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VERSITA

RESEARCH NOTE

# Vaccination of rats against the rodent hookworm *Nippostrongylus brasiliensis* with a recombinant superoxide dismutase fails to protect against infection

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## Abstract

Anti-oxidant enzymes including superoxide dismutase (SOD) protect cells from damage by oxygen radicals produced during respiration. There is also a substantial body of evidence that anti-oxidant enzymes facilitate the survival of parasitic helminths, including gastrointestinal nematodes, within the host. Superoxide dismutase has been shown to be released by a variety of parasitic helminths and may protect them from host mediated oxidative immune responses. As it may play a parasite protective role during infections SOD has been investigated as a vaccine candidate in a range of helminth parasites including *Schistosoma mansoni*, *Acanthocheilonema viteae* and *Haemonchus contortus*. Here, we sought to evaluate the protective potential of SOD against the rat hookworm *Nippostrongylus brasiliensis*, a commonly utilised laboratory infection, as a vaccination model. A cytosolic SOD from this parasite, with high sequence homology to a putative extracellular form of the enzyme was cloned and then expressed in bacteria. The resultant recombinant protein was assessed for enzyme activity and used to immunise rats prior to a single challenge infection with the parasite. No protection was observed and monitoring systemic and mucosal antibody responses and mast cell protease levels in superoxide dismutase vaccinated rats suggested that this recombinant SOD was only weakly immunogenic.

## Keywords

*Nippostrongylus brasiliensis*, nematode, hookworm, superoxide dismutase, recombinant vaccine

A substantial body of evidence suggests that helminths, including gastrointestinal nematodes, utilise anti-oxidant enzymes to facilitate their survival within the host. Elevated levels of key anti-oxidant enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) have been correlated with *Nippostrongylus brasiliensis* persistence in the intestine of its rodent host (Smith and Bryant 1986, Batra *et al*. 1990, Knox and Jones 1992). Increased free-radical production by peritoneal leukocytes was associated with the rejection of *N. brasiliensis* and *Fasciola hepatica* (Smith and Bryant 1989a, Smith *et al*. 1992) while rejection of the former was inhibited by the administration of anti-oxidants (Smith and Bryant 1989b). Several anti-protozoal agents (Docampo and Moreno 1986, Smith *et al*. 1988) and some anthelmintic agents may act as free-radical generators (Bennet-Jenkins and Bryant 1996). Taken together, these reports suggest that free-radical generation may be an important effector mechanism for worm expulsion. Thus, parasite free-radical scavenging enzymes such as SOD may be crucial for parasite survival and may be appropriate targets for the development of novel immune or chemical based control procedures.

There is evidence that helminth parasites secrete SOD, extracellular forms of the enzyme have been identified in various nematode species (Batra *et al*. 1990, Callahan *et al*. 1993, Henkle-Dührsen *et al*. 1994, Tang *et al*. 1994, Liddell and Knox 1998). SODs may participate in immune evasion of infection and hence be potential target molecules for anti-helminth vaccines. Notably, sera from cattle vaccinated against the bovine lungworm *Dictyocaulus viviparus* using the irradiated larval vaccine Dictol recognised and neutralised the activity of the parasite excreted/secreted SOD (Britton *et al*.

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1994). Moreover, worm burdens in jirds vaccinated with a recombinant extracellular SOD from the filarial nematode *Acanthocheilonema viteae* were reduced by 30% compared to controls, the antigen being delivered orally as a live vaccine in aroA attenuated *Salmonella typhimurium* (Latteman *et al*. 1999). In addition, vaccination of mice with naked DNA constructs containing *Schistosoma mansoni* Cu/Zn cytosolic SOD gave significant levels of protection against infection compared with a control group (LoVerde *et al*. 2004). Vaccination with SOD but not with GPX also resulted in elimination of adult worms (LoVerde *et al*. 2004). Lambs vaccinated with an enzymically active recombinant cytosolic SOD from *H. contortus* were partially protected (20% reduction in worm burden) against a challenge infection (Liddell and Knox 1998).

Here, we sought to evaluate the protective potential of SOD against *N. brasiliensis* as a vaccination model. A cytosolic SOD (SODc) with high homology to a putative extracellular form was cloned and then expressed in bacteria. The resultant recombinant protein was assessed for enzyme activity and used to immunise rats prior to a single challenge infection with the parasite.

Larvae (L3 stage) and adult worms were collected from faecal culture or rat intestine respectively using a Baermann apparatus. Somatic proteins were extracted in ice cold PBS using a glass/glass homogeniser and separated using 10% SDS-polyacrylamide gel electrophoresis under reducing conditions. Separated proteins were then transferred onto a PVDF membrane and probed with serum raised against *H. contortus* SOD (Liddell and Knox 1998), previously shown to recognise *N. brasiliensis* SOD (data not shown).

Antiserum to *H. contortus* SODc recognised two protein bands on blots of L3 and adult *N. brasiliensis* somatic extracts (Fig. 1, panel B, lanes 1 and 2, respectively). These were visible as a doublet with the lower molecular weight protein, being more abundant. In the L3 extract the both lower and higher molecular weight proteins appeared more abundant compared with the adult, consistent with the higher SOD activity demonstrated in extracts from the L3 (Knox and Jones 1992). This difference in activity may be ascribed to a higher rate oxidative metabolism in the more active L3 (Knox and Jones 1992). In addition, the larger molecular weight protein of the L3 appeared to separate at a slightly different size to that of the adult, possibly indicating different isoforms of the secreted protein, as described previously (Knox and Jones 1992).

The SODc gene sequence was amplified by rapid amplification of cDNA ends (RACE) PCR. Parasites isolated as described above were snap frozen in liquid $N_2$, and stored at –80°C until used for total RNA extraction using standard methods. An *N. brasiliensis* adult cDNA was prepared using a SMART cDNA synthesis kit (Clontech, PT 3001/PR92334, K1051-1). Gene specific primers for *N. brasiliensis* SOD were designed based on partial *N. brasiliensis* SOD sequences obtained by PCR, using degenerate primers based on conserved regions in alignments with other nematode SOD (data not shown). The 5' gene sequence was isolated using standard reaction conditions with a gene-specific antisense primer (5'-GTG ATT TCG AAA TGA GCA ACA CCA CTG GC-3') and a cDNA 5' incorporated oligonucleotide, SMART III (5'-AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ATG GCC GGG-3'). An overlapping 3' gene sequence was amplified by using a gene-specific sense primer (5'-CTG TAT CTC TTG GG ACC TCA CTT TAA-3') and an antisense primer against a cDNA 3' incorporated oligonucleotide, CDS III (5'-ATT CTA GAG GCC GAG GCG GCC GAC ATG-d(T).

DNA sequencing was performed on amplification products cloned in pGEM-T (Promega, A3600) and sequence analysis

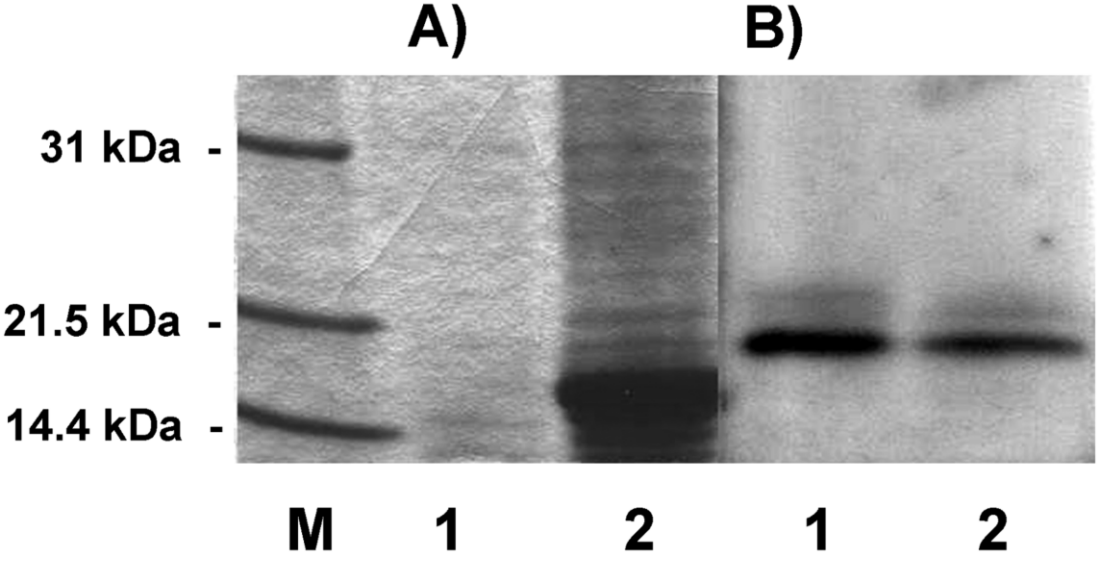


**Fig. 1.** Panel **A)** a Coomassie stained PAGE gel and **B)** the corresponding Western blot of: (**1**) L3 and (**2**) adult *N. brasiliensis* somatic extracts developed with anti-*H. contortus* SODc serum and ECL+ Plus. The dark bands on the blot indicate the presence of SOD isoforms in the somatic extracts

was carried out using online tools (http://ca.expasy.org/prosite/; http://www.ebi.ac.uk/).

An open reading frame encoding a 159 amino acid protein was derived and confirmed by sequencing full-length PCR products and alignment with other nematode SOD proteins (Fig. 2). The predicted sequence showed high levels of homology to other nematode cytosolic superoxide dismutase (SODc) sequences (Fig. 2) and contained two regions characterised as Cu/Zn SOD signatures. The first of these contained the copper atom binding site and the second a conserved cysteine residue involved in disulfide bonding. The sequence did not contain an N-terminal leader sequence enabling extracellular transport, which is indicative of protein secretion and which is common to extracellular SOD (SODe) of other nematodes (Henkle-Dührsen *et al*. 1994, Liddell and Knox 1998).

Western blot analysis of L3 and adult parasite extracts (Fig. 1, panel B) indicated that an extracellular form of SOD (SODe) may be produced by *N. brasiliensis* and release of SOD by *N. brasiliensis* during *in vitro* culture has been described (Batra *et al*. 1993). Other work, however, has demonstrated that a putative extracellular form and the cytosolic form, described here, are essentially identical (Young 1996, Ball, unpublished data).

A full-length cDNA encoding SOD was PCR amplified with *Bam*H1 and *Eco*R1 restriction sites incorporated into the sense (5'-CGT AG$^{\downarrow}$G ATC CGA TGA GCA ACG GAG CAG TG-3') and antisense (5'-GTC TAG$^{\downarrow}$ AAT TCA GTC ACT GGG GAG CAG C-3') primer permitting cloning in frame into the pET22b + bacterial expression vector (Novagen, 69744-3). The constructs were confirmed by PCR and protein expression was induced in BL21 competent cells (Stratagene, 200133). The recombinant SOD was subsequently purified from soluble bacterial culture fractions by low pressure ion exchange column chromatography using a Mono Q Sephadex column and eluted in 10 mM Tris/HCl pH 7.5 (Liddell and Knox 1998). A protein of approximately 20 kDa was abundant in protein fractions from induced BL21 cells containing the SOD insert (Fig. 3, panel A). The purified protein was recognised on a Western blot by serum raised against *H. contortus* SODc (Liddell and Knox 1998; Fig. 3, panel B) and demonstrated SOD activity visualised with an in-gel staining procedure (Beauchamp and Fridovich 1971; Fig. 3, panel C). Activity was inhibited by the addition of 2 mM EDTA (Fig. 3, panel C), a chelating agent which inhibits Cu/Zn SOD. In addition, serum IgG from rats repeatedly infected with 200 L3 larvae over the course of 4 weeks recognised recombinant SOD on a blot, present at approximately 20 kDa (Fig. 3, panel D).

Seven male Wistar rats 2–3 months old were immunised subcutaneously with 10 µg SOD in 0.2 ml PBS with 10 µg Quil A as adjuvant (Superfos Biosector, Batch L77-194). Another group of seven rats were not immunised and one further control group was immunised with 10 µg Quil A alone. Boosts of the same vaccination dose were given at 4 and 8 weeks. Two weeks after the final boost, all groups were given a single challenge infection of 25 *N. brasiliensis* L3 larvae in 0.2 ml PBS injected subcutaneously above a hind limb (Ball *et al*. 2007). Challenge doses for the experiment were chosen to represent a low level (sub-acute) single challenge dose (Ball *et al*. 2007).

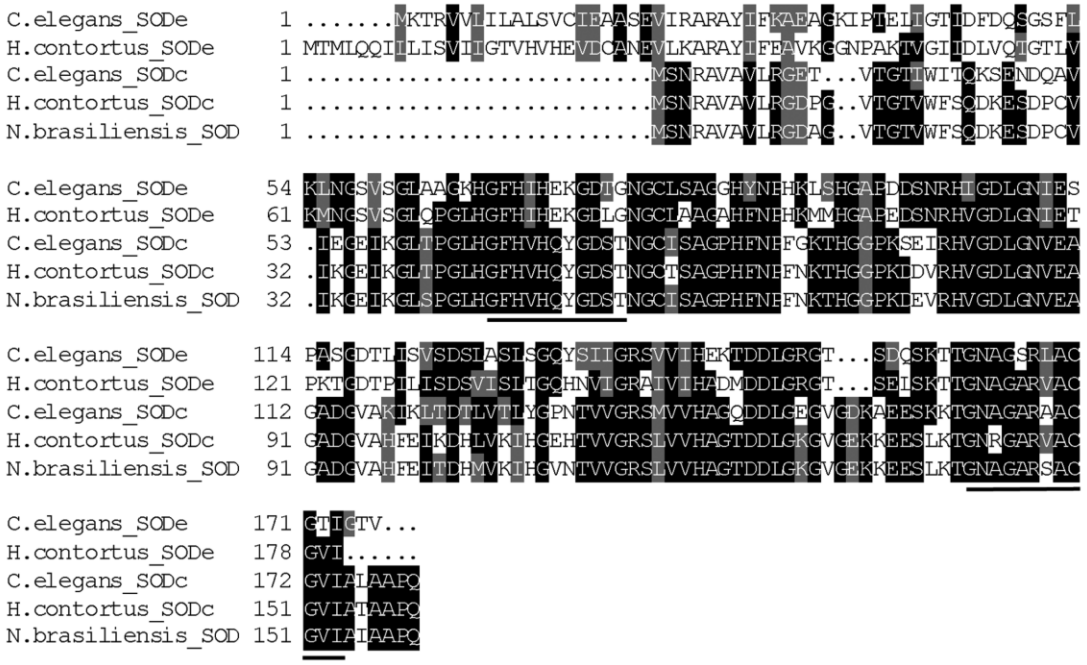


**Fig. 2.** The predicted *N. brasiliensis* SOD protein sequence aligned against other known nematode SOD protein sequences from: *Caenorhabditis elegans* and *H. contortus*. The level of amino acid homology is indicated by shading: ■ – 100%; ■ – conserved substitution. Suffix C and E refer to cytoplasmic and extracellular homologues of the enzyme respectively. Underlined residues are conserved Cu/Zn superoxide dismutase signatures

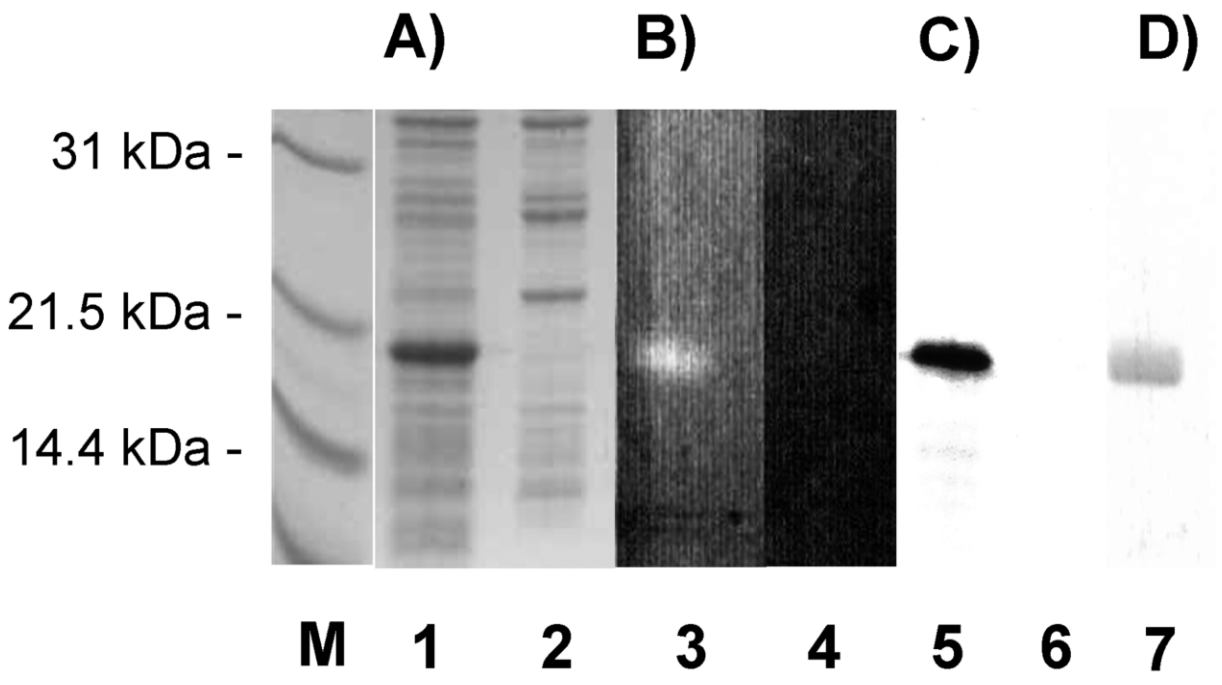


**Fig. 3.** Panel **A)**. Coomassie blue stained PAGE gel analysis of: (**1**) Protein extract from cells transformed with the SOD insert; (**2**) extract from cells transformed with pET22b + only (control). The expressed protein is evident at ~20 kDa. Panel **B)**. Immunoblot of a similar gel to panel A) probed with rabbit anti-*H. contortus* SOD antiserum; (**3**) the brown banding indicates the recognition of recombinant SOD but (**4**) not in a pET22b + control. Panel **C)**. (**5**) A PAGE gel of the *N. brasiliensis* recombinant SOD protein stained for SOD activity. Enzymically active SOD protein is visible as white/clear patches against the dark background; (**6**) SOD activity at 20 kDa was inhibited by the addition of 2 mM EDTA a chelating metalo-enzyme inhibitor. Panel **D)**. Recombinant SOD blotted on PVDF and probed with serum from a hyper-immune rat; (**7**) strong reactivity was evident at ~20 kDa, coincident with the SOD protein

The progress of infection was monitored for 11 days using faecal egg counts (FEC) measured using a modified saline flotation method (Jackson 1974). Results were expressed as eggs/g faeces for each cage of 2–3 animals giving a mean of 3 samples for each group. A cumulative mean egg output for the course of infection was calculated and compared between groups using analysis of variance (one way ANOVA). Egg counts from control and vaccinated groups are shown in Figure 4. The mean FEC from animals vaccinated with SOD was slightly elevated compared to that of the controls, but the difference was not significant. At necropsy on day 11 post infection, no adults were detected in the small intestine of any of the animals.

Antigen-specific immunoglobulins in serum samples, collected by tail bleed during each trial following the third immunisation and at necropsy (post challenge) were measured using an enzyme-linked immunosorbent assay (ELISA; Ball *et al*. 2007). Mucosal extracts were prepared for ELISA analysis as described previously (Ball *et al*. 2007). ELISA plates were coated with 1 µg/ml of SOD and Ig isotypes detected using secondary antibodies specific for rat IgG (Sigma, R-9037), IgG1 (Serotec, MCA194), IgG2a (Sigma, R-0761), IgA (Sigma, R-0636) and IgE (Serotec, MCA193). Specific antibody levels were expressed as the titre required to obtain the mean OD of a dilution of control sera (1 in 20) or mucosal homogenate (10 mg/ml). The specificity of antibody from immunised animals for the immunising antigens was confirmed by Western blot (data not shown).

The level of recombinant SOD specific antibody of the various isotypes measured in the serum of SOD vaccinated animals was not significantly different from the control or adjuvant immunised group with titres ranging from 0 to 357 (Fig. 5). The two highest responders were weakly positive as judged by immunoblot and diamino-benzidine staining although the intensity of the reaction was too weak for photographic reproduction (data not shown). No SOD specific antibody was detected in the mucosae of vaccinated and control animals at necropsy, 11 days after infection (data not shown).

As a group of seven rats immunised subcutaneously with a recombinant acetylcholinesterase (AChE) in an identical experiment had AChE specific antibody titres in the range 2,800 to 5,000 (Ball *et al*. 2007), it was therefore surprising that here vaccination with recombinant SOD failed to stimulate consistent antibody responses of the same magnitude in recipient animals.

This observation, in isolation, would indicate that the SOD might be only weakly immunogenic, perhaps due to MHC restriction of antigen recognition as has been described previously in rodents during *N. brasiliensis* infection (Kennedy *et al*. 1990, 1991). However, this is unlikely as the recombinant protein was recognised by serum from a hyper-immune Wistar rat repeatedly infected with *N. brasiliensis* L3 (Fig. 3,

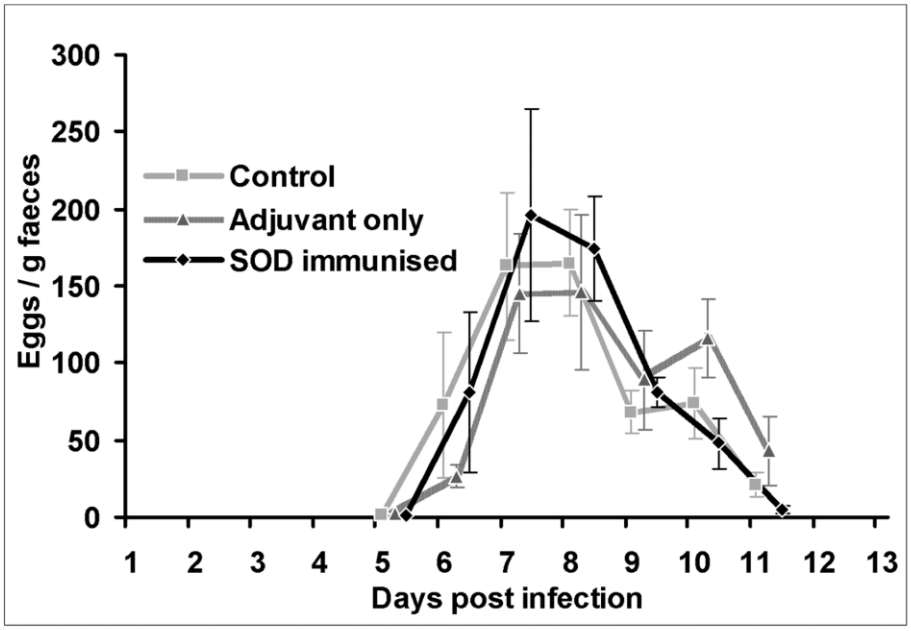


**Fig. 4.** The faecal egg count from control (unvaccinated) and adjuvant and SOD vaccinated animals over 11 days following challenge infection with 25 *N. brasiliensis* L3 larvae. The data represent the mean of faecal egg counts performed on three pooled samples from each group at each timepoint (± the standard error). There were no significant differences between the groups

panel D), suggesting that the native protein was immunogenic during infection, and a further four similar infection sera had anti-SOD titres ranging from 1707 to 4642 in the same ELISA used here (data not shown). When MHC restriction of antigen recognition was tested by immunising a second strain of rat in the same manner these also showed no antibody response and no protection from infection indicating that this is unlikely to be the cause for the lack of response (data not shown).

Another possibility is that the recombinant *N. brasiliensis* SODc was only weakly immunogenic because it is closely related at the sequence level to rat SOD (58% identity; 73% similarity). However, Carvalho-Queiroz *et al*. (2004) undertook an analysis of parasite-specific B epitopes in a cytosolic SOD derived from *S. mansoni*, the enzyme being considered as a potential vaccine component (LoVerde *et al*. 2004). This work showed that BALB/c mice developed high levels of antibodies to a peptide spanning residues 64 to 83 (*S. mansoni* numbering). This region lies between two SOD signature sequences and contains 3 conserved residues required for Cu/Zn ion binding. The *S. mansoni* SOD showed 58/59% identity and 65/64% similarity to the murine and human enzymes over the entire SOD sequence indicating that the *N. brasiliensis* SOD is not unusually identical to the rat enzyme. Similar identity and similarity figures were obtained when the region corresponding to the *S. mansoni* 64 to 83 residue region was analysed (data not shown). Therefore, this sequence analysis does not highlight any obvious reasons why the *N. brasiliensis* SOD should be so weakly immunogenic but nonetheless, is an important observation in the context of recombinant protein vaccine testing.

Rat mast cell protease II (RMCP II) levels, an indicator of mast cell activation, were quantified from sera and mucosal tissues by ELISA as outlined in Miller *et al*. (1983), using a RMCP II ELISA kit (Moredun Scientific Limited) according to manufacturer's instructions. RMCP II levels in serum or small intestinal mucosa of SOD vaccinated animals at necropsy did not significantly differ from control animals, ranging between 800–1,000 ng/ml and 50–100 ng/mg soluble protein, respectively.

In summary, vaccination of rats with a recombinant Cu/Zn SOD enzyme of *N. brasiliensis* failed to stimulate a marked host immune response and vaccination did not confer protection from infection, as judged by comparing the cumulative faecal egg outputs from vaccinated and non-immunised animals. This is an important result in the context of anti-parasite recombinant protein vaccine development. SOD has been proposed as a vaccine candidate in *S. mansoni* infection (LoVerde *et al*. 2004) and has also been attracting attention in livestock nematodes (Liddell and Knox 1998). For many parasites, recombinant protein production and testing in a laboratory model is the first step in vaccine development. The lack of immunogenicity observed here may reflect subtle conformational changes arising from aberrant recombinant protein folding but not affecting enzymatic activity. Alternatively, different responses to native and recombinant SOD may reflect antigen presentation and T-cell priming. In the course of natural infection, antigen presenting cells in the skin, lungs and gastrointestinal tract will be exposed to parasite-derived SOD as well as numerous other protein and non-protein antigens. This combination of *N. brasiliensis* antigens has a demonstrated ad-

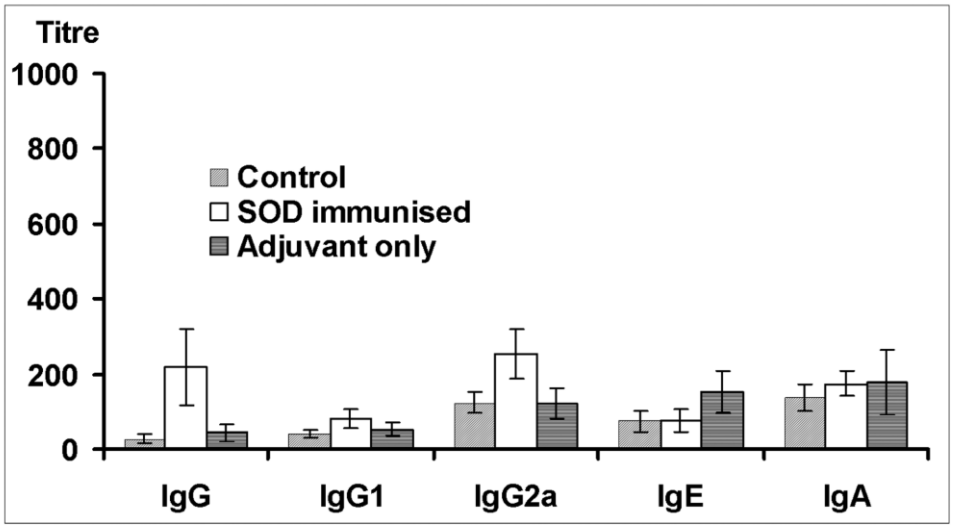


**Fig. 5.** Serum antibody responses of rats to vaccination with *N. brasiliensis* SODc in Quil A, adjuvant alone and unvaccinated control rats. Only IgG2a levels were significantly elevated in vaccinates compared to controls for all the isotypes tested

juvant capacity and enhances immunogenicity and the development of protective type 2 (Th-2) responses to co-administered antigens (Holland *et al*. 2000). By contrast, the recombinant SOD was purified, with a few minor contaminating bacterial proteins (not shown), and given subcutaneously with Quil A and this may restrict immunological processing of the recombinant SODc compared to exposure to the native enzyme in the course of natural infection. The findings here highlight a requirement for a full analysis of the responses of vaccine recipients within and between laboratory animal strains used to conduct the work.

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