

A hookworm allergen which strongly resembles calreticulin

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SUMMARY

Immoglobulin E-rich plasma from patients from Papua New Guinea infected with Necator americanus has been used to probe an adult N. americanus cDNA library for the presence of hookworm allergens. Using this approach, one hookworm allergen has been identified as calreticulin, which was subsequently expressed in Escherichia coli. Little serological cross reactivity was seen between the recombinant calreticulins of this hookworm and its host. Prospective roles for hookworm calreticulin in the host-parasite relationship are discussed in depth.

Keywords calreticulin, allergen, *Necator americanus*, IgE

INTRODUCTION

Some metazoan parasites are highly allergenic, and schistosomes and hookworms are good examples of parasitic organisms which induce predominant T-helper 2 (Th2) immune responses in humans at key stages of their life cycles (reviewed by Pritchard *et al.* 1997). Although Th1 induced immune responses, supported by the early presence of interleukin (IL)-12 and interferon- α (Romani *et al.* 1997) are of undoubted importance in controlling the migratory larval stages of some helminth parasites (Pritchard & Wilson 1997) it seems that Th2 driven responses, characterized by IL-4 and IL-5 secretion by activated T and other cells, and by the production of immunoglobulin (Ig)E and the induction of eosinophilia, evolved as part of the immunological armoury against helminth parasites (Pritchard *et al.* 1997). For example, patients with high levels of IgE to *Necator americanus* harbour smaller and less fecund parasites (Pritchard *et al.* 1995). It is therefore of immunoparasitological interest to determine the molecular targets of such potentially protective, and diagnostic (Pritchard & Walsh 1995), immune responses against human hookworms.

In the present paper, IgE-rich plasma from patients infected with *N. americanus* was used to screen cDNA libraries made from adult stages of this parasite; this procedure has identified an allergen with homology to calreticulin. The significance of this finding to the host–parasite relationship is discussed.

MATERIALS AND METHODS

Plasma samples and serological analysis

It was immunologically important to maintain the physical integrity of hookworm-reactive IgE, the antibody probe used in this study, given the lability of its Fc ϵ (immunoreactive with anti-IgE) domains. Consequently, plasma samples were taken with informed consent from 135 individuals

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Received: 6 March 1998

Accepted for publication: 25 January 1999

known to be infected with *N. americanus* (Pritchard *et al.* 1990, 1992), separated on site in Kebasob village, and frozen at -20°C at the Gaubin Mission Hospital on Karkar Island. Unavoidably, the samples were thawed once in transit by copra boat to mainland Papua New Guinea, where they were frozen once more at the Papua New Guinea Institute of Medical Research, Madang, and shipped on dry ice to Nottingham. Samples were still frozen on arrival and were stored at -80°C until screening began.

Samples were subsequently screened by enzyme-linked immunosorbent assay (ELISA) for their IgE reactivity against adult *Necator* ES antigen (Pritchard *et al.* 1992) and shown to have retained their activity. To compare the reactivity between samples, the reactivity of the plasma pool ($n=135$) was ascribed a value of 1000 units. The 135 samples were then assigned into categories of reactivity (Figure 1a), and the eight most reactive samples (OD 0.2–0.72) were then subselected for screening purposes. To determine the specificity of the assay for IgE binding, a number of competitive blocking procedures were included, as indicated in the legend to Figure 1. The Western blot procedure was conducted as described previously for hookworm antigens (Carr & Pritchard 1986). To control for the presence of IgE binding proteins in worm secretions and extracts, blots were also developed with myeloma IgE in comparison with post-infection plasma.

Construction and screening of adult *N. americanus* cDNA library

Briefly, adult hookworms were allowed to detach from the intestines of infected 35-day-old DSN hamsters, then washed for 4 h in several changes of sterile RPMI 1640 supplemented with penicillin (100 U/ml^{-1}) and streptomycin ($100\text{ }\mu\text{g/ml}^{-1}$). This procedure removes regurgitated host tissue, ensuring that subsequent mRNA is of parasite origin only. Worms were then extracted in guanidinium thiocyanate/phenol/chloroform in a single step procedure, prior to precipitating total RNA (Carr & Pritchard 1986). 0.536 mg of total RNA ($A_{260/280}$ 1.86) was then passed over columns of poly dT cellulose, and the poly A⁺ mRNA eluted ($A_{260/280}$ 2.0).

An adult *N. americanus* cDNA library was constructed in λ ZAP express (Stratagene—ZAP express TM cDNA Gigapack III Gold Cloning Kit). 6 μg of poly A⁺ RNA were used as the initial template, and the resultant cDNA's size—selected through Sephacryl S-500 spun columns. A library consisting of high MW cDNAs (termed N1–N3, terminology included for those who have received gifts of this library), as assessed by polyacrylamide gel electrophoresis (PAGE), was made with a titre of 10^9 pfu (plaque forming unit)/ml and frequency of recombination of 79%.

The library was screened using a standard procedure, with 2×10^6 pfu plated for the primary screen, with the PNG plasma pool applied overnight at 1:20, and the anti-IgE applied for 2 h at 1:1000; prolonged incubation was necessary, to obtain a strong signal. Further details are given in the legends to the figures. Positive pfu were taken to a quaternary screen, and then excised into the pBK-CMV phagemid (Statagene) for sequencing, which was conducted using purified plasmid DNA and a Perkin-Elmer Applied Biosystems automated sequencer.

Gene sequencing and analysis

Double stranded cDNA from clone *Na-cal-1* was sequenced using universal forward and reverse primers and subsequent gene-specific primers; sequencing was performed using the AmpliTaq DyeDeoxy Terminator Cycle Sequencing System (Applied Biosystems, Foster City, CA, USA) and an automated ABI Prism 377 DNA sequencer (Applied Biosystems). Analyses of nucleotide and deduced amino acid sequences were performed using the Mac Vector 4.5.1 software program (Eastman Kodak Company, New Haven, CT, USA). Database searches were performed using the BLAST (Altschul *et al.* 1990) server to search a nonredundant set of databases. Sequence alignments were performed using the GCG PILEUP program (Genetics Computer Group, Madison, WI, USA) and Boxshade server. The translated protein sequence was analysed using the Analyse Signalase (Eastman Kodak Company) program in order to identify a putative signal peptide and corresponding cleavage point (Von Heijine 1986).

Phylogram

The aligned proteins of *Na-cal-1* and other calreticulin-like proteins were subjected to phylogenetic analysis using maximum parsimony implemented in PAUP (volume 3.1.1; Swofford 1991). Bootstrap support for the shortest tree found by heuristic search was assessed using 100 replicates.

Construction of plasmid expressing calreticulin (without signal sequence)

For the expression and isolation of recombinant protein, a 6 xHis-system (Qiagen) was used, consisting of the expression vector pQE10 and the repression plasmid pREP.

Calreticulin cDNA was cleaved from the pBK-CMV phagemid and subcloned into the expression vector pQE10 (Qiagen). In order to express calreticulin within the *Escherichia coli* cell, the open reading frame of the mature protein was fused with the histidine-tag encoded by the pQE10 vector, thus deleting the predicted N-terminal

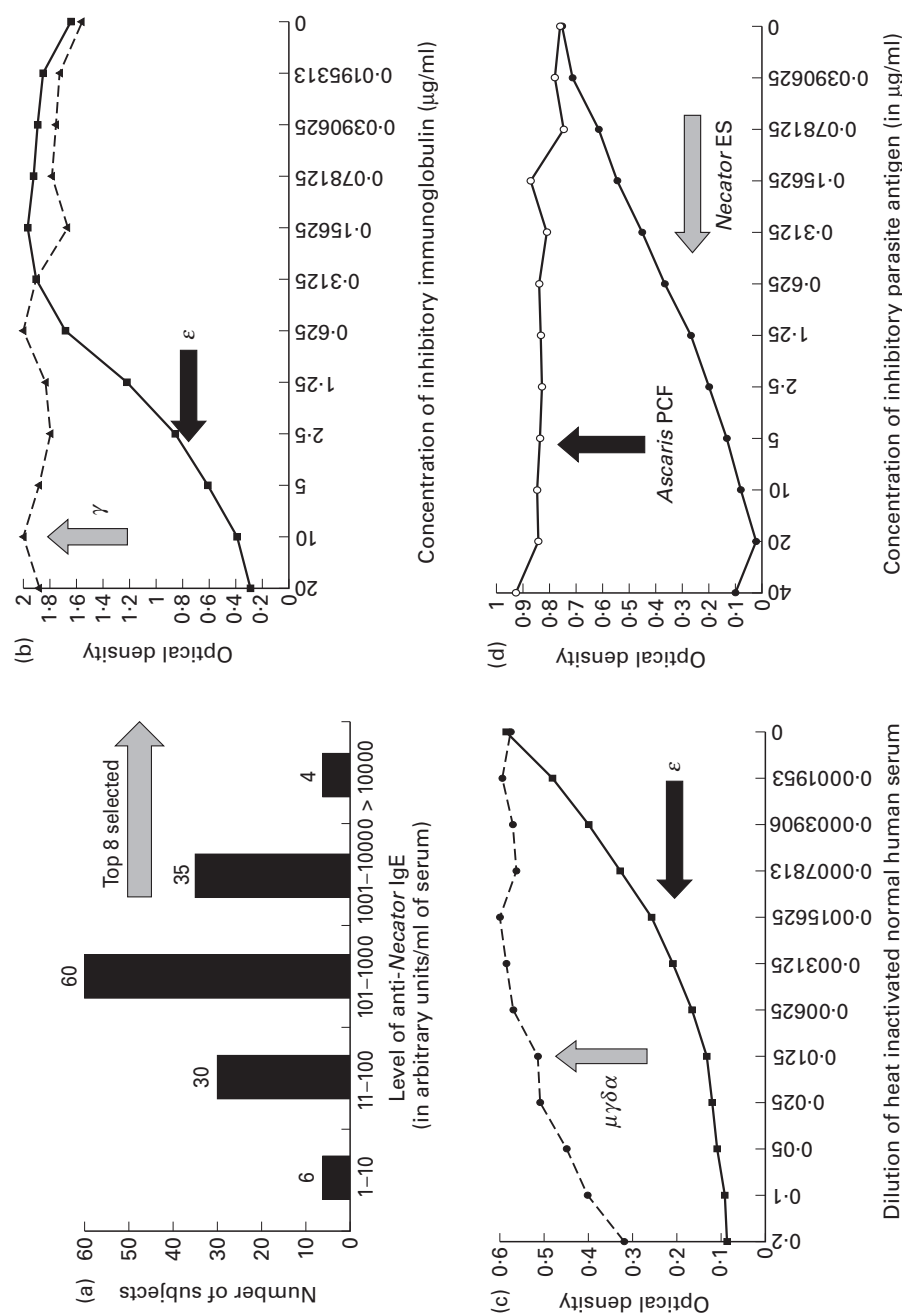
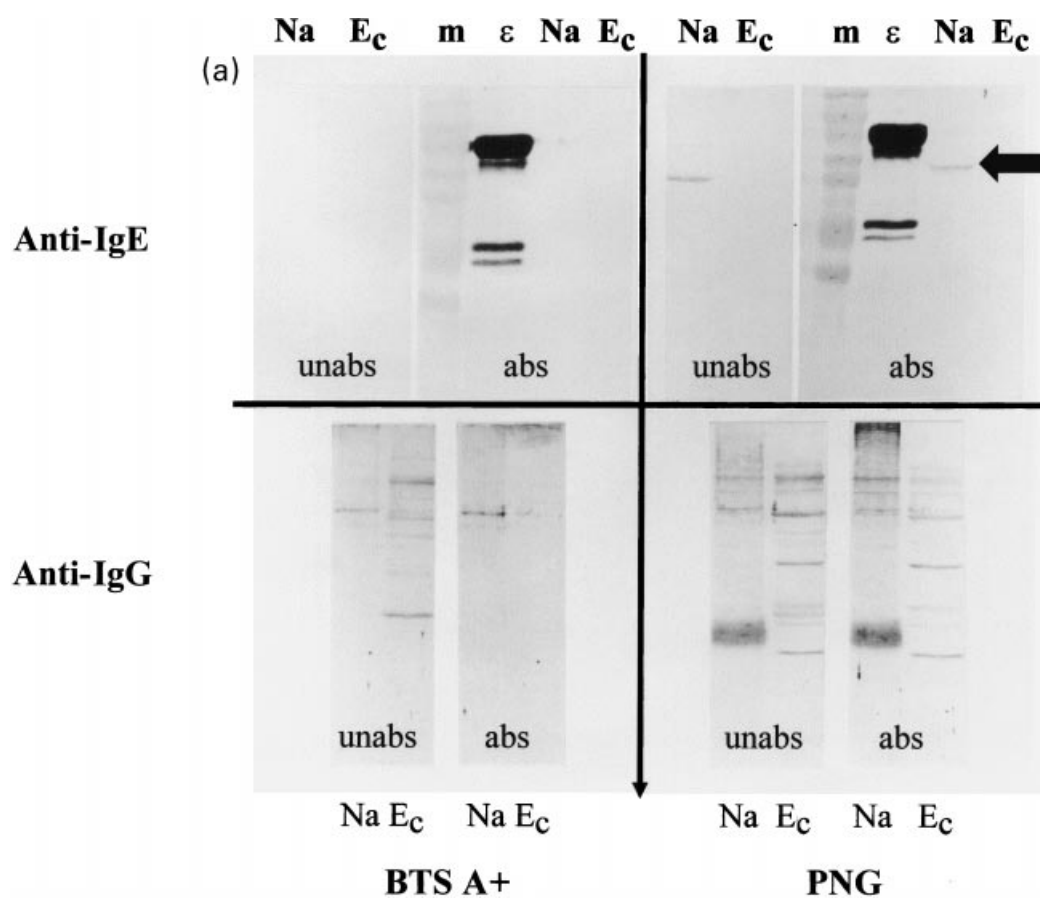


Figure 1 (a,b) One hundred and thirty-five plasma samples from hookworm-infected patients from Papua New Guinea (PNG) were screened in ELISA using, sequentially, *Necator americanus* adult ES antigen (5 μg/ml), a primary plasma dilution of 1:20 (in phosphate-buffered saline – PBS, pH 7.4 containing 0.05% Tween) and a 1:1000 dilution of alkaline phosphatase-conjugated monoclonal anti-human IgE (Sigma). In comparative terms, the eight most reactive individual plasma samples were then pooled, and used to assess the specificity of the ELISA. The IgE ELISA (OD 405 nm) against *Necator* antigen was 'blocked' with either soluble myeloma IgE or IgG, each diluted down from a 20 μg/ml stock. IgG (▲—▲) completely failed to inhibit the assay; in contrast (■—■) IgE showed a dose-dependent inhibition of the ELISA. (c) Heated normal human serum (●—●) containing all antigenically-reactive isotypes (γ, α, μ, δ), except ε also failed to inhibit the assay, except at very high concentrations. Myeloma IgE (■—■) used in the same concentration range as (b), again inhibited the assay in a dose-dependent fashion. (d) The assay was inhibited by soluble *N. americanus* excretory-secretory antigen (●—●), but was not inhibited by the ABA-1 containing pseudocoelomic fluid from *Ascaris lumbricoides* (○—○), a parasitic nematode which is also endemic to the study population (Pritchard *et al.* 1990).

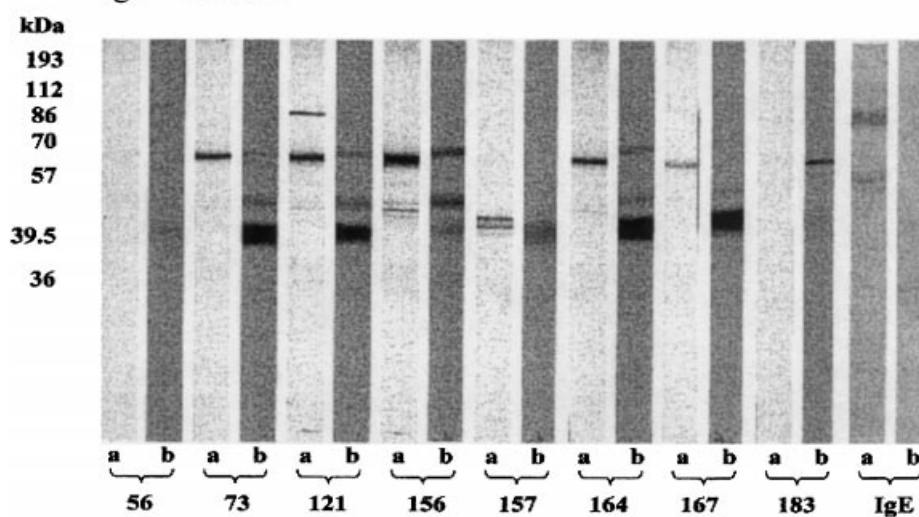


(b) **Western**

a = extract

b = ES

IgE = control



signal sequence of calreticulin. For this purpose, fragments of the flanking sequences left and right to the signal peptide coding sequence were amplified by polymerase chain reaction (PCR) using the primers 322-forward, 5'-CGTAT-CACGAGGCCCTTTTCG-3' (20-mer) and CARE-reverse, 5'-TTGAAGTAGACCTCCGCTACATGGTGATGGTGA TGGTG-3' (38-mer) for the N-terminal part and the primers CARE-forward, 5'-CACCATCACCATCACCATG-TAGCGGAGGTCTACTTCAA-3' (38-mer) and pQE-reverse, 5'-GAGTTCTGAGGTCATTACTG-3' (20-mer) for the C-terminal part. Since CARE-forward and CARE-reverse have an overlapping complementary sequence, the two resulting purified PCR fragments could be annealed via these sticky ends upon denaturation and the single-stranded regions filled in by DNA polymerase. The resulting fragment was amplified in a second PCR step using only the flanking primers p322-forward and pQE-reverse. The final PCR-product as well as pQE10-Cal were digested with *Hind*III and *Eco*RI, the resulting 249 bp PCR fragment and the 4630 bp vector fragment isolated by PAGE, ligated and used to transfect competent *E. coli* M15 [pREP] cells. Colonies carrying the correct plasmid pQE-Cal-His6 were identified by restriction analysis of isolated plasmid DNA.

Expression and purification of recombinant calreticulin

Talon resin (Clontech), which contains a cobalt ligand to bind 6xHis proteins, was used for purification of recombinant calreticulin.

E. coli M15 [pREP4], harbouring pQE-Cal Δ Sig, were grown over-night at 37°C in LB broth containing kanamycin (30 mg/l) and ampicillin (200 mg/l). The culture was diluted 1:10 in LB broth and IPTG added to a final concentration of 2 mM to induce the lac promoter. After being vigorously shaken for 4 h at 37°C, 10 ml of the culture were harvested. The cells were suspended in 5 ml of a lysis buffer (0.1% Triton-X100, 20 mM Tris/HCl pH 8.0, 100 mM NaCl) and lysozyme was added to a final concentration of 0.5 mg/ml. The suspension was incubated at room temperature for 30 min. The cells were sonicated on ice at 200 W for 10 s bursts with intervals of 20 s for 10 cycles, to shear the bacterial chromosomal DNA. All subsequent steps of

purification were carried out at 4°C. After centrifugation of the lysate for 10 min at 12000 *g* the supernatant was added to 100 μ l of Talon resin and gently shaken for 1 h. The matrix was collected by centrifugation for 2 min at 5000 *g* and washed with 5 ml lysis buffer 3 times for 15 min each by gentle shaking. The protein was eluted by adding 80 μ l lysis buffer and 20 μ l 0.5 M ethylenediaminetetraacetic acid solution to the matrix and gently shaken for 30 min; the procedure was repeated for a second sample.

Serological cross reactivity with recombinant human calreticulin

Recombinant human calreticulin, and its N, P and L domains were kindly provided by Dr Paul Eggleton, expressed and purified as described by Kishore *et al.* (1997b). A full methodology is provided in Figure 5(b).

RESULTS

Development of an IgE-specific probe

Before proceeding to screen cDNA libraries, and to avoid the selection of 'false positive' pfus (i.e. nonallergens), it was important to develop a screening system which was proven to be specific for the IgE-based detection of antigens (allergens). Initially, to develop such a selective probe, an ELISA assay for reactivity to *Necator* antigens was tested to be IgE specific; the addition of myeloma IgE effectively competed with the monoclonal monospecific anti-IgE (Sigma) used in the assay in a dose-dependent fashion (Figure 1b), whereas human IgG, used at the same concentration range, failed to interfere with the assay. Furthermore, the assay was not inhibited to a great extent by heat-treated normal human serum (Figure 1c), which contains all immunoglobulin isotypes, apart from antigenically recognizable IgE, whose Fc ϵ domain is labile to the denaturing temperature used. These data supported the manufacturer's claims for the specificity of their reagent; not all anti-IgE reagents tested by us reached this standard; hence, it is crucial that such prior screening is conducted for selective allergen identification.

As we were targeting allergens from *Necator*, and not those from sympatric parasitic nematodes, the species

Figure 2 (a) Western blot of *N. americanus* (Na) antigen (20 μ g) *E. coli* (*E.*) lysate (20 μ g) and myeloma (ϵ) IgE (5 μ g), probed with plasma (1:20) from non-infected individuals (A+, Blood Transfusion Service) before (unabs) and after absorption (abs) with soluble *E. coli* lysate (5 mg/ml), or plasma (PNG) from hookworm-infected patients treated in an identical fashion. The upper panel was probed secondarily with anti-IgE, while the lower panel was probed with anti-IgG, each at a dilution of 1:1000. The heavy chain of IgE is approximately 70 kDa, and the molecular mass markers (m) are 200, 85, 66, 56, 39 and 35 kDa. The reactive band in the *Necator* antigen lane (arrowed) is approximately 60 kDa. (b) Western blots (approximately 10 μ g of protein per lane) of adult *N. americanus* homogenate (a) and ES products (b) were probed with individual human sera (diluted 1:20) and myeloma IgE (100 μ g/ml). Blots were developed using sheep anti-human IgE-alkaline phosphatase conjugate (diluted 1:1000). Numbers refer to individual serum samples collected from a field site on Karkar Island, Papua New Guinea. Molecular mass markers are indicated.

Figure 3 (a) DNA sequence, and predicted translation of *Nq-cal-1*. The signal sequence has a single underline, the putative C1q binding sites are double underlined, and the N and P-domain repeats highlighted (light shading, A repeats; dark shading, B repeats).

(b)

Na-cal-1	1	...MRSLVALLPVLCIAVAEVYFKEEF	LD	DSWKEKRWVQSKHKS	S	DYGEFVLSAGKYFGD
C.elegans.cal	1	...MKSLCLLAI VAVVSAEVYFKEEF	ND	ASWEKRWVQSKHKS	D	DFGAFKLSAGKFPDV
Ov.RAL-1	1	...MQLSLVGLVCFSAINAKIYFKEEF	SD	DDWEKRWVQSKHKS	D	DFGKWEISHGKFGD
Human.cal	1	MLLSVPLLLGLLAVAEPAVYFKEEF	LD	GDGWTSTSRWVQSKHKS	S	DFGKFVLSGKFGD
Drosophila.cal	1	MMWCKTVIIVLLATVGFI SAEVYFKEEF	ND	ENENWEDTWTYFKEEF	ND	MMWCKTVIIVLLATVGFI SAEVYFKEEF
S.mansoni.cal	1	..MLSI LLLTLLLSKYALGHEVWFSE	TF	PN	..ESIENWVQSTYNAEKQGE	FKVEAGKSPVD
Na-cal-1	56	ATRDQGMKTSQDAKFYSRAAKFPKA	FSNKGKTV	VIQFTVKHEQ	GIDCGGGYVK	VMSSDV
C.elegans.cal	55	ESRDQGLQTSQDAKFYSRAAKFDK	DSNKGKTV	VIQFTVKHEQ	GIDCGGGYVK	VMRADAD
Ov.RAL-1	57	AVKDKGLQTSQDAKFYSRAAKFDK	FSNKGKTV	VIQFTVKHEQ	DIDCGGGYVK	LMASDVN
Human.cal	60	EKKDKGLQTSQDAKFYSRAAKFDK	FSNKGKTV	VIQFTVKHEQ	DIDCGGGYVK	LPNSLD
Drosophila.cal	60	AEADKGLQTSQDAKFYSRAAKFDK	FSNKGKTV	VIQFTVKHEQ	DIDCGGGYVK	LPNSLD
S.mansoni.cal	57	PIEDLGLKTTQDAKFYSRAAKFDK	FSNKGKTV	VIQFTVKHEQ	DIDCGGGYVK	LPNSLD
Na-cal-1	116	LSDFHGETPYNVIMFGPDDICGP	TKKVVHDI	FYSYKGNHLLIKKDI	RCCKDDE	LTHLYTLILNP
C.elegans.cal	115	LGSDFHGETPYNVIMFGPDDICGP	TKKVVHDI	FYSYKGNHLLIKKDI	RCCKDDE	LTHLYTLILNP
Ov.RAL-1	117	LEDSHGETPYNVIMFGPDDICGP	TKKVVHDI	FYSYKGNHLLIKKDI	RCCKDDE	LTHLYTLILNP
Human.cal	119	QTDHGDSEYNIMFGPDDICGP	TKKVVHDI	FYSYKGNHLLIKKDI	RCCKDDE	LTHLYTLILNP
Drosophila.cal	119	QTDHGDSEYNIMFGPDDICGP	TKKVVHDI	FYSYKGNHLLIKKDI	RCCKDDE	LTHLYTLILNP
S.mansoni.cal	117	PKKFHGESPYKIMFGPDDICGMAT	TKKVVHDI	FYSYKGNHLLIKKDI	RCCKDDE	LTHLYTLILNP
Na-cal-1	175	DNT.EVQIDGGEKVESGLES	DWDLLP	PKKIKDPDAKKPEDWDER	REY	IDDADKKKPEDWDK
C.elegans.cal	174	DNTYEVKIDGGEKVESGLES	DWDLLP	PKKIKDPDAKKPEDWDER	REY	IDDADKKKPEDWDK
Ov.RAL-1	177	DNTYEVQIDGGEKVESGLES	DWDLLP	PKKIKDPDAKKPEDWDER	REY	IDDADKKKPEDWDK
Human.cal	179	DNTYEVKIDGGEKVESGLES	DWDLLP	PKKIKDPDAKKPEDWDER	REY	IDDADKKKPEDWDK
Drosophila.cal	179	DNTYEVKIDGGEKVESGLES	DWDLLP	PKKIKDPDAKKPEDWDER	REY	IDDADKKKPEDWDK
S.mansoni.cal	177	NKKYEVLDNAKVEEGSLED	DDMLP	PKKIKDPDAKKPEDWDER	REY	IDDADKKKPEDWDK
Na-cal-1	234	PEHIPDPDAKKPEDWDD	EMDGEWEP	PMIDNP	EYKGEWKP	KQIKNPAYKGGWIHP
C.elegans.cal	234	PEHIPDPDAKKPEDWDD	EMDGEWEP	PMIDNP	EYKGEWKP	KQIKNPAYKGGWIHP
Ov.RAL-1	237	PEHIPDPDAKKPEDWDD	EMDGEWEP	PMIDNP	EYKGEWKP	KQIKNPAYKGGWIHP
Human.cal	239	PEHIPDPDAKKPEDWDD	EMDGEWEP	PMIDNP	EYKGEWKP	KQIKNPAYKGGWIHP
Drosophila.cal	239	PEHIPDPDAKKPEDWDD	EMDGEWEP	PMIDNP	EYKGEWKP	KQIKNPAYKGGWIHP
S.mansoni.cal	237	PKTIPDMDAKKPEDWDD	AMDGEWEP	PQKDNPEYKGEW	TPRRID	NPYKGEWKP
Na-cal-1	294	YTPDDDELVLKDWGAIGFDLWQVKS	GTIFDNI	ITDSVEEA	EAHAAET	FDKLPVKEKELK
C.elegans.cal	294	YTPDDDELVLKDWGAIGFDLWQVKS	GTIFDNI	ITDSVEEA	EAHAAET	FDKLPVKEKELK
Ov.RAL-1	297	YTPDDDELVLKDWGAIGFDLWQVKS	GTIFDNI	ITDSVEEA	EAHAAET	FDKLPVKEKELK
Human.cal	299	YSPDPSIYADNFGVGLDLWQVKS	GTIFDNI	ITDSVEEA	EAHAAET	FDKLPVKEKELK
Drosophila.cal	299	YTPDDDELVLKDWGAIGFDLWQVKS	GTIFDNI	ITDSVEEA	EAHAAET	FDKLPVKEKELK
S.mansoni.cal	297	YKHPPELYVLNDIGYVGFDLWQVKS	GTIFDNI	ITDSVEEA	EAHAAET	FDKLPVKEKELK
Na-cal-1	354	EKADEENRKKMEEA	KKQEE	EKKKEE	KEEKEE	DEEKEE
C.elegans.cal	354	EKADEENRKKMEEA	KKQEE	EKKKEE	KEEKEE	DEEKEE
Ov.RAL-1	357	EKADEENRKKMEEA	KKQEE	EKKKEE	KEEKEE	DEEKEE
Human.cal	359	EKADEENRKKMEEA	KKQEE	EKKKEE	KEEKEE	DEEKEE
Drosophila.cal	358	EKADEENRKKMEEA	KKQEE	EKKKEE	KEEKEE	DEEKEE
S.mansoni.cal	357	QSSAKDDKEEA	EETKEE	KEEKEE	KEEKEE	DEEKEE

Figure 3 (b) Pileup of the predicted protein encoded by *Na-cal-1* and other calreticulin-like proteins. Genbank accession numbers: *N. americanus* calreticulin, A1006790; *C. elegans* calreticulin, X59589; *O. volutus*, RAL-1-M20565; *Drosophila melanogaster* calreticulin, X64461; *S. mansoni* calreticulin, M93097.

specificity of the assay (Pritchard & Walsh 1995) was confirmed; soluble *Necator* ES inhibited the ELISA assay while soluble *Ascaris* PCF, the only other known allergenic nematode parasite of this population (Pritchard *et al.* 1990) failed to show any inhibitory activity (Figure 1d). Having established the specificity of the ELISA for *Necator* IgE, the 8 most reactive samples from the study population (OD range 0.2–0.72) were pooled and used to screen a Western blot loaded with myeloma IgE (positive control for anti-IgE), *N. americanus* homogenate (target) and solubilized *E. coli* (the library is expressed in *E. coli*; this is control for background activity to the host bacterium). Before probing, the plasma pool ($n=8$), and second antibody, were rigorously absorbed with a soluble preparation of lysed *E. coli* (5 mg/ml final concentration).

The results of the Western blot are shown in Figure 2(a). The upper panel, developed with anti-IgE, indicates the reactivity of a plasma pool from nonexposed individuals (A+, Blood Transfusion Service), before (unabs.) and after (abs.) absorption with *E. coli*, then subsequently probed with anti IgE. A+ background activity was absent. The second antibody was shown to be strongly reactive to IgE.

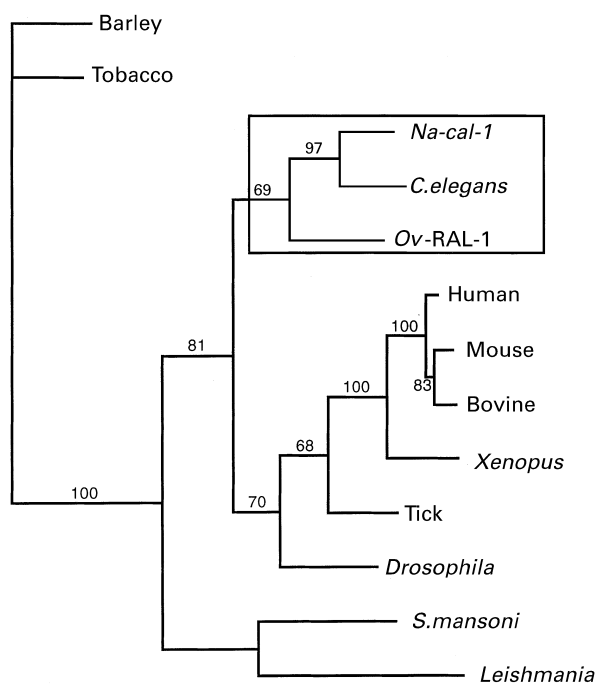


Figure 4 Phylogram showing the relationship between the predicted protein encoded by *Na-cal-1* and other calreticulin-like molecules. Nematode sequences are boxed. GenBank accession numbers: mouse calreticulin, X14926; bovine calreticulin, L13462; *Xenopus laevis* calreticulin, X67597; tick calreticulin, U07708; *Leishmania donovani* calreticulin, U49191. Percentage support for each node is shown on the preceding branches. Nodes with less than 50% support are collapsed to form polytomies — cross reactivity.

However, the second antibody did not react with myeloma preparations representing the remaining immunoglobulin isotypes (data not shown).

When the procedure was repeated for post-hookworm infection plasma from Papua New Guinea (PNG), substituting for A+, a significant reaction was seen against the hookworm preparation, predominantly around 50 kDa. No background was seen against *E. coli*; this is an important observation, as the library to be screened is expressed in *E. coli*.

In comparison, probing blots with a second antibody to IgG revealed a more complex pattern of reactivity (Figure 2a, lower panel). Background reactivity against *E. coli* was strong, and difficult to remove by absorption, making it impossible to screen libraries with PNG plasma and IgG probes. The hookworm antigen recognition profile with IgG was typical of that seen on previous occasions (Carr & Pritchard 1986) with a predominant antigen seen at 32.5 kDa. A+ plasma also showed a degree of IgG reactivity against *N. americanus*, to antigens of higher molecular mass.

Significantly, when individual samples were tested for reactivity against hookworm secretions and whole worm extracts, differential patterns were seen, suggesting that a number of allergenic molecules exist (Figure 2b). Furthermore, attempts to develop blots directly with myeloma IgE were unsuccessful, confirming the reactivity of the IgE probe for allergens in worm extracts and secretions, and the absence of reactivity due to IgE-binding proteins.

Probing cDNA library: *Na-cal-1* encodes a calreticulin-like molecule

Having established the specificity of the commercial anti-IgE in ELISA for the detection of IgE antibodies against *N. americanus* adult homogenate, and the strength and relative exclusivity of the 'anti-allergen' signal on Western blot, cDNA libraries were successfully probed and positive pfu taken to quaternary screen excision, and sequencing. Consequently the DNA sequence of the allergen designated *Na-cal-1* is shown in Figure 3(a) (EMBL Nucleotide sequence database accession AJ006790). The protein encoded by *Na-cal-1* cDNA has strong homology to mammalian calreticulins and calreticulin-like proteins from a range of phyla. *Na-cal-1* is 401 amino acids including a signal peptide of 16 amino acids and has a predicted molecular weight of 46.5 kDa, although calreticulin runs atypically at 60 kDa on SDS-PAGE (Opas *et al.* 1996, Spiro *et al.* 1996). Proteins of similar molecular mass were observed in Western blotting with IgE from post-infection plasma (Figure 2b). *Na-cal-1* shares 79%, 70% and 66% identity with calreticulin-like protein from the free-living nematode *Caenorhabditis elegans*, Ov-RAL-1 from the filarial nematode

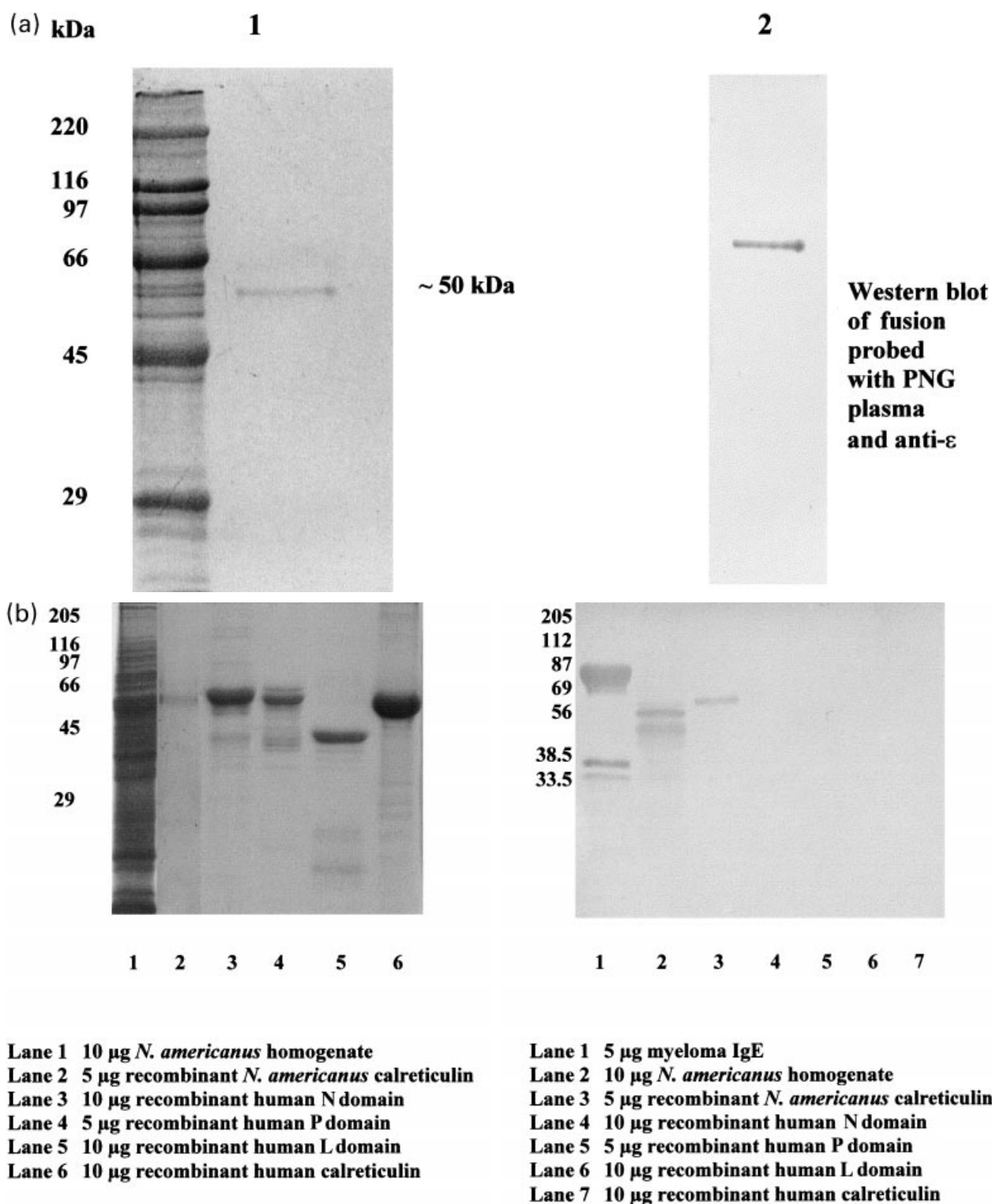


Figure 5 (a) Expression of recombinant calreticulin. The cDNA for *Na-cal-1* was subcloned into pQE10, expressed as a 6 His-tagged fusion, the fusion protein solubilized in 8 M urea, and analysed by SDS-PAGE. The 50 kDa fusion polypeptide was recognized by IgE from post infection plasma. (b) Lack of serologic cross-reactivity between recombinant human and hookworm calreticulin. Western blots were conducted with recombinant human calreticulin, its N, P and L domains (fused to maltose-binding protein, Kishore *et al.* 1997b), adult *N. americanus* homogenate, recombinant *Necator* calreticulin, and post *N. americanus* infection plasma (developed with anti-IgE as described above). No cross reactivity was seen between recombinant calreticulins from host or parasite. Panel 1, Coomassie Blue; panel 2, Western blot with anti-IgE.

Onchocerca volvulus and human calreticulin, respectively. *Drosophila* and *S. mansoni* calreticulins are also included for comparison (Figure 3b). Phylogenetic analysis of *Na-cal-1* and other calreticulin-like molecules showed strong support for a distinct clade containing the nematode proteins (Figure 4). Further *Necator* allergens have since been identified as having homology to a globin (body wall isoform) of *Nippostrongylus brasiliensis* and a *C. elegans* ubiquitin conjugating enzyme.

When the cDNA was expressed in *E. coli*, the affinity purified 6 his-tagged fusion protein resolved at approximately 50 kDa on SDS-PAGE, and was recognized in IgE-specific Western blot (Figure 5a). The recombinant protein will lack the tertiary structure of the native molecule, and might be expected to resolve more closely to its true molecular mass. No cross-reactivity was seen with recombinant human calreticulin or its N, P and L domains (Figure 5b), although patient IgE continued to recognize *N. americanus* homogenate and recombinant *Necator* calreticulin.

DISCUSSION

Allergens are, by definition, antigens recognized by IgE. Therefore, the development of specific serological probes, based on the recognition of antigens by IgE, allows the identification and characterization of allergens. This approach was used to identify an antigen from the highly allergenic human pathogen *N. americanus*, and exploited the elevated levels of specific IgE (Pritchard *et al.* 1993) in the plasma of patients from a geographical location where this parasite is highly endemic. By establishing what is considered to be an IgE-specific ELISA, and by fine-tuning the system to obtain a very strong signal on Western blot, a cDNA library made from the adult stage of the parasite has been screened, and a positively identified pfu cloned for sequencing.

Clones screened in this way for immuno-reactivity have been identified as calreticulin. Sequence analysis revealed the presence of characteristic P-domain repeats (for example; calreticulin repeats A: amino acid sequence PXXIXDPDAXKPEDWDE, and repeats B: amino acid sequence GXWXPPIXNPXYX) and a KDEL-like ER retrieval signal at the C-terminus (Krause & Michalak 1997). Furthermore, the putative N-domain C1q binding motifs, VQSKHKSDY (IESKHKSDY), FSYKGKN (FNYKGKN) and KDIRCKDD (KDIRCKDD — most potent inhibition of C1q haemolysis — $I_{50} = 150 \mu\text{M}$) (Eggleton and Reid, personal communication) and P-domain equivalents, WDEREYIDD (WDERAKIDD) seem relatively well conserved (human homologues in parenthesis homology in italics). Significantly, the *Necator* sequence lacks the human N-domain DEEKDKG Clq binding motif,

and shares an overall identity of 66% with its human counterpart, compared with 79% with *C. elegans* and 70% with *O. volvulus* calreticulins. The homology with human calreticulin was insufficient to counteract the antigenicity of *Necator* calreticulin in human patients.

Having established that the molecule recognized by post-infection IgE is almost certainly calreticulin, the role of this molecule in the host-parasite relationship needs to be addressed. What is calreticulin? Calreticulin is an abundant, high capacity Ca^{2+} -binding protein found in every cell of higher organisms (Krause & Michalak 1997).

Numerous functions have been suggested for calreticulin. It may have a role as a molecular chaperone in the endoplasmic reticulum. (Nanseef *et al.* 1995, Peterson *et al.* 1995), be involved in Ca^{2+} signalling, acting as a Ca^{2+} buffer (Mery *et al.* 1996), contribute to the regulation of cell adhesion (Opas *et al.* 1996, Coppolino *et al.* 1997) and be involved in the regulation of gene expression (Michalak *et al.* 1996).

Calreticulin function also has implications in parasite biology (Nakhasi *et al.* 1998). Calreticulin is found in nematodes and, for example, constitutes the RAL-1 41 kDa larval antigen of *O. volvulus* and a 58-kDa antigen from *Schistosoma japonicum* (Khalife *et al.* 1994). The fact that, in the present paper, a calreticulin-like molecule is recognized by post-infection plasma would strongly suggest that it is secreted during feeding by the adult parasite in the gut lumen. Calreticulin is found in secretions, for example in flea saliva (Jaworski *et al.* 1996) and the analysis of culture supernates from adult worms, using $^{45}\text{Ca}^{2+}$, should indicate whether a calcium-binding protein is indeed secreted by this pathogen (Opas *et al.* 1996). Meanwhile, we can only speculate on its presence in secretory products, its role in the host-parasite relationship, and the molecular basis for its allergenicity. For example, it is released by neutrophils during inflammation, can act as an auto-antigen in SLE (Kishore *et al.* 1997a,b), and is contained in the lytic granules of cytotoxic lymphocytes (Dupuis *et al.* 1993). Once released, it may alter immune function by binding to the Clq component of the complement system. On the basis of this information, hookworm calreticulin can be ascribed an immunomodulatory function, given its Clq binding domains (above) and hookworms are renowned for their durability in the face of potentially powerful immune responses (Hotez & Pritchard 1995, Pritchard 1995). This is a phenomenon which can be investigated experimentally with native or 'active' recombinant calreticulin.

Hookworms also have an antihaemostatic action (Pritchard 1995, Cappello *et al.* 1996, Stanssens *et al.* 1996) and hookworm calreticulin may possess such potential (Stewart *et al.* 1997) if secreted into the intestinal

tissues. But why should calreticulin be allergenic? Several hypotheses have been put forward to explain the molecular basis for allergenicity, and given the disparate physico-chemical nature of allergens, there is little doubt that several hypothesis can be catered for. Given the tendency for calreticulin-like molecules to associate with components of the complement system, it is possible that the parasite allergen forms almost pseudo-immune complexes with complement components, leading to aberrant antigen presentation and the skewing of T cell development to Th2 (Pritchard *et al.* 1997).

Calreticulin has the potential to act as a molecular chaperone for hookworm ES products (as it does, for example, for endomannosidase in the Golgi, Spiro *et al.* 1996), which are glycosylated and react strongly with wheat-germ agglutinin; which is indicative of the presence of GlcNAc (*N*-acetyl glucosamine). Whether such chaperoned complexes would be more allergenic is unknown. The lectin properties of calreticulin (a property of the P-domain) could also result in the cross-linking of cell-surface molecules involved in the regulation of IgE synthesis, e.g. CD21 and mIgE by sCD23. (Hewitt *et al.* 1995) CD4 T cells can also be directly induced to produce cytokines by lectins of appropriate specificity (Blasco *et al.* 1995), and some parasite lectins induce Th2 activity (Talamas Rohana *et al.* 1995). Furthermore, given the sugar-specificity of the lectin domains of calreticulin (Glc₁, Man₉ GlcNAc₂, Krause & Michalak 1997) and the pattern of glycosylation of human IgE, which is thought to possess high mannose side chains as well as a mannose and GlcNAc-rich biantennary side chain complex (Baenziger *et al.* 1974a,b), it is also conceivable that hookworms calreticulin is able to directly and innately induce Type 2-cytokine production from human basophils and mast cells in the manner already described for proteinase allergens (Machado *et al.* 1996). In essence, the secretion into the gastrointestinal mucosa of allergens such as calreticulin, in the presence of powerful pro-allergenic proteinases, could result in the creation of a super-allergenic Type 2 cytokine milieu. It is clear therefore that the identification of calreticulin as a hookworm allergen forms the basis for a series of powerful experiments to provide data which may increase our understanding of the ways parasitic helminths dysregulate the IgE network. Furthermore, the implication that IgE responses to *N. americanus* may be host-protective and, possibly, of diagnostic value (Pritchard & Walsh 1995) paves the way for the exploitation of recombinant hookworm calreticulin as a vaccine and/or diagnostic reagent. An 'venom allergen-like' molecule cloned from another hookworm, *Ancylostoma caninum* (Hawdon *et al.* 1996) acts as a protective antigen in vaccination experiments in mice (Ghosh *et al.* 1996).

ACKNOWLEDGEMENTS

The authors would like to thank Rupert Quinnell, Mari Nowell, James Griffin and Andrew Raiko for their invaluable help in Papua New Guinea, and Dr Franco Falcone and Helmut Haas (Borstel, Germany) for their input into the discussion of lectin activity. We are also indebted to Dr Paul Eggleton and Dr K.B.M.Reid of the MRC Immunochemistry Unit, University of Oxford, for the gift of recombinant human calreticulin and related antisera, and for their valuable advice. Dr Matthias Eberl of Giessen provided the first recombinant calreticulin sample. The project was supported by the award of a Research Leave Fellowship to D.I.Pritchard by the Wellcome Trust, which was held at the CMHT, Leicester, UK. I thank the staff of this centre for their help, advice and cooperation during my enjoyable and productive stay.

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