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Stage-specific antigens of *Nematospiroides dubius*

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Summary. The results presented here demonstrate conclusively for the first time that *Nematospiroides dubius* exhibits stage specificity in the expression of cuticular antigens, and infer that the stage specific antigens demonstrable on L4 stages 6 days following infection account for the immunity generated by abbreviated infection regimes.

L4 stages 6 days following infection appeared to possess a stage-specific surface molecule (MW 16,000) which either disappeared on maturity to adults or somehow became unavailable to the surface probes used in this study. A molecule of similar molecular size was recognized by sera raised in mice immunized either by the administration of irradiated larvae or by using an antihelminthic-abbreviated infection and also by sera from jirds which had recently expelled *N. dubius*. The immune derivation of these sera supports the hypothesis that the molecule having a MW of 16,000 is functionally immunogenic.

A monoclonal antibody (29-1-D1) was used to demonstrate a further stage specific molecule (MW 65,000), this time on the surface of adult *N. dubius*. The application of biochemical techniques suggested that further heterogeneity existed in surface molecules resolving between 60,000–70,000 on SDS-PAGE and a hypothesis was formulated in an attempt to explain this phenomenon.

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INTRODUCTION

The discovery of stage-specific antigens of gastrointestinal helminths had added considerably to our understanding of the immunology of parasitic diseases (Maizels, Philipp & Ogilvie, 1982). However, investigations of stage-specificity have been largely limited to species against which effective immunity can be generated, e.g. *Trichinella spiralis, Nippostrongylus brasiliensis*. Helminth parasites which cause chronic infection of the gastrointestinal tract can feel relatively neglected in this respect despite the fact that such information would be highly relevant in explaining how those parasites evade host immunity and possibly pertinent to the development of effective vaccination procedures.

Nematospiroides dubius is a murine helminth parasite which resides for extensive periods in the gastrointestinal tract, apparently in defiance of the immune system. Consequently, this infection has been used as a model for chronic gastrointestinal nematode infections of man, for example *Ancylostoma duodenale* and *Necator americanus* (Bartlett & Ball, 1972). The ability of *N. dubius* to survive for periods of up to 9 months or more (Ehrenford, 1954) prompted studies into the possibility that adult stages suppressed the immune system of the host, and this has proven to be the case against both homologous and heterologous antigenic stimulation (Behnke, Hannah & Pritchard, 1983; Shimp, Crandall & Crandall, 1975). However, immunity can be generated against *N. dubius* using either

irradiated larvae (Hagan, Behnke & Parish, 1981) or antihelminthic truncated infections (Lueker & Hepler, 1975), in neither of which the host experiences the adult stages of the parasite. This suggests that immunity is generated by, and directed against, L4 stages of the parasite's life cycle.

The present study (using conventional post-infection sera and monoclonal antibodies) was initiated to investigate the possibility that stage-specific immunogens are present in or on *N. dubius*. It was also considered relevant to determine whether sera from the Mongolian gerbil (*Meriones unguiculatus*), a species which rejects *N. dubius* (Hannah & Behnke, 1982), was capable of recognising unique antigens on the parasite surface.

MATERIALS AND METHODS

Parasitology

The origin and maintenance of our strain of *N. dubius*, and the methods used for infection and recovery of the various life-cycle stages, have been described elsewhere (Pritchard *et al.*, 1983).

Immune sera

Immune mouse sera (IMS) were raised in CFLP mice using the multiple infection schedule (Behnke & Parish, 1979a). Primary infection sera (PrS) were collected as described by Pritchard, Behnke & Williams (1984b). Mice were immunized with irradiated larvae (~L3) or antihelminthic-abbreviated (Pyr) schedules as described previously (Hagan, *et al.*, 1981; Pritchard *et al.*, 1984b).

Immune jird sera were collected 35 and 63 days following a single infection with 200 L3 larvae of *N. dubius*. The worm burden was zero at bleeding on day 35.

Agar-gel diffusion (Ouchterlony)

Immunoprecipitins were detected in 1% agarose (0·2 M Tris buffer, pH 8·3) containing 3% PEG 6000.

Surface-labelling, immunoprecipitation and SDS-PAGE

Parasite antigens were analysed biochemically using the techniques described above following the methods of Philipp, Parkhouse & Ogilvie (1980) and Pritchard *et al.* (1983). Briefly, freshly acquired parasite stages were washed extensively prior to labelling with I^{125} using chloramine T, Bolton–Hunter reagent (Bolton & Hunter, 1973) or lodogen (Fraker & Speck, 1978). Labelled parasites were homogenized in the presence of protease inhibitors and centrifuged for 30 min at 11,000 *g* (4°). Supernates were routinely assessed for labelling efficiency and counts were always between 30–64% TCA precipitable. Immunoprecipitates manufactured by reacting polyclonal or monoclonal reagents with parasite homogenates were treated as described by Pritchard *et al.* (1983) and autoradiographs performed on completed gels analysed by SDS-PAGE. Slab gels consisting of a 12% running gel and 3% stacking gel were used unless otherwise stated. V8 protease was used against excised 65,000 MW bands of adults and L4 homogenates at a ratio of 2·5 μg protease to approximately 5000 c.p.m. labelled protein. The V8 and excised 65,000 MW bands were co-electrophoresed into the stacking gel and allowed to react at the stacking gel-running gel interphase for 30 min at RT.

Monoclonal antibodies

Monoclonal antibodies to *N. dubius* antigens were prepared by fusing immune mesenteric lymph node cells from Balb/c mice (immunized as described by Behnke & Parish, 1981) with the NS.1 plasmacytoma cell line (Kohler & Milstein, 1975). Positive cultures were identified by RIA against adult and L4 antigens of *N. dubius* (the clonality of positive cultures against worm antigen was determined by immunoblotting or IEF overlay techniques).

RESULTS

The antigen specificity of primary infection sera (PrS) and immune sera (IMS) in gel diffusion and SDS-PAGE electrophoresis

Sera were tested initially for reactivity against homogenates of life-cycle stages of *N. dubius* by gel diffusion in agar (Fig. 1). The order of reactivity for primary sera (PrS) was adult homogenate > d6L4 > d4L4. There was little reactivity against L3 homogenate (Fig. 1a). In contrast, immune sera (IMS) reacted most strongly against d6L4 homogenate, followed by AH > d4L4 > L3. The differential reactivity of PrS and IMS is again shown in Fig. 1b, where a direct comparison is illustrated. The latter analysis also

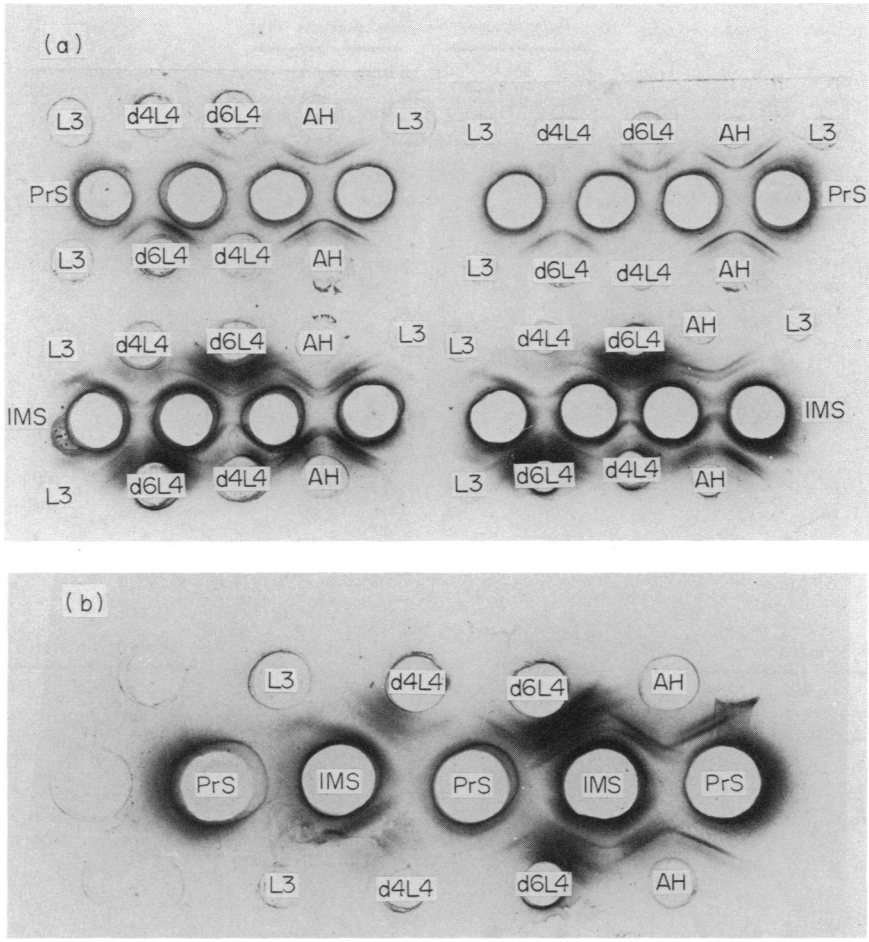

Figure 1. The reactivity of primary infection sera (PrS) and immune sera (IMS) against homogenates of the life cycle stages of *N. dubius*. Sera were analysed for reactivity by gel-diffusion against L3, d4L4, d6L4 and adult (AH) homogenates. The protein concentration of each homogenate was adjusted to 2 mg protein/ml.

suggested that immune sera recognises distinct antigens from d6L4 stages. To investigate this further, immunoprecipitates were prepared between immune sera and the range of homogenates. These precipitates were analysed by conventional SDS-PAGE electrophoresis and the resulting gel is shown in Fig. 2a.

It was seen that *N. dubius* homogenates were, not surprisingly, very heterogeneous (lanes 4–6) and that only a small number of these proteins were precipitated by immune sera (7–9). Antibody to *N. dubius* in immune sera is largely restricted to the IgG_1 isotype (Pritchard *et al.*, 1983), and the subtraction of heavy and light chain (lane 3) from the complexes allowed the antigenic composition of the precipitates to be evaluated against the known molecular weight markers (lanes 1 and 10).

It appears that an antigen of similar molecular size exists between the three stages of the life cycle analysed here, and that this antigen (under reducing conditions) has a molecular weight of approximately 14,000 (lanes 7–9, Fig. 1a). However, d6L4 larvae also possess an additional antigen with a molecular weight of approximately 16,000 (arrowed-lane 8).

A repeat run of freshly made immunoprecipitates again revealed this additional antigen, and also confirmed the lack of this antigen on L3 stages (Fig. 2b).

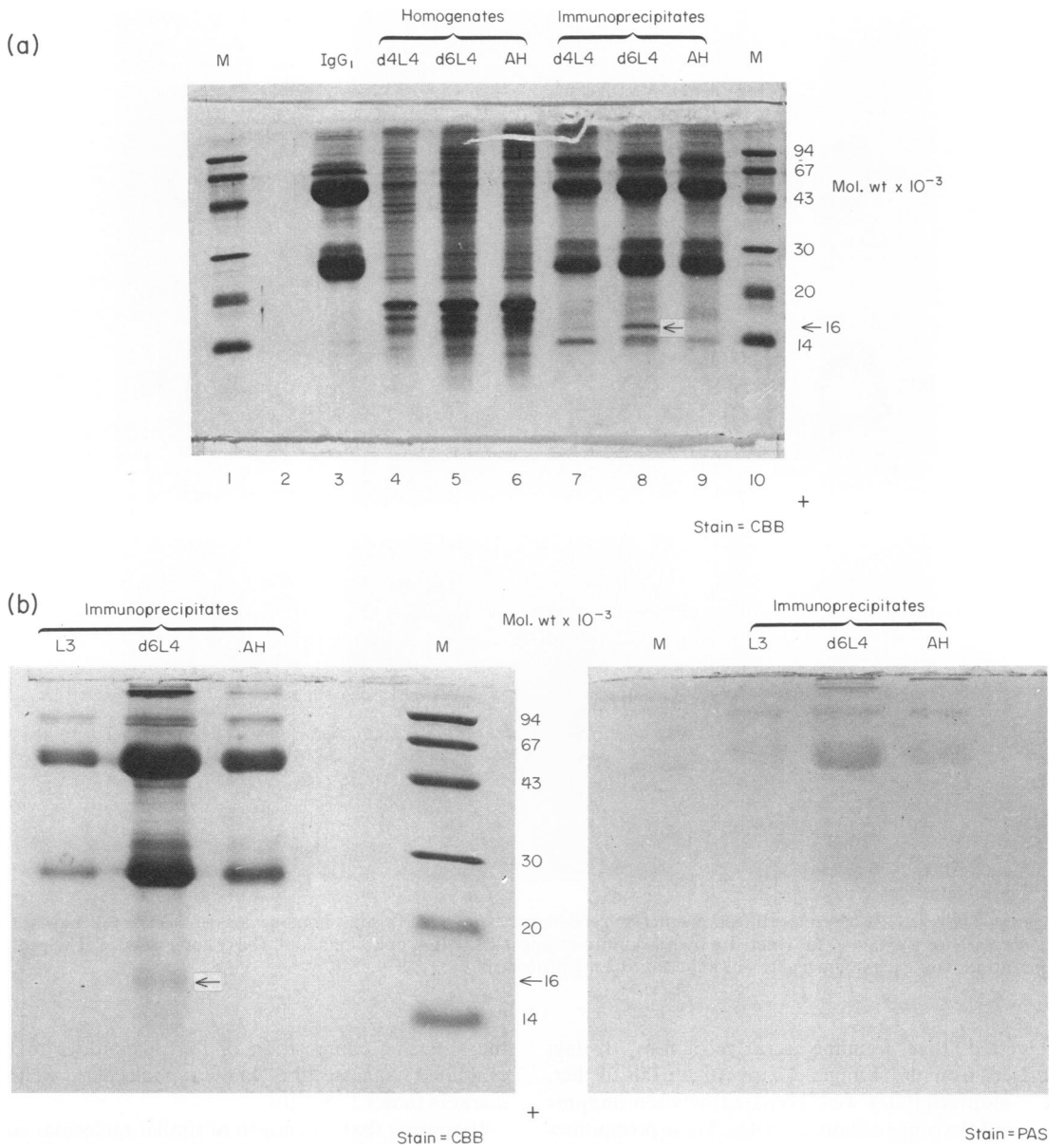

Figure 2. The antigen specificity of immune sera against homogenates of the life cycle stages of *N. dubius*. Immunoprecipitates manufactured between immune sera and the antigens of *N. dubius* were solubilized at 100° in 0·0625 M Tris buffer containing 2% SDS and 5% 2-mercaptoethanol. Samples were resolved by SDS-PAGE using a 3% stacking gel (pH 6·8) and a 12% running gel (pH 8·8) at constant voltage (60v) overnight. (a) A comparison of the immunoprecipitates and their unreacted components. IgG_1 = immunoglobulin purified from immune serum by affinity chromatography on Staph A-Sepharose; M = low MW markers. Stain = Coomassie Brilliant Blue (CBB). (b) The above procedure was repeated using fresh immunoprecipitates to include immunoprecipitated L3 homogenate. The gel was stained with Coomassie Brilliant Blue (CBB) and Schiff's reagent for carbohydrate (PAS).

PAS staining of a parallel run suggested that the apparent stage-specific antigen of d6L4 larvae was unlikely to be a glycoprotein (Fig. 2b).

Surface labelling studies

The disadvantage of the approach described above is that it excludes the possibility that there might exist parasite antigens of the same or similar molecular weight to antibody components. To overcome this, intact d6L4 or adult worms were radio-labelled and subsequently solubilized prior to immunoprecipitation. This ensured that any bands appearing in the precipitates on SDS-PAGE gels following autoradiography were of parasitic origin. The labelled immunoprecipitated surface antigens (*SAg) were generally counted on a gamma counter prior to analysis by SDS-PAGE (Table 1). It can be seen that sera of immune derivation appeared to react more potently against d6L4 *SAg than against adult *SAg.

Analysis by SDS-PAGE and autoradiography

Figure 3 shows the results of an experiment in which the parasite surface was labelled by two distinct methods (Bolton–Hunter reagent—BH; or lodogen—10D) and the labelled antigens precipitated using either primary infection sera (PrS) or immune sera (IMS). In general, the immunogenic determinants of adults were more readily labelled by Bolton–Hunter reagent, indicating the presence of free amino groups at the cuticular surface and the presence of amino acids such as lysine, arginine, asparagine and glutamine. In contrast, the immunogenic determinants of larval forms labelled more readily using iodogen, indicating the presence of tyrosyl residues. Although traces of immunogenic material labelled by iodogen were detectable on adults, and traces of immunogenic Bolton–hunter-labelled material detectable on larvae, the overriding message was that considerable biochemical differences existed between the cuticular surfaces of each stage. A more detailed summary of the results is given below.

Reactivity vs *d6L4*

Immune serum was more potent than primary serum in detecting an antigen of MW 65,000 (cf. immunoglobulin heavy chain), and this antigen was more heavily labelled using iodogen (lanes 1, 2, 5, 6). Immune sera also detected additional specificities in the low molecular weight range.

Reactivity vs *adults*

Primary sera and immune sera detected a similar antigenic spectrum in terms of molecular weight, with primary sera possibly more potent. In this case, the Bolton–Hunter reagent labelled a more heterogeneous range of proteins. Note again the presence of an

Table 1. The relative efficiency of sera to immunoprecipitate I^{125}-labelled surface antigens (*SAg) of L4 larvae and adults of *N. dubius*

	I^{125} *SAg (ct/min) in immunoprecipitate	
	D6L4	Adult
Normal mouse sera (NMS)	1954	1980
Primary infection sera (PrS)	1499	2470
Immune mouse sera (IMS)	83869	24253
Irradiated L3 (~L3)	9004	6669
Anti-helminthic-abbreviated (Pyr)	52857	16814
Normal jird sera (NJS)	2643	2140
Immune jird sera (IJS 35)	60956	16423
(IJS 63)	37171	7986
Monoclonal (29-1-D1)	1022	20054
*SAg labelling efficiency (IODOGEN)		% TCA precipitable
c.p.m. 10 μl d6L4 *SAg = 99,568		55
c.p.m. 10 μl ADULT *SAg = 131,172		57

Table 2. The relative intensity of the IODOGEN-labelled 65,000 MW band on autoradiographs following immuno-precipitation with primary serum or immune serum

Primary serum + L4 65 kd band	+
Immune serum + L4 65 kd band	+++
Primary serum + AD 65 kd band	+
Immune serum + AD 65 kd band	+

antigen of MW 65,000 in adults labelled by the iodogen method. The intensity of this band was similar to that seen with primary sera vs L4's. The relative intensity of the 65,000 MW band was arbitrarily scored as illustrated in Table 2.

L4's vs *adults*

Immune sera detected specificities in L4's which were absent in adults and not detected by primary sera, i.e. stage-specific antigens. This antigen had a molecular weight of approximately 16,000 (Fig. 3).

It is also worth noting at this stage that no major antigenic molecules were detected on L3 larvae by immune sera following iodination by either Bolton–Hunter or chloramine-T (sheathed and exsheathed). Also, normal mouse sera had negligible precipitin activity and when present was directed against a very weak band in the 65,000 MW region.

The activity of sera taken from animals immunized using irradiated larvae or antihelminthic-truncated infection

As immunity generated by irradiated larvae or abbreviated infections is claimed to act against L4 stages, it was of interest to determine whether sera taken from animals immunized following the procedures outlined above preferentially recognised antigens present on the surface of L4 stages.

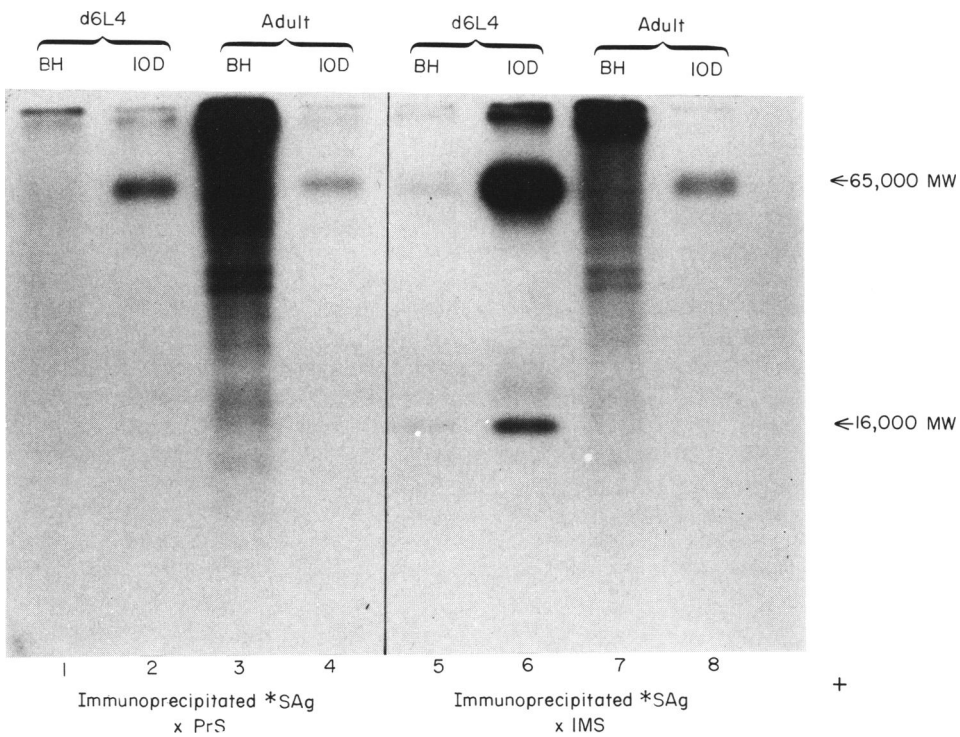

Figure 3. The reactivity of primary infection sera and immune sera against iodinated surface proteins (*SAg) of d6L4 or adult (AD) *N. dubius*. Freshly acquired stages were labelled using Bolton–Hunter (BH) reagent or iodogen (10D) and the solubilized surface proteins immunoprecipitated using appropriated sera. An autoradiograph of SDS-PAGE analysis of the resulting precipitates is shown.

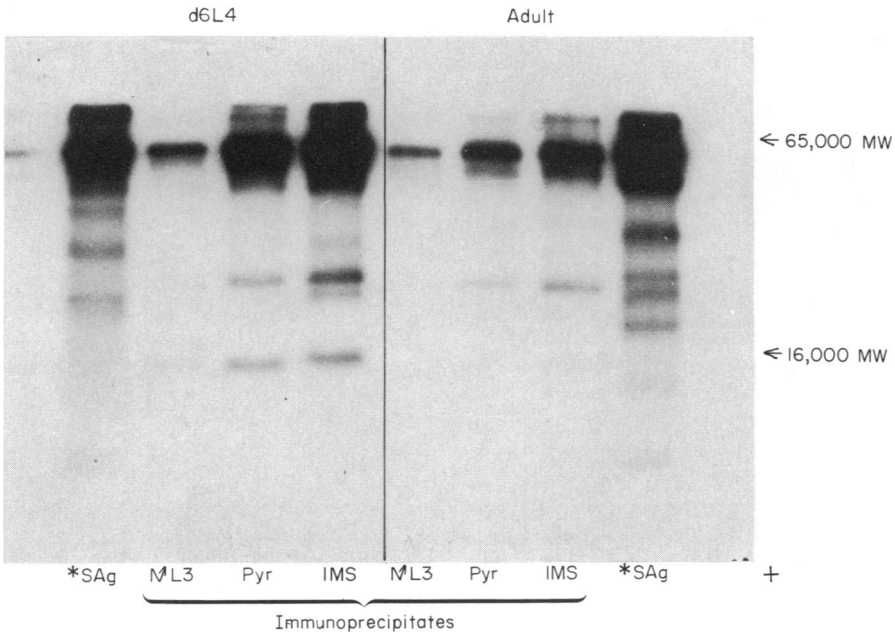

Figure 4. The antigens of *N. dubius* recognised by sera from mice immunized using irradiated L3 larvae or an anti-helminthic abbreviated infection. Iodinated surface proteins (*SAg) of d6L4 or adult *N. dubius* were immunoprecipitated using sera from mice immunized using irradiated larvae (~L3), anti-helminthic abbreviated infection (Pyr) or the multiple immunisation regime to produce immune mouse sera (IMS). An autoradiograph of SDS-PAGE analysis of these precipitates is shown in direct comparison to the original labelled surface proteins.

The results of this experiment are shown in Fig. 4, where it can be seen that sera from mice immunized using an antihelminthic-abbreviated infection (Pyr) recognised the stage-specific (16,000 MW) antigen on d6L4 larvae (cf. IMS) and failed to recognize an antigen of similar molecular weight on adult worms. Sera from mice immunized using irradiated larvae (~L3) also reacted, albeit weakly, with this antigen on d6L4 larvae but similarly failed to react against adult worms.

The heavily-labelled band at approximately 65,000 MW was again evident in all precipitates and may suggest the presence of a common antigen between d6L4 larvae and adults as donors of sera resulting from irradiated infections or abbreviated infections had little opportunity to experience adult stages of the life cycle. Other observations worthy of note from this experiment were the apparent lack of the 16,000 MW antigen in the native d6L4 labelled-surface molecules and also the presence of an additional and perhaps common antigen at approximately 30,000 MW.

The antigens of *N. dubius* recognized by the immune jird (*M. unguiculatus*)

Sera taken from jirds following the expulsion of *N. dubius* (immune sera, days 35, 63) were assayed for their ability to immunoprecipitate surface-labelled antigens of the same parasite, and the results of this experiment are shown in Fig. 5. It can be seen that normal jird sera failed to react with either d6L4 or adult homogenate, and that immune sera reacted against a 65,000 MW band in both d6L4 and adult homogenate but with lower molecular weight bands (Ω16,000) only in the L4 homogenate (arrowed). The corresponding profiles of mouse immune serum against d6L4 and adult homogenates is shown for comparison.

The microheterogeneity of *N. dubius* surface antigens of 60,000–70,000 MW

Although adult and L4 *N. dubius* both have a major band at approximately 65,000 MW (by iodogen),

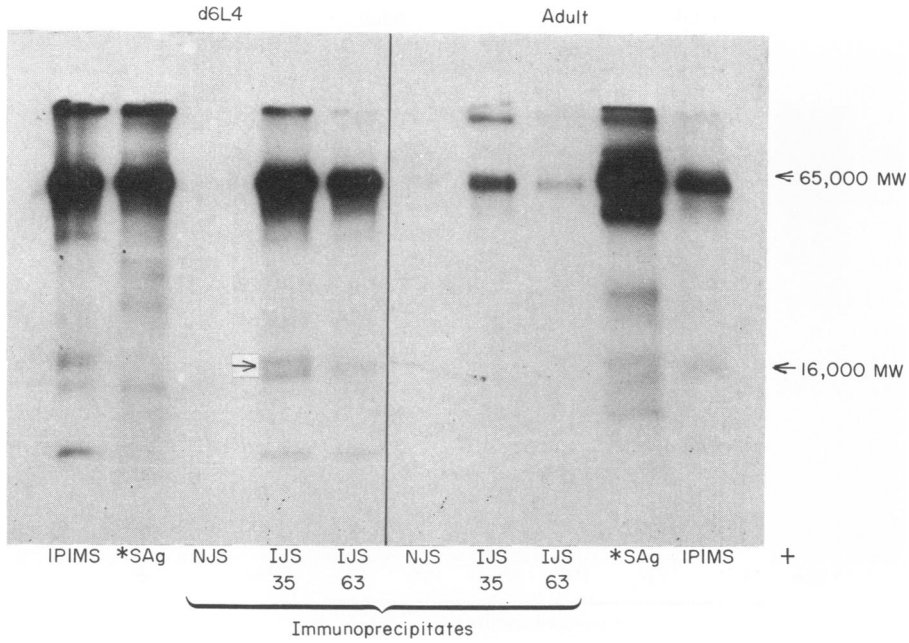

Figure 5. The antigens of *N. dubius* recognized by the immune jird (*Meriones unguiculatus*). Iodinated surface proteins (*SAg) of d6L4 or adult (AD) *N. dubius* were immunoprecipitated using sera taken from jirds 35 and 63 days following infection (IJS) with 200 L3 larvae (post-expulsion). The resulting immunoprecipitates were analysed by SDS-PAGE (autoradiograph shown) and compared to the *SAg precipitated by immune mouse sera (IMS), normal jird sera (NJS) and the original *SAg.

immune sera are selectively stronger against the L4 band as shown in Fig. 3. Thus, although the bands initially appear to be biochemically similar, they may be immunologically different. For example, at least three, and possibly four, species of molecule can be discriminated in the adult 60,000–70,000 MW range if bands are eluted from polyacrylamide rod gels and fractions re-run (R.M. Maizels, unpublished data). This microheterogeneity within the 60–70 kd range was confirmed using a range of monoclonal antibodies raised by infection. A large proportion of these antibodies, and in particular 29-1-D1, recognized a band of 65,000 MW in adults but failed to recognize a similar band on d6L4 stages (Fig. 6a, Table 1). In addition, the V8 protease digest spectrum of the 65,000 MW band from d6L4's was different to that of the 65,000 MW band from adults (Fig. 6b) suggesting differential susceptibility to the protease and different amino acid composition; therefore it is quite possible that stage-specific antigens also exist at the 60,000–70,000 MW range. The V8 protease digested the 65,000 MW band from L4 larvae into two major fragments of approximately 49,000 and 36,000, whilst it only digested a 49,000 fragment from the original 65,000 band of adults, with some material remaining apparently undigested.

DISCUSSION

Experimental evidence has been presented in the past to suggest that *N. dubius* possesses stage-specific antigens. However, this evidence has been largely circumstantial and has depended either on absorption experiments using worm homogenates and immune sera (Pritchard *et al.*, 1983) or on the demonstration that incomplete infections (irradiated or anti-helminthic abbreviated) lacking adult stages were highly effective in stimulating immunity (Hagan *et al.*, 1981; Pritchard *et al.*, 1984b) whilst adults were positively detrimental to the development of immunity (Behnke *et al.*, 1983; Pritchard Ali & Behnke, 1984a). Direct experimental evidence was therefore required to clarify this point.

Following the demonstration in the present study that immune sera seemed to preferentially precipitate d6L4 homogenates, and that a stage-specific antigen

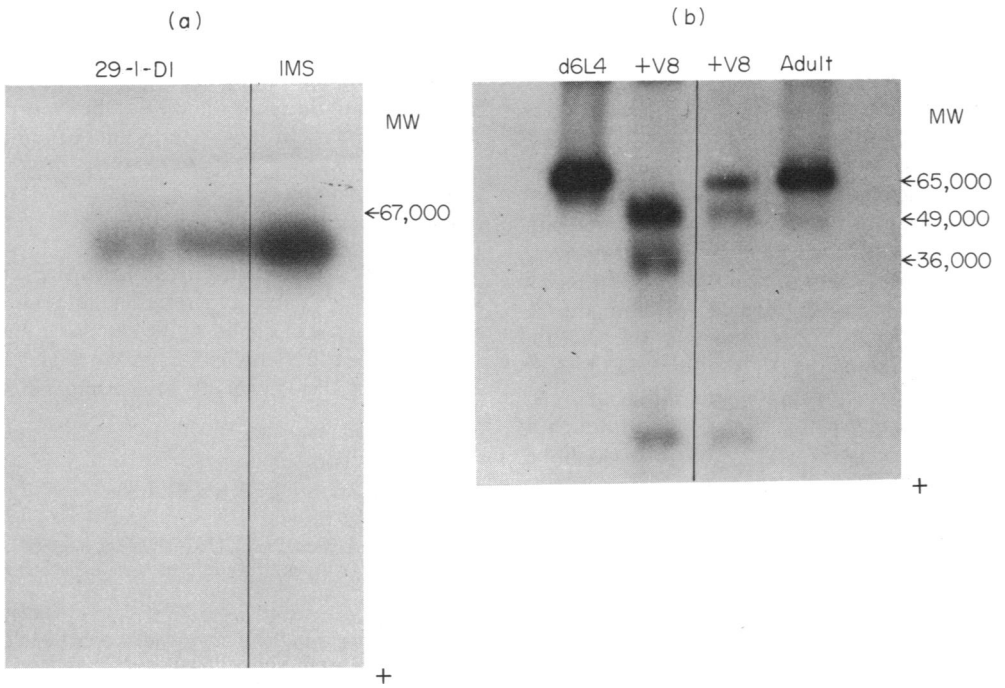

Figure 6. The heterogeneity of *N. dubius* antigens of 60,000–70,000 MW on SDS-PAGE. (a) An autoradiograph of the surface antigens of adult *N. dubius* immunoprecipitated by monoclonal antibody 29-1-D1 as compared to the antigens immunoprecipitated by immune mouse sera (IMS) (only 65,000 MW band shown for IMS). (b) An autoradiograph of the V8 protease digest spectra of the excised 65,000 MW surface antigens (*SAg) of d6L4 and adult (AD) *N. dubius*.

was demonstrable at 16,000 MW by SDS-PAGE analysis of conventional immunoprecipitates, surface-labelling analysis was applied in an attempt to understand the antigenic complexity of this parasite species.

The initial observation that immunogenic molecules on d6L4 stages were more prone to labelling with iodogen, whilst adults were more efficiently labelled by Bolton–Hunter reagent, immediately suggested differences between the surfaces of the relative parasitic stages. Following iodogen-labelling, immune sera were more potent in recognizing a 65,000 MW band on d6L4 larvae than on adults (cf. immunoprecipitate c.p.m.) and also detected an extra specificity at 16,000. It would therefore appear that sera from immune mice recognize a stage-specific antigen on the surface of d6L4 larvae. This stage-specific antigen co-migrated on SDS-PAGE with the stage-specific antigen immunoprecipitated from d6L4 homogenate, and may represent the same molecule.

The question which arose at this stage was whether this 16,000 MW molecule was functionally immunogenic. Although this question cannot be answered satisfactorily until the protein is purified and used in immunization experiments, the functional immunogenicity of this molecule was suggested by a number of experimental observations. Firstly, this molecule was not recognized by sera from animals undergoing a chronic infection with *N. dubius* (primary sera). In addition, a 16,000 MW surface molecule was precipitated by a number of separate sera of immune derivation. For example, it was recognized by sera from mice immunized using irradiated larvae or using an antihelminthic abbreviated infection regime. It was also recognized by sera from the Mongolian gerbil, a species which expels primary infections of *N. dubius*. So it would appear that the 16,000 MW molecule is recognized in a number of immune situations.

The ability of immune sera to precipitate an iodogen-labelled 65,000 MW determinant from the surface of L4 larvae seemingly more efficiently than from adults could either mean that the molecule is expressed to a greater extent on the larval stage or that

heterogeneity exists within this rather intense band and that further stage-specificity may be the cause of differential immuno-precipitation. However, it is also likely that determinants common to the two stages migrate at approximately 65,000 MW, as sera from mice immunized by irradiated or antihelminthic-abbreviated infections precipitated a 65,000 MW determinant from adult material. Heterogeneity within this molecular weight region was confirmed by the fortuitous production of a monoclonal antibody which recognised a stage-specific determinant at 65,000 MW on adults. In addition, the V8 protease digestion spectrum of the 65,000 MW band from larvae was sufficiently dissimilar to that from adults to suggest heterogeneity within this region, although a dose response experiment with V8 protease would be required to confirm this observation.

Therefore, it would appear to date that *N. dubius* larvae possess a stage-specific determinant 6 days following infection, and that a further stage-specific antigen is acquired at adulthood. This obvious biochemical difference between the stages may reflect the contrasting environments occupied by each stage, as the larval forms are essentially tissue dwelling whilst adults are inhabitants of the duodenal lumen. It is tempting to speculate at this stage that the larval stage-specific antigen is functionally immunogenic and triggers an effective immune response (immunity against *N. dubius* is expressed against tissue-dwelling larvae; Behnke & Parish, 1979b), only to be lost or masked upon maturity to the evasive adult stage.

The complexity of *N. dubius* immunogenic determinants around 65,000 MW is not as easy to explain at this stage, although a reasonable working hypothesis can be formulated. It is possible that a common molecular backbone (65,000 MW) is initially shared between the two stages studied. However, the d6L4 stage has a relatively tyrosine (iodogen-labelling) and glutamic acid (V8-protease digest spectrum) rich moiety which is lost or masked on maturity, whilst the adult stage expresses a greater propensity of free amino groups at its surface (Bolton–Hunter labelling) and acquires an additional immunogenic determinant (monoclonal 29-1-D1). In this context, Parkhouse, Philipp & Ogilvie (1981), using *Trichinella spiralis*, demonstrated that the glycosylation of proteins was a major cause of antigenic heterogeneity, and the possibility that this occurs with *N. dubius* should be considered.

To conclude, it appears that *N. dubius* does indeed express stage-specific antigens at the cuticular surface. As with many immunological studies, the data presented poses more questions than it answers. However, it would appear that a significant advance has been made into the understanding of the evasion of the immune response by a gastrointestinal helminth.

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