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IgG4-reactive low molecular weight antigens from *Setaria digitata* adult parasites have immunodiagnostic potential in lymphatic filariasis

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Development of sensitive as well as low-cost immunodiagnostics for detection of infected individuals would be an important step towards reaching the goal of lymphatic filariasis elimination by 2020. In an earlier study, using soluble antigens of adult *Setaria digitata*, we have shown that an antigen fraction in the molecular weight range 14 to 20 kDa can induce differential Th1/Th2 immune response in the PBMC of the endemic normal (EN) and asymptomatic microfilaraemic (ASM) individuals residing in bancroftian filariasis endemic areas. In the present study, using 2D immunoblot we are able to show that two antigens of 17 and 18 kDa molecular weight and pI of 5–5.5 are recognized by the IgG4 isotype antibodies, a marker for active parasite infection, present in the sera of ASM but not in that of EN individuals. Elevated antigen-specific IgG4 antibodies in the blood are the indicators of circulating parasite in infected individuals who do not have apparent clinical symptoms of filariasis. Thus, we suggest that these two antigens can be explored for the immunodiagnosis of lymphatic filariasis.

Keywords: IgG4 isotype immunoblot, immunodiagnosis, low molecular weight antigens, lymphatic filariasis, *Setaria digitata*.

LYMPHATIC FILARIASIS (LF) is recognized as one of the world's most disabling diseases[1]. The disease is caused in humans by infection with the filarial nematode *Wuchereria bancrofti*, *Brugia malayi* or *Brugia timori* and continues to be a major public health problem in many of the tropical countries. The World Health Organization[2] has declared lymphatic filariasis to be the second leading cause of permanent and long-term disability worldwide and has targetted to eliminate LF globally by 2020. The disease manifests itself in a broad clinical spectrum. Individuals living in a filaria endemic area can be categorized on the basis of their clinical symptoms and parasitological as well as immunological profile into three broad groups, namely endemic normals (EN), asymptomatic microfilaremic individuals

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(ASM) and chronic filarial patients (CH). The EN individuals despite living for a long time in a filaria endemic area and getting exposed to filarial parasite infective larvae through repeated mosquito bites, do not show any clinical symptoms of filariasis, do not have microfilariae or circulating filarial antigens (CFA) in their blood and do not have high titres of filaria parasite antigen-specific IgG4 isotype antibodies in their serum. The ASM individuals also do not show any apparent clinical symptoms of filariasis, while in most of them there is sub-clinical lymphatic damage[3,4]. They have microfilariae and CFA in their blood and have high titres of filaria parasite antigen-specific IgG4 isotype antibodies in their serum[5]. The chronic filarial patients manifest obstructive filarial pathology and may or may not have microfilariae and CFA in their blood[3,4].

For successful mass chemotherapy programmes in countries like India, which account for about 40% of the global prevalence of LF[6], development of immunodiagnostic methods that are cheap and sensitive and can be used for rapid detection of infected individuals is an important requirement. Since *W. bancrofti* antigens are scarce, heterologous parasite antigens have been often used to analyse the immune response of humans in filariasis. The cattle filarial nematode, *Setaria digitata* is similar to the *W. bancrofti* parasite in its morphology, histology, response to drugs and antigenicity[7,8]. Our earlier study had shown that the 14 to 20 kDa antigen fraction of adult *S. digitata* could induce differential Th1/Th2 immune response in the PBMC of EN individuals in comparison to ASM individuals[8]. Such differential response is mediated by cytokines, but the biochemical nature of the antigens that induce such differential cytokine response is not known. It is well established that antibody responses to filarial parasites in humans are dominated by the IgG4 subclass[9–11], which is known to be a marker of active infection[5]. The parasite antigen-specific antibody isotype balance is greatly influenced by the clinical nature of the disease[12–15]. In the present study, using 2D immunoblot, we have tried to identify the IgG4-reactive antigens present in the 14–20 kDa fraction of *S. digitata* adult soluble antigens.

Healthy human volunteers from both sexes and in the age group of 20 to 45 years (mean age: 25 years) residing in Khurda district, Orissa, India (within 20° lat. and 86° long.), which is endemic for bancroftian filariasis[8], were selected for the present study. They were screened for their microfilaremia status by examining finger-prick blood samples collected from them around midnight. Sera of those individuals who were negative for microfilariae were examined for the presence of CFA by Og4C3 ELISA[16] as recommended by the manufacturer (JCU Tropical Biotechnology Pvt Ltd, Brisbane, Australia). The limit for positivity was 100 U of Ag per ml. EN individuals who were negative both for microfilariae and circulating filarial antigen were longitudinally followed up for at least three years to establish their truly infection-free status. Only those individuals who had no microfilariae or circulating filarial antigen in their blood and did not have high titres of filarial parasite antigen-specific IgG4 isotype antibodies in serum and were healthy without any clinical symptoms of filariasis at the end of three years were selected as EN subjects. Individuals who were microfilariae-positive, had high levels of circulating filaria antigens in their blood and had high titres of filaria parasite antigen-specific IgG4 isotype antibodies but were otherwise healthy and free from clinical symptoms of filariasis were selected as ASM individuals. EN as well as ASM individuals were included in the study after obtaining their informed consent.

Venous blood collected from EN and ASM individuals in the study population was allowed to clot, serum samples were aspirated out and centrifuged. The clear supernatants were aliquoted and were stored at –20°C till further use.

Live *S. digitata* adult parasites were collected from peritoneal cavity of freshly slaughtered cattle, and washed with sterile PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4), pH 7.3 to remove adherent cells and serum proteins. After washing, the worm extract was prepared by disrupting the parasites in PBS containing protease inhibitors (1 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM TPCK, 0.2 mM TLCK) and 1.5% *n*-octyl glucoside, in a ground-glass homogenizer[11]. The extract was allowed to stand for an hour, after which it was centrifuged at 10,000 rpm for 30 min and supernatant was collected. The entire operation was carried out at 4°C. Protein concentration of the extract was measured according to Lowry[17]. The extracts were aliquoted and stored frozen at –70°C.

The parasite antigen extract was analysed using 2D polyacrylamide gel electrophoresis (2D PAGE). Isoelectric focusing (first dimension run) was performed in a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Richmond, CA) following standard protocol[18], as given in the manufacturer's instructions. CHAPS was used as detergent. The antigen extract (50–60 μg protein) was loaded into the sample reservoirs. After the first-dimension run, the gels were equilibrated with second-dimension sample buffer for 10–15 min at room temperature and loaded onto the slab gel for the second-dimension run. The IEF gels not used immediately were stored at –70°C in sample equilibration buffer. SDS–PAGE (second-dimension run) was carried out in a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories) using a 5% (w/v) stacking gel and 10% (w/v) separating gel[19]. After electrophoresis, the gels were either equilibrated in transfer buffer for 5 min to be transferred to nitrocellulose (NC) membrane for 2D Western blot or were stained with Coomassie blue R-250 for visualization of the protein spots. The pI and molecular weight of the protein bands were determined with reference to standards (SIGMAMARKER, M-4038) and Bio-Rad 2D PAGE markers.

Determination of antigen-specific IgG4 isotype antibody titre, in the sera of ASM ($n = 20$) and EN ($n = 20$) individuals, was carried out by ELISA using *S. digitata* adult

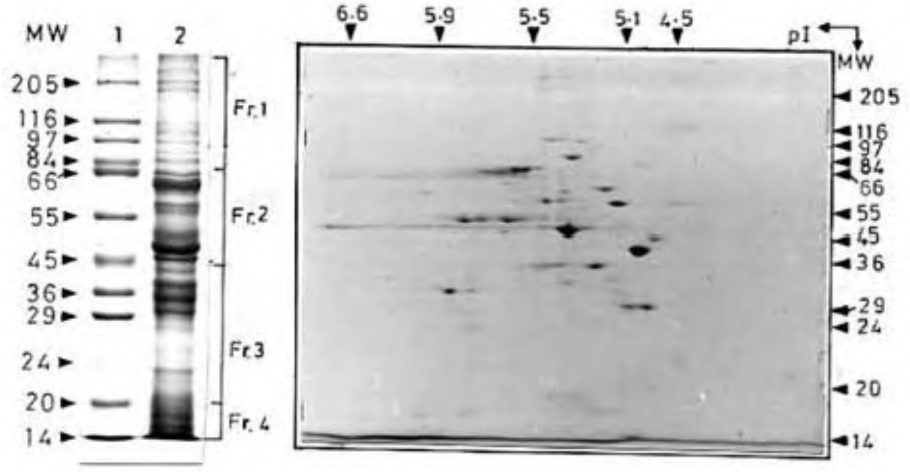

Figure 1. 2D PAGE analysis of *Setaria digitata* adult (mixed) soluble antigens. Isoelectric focusing in the first dimension followed by SDS–PAGE (Laemmli, 10% acrylamide) in the second dimension separated the antigens. 2D SDS–PAGE markers (BIO-RAD) were also run with the samples. The side lane shows SDS–PAGE profile of the antigens. Proteins/polypeptides were visualized by staining with Coomassie blue.

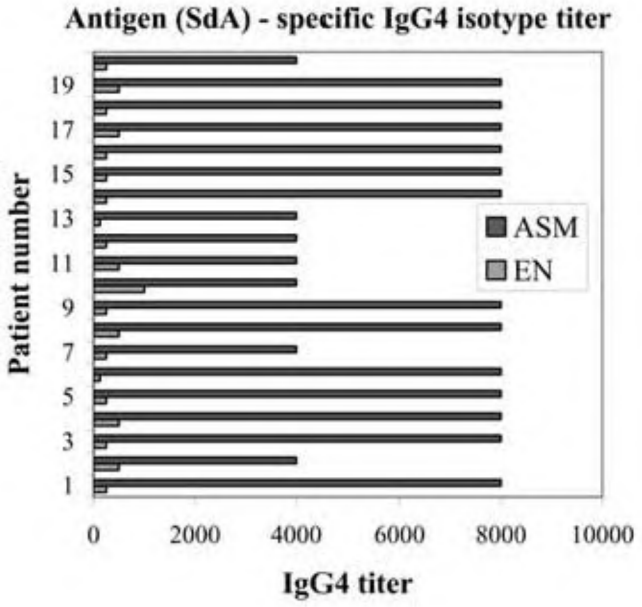

Figure 2. Titer of *S. digitata* adult soluble antigen (SdA)-specific IgG4 isotype in sera of EN ($n = 20$) and ASM ($n = 20$) individuals, determined by ELISA.

soluble antigens[11]. EN and ASM sera were used as primary antibodies at serial dilution. The following antibodies were used at the respective dilutions: mouse anti-human IgG4 (1/5000), peroxidase conjugated goat anti-mouse Ig (1/7500) (Sigma, USA). ABTS (Sigma) and H_2O_2 were used for development of colour and microplate (Nunc) was read in a microplate reader (BIO-RAD Model 550) at 405 nm.

S. digitata adult, soluble antigens separated on 2D gels (Bio-Rad Mini-PROTEAN II) were transferred to NC membrane (0.2 m pore size)[20]. The membrane containing separated antigens was blocked with TBS-T containing 2% BSA for 4 h at room temperature (RT). EN and ASM sera were used at 1/50 dilution and the membranes were incubated overnight (RT). Mouse anti-human IgG4 monoclonal antibodies (Sigma) were added at a dilution of 1/5000. Peroxidase-conjugated goat anti-mouse immunoglobulins (Sigma) diluted 1/10,000 was added for an hour at RT, before final washing and addition of 0.06% DAB in 100 mM Tris-Cl pH 7.6 with 0.1% H_2O_2 for 10 to 15 min until colour developed.

Profile of the *S. digitata* adult soluble antigens was analysed using 1D and 2D gel electrophoresis (Figure 1). As seen by Coomassie blue staining of SDS–PAGE-separated proteins, there are a large number of antigens ranging in molecular weights from 200 to 14 kDa. The 2D SDS–PAGE profile shows that the antigens have pI ranging from 6.6 to 4.6. It is known that parasite antigen-specific IgG4 isotype is the predominant antibody present in the sera of ASM individuals compared to that of EN individuals. We also observed high titres of IgG4 isotype antibodies in the sera of ASM individuals compared to EN individuals (Figure 2). It has been demonstrated that in individuals with elevated IgG4, the B-cell compartment in PBMC carries cells that are already committed to IgG4 production and these committed cells can be stimulated to release large amounts of IgG4 in an IL-4- and IL-13-independent manner[21]. The balance between the IgG4 and IgE influences the outcome of the infection.

The antigens reacting with the IgG4 antibodies present in the sera of ASM and EN individuals were analysed by 2D immunoblot (Figure 3). The ASM sera recognized

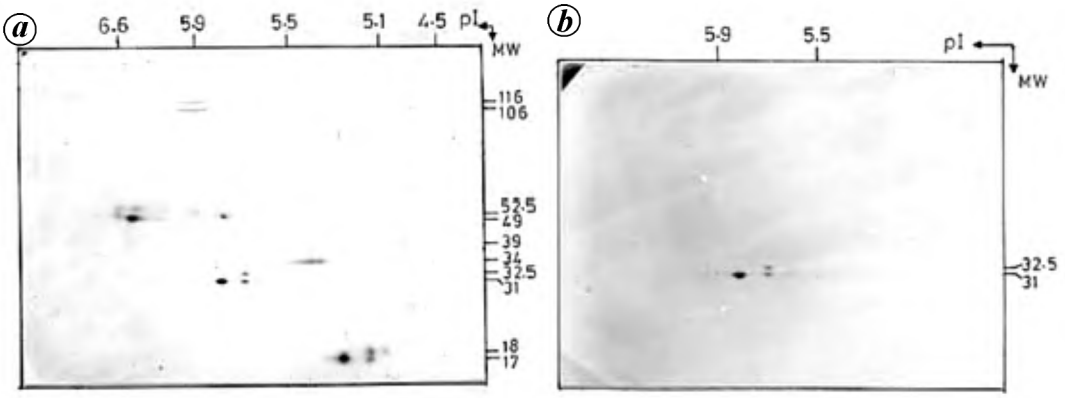

Figure 3. 2D immunoblot. 2D PAGE was carried out. After the second dimension run, proteins were electrophoretically transferred to nitrocellulose membrane and probed with ASM sera (***a***) or EN sera (***b***) at a dilution of 1/50 to obtain IgG4 isotype antibody reactive *S. digitata* adult soluble antigens.

larger number of antigens than the EN sera. The IgG4 antibodies present in the sera of ASM individuals (12 out of 12 tested) reacted with a set of low molecular weight, viz. 52, 49, 39, 34, 32.5, 31, 18 and 17 kDa antigens besides the high molecular weight, viz. 116 and 106 kDa antigens (Figure 3 *a*). The sera from EN individuals (10 out of 12 tested) however, recognized only two antigens, viz. 32.5 and 31 kDa (Figure 3 *b*). Some EN sera (2 out of 12 tested) recognized a 97 kDa antigen (immunoblot not shown).

Human antibody response to filarial parasite antigens tends to be dominated by IgG4 subclass of antibodies, which is the least abundant antibody isotype present in normal serum[10,22]. IgG4 acts as a blocking antibody and competes with IgE antibodies for allergen and thus dampens IgE-mediated immune reactivity[13,23–25]. This isotype antibody plays an important role in modulating IgE-mediated allergic responses *in vivo* and have been implicated in the development of immunopathology as well as establishment of successful host–parasite relationships in helminthic infections[14,22].

Low molecular weight antigens are reported to be selectively recognized by the sera of ASM individuals in filariasis[8,20,26]. These observations are supported by Western blot data generated using sera of persons infected with *W. bancrofti*[12], *B. malayi*[20,26], *O. volvulus*[27–29] and *S. digitata* parasite[8]. A similar phenomenon was also reported in schistosomiasis[30]. In onchocerciasis, it is known that low molecular weight antigens perform well in serodiagnostic tests, due to good recognition of these smaller antigens by the sera of the patient[31–33]. Several explanations have been offered for this. The smaller proteins may be heavily represented in the secretion of parasites, which are most readily exposed to the host immune system; they may form a related family of antigens capable of inducing strong Th2 response and most speculatively, filarial parasites may interfere with the processing of larger proteins, thereby accentuating the response to antigens with low molecular weight[20]. Such low molecular weight antigens have also been shown by us to induce a differential Th1/Th2 cytokine response in the PBMC of ASM individuals[8]. EN individuals show a preferential Th1 cytokine response (high IFN-γ, IL-12 and low IL-4, IL-10), whereas ASM individuals show a preferential Th2 cytokine response (high IL-4, IL-10 and low IFN-γ, IL-12) when the PBMC of individuals of these two categories was sensitized by *S. digitata* adult soluble antigen fraction 5 (14–20 kDa antigens) *in vitro*[8]. In the present study, using 2D immunoblot we could show that there are two IgG4-reacting antigens in this antigenic fraction, viz. 17 and 18 kDa. The potential of the antigens identified in the present investigation needs to be evaluated in a larger number of sera samples from filariasis endemic areas.

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Molecular characterization of specialty mushrooms of western Rajasthan, India

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Eighteen specialty mushroom germplasm accessions were collected from Udaipur (Rajasthan) and characterized using DNA fingerprinting and ribosomal rRNA gene sequencing. Phylogenetic analyses based on RAPD profiles and nucleotide sequence of 5.8S rRNA gene along with its spacer regions revealed variation of inter-generic and intra-species isolates among accessions. Based on ITS sequence polymorphism, seven isolates were identified as *Podaxis pistillaris*, four as *Phellinus igniarius*, one as *Gymnopilus subearlei* and six as *Phellorinia herculea*. The similarity matrix revealed very high intra-species homology (99.5–100%) and significant inter-generic diversity (21.2–37.7%). *G. subearlei* and *P. herculea* have been discovered as new additions to the Indian basidiomycetes biodiversity.

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