologically exposed on the surface, confirm data from experiments in which immune sera are absorbed with living

worms. Such absorption reduces the titre of reactivity with surface labelled preparations by 75%, and immunoprecipitation of all bands are proportionately reduced.

A male-specific surface molecule

The 90,000 component proved particularly interesting in that it is found only on male adult worms (Fig. 4). A comparison of parallel immunoprecipitates from male and female adults indicates that this protein is also recognized by the host and specifically bound by antibody. No other species of nematode has yet been
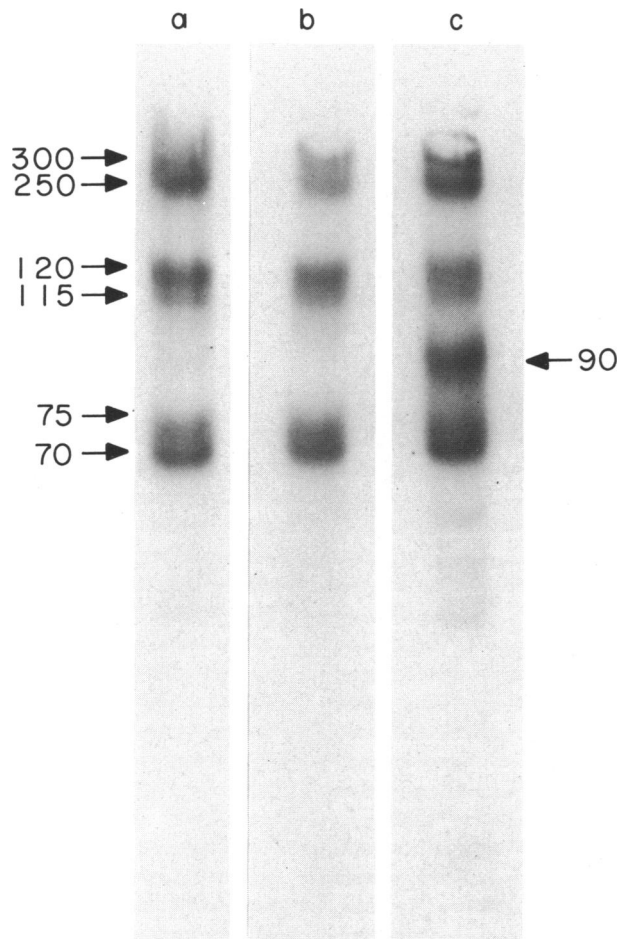

Figure 4. Male adult worms possess a sex-specific surface component of 90,000 mol. wt. Adult worms unseparated according to sex were radiolabelled and soluble components analysed by SDS-PAGE (a). When adults were divided into male and female worms and labelled separately, gel analysis shows that female worms do not possess the 90,000 mol. wt. components which is well represented on the surface of male worms (c).

reported to possess sex-restricted antigens in this manner.

A summary of the major surface antigens described in the preceding sections is presented in Fig. 5. This presents data from three larval phases and both adult sexes, in a diagrammatic form, derived from the results given in Figs 1–4.

Stability of adult antigenic profile under different conditions of immunity

One of the fascinating aspects of *N. brasiliensis* infection is the ability of the parasite to adapt to host immunity, although except for 'trickle' infections, such adaptation does not prevent expulsion (Jenkins & Phillipson, 1972; Jenkins, 1972, 1974; Ogilvie & Love, 1974; Ogilvie, 1974). Having characterized the major adult surface antigens we then inquired into the constancy or variability of those antigens from worms recovered at different stages of an infection in hosts of different immune status. As well as 'immune adapted' worms taken early in a secondary infection (Ogilvie, 1969), we examined 'antibody-damaged' worms taken
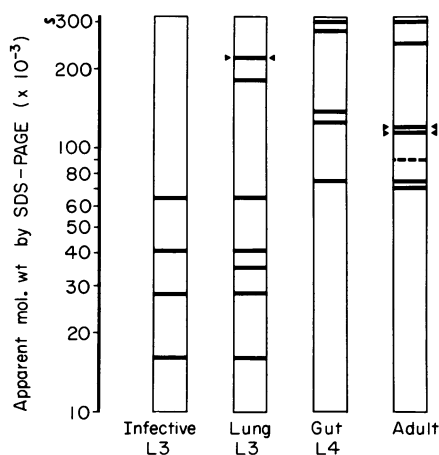
Figure 5. Diagrammatic summary of the major surface antigens of larval and adult *N. brasiliensis*. Data from a total of fifteen separate experiments with infective larvae, six with lung and five with gut larvae, and eighteen with adult worms, are summarized in this diagram. The apparent molecular weight ($\times 10^{-3}$), notwithstanding the great degree of error in estimating sizes over 200,000, are infective larvae—16, 28, 41 and 65; lung L3—16, 28, 35, 41, 65, 180 and 220; intestinal L4—75, 100, 110, 280 and 300; and adult—70, 75, 115, 120, 250 and 300. In addition adult males bear a 90,000 component (– – –). Three components are lost on reduction (▶ ◀), the lung stage 220,000 band and the adult doublet of 115–120,000 mol. wt.

later in an infection of naive rats (Ogilvie & Hockley, 1968). 'Trickle'-adapted worms were recovered at different points in an established long term infection for comparison. Finally, adult worms from a murine infection were examined in view of the more rapid effect of immunity in this host (Jacobson & Reed, 1974). The results, presented in Fig. 6, show that at the simplest level of comparison, by molecular weight, there is no substantial alteration in surface profiles under any of these conditions. Some minor variations are seen, in particular with respect to a band of 150,000 mol. wt, but all of these small differences are of the order seen between preparations of 8 day primary infection worms.

Time course of humoral response to surface antigens

Surface labelled molecules can readily be used to assay levels of humoral anti-parasite antibody (Philipp *et al.*, 1981), and the course of an infection thereby followed. Figure 7 presents the time course of anti-surface antibodies in infected rats, expressed in terms of radiolabelled antigen bound by serum samples taken at different points in time. This assay is more sensitive than those based on somatic extracts (Poulain, Luffau & Pery, 1976; Sinski & Holmes, 1977) because high levels of antigen binding are detected as early as day 10 with surface labelled preparations. Antibody to larval parasites follows similar kinetics, although poorer specific precipitation ratios make these tests less sensitive. As shown in Table 1, humoral levels of anti-larval antibody are also very persistent despite the brief duration of presentation of larval antigens to the host immune system.

All adult surface antigens appear to be recognized simultaneously by the immune system, in that all bands are immunoprecipitated by sera taken from rats infected from 10 to 94 days previously. Differential responsiveness to separate surface antigens has however, been reported in *T. spiralis* infections of the mouse (Jungery & Ogilvie, 1981). These experiments measured total antibody activity regardless of class or subclass. It is interesting to note however that the IgE response to *N. brasiliensis* follows different kinetics (Jarrett, Haig & Bazin, 1976); this isotype peaks later at day 34, decays markedly thereafter, and is strongly anamnestic.

Stage-specificity demonstrated by single stage infection

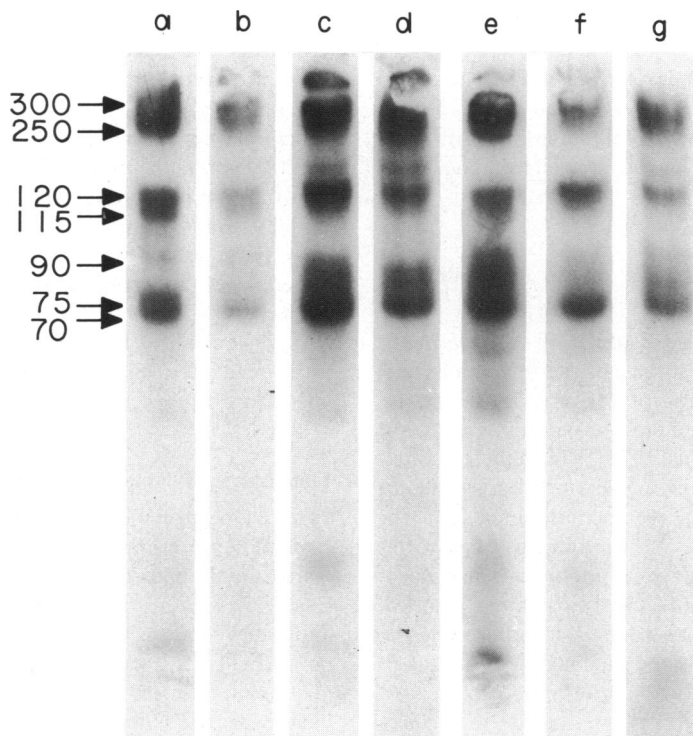Although the extensive diversity of surface antigens

Figure 6. Comparison of surface components of adult worms after infections of different status. Adult worms taken 8 days after primary infection (a) are compared with 'antibody damaged' parasites at day 11 (b) and day 14 (c), or 'immune-adapted' worms 7 days after a challenge infection (d). 'Trickle-adapted' parasites were analysed 3 days after the final infection, i.e. 28 days following initial infection (e) and 15 days later, i.e. 43 days following initial infection (f). Adult worms from a murine infection were also examined (g).

between the stages described above is strong evidence of antigenic stage specificity in *N. brasiliensis*, some question remained whether gut L4 and adult worms shared any antigenic determinants. In addition to the two surface bands of similar molecular weight, the rapidity of the antibody response to adult antigens (Fig. 7) could be interpreted as a reflection of antigenic sharing with earlier stages.

This question was examined by infecting rats with mature adult worms by an intraduodenal transfer (Ogilvie, 1965). Sera from these rats were compared with sera from rats undergoing the full course of infection in tests against antigens from the four life cycle phases under study. Table 2 summarises a series of experiments at one time point, showing anti-adult activity comparable with a secondary immune serum, but no significant reactivity to any of the larval antigens in sera from rats infected with adults alone. Later bleeds continued to show only anti-adult acti-

vity, and a further comparison of sera taken over the course of three months following transfer shows that this mode of infection stimulates high levels of stage-specific antibody (Fig. 8).

A second approach generated a single stage infection by immunizing rats with heavily irradiated larvae. Our preliminary experiments were in concordance with the reports of Jennings, Mulligan & Urquhart (1963) and Prochiazka & Mulligan (1965) that larvae irradiated with 150 krad or more failed to develop into adult worms. Accordingly, rats subjected to two 'infections' of 5000 irradiated (150 krad) larvae were bled and tested for reactivity to larval and adult antigen. Seven days after challenge, antibody to L3 antigen was detected but no significant reactivity towards adult antigen was found (see Fig. 8). Following subsequent challenge, anti-adult antibody levels began to rise, although sera remained predominantly reactive to L3 antigens. However, this may well reflect
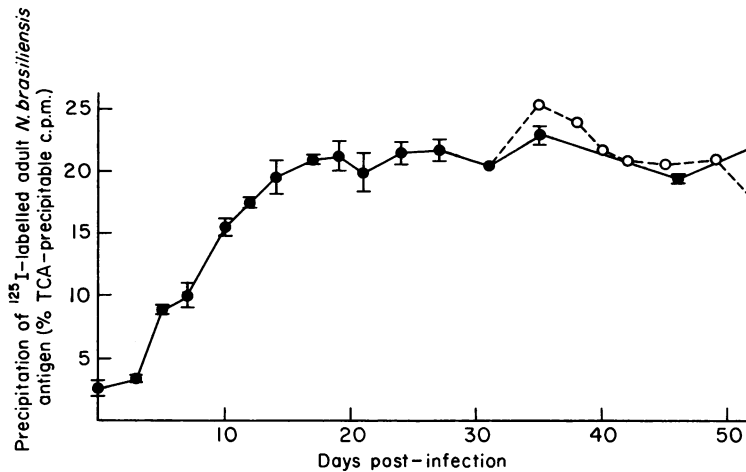
Figure 7. Time course of the humoral immune response to *N. brasiliensis* adult surface antigens. Immunoprecipitation analysis c. serial serum samples from *N. brasiliensis* infected S/D rats was carried out in radiolabelled adult antigen over the course of a primary infection (—●—) or following a secondary challenge (– –O– –).

Table 2. Serum from rats receiving adult worms does not react with surface antigens from earlier stages

	Normal rabbit serum	Transferred (adults only) serum	Full course infection serum	Ratio: transferred/ Normal	Ratio: full course/ Normal
Infective L3 ^{125}I-Ag	1·1±0·1	2·1±0·4	3·7±0·2	1·9	3·4
Lung L3 ^{125}I-Ag	5·0±2·2	6·3±2·9	21·1±5·7	1·3	4·2
Gut L4 ^{125}I-Ag	9·6±5·5	9·1±4·6	36·0±15·4	0·9	3·7
Adult ^{125}I-Ag	3·9±2·0	28·2±5·3	32·5±11·8	7·2	8·3

Data presented are the precentage of TCA-precipitable input c.p.m. precipitated from each antigen by each serum sample. Results summarize two (infective L3), four (lung and gut larvae), or five (adult) immunoprecipitation experiments, excluding that presented in Fig. 8.

the development of a few worms to the adult stage in later infections.

DISCUSSION

The definition of major antigens is particularly important for a parasite such as *N. brasiliensis*, which has been the subject of intensive immunological studies. In approaching this question we have been particularly interested in two aspects of the parasite's antigenic character: stage-specificity, and adaptive variation in response to host immunity. In the course of these

studies we have also observed a number of other facets of *N. brasiliensis* antigens which may have a bearing on this intricate host-parasite relationship.

We should first discuss the evidence that our techniques satisfy our approach of selectively labelling only molecules which are exposed on the cuticular surface. Identical methods applied to *T. spiralis* have been shown by autoradiography to label only surface molecules (Parkhouse *et al.*, 1981), and confirmatory results have been obtained with *Litomosoides carinii* (Philipp, Worms & McLaren, unpublished). A formal demonstration that the antigens described here are all located on the surface await similar autoradiographic
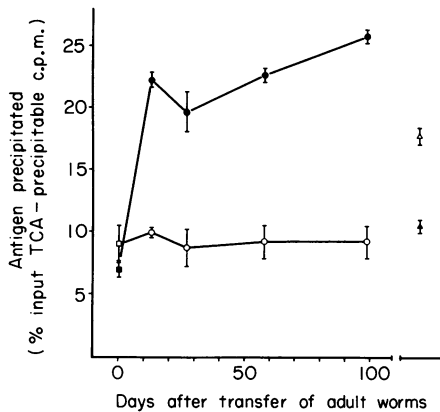
Figure 8. Stage-specificity of the humoral immune response. Immunoprecipitation analyses were carried out on sera from single stage infection using antigens from adult (solid symbols) or infective larval (open symbols) surface labelled preparations. The level of reactivity of normal rat serum to adult antigen (■) and L3 antigen (□) are plotted at day 0. The humoral response of rats following intraduodenal transfer of adult worms has been measured against adult antigen (●) and L3 antigen (○). Finally, serum from rats 'infected' twice with heavily irradiated (150 Krad) larvae has been analysed for reactivity to adult (▲) and L3 (△) antigens. Values given are percentages of initial TCA-precipitate c.p.m. in each assay tube, being the mean and standard deviation from duplicate determinations.

analysis. However, the analogy with evidence from other species is strengthened by the following results: (i) absorption of antisera with living worms reduces binding to surface labelled antigens. (ii) Haemoglobin, located within the cuticle but beneath the surface, is not labelled. (iii) Iodination of moulting worms produces labelled molecules characteristic only of the exterior cuticle. (iv) Surface labelled antigens can be immunoprecipitated by allowing antibody to bind to living adult worms before extraction and coprecipitation. In addition, as argued by Parkhouse et al. (1981) in the case of T. spiralis, the paucity of molecules labelled and their restriction to a single stage of the life cycle would not be consistent with an extensively penetrating radiolabel.

The converse reservation, that not all surface antigens have been detected, is probably more pertinent. We have strong grounds for concluding that these techniques do not reveal the major surface antigen(s) of infective larvae which are the target of powerful antibody-mediated leucocyte adherence (Mackenzie et al., 1980). Low molecular weight antigens, polysaccharides or proteins with no avail-

able tyrosine residues will have been omitted from our analysis.

For the three stages of the life cycle we have examined, surface labelled molecules are stage-specific. In general this is clear from the molecular weight distribution of solubilized molecules on polyacrylamide gels, and from the differential susceptibility of certain bands to reductive degradation. Where bands of similar molecular weight coincide between intestinal larvae and adult worms, stage-specificity has been confirmed by raising antisera specific for the adult (by intraduodenal transplantation of adult worms) which do not react with intestinal L4 antigens.

In addition to the restriction of surface antigens to a single stage, it is most interesting to note the appearance of antigens within the L3 stage before the first post-infection moult. Our results do not distinguish whether these larvae undergo a complete replacement of surface proteins once they invade the host, or, since there are several bands of like molecular weight from cultured and parasitic L3, there is simply an acquisition of proteins of 180,000 and 220,000 mol. wt. In either case, these data reaffirm the view that the nematode cuticle does not present a static profile between moults, but is subject to change according to age (Philipp et al., 1980) or environment. These acquired surface molecules are unlikely to be of host origin because they stimulate a persistent host antibody response, and the same may be said of surface antigens which appear later in the life cycle.

The transition between pre- and post-infection L3 is the first of three extensive changes in the parasite surface, two of which occur in the first 48 hr of infection. Does this rate of replacement reflect an immunologically orientated adaptation, by which the parasite keeps ahead of the host's immune response, or are each set of antigens functional molecules required for parasite survival in each different environment?

N. brasiliensis shares with *T. spiralis* a sharp stage-specificity of surface antigens, but these antigens themselves are very different from those of *T. spiralis*. The surface of parasitic *N. brasiliensis* worms carry more components accessible to radioiodination than are found on *T. spiralis* (Parkhouse et al., 1981; Philipp et al., 1980, 1981). However, unlike *T. spiralis* relatively few of these proteins are composed of disulphide-linked subunits, the only bands lost on reduction in 2-mercaptoethanol being the 220,000 mol. wt component on lung stage L3 and the doublet of 110–115,000 mol. wt on the surface of adult worms. The adult antigens differ further in this species in that

they are soluble in PBS and do not require deoxycholate detergent for their efficient extraction; indeed incubation of labelled worms in culture medium yields soluble adult antigens. Finally, the free-living L3 are not readily analysed by these techniques, raising the possibility that in this instance the surface antigens are non-proteinaceous glycoconjugates or part of the cuticle matrix which is not susceptible to detergent solubilization.

The extensive studies with *N. brasiliensis* by many groups of investigators have allowed us to approach another series of interesting questions. Since the conditions for parasite survival or expulsion in the rat host, and the effects of the host immune response on adult worms over a period of time have been well documented (Jarrett, Jarrett & Urquhart, 1966; Ogilvie & Hockley, 1968; Ogilvie & Jones, 1971; Jenkins, 1972, 1974; Love & Ogilvie, 1975) we can readily examine the surface profile of antigens borne by adult worms recovered at different times or conditions of infection. Thus, antibody-damaged worms on the point of expulsion (Ogilvie & Hockley, 1968) can be compared with healthy adults at their most fertile period of tenure, and with parasites adapted to prolonged establishment in the gut by a 'trickle' infection of the host (Jenkins & Phillipson, 1972; Jenkins, 1972, 1974).

We were unable to demonstrate any gross alteration in the molecular weight of worm surface antigens in any of the host-parasite conditions examined. If it is true that surface antigens are protective, a simple deletion of surface molecules cannot account for the persistence of trickle-adapted parasites. However, more subtle changes in parasite molecular properties are known to occur under such conditions, particularly to the electrophoretic pattern of acetylcholinesterase (Edwards, Burt & Ogilvie, 1971; Ogilvie, 1974; Ogilvie & Love, 1974), and to the overall immunogenicity of adapted worms (Ogilvie, 1969; Jenkins, 1972). It may therefore be necessary to analyse these surface antigens with a more sophisticated two-dimensional gel electrophoresis system (O'Farrell, Goodman & O'Farrell, 1977).

In summary, our results tell us much about the events in *N. brasiliensis* infection but shed little light on how these contribute to the overall picture of a successful, but limited, parasitic episode. At each point in time at which we have looked, the parasite bears surface molecules which are recognized by the host and which should act as targets for the antibody produced. The larval antigens may be replaced

rapidly, but the adult antigens appear to be surprisingly stable in view of the variety of consequences of different infection regimens. Perhaps this latter finding supports other suggestions of a relatively unimportant role of serum antibody (Jacobson, Reed & Manning, 1977), and that it is cell-mediated immunity, and the manipulation of that arm by the parasite which is the decisive factor in this nematode infection.

REFERENCES

AMBLER J. & ORR T.S.C. (1972) Studies on an allergenic component extracted from *N. brasiliensis*. *Immunochemistry*, **9**, 263.

BOLTON A.E. & HUNTER W.M. (1973) The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **133**, 529.

BURT J.S. & OGILVIE B.M. (1975) *In vitro* maintenance of nematode parasites assayed by acethylcholinesterase and allergen secretion. *Exp. Parasitol.* **38**, 75.

BUTTERWORTH A.E., STURROCK R.F., HOUBA V., MAHMOUD A.A., SHER A. & REES P.H. (1975) Eosinophils as mediators of antibody-dependent damage to schistosomula. *Nature (Lond.)*, **256**, 727.

DAY K.P., HOWARD R.J., PROWSE S.J., CHAPMAN C.B. & MITCHELL G.F. (1979) Studies on chronic versus transient intestinal nematode infections in mice. 1. A comparison of responses to excretory/secretory (ES) products of *Nippostrongylus brasiliensis* and *Nematospiroides dubius* worms. *Parasite Immunol.* **1**, 217.

DENHAM D.A. (1969) Secretion of metabolic antigen by *Nippostrongylus brasiliensis in vitro. J. Parasitol.* **55**, 676.

EDWARDS A.J., BURT J.S. & OGILVIE B.M. (1971) The effect of immunity upon some enzymes of the parasitic nematode *Nippostrongylus brasiliensis*. *Parasitology*, **62**, 339.

GAHMBERG C.G. & HAKOMORI S.-I. (1973) External labelling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes. *J. biol. Chem.* **248**, 4311.

JACOBSON R.H. & REED N.D. (1974) The immune response of congenitally athymic (nude) mice to the intestinal nematode *Nippostrongylus brasiliensis*. *Proc. Soc. exp. Biol. Med.* **147**, 667.

JACOBSON R.H., REED N.D. & MANNING D.D. (1977) Expulsion of *Nippostrongylus brasiliensis* from mice lacking antibody production potential. *Immunology*, **32**, 867.

JARRETT E.E.E., HAIG D.M. & BAZIN H. (1976) Time course studies on rat IgE production in *N. brasiliensis* infection. *Clin. exp. Immunol.* **24**, 346.

JARRETT E.E.E., JARRETT W.F.H. & URQUHART G.M. (1966) Immunological unresponsiveness in adult rats to the nematode *Nippostrongylus brasiliensis* induced by infection in early life. *Nature (Lond.)*, **211**, 1310.

JARRETT E.E.E., JARRETT W.F.H. & URQUHART G.M. (1968) Quantitative studies on the kinetics of establishment and expulsion of intestinal nematode populations in susceptible and immune hosts. *Nippostrongylus brasiliensis* in the rat. *Parasitology*, **58**, 625.

JENNINGS F.W., MULLIGAN W. & URQUHART G.M. (1963) Variables in X-ray 'inactivation' of *Nippostrongylus brasiliensis* larvae. *Exp. Parasitol.* **13**, 367.

JENKINS D.C. (1972) *Nippostrongylus brasiliensis*: observations on the comparative immunogenicity of adult worms from primary and immune-adapted infections. *Parasitology*, **65**, 547.

JENKINS D.C. (1974) *Nippostrongylus brasiliensis*: observations on factors affecting the establishment of secondary worm populations in rats. *Parasitology*, **68**, 13.

JENKINS D.C. & PHILLIPSON R.F. (1972) Evidence that the nematode *Nippostrongylus brasiliensis* can adapt and to overcome the effects of host immunity. *Int. J. Parasitol.* **2**, 353.

JONES V.E. & OGILVIE B.M. (1971) Protective immunity to *Nippostrongylus brasiliensis*: the sequence of events which expels worms from the rat intestine. *Immunology*, **20**, 549.

JUNGERY M. & OGILVIE B.M. (1981) The reaction of high and low responder mice to the surface antigens of *Trichinella spiralis*. In : *Trichinellosis* (ed. by C. W. Kim, E. J. Ruitenberg & J. S. Teppema), p. 71. Reedbooks, Chertsey, Surrey.

KELLER R. & KEIST R. (1972) Protective immunity to *Nippostrongylus brasiliensis* in the rat. Central role of the lymphocyte in worm expulsion. *Immunology*, **22**, 767.

KELLY J.D. & DINEEN J.K. (1972) The cellular transfer of immunity to *Nippostrongylus brasiliensis* in inbred rats (Lewis strain). *Immunology*, **22**, 199.

LAEMMLI U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*, **227**, 680.

LEE D.L. (1969) Changes in adult *Nippostrongylus brasiliensis* during the development of immunity to this nematode in rats. I. Changes in ultrastructure. *Parasitology*, **59**, 29.

LOVE R.J. & OGILVIE B.M. (1975) *Nippostrongylus brasiliensis* in young rats. Lymphocytes expel larval infections but not adult worms. *Clin. exp. Immunol.* **21**, 155.

MACKENZIE C.D., JUNGERY M., TAYLOR P.M. & OGILIVIE B.M. (1980) Activation of complement, the induction of antibodies to the surface of nematodes and the effect of these factors and cells on worm survival *in vitro*. *Europ. J. Immunol.* **10**, 594.

MACKENZIE C.D., PRESTON P.M. & OGILVIE B.M. (1978) Immunological properties of the surface of nematodes. *Nature (Lond.)*, **276**, 826.

MAIZELS R.M., PHILIPP M. & OGILVIE B.M. (1982) Molecules on the surface of parasitic nematodes as probes of the immune response in infection. *Immunol. Rev.* **61**, 109.

MILLER H.R.P. (1980) Expulsion of *Nippostrongylus brasiliensis* from rats protected with serum. I. The efficacy of sera from singly and multiply infected donors related to time of administration and volume of serum injected. *Immunology*, **40**, 325.

MULLIGAN W., URQUHART G.M., JENNINGS F.W. & NEILSON J.T.M. (1965) Immunological studies on *Nippostrongylus brasiliensis* infection in the rat: the 'self-cure' phenomenon. *Exp. Parasitol.* **16**, 341.

MURRAY M., ROBINSON P.B., GRIERSON C. & CRAWFORD R.A. (1979) Immunization against *Nippostrongylus brasiliensis* in the rat. *Acta Tropica*, **36**, 297.

NAWA Y. & MILLER H.R.P. (1978) Protection against *Nippostrongylus brasiliensis* by adoptive immunization with immune thoracic duct lymphocytes. *Cell. Immunol.* **37**, 51.

O'FARRELL P.Z., GOODMAN H. & O'FARRELL P.H. (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell*, **12**, 1133.

OGILVIE B.M. (1965) Role of adult worms in immunity of rats to *Nippostrongylus brasiliensis*. *Parasitology*, **55**, 325.

OGILVIE B.M. (1969) Immunity to *Nippostrongylus brasiliensis*. In: Nipponstrongylus *and* Toxoplasma, Symposia of the British Society of Parasitology (Ed. by A. E. R. Taylor), vol. 7, p. 31. Blackwell Scientific Publications, Oxford.

OGILVIE B.M. (1974) Antigenic variation in the nematode *Nippostrongylus brasiliensis*. In: *Parasites in the Immunized Host: Mechanisms of Survival*, Ciba Foundation Symposium 25, p. 81. Elsevier/North Holland, Amsterdam.

OGILVIE B.M. & HOCKLEY D.J. (1968) Effects of immunity on *Nippostrongylus brasiliensis* adult worms: reversible and irreversible changes in infectivity, reproduction and morphology. *J. Parasitol.* **54**, 1073.

OGILVIE B.M. & JONES V.E. (1968) Passive protection with cells or antiserum against *Nippostrongylus brasiliensis* in the rat. *Parasitology*, **58**, 939.

OGILVIE B.M. & JONES V.E. (1971) *Nippostrongylus brasiliensis*: a review of immunity and the host/parasite relationship . *Exp. Parasitol.* **29**, 138.

OGILVIE B.M. & LOVE R.J. (1974) Cooperation between antibodies and cells in immunity to a nematode parasite. *Transplantn. Rev.* **19**, 147.

OGILVIE B.M., PHILIPP M., JUNGERY M., MAIZELS R.M., WORMS M.J. & PARKHOUSE R.M.E. (1980) The surface of nematodes and the immune response of the host. In: *The Host-Invader Interplay* (Ed. by H. Van den Bossche), p. 99. Elsevier, North-Holland Biomedical Press, Amsterdam.

PARKHOUSE R.M.E., PHILIPP M. & OGILVIE B.M. (1981) Characterisation of surface antigens of *Trichinella spiralis* infective larvae. *Parasite Immunol.* **3**, 339.

PETIT A., PERY P. & LUFFAU G. (1980) Purification of an allergen from culture fluids of *Nippostrongylus brasiliensis*. *Mol. Immunol.* **17**, 1341.

PHILIPP M., PARKHOUSE R.M.E. & OGILVIE B.M. (1980) Changing proteins on the surface of parasitic nematode. *Nature (Lond.)*, **287**, 538.

PHILIPP M., TAYLOR P.M., PARKHOUSE R.M.E. & OGILVIE B.M. (1981) Immune response to stage-specific surface antigens of the parasitic nematode *Trichinella spiralis*. *J. exp. Med.* **154**, 210.

POULAIN J., LUFFAU G. & PERY P. (1976a) *Nippostrongylus brasiliensis* in the rat: immune response in serum and in intestinal secretions. *Ann. immunol. Inst. Pasteur*, **127C**, 215.

POULAIN J., PERY P. & LUFFAU G. (1976b) Protection of rats

against *Nippostrongylus brasiliensis* with worm antigens by oral administration. *Ann. Immunol. Inst. Pasteur*, **127C**, 209.

PROCHAZKA Z. & MULLIGAN W. (1965) Immunological studies on *Nippostrongylus brasiliensis* infection in the rat: experiments with irradiated larvae. *Exp. Parasitol.* **17**, 51.

SHARPE M.J. & LEE D.L. (1981) Observations on the structure and function of the haemoglobin from the cuticle of *Nippostrongylus brasiliensis* (Nematoda). *Parasitology*, **83**, 411.

SINSKI E. & HOLMES P.H. (1977) *Nippostrongylus brasiliensis*: systemic and local IgA and IgG immunoglobulin responses in parasitized rats. *Exp. Parasitol.* **43**, 382.

SOULSBY E.J.L. (1963) The nature and origin of the functional antigens in helminth infections. *Ann. N.Y. Acad. Sci.* **113**, 492.

WILSON R.J.M. (1967) Homocytotropic antibody response to the nematode *Nippostrongylus brasiliensis* in the rat—studies on the worm antigen. *J. Parasitol.* **53**, 752.

YOKOGAWA S. (1922) The development of *Heligmosomum muris* Yokogawa, a nematode from the intestine of the wild rat. *Parasitology*, **14**, 127.