of mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Supplementary Table 3). The parasite rRNA levels were measured with the copy number-based standard curve ($R > 0.99$), and they were at least 100000-fold less than the mouse GAPDH mRNA levels. Parasite 18S rRNA was detected in all liver samples examined, except for the sample from the animal inoculated with formalin-inactivated sporozoites (data not shown). There was no significant difference in the liver parasite burden, which was quantified for C-type (sporozoite-type) 18S rRNA, between groups of mice infected with WT ($5.40 \pm 2.55 \times 10^{-5}$, $n = 4$) and Prx-WT ($5.03 \pm 1.22 \times 10^{-5}$, $n = 5$) ($P = 0.89$) and with WT and Prx-KO1 ($4.81 \pm 1.98 \times 10^{-5}$, $n = 6$) ($P = 0.86$). There was also no significant difference in the liver parasite burden, which was quantified for A-type (asexual-type) 18S rRNA, between groups of mice infected with WT ($2.24 \pm 0.97 \times 10^{-5}$, $n = 4$) and Prx-WT ($1.58 \pm 0.44 \times 10^{-5}$, $n = 5$) ($P = 0.52$) and with WT and Prx-KO1 ($1.86 \pm 0.85 \times 10^{-5}$, $n = 6$) ($P = 0.78$). The results of our animal infection experiments suggested that the Prx-KO population requires a longer pre-patent period to appear in mouse erythrocytes than the WT population. This finding indicates that Prx-KO has a defect in liver-stage infection because this population can multiply in erythrocytes with efficiency similar to that of WT [10]. However, the results of quantitative RT-PCR experiments, which targeted the parasite C-type 18S rRNA, suggested that Prx-KO does not affect sporozoite invasion into the mouse liver parenchyma. Zhu et al. [16] suggested that invasion of liver cells by sporozoites and transformation to the exo-erythrocytic form (EEF) can trigger the ribosome switch. In the present study, the ribosome switch from C to A was detected in all liver samples examined (data not shown). However, there was also no significant difference in the liver parasite burden, which was quantified for A-type 18S rRNA, between groups of mice infected with WT and Prx-KO (Supplementary Table 3). These results suggested that Prx-KO sporozoites could invade liver parenchyma and initiate EEF development with similar efficiency to WT during the early stage of liver infection. This finding suggests that the Prx-KO population can produce a few but intact sporozoites with normal activity to initiate the liver stage development. If this is the case, it is presumed that Prx-KO parasites have a defect in EEF development after the early stage of liver infection. *pbtpx-1* may be involved in development during exponentially multiplying parasites stages, such as sporozoites and EEF. The 2nd generation Prx-KO parasites have the same defect in gametocytes production as that of the 1st generation [10] (data not shown). The activity of its sporozoite production should be confirmed.

Although the specific mechanism by which disruption of *pbtpx-1* leads to DNA damage and reduces maturation of oocysts remains to be elucidated, the present findings suggest that the parasite antioxidant system contributes to sporozoite development during the insect stage. Further studies to clarify the role of TPx-1 in sporozoite development will provide further insights into the contribution of this antioxidant protein to the insect-stage development of malaria parasites and may provide novel transmission-blocking strategies.

Acknowledgments

We are very grateful to Dr. A.P. Waters of Glasgow University, UK for providing targeting vector pMD204, to the Malaria Research and Reference Reagent Resource Center (MR4/ATCC) and to Dr. M. Yuda of Mie University, Japan for kind advice regarding gene targeting. We are also grateful to Dr. T. Ishino of Institut Pasteur, France for comments regarding insect stage experiments. This work was supported by a Grant-in-Aid for Scientific Research (16590351 and 18590412 to S.I.K.) from the Japan Society for the Promotion of Science; Grants-in-Aid for Scientific Research (16390126, 18406009 to M.T., and 18659120 to M.A.) and Scientific Research on Priority Areas (16017273 to T.T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and a grant for Precursory Research for Embryonic Science and Technology from the Japan Science and Technology Agency (to S.I.K.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2008.03.002.

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PARINT-00607; No of Pages 4

Parasitology International xxx (2008) xxx–xxx

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Short communication

An *Entamoeba* cysteine peptidase specifically expressed during encystation

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ARTICLE INFO

Article history:
Received 13 June 2008
Received in revised form 11 July 2008
Accepted 19 July 2008
Available online xxxx

ABSTRACT

Protozoan parasites of the genus *Entamoeba* possess a considerable number of cysteine peptidases (CPs), the function of most of these molecules for amoeba biology needs to be established. In order to determine whether CPs may play a role during *Entamoeba* stage conversion from trophozoites into cysts and vice versa, expression of *cp* genes was analysed in the reptilian parasite *Entamoeba invadens*, a model organism for studying *Entamoeba* cyst development. By homology search, 28 papain-like *cp* genes were identified in public *E. invadens* genome databases. For eight of these genes the expression profiles during stage conversion was determined. By Northern blot analysis, transcripts for *eicp-a9*, *-b7*, *-b8* and *-c2*, respectively, were detected neither in trophozoites or cysts nor at any of the point of times analysed during stage conversion. On the other hand, *eicp-a5* is constitutively expressed during all developmental stages, whereas *eicp-a3* and *eicp-a11*, respectively, are trophozoite-specific. Only *eicp-b9* was found to be cyst-specific as it is expressed exclusively 18 to 28 h after cyst induction. Cyst-specific expression was confirmed by immunofluorescence microscopy of the corresponding protein EiCP-B9. In immature cysts, the molecule is located in structures that accumulate near the cyst wall, but which are uniformly distributed in mature cysts. The precise function of EiCP-B9 during *Entamoeba* encystation remains to be determined. However, colocalisation studies with an *Entamoeba* marker for autophagosomes suggest that EiCP-B9 is not associated with *Entamoeba* autophagy.

Entamoeba species have a relatively simple life cycle, which comprises two developmental stages, the dividing trophozoite stage present within the infected host and the transmissible environmentally resistant cyst stage. Most studies on *Entamoeba* biology have been performed with *Entamoeba histolytica* as it is the only *Entamoeba* species that is pathogenic to humans. However, most of these studies have focused on the trophozoite stage, because *E. histolytica* trophozoites can be easily grown in culture but for reasons so far unknown, *E. histolytica* does not efficiently encyst *in vitro*. The *Entamoeba* model for studying en- or excystation is *Entamoeba invadens*, a reptilian parasite, which has a similar life cycle as *E. histolytica* but which efficiently forms cysts *in vitro*. In some of its reptilian hosts, *E. invadens* causes liver and intestinal damage similar to the pathology of *E. histolytica*-infected individuals. Moreover, *E. invadens* forms quadrinucleated cysts indistinguishable from those of *E. histolytica*. Thus, it is predicted that the molecular mechanisms responsible for stage conversion are similar between both parasites.

Previous studies in a variety of protozoan parasites including *E. histolytica* have shown that peptidases play a role in diverse cellular processes such as growth, differentiation, cell cycle progression or host cell invasion (for review [1–3]). In addition, peptidases and in particular cysteine peptidases (CPs) have been found to be involved in en- or excystation in a number of cyst-producing protozoans such as *Cryptosporidium parvum* [4], *Sterkiella historiomuscorum* [5], *Giardia muris* [6] and *Giardia lamblia* [7,8]. Especially for *E. invadens* it has been shown that cysteine peptidases participate on the excystation and metacystic development as well as in encystation processes. Makioka and colleagues ascertained that incubation of cysts with the cysteine peptidase inhibitors Z-Phe-Ala-DMK and E-64d block excystation and metacystic development [9]. In addition, incubation with 5 μM E-64d has only little influence on trophozoite growth (20% reduction of the growth rate), but the encystation process is highly inhibited (60% less cyst formation in comparison to the control: unpublished data).

In the genome of *E. histolytica* 86 genes coding for proteolytic enzymes have been identified. These comprise homologs to cysteine, serine, aspartate and metallo peptidases, respectively. A total of 20 of these amoeba peptidase genes are significantly expressed during standard axenic cultivation of the parasite whereas the remaining are not expressed or expressed at very low levels. Of the 37 *E. histolytica* genes that encode *cps*, only 8 are expressed in cultured trophozoites [10]. Thus, it is of question whether at least some of these peptidases may have a specific role during *Entamoeba* en- or excystation.

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doi:10.1016/j.parint.2008.07.002

Please cite this article as: Ebert F, et al, An *Entamoeba* cysteine peptidase specifically expressed during encystation, Parasitol Int (2008), doi:10.1016/j.parint.2008.07.002

In contrast to the *E. histolytica* genome, which is completely sequenced, an approximately 0.5-fold sequence coverage of the *E. invadens* genome is published [11,12]. By analysing the latest whole genome shotgun reads of *E. invadens* (taxid:33085, NCBI), we identified 28 putative cysteine peptidase genes. These comprise 7 homologs to the *E. histolytica* cysteine peptidase family A (EhCP-A) (*cp-a2, -a3, -a5, -a8, -a9, -a10, -a11*), 8 homologs to EhCP-B (*cp-b1, -b3, -b5, -b6, -b7, -b8, -b9, -b10*) as well as 13 homologs to EhCP-C (*cp-c1* to *-c13*) (Table 1). In contrast to the study by Wang and colleagues we did not find homologs to *ehcp-a4* or *ehcp-b2* within the analysed *E. invadens* dataset [12].

For eight of the identified *E. invadens cp* genes (*eicp-a3, -a5, -a9-, a11, -b7, -b9, -b10,* and *-c2*) expression profiles in trophozoites and cysts as well as during stage conversion was determined by Northern blot analysis using total RNA of the *E. invadens* strain IP-1 (obtained from American Type Culture Collection). Amoebae were cultured in axenic TYI-S-33 medium at 24 °C [13]. Cyst formation was induced by transfer of trophozoites to LG-medium (TYI-S-33 medium without glucose) [14]. Cells were harvested at various time points during cyst development until maturation of cysts was completed (usually within 48 h of cultivation in LG-medium). Those cells that did not form cysts were lysed by treatment with 0.1% sarcosyl (*N*-laurylsarcosine) in order to obtain pure cyst fractions. The integrity of the cysts was proven by cell wall staining using Calcofluor white (Sigma), a chitin-staining reagent. After washing twice with PBS, cells were centrifuged (500×*g* for 5 min) and the pellets were shock frozen in dry ice/ethanol and stored at −80 °C until RNA was extracted. For excystation experiments, fractions of pure cysts were washed in TYI-S-33 medium. Subsequently, 4×10^5 cysts were seeded in 50 ml culture flasks and grown in complete TYI-S-33 medium at 24 °C for various point of times. Total RNA from trophozoites or cysts was isolated using the RNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen). For Northern blotting, 10 µg of total RNA was separated on agarose gels and transferred to nylon membranes (Hybond-N, Amersham Bioscience). Blots were sequentially hybridised with radiolabelled DNA probes representing coding regions of the various *E. invadens cp* genes (Table 1). The results indicated that four of the eight genes analysed, namely *eicp-a9, -b7, -b8,* and *-c2*, are not expressed in significant quantities neither in cysts or trophozoites nor at any of the time points analysed during stage conversion (data not shown). In contrast, *eicp-a5* is constitutively expressed at almost the same levels in both parasite stages as well as during stage conversion, whereas *eicpa3, eicpa11* and the *E. invadens* actin gene (*eiactin*) are preferentially expressed in trophozoites, as transcripts of the latter three genes disappear at early time points during encystations (<24 h) and reappear, with some differences in kinetics, about 12 to 48 h after induction of excystation (Fig. 1A, B). Interestingly, a completely different picture was observed when the expression profile of *eicp-b9* was analysed (Fig. 1A–C). This gene revealed a sharp time-dependent expression during encystation only. Transcripts of *eicp-b9* were seen between 18 and 28 h after initiation of encystation but not at any point of time outside this period nor during excystation or in trophozoites (Fig. 1A–C). Primary structure analysis of the corresponding protein indicated that EiCP-B9 consists of 448 amino acid residues with a calculated molecular mass of 50,584 kDa. It contains a predicted signal sequence of 16 amino acid residues and an N-terminal cathepsin pro-peptide inhibitor domain (I29; between aa 30 and 85). An I29 domain is found in all *E. histolytica* peptidases belonging to subfamilies A or B. It is suggested that this domain acts as a pro-peptide. At present, the exact size of the EiCP-B9 pro-peptide or of the mature enzyme is unknown. By comparing the sequence with the *E. histolytica* cysteine peptidases EhCP-A1, -A2 and -A5, for which the N-terminus of the mature enzyme is known, it can be predicted that the pro-peptide of EiCP-B9 is cleaved between amino acid 132 and 133, resulting in a mature protein with a molecular mass of approximately 35 kDa.

Based on the result of time-dependent expression of *eicp-b9* it was of interest whether this is also reflected at the protein level. Therefore, Western blot analysis using an antibody raised against the recombinantly expressed EiCP-B9 was performed. For this, a 951 bp fragment encoding the putative mature form of EiCP-B9 was amplified from the genomic *E. invadens* DNA using the oligonucleotides EiCP-B9-S31 (5′ GGC ATA TGC TGC CAA AAT ACC ACG CCT ACT G '3) and EiCP-B9-AS29 (5′ GGG GAT CCG TTT TTG GTC TGA TTG CAA CT '3). The DNA fragment was cloned into the prokaryotic expression plasmid pJC45 [15]. Following transformation in *Escherichia coli* BL21(DE3) (pAPlacIQ) the protein was expressed according to standard procedures. Recombinant EiCP-B9 (rEiCP-B9) was isolated under denaturating conditions using Ni-NTA resin according to the manufacturer's recommendations (Qiagen, Hilden, Germany). The protein has a molecular mass of approximately 37 kDa, which corresponds to the calculated molecular mass of EiCP-B9 with 35 kDa plus His-tag. For generation of antibodies 100 µg rEiCP-B9 was injected into a mouse, followed by two booster injections. For Western blot analyses trophozoites and pure cysts, harvested after 20, 24, 32, 40 and 48 h cultivation in LG-medium, were sonicated in the presence of the cysteine peptidase inhibitor E-64 (final concentration 60 µM) and centrifuged at 50,000×*g* at 4 °C for 30 min. Before separation on a 12% SDS-PAGE (50 µg protein/lane) the extracts were incubated in SDS sample buffer (125 mM Tris–HCl pH 6.8, 20% glycerine, 20% SDS, 100 mM dithiothreitol (DTT), 0.001% bromophenolblue) at 95 °C for 5 min. Western blot analysis was carried out by using the semidry blotting technique with 25 mM Tris, 192 mM glycine, and 0.1% SDS as blotting buffer. Polyclonal mice

Table 1
Cysteine peptidases of *E. invadens*

Protein name	Accession-no.	ORF (bp)	Significant sequence identity to cysteine peptidases of *E. histolytica* (EhCP)
EiCP-A2	AANW02000574	954	65% to EhCP-A2
			61% to EhCP-A1
			58% to EhCP-A7
EiCP-A3[a]	AANW02000038	966	74% to EhCP-A3
EiCP-A5[a]	AANW02000277	951	64% to EhCP-A5
			58% to EhCP-A4
			55% to EhCP-A6
EiCP-A8	AANW0200018	1020	59% to EhCP-A8
			28% to EhCP-A12
EiCP-A9[a]	AANW02001013	996	46% to EhCP-A9
EiCP-A10	AANW02001528	1284	76% to EhCP-A10
EhCP-A11[a]	AANW02000008	999	40% to EhCP-A11
EiCP-B1	AANW02000161	1124	43% to EhCP-B1
			43% to EhCP-B2
EiCP-B3	AANW02000250	1503	51% to EhCP-B3
			47% to EhCP-B4
EiCP-B5	AANW02000072	756	48% to EhCP-B5
EiCP-B6	AANW02002207	1011	53% to EhCP-B6
EiCP-B7[a]	AANW02000450	1656	48% to EhCP-B7
EiCP-B8[a]	AANW02000897	2337	43% to EhCP-B8
EiCP-B9[a]	EU750752	1344	63% to EhCP-B9
	AANW02001582	774	
EiCP-B10	AANW02001126	582	47% to EhCP-B10
	AANW02001420	1437	48% to EhCP-B10
EiCP-C1	AANW02000003	1266	62% to EhCP-C1
EiCP-C2[a]	AANW02000028	954	68% to EhCP-C2
EiCP-C3	AANW02001069	1548	72% to EhCP-C3
EiCP-C4	AANW02000661	798	76% to EhCP-C4
EiCP-C5	AANW02000256	1464	60% to EhCP-C5
EiCP-C6	AANW02000026	1500	64% to EhCP-C6
EiCP-C7	AANW02000183	1653	69% to EhCP-C7
EiCP-C8	AANW02000285	1491	66% to EhCP-C8
EiCP-C9	AANW02000112	1620	65% to EhCP-C9
EiCP-C10	AANW02000256	1464	58% to EhCP-C10
EiCP-C11	AANW02000914	1494	42% to EhCP-C11
EiCP-C12	AANW02000655	978	44% to EhCP-C12
EiCP-C13	AANW02000183	1653	63% to EhCP-C13

[a]Cysteine peptidases used as probe for Northern blot analyses.

Please cite this article as: Ebert F, et al, An *Entamoeba* cysteine peptidase specifically expressed during encystation, Parasitol Int (2008), doi:10.1016/j.parint.2008.07.002

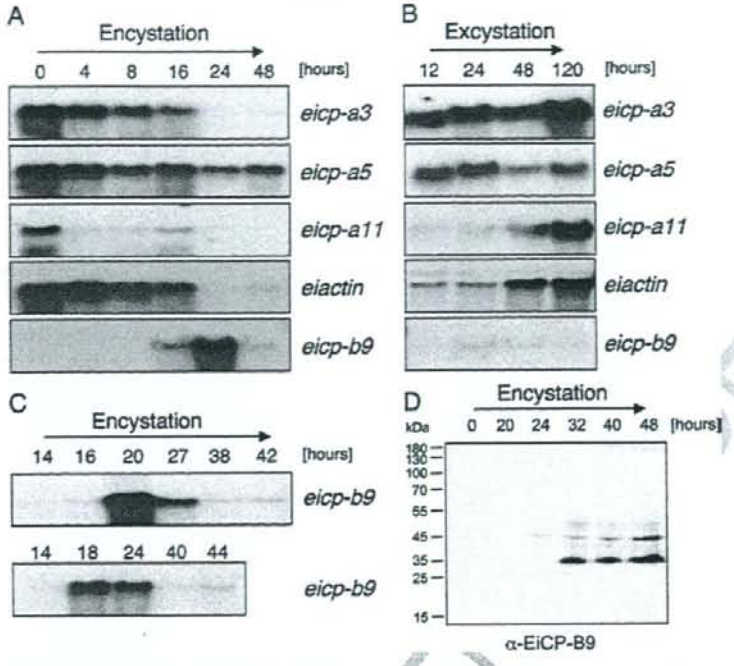

Fig. 1. Expression of *E. invadens* cysteine peptidase genes during stage conversion. A–C. Shown are the results from Northern blot analyses. Total cellular RNA isolated at different point of times after induction of en- and excystation was separated on formaldehyde-agarose gels, blotted onto nylon membrane and hybridised sequentially with coding sequences of the various *E. invadens cp* or actin genes as indicated. D. Western blot analysis of *E. invadens* lysates from cells isolated at different point of times after cyst induction. Lysates were fractionated by 12% SDS-PAGE under reducing conditions, and transferred to nitrocellulose membrane. The blot was developed with anti-EiCP-B9 polyclonal antibodies (1:500 dilution). As secondary antibody a horseradish peroxidase (HRP)-conjugated α-mouse IgG (1:1000; Sigma-Aldrich, Muenchen) was used. Proteins were visualised with ECL (Amersham ECL plus Western blotting detection reagents; GE Healthcare). Molecular mass standards are indicated on the left.

antisera (α-rEiCP-B9, 1:500) and as secondary antibody a horseradish peroxidase (HRP)-conjugated α-mouse IgG (1:1000; Sigma-Aldrich) were used to detect the protein using ECL (Amersham ECL plus Western blotting detection reagents; GE Healthcare). The antibody reacted specifically with a 35 kDa protein only in the late stages of the encystation process (Fig. 1D). Whereas the respective RNA was detected 16 h after initiation of encystation, the corresponding protein was detected 14 h later (Fig. 1D). In addition to the 35 kDa mature enzyme, a second faint band of about 47 kDa was detected on Western blots, which might represent the unprocessed peptidase, which has a calculated molecular mass of 49 kDa (Fig. 1D).

In addition to *eicp-b9*, a few other genes have been described in recent years that are differentially expressed during *Entamoeba* encystation. These comprise genes for chitin synthases [16], chitinases [17], ubiquitin [18] and a gene expressing a developmentally regulated transcript (105–122 transcript) of unknown function, which peaks at 22 h after cyst induction [14]. Moreover, differentially expressed cyst-specific lectins have been identified, which are present at different phases during encystations [19]. It is noteworthy that similar to *eicp-b9* these differentially expressed genes are upregulated within a relative short time frame around 24 h after cyst induction. During this period various fundamental events may occur, which result in drastic changes of organelle structures and cellular composition. The recycling of cellular components is achieved by the process of autophagy, which may play an important role during *Entamoeba* growth and stage conversion, respectively, as the formation of autophagosomes has been recently described in *E. invadens* at logarithmic phase of growth as well as during encystation [20]. Likewise, autophagy occurs during stage conversion from promastigotes to amastigotes in *Leishmania mexicana*. Interestingly, in this protozoan species the two lysosomal cysteine peptidases CPA and CPB facilitate effective autophagy [21]. To determine, whether EiCP-B9 may play a role in autophagy of *Entamoeba*, immunofluorescence microscopy (IFA) was performed in order to visualise the subcellular localisation of the enzyme. Amoebae were analysed with anti-EhCP-B9 antiserum or with an antiserum raised against the *E. histolytica* autophagosome marker Atg8 (autophagy related gene 8). For the IFAs, cells were fixed at room temperature for 30 min in PBS containing 3% paraformaldehyde and subsequently permeabilised with 0.05% saponin (PBSS). Prior to fixation cysts were additionally permeabilised by sonication for 30 s. Cells were incubated at room temperature for 1 h with antisera against EiCP-B9 (1:250 dilution) or against Atg8 (1:500 dilution). Utilised secondary antibodies (1:400) were Alexa-594 coupled α-rabbit and Alexa-488 coupled α-mouse antibodies. Subsequently, cells were mounted on glass slides and examined under 6300× magnification. For deconvolution microscopy images of selected cells were captured with a 63× oil immersion lens in a UV equipped Leica DM RB microscope with 0.2-µm-diameter step Z sections. Deconvolved Z sections were examined for colocalisation of Atg8 and EiCP-B9 staining with the Openlab 4.0.4 program. Adobe Photoshop 7.0.5 was used for additional processing of the images. The results from IFA indicate that anti-EiCP-B9 antiserum does not specifically react with *E. invadens* trophozoites as only a faint unspecific background labelling is observed in this parasite stage. However, in agreement with the results from Western blot analysis, anti-EiCP-B9 clearly visualises structures present in immature cysts, about 28 h after cyst induction. At this time-point an accumulation of signals is seen near the cyst wall, whereas in mature cysts EiCP-B9 is localised in distinct structures, distributed over the entire cell (Fig. 2).

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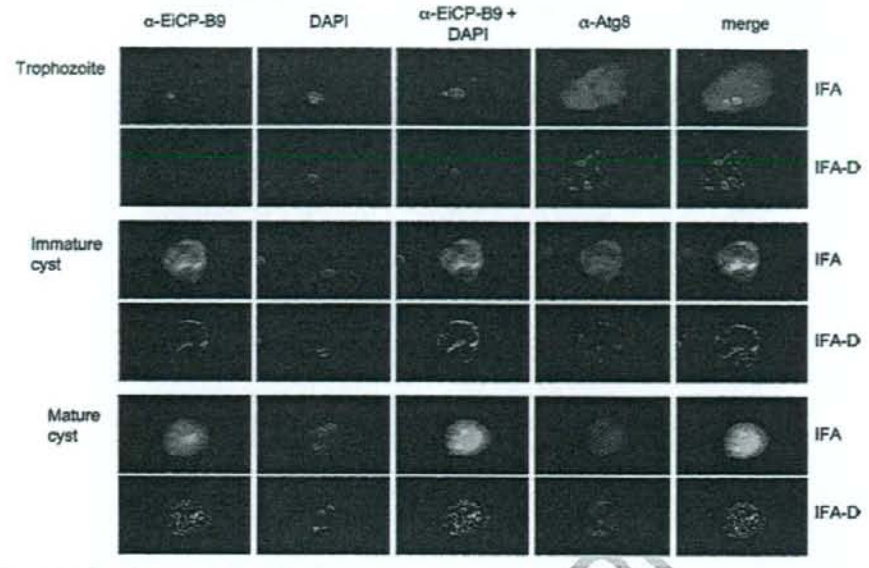

Fig. 2. Intracellular localisation of EiCP-B9 and Atg8 in *E. invadens* by IFA. IFAs were performed with antisera raised against EiCP-B9 and Atg8, respectively. As secondary antibodies Alexa-488 coupled α-mouse antibodies (green) and Alexa-594 coupled α-rabbit antibodies (red) were used. IFA-D. Images obtained by deconvolution microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Colocalisation with anti-Atg8 indicated that in contrast to EiCP-B9, the autophagosome-like structures are present in trophozoites during the late logarithmic phase of growth, as described by Picazarri et al. [20]. However, both EiCP-B9 and Atg8 are detected in mature and immature cysts, respectively, but only in immature cysts partial colocalisation is observed (Fig. 2). These results suggest that EiCP-B9 is not involved in *Entamoeba* autophagy. Thus, the precise role of EiCP-B9 during the process of *Entamoeba* encystation remains to be determined.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Br1744/7-1).

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Please cite this article as: Ebert F, et al, An *Entamoeba* cysteine peptidase specifically expressed during encystation, Parasitol Int (2008), doi:10.1016/j.parint.2008.07.002

"Inject-Mix-React-Separate-and-Quantitate" (IMReSQ) Method for Screening Enzyme Inhibitors

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Many enzymes involved in regulatory cellular processes are considered attractive therapeutic targets and their inhibitors are potential drug candidates.[1] Screening of large combinatorial libraries for enzyme inhibitors is pivotal to identifying large numbers of hit compounds for the development of enzyme-targeting drugs. While large combinatorial libraries of thousands of compounds are available,[2] efficient and economical methods suitable for screening them are required. A method suitable for screening large combinatorial libraries should preferably: (i) consume nanoliter volumes of the reactant solutions and (ii) be applicable to regulatory enzymes. To the best of our knowledge, none of the existing methods satisfy the two requirements together. Here, we introduce an Inject-Mix-React-Separate-and-Quantitate (IMReSQ) method for screening enzyme inhibitors, that meets the two requirements. The concept of the method is shown in **Fig. 1**. First, nanoliter volumes of substrate, candidate inhibitor, and enzyme solutions are injected separately (from microliter volumes in capped vials) by pressure into a capillary as separate plugs without the need of nanoliter-scale liquid handlers. Second, the plugs are mixed inside the capillary by transverse diffusion of laminar flow profiles (TDLFP).[3] Third, the reaction mixture is incubated to form the enzymatic product. Fourth, the product is separated from the substrate inside the capillary by electrophoresis. Fifth, the amounts of the product and substrate are quantitated. In this proof-of-principle work, we applied the method to study inhibition of recently-cloned protein farnesyltransferase (FT) from parasite *Entamoeba histolytica* (*Eh*); this enzyme is a potential therapeutic target for anti-parasitic drugs.[4,5] We identified three previously unknown inhibitors of *Eh*FT and proved that IMReSQ could be used for accurately ranking the potencies of inhibitors.

Methods for screening enzyme inhibitors can be divided into two broad categories: homogeneous, which monitor product formation without its physical separation from the substrate, and separation-based, which separate the product from the substrate by means of chromatography or electrophoresis prior to its quantitation. Recent advances in printing chemical libraries made it possible to transfer homogeneous methods from microtiter plates to microarrays, which require only nanoliter volumes of reagents.[6] Microarrays, however, require substrates that do not fluoresce before being converted into fluorescent products. Such fluorogenic substrates are not available for the majority of regulatory enzymes, e.g. prenyltransferases, glycosyltransferases, and kinases. In separation-based methods, simple fluorescently-labeled substrates can be used instead of fluorogenic substrates. Fluorescently-labeled substrates are available for many regulatory enzymes.[7] If separation is carried out in a narrow-bore capillary, only nanoliter volumes of reaction mixtures are consumed. Due to the lack of a generic way of mixing solutions inside the capillary, however, the reaction mixture must be prepared in a vial outside the capillary with a volume of at least several microliters. This work was inspired by the insight that TDLFP can be used to mix nanoliter volumes of enzyme, substrate, and candidate inhibitor, injected into the capillary as separate plugs. Thus far, TDLFP had been used to mix two reactants only.[3] Here, we demonstrate the TDLFP-based mixing of four reactants – enzyme, two substrates, and inhibitor – followed by enzymatic product formation, separation of the product from the substrate, and quantitation of the formed product and remaining substrate.

The *Eh*FT enzyme was a recombinant protein produced as described elsewhere.[5] The enzyme transfers the farnesyl group from farnesyl pyrophosphate (substrate 1, S1) to a fluorescently-labeled pentapeptide (substrate 2, S2), which mimics Ras protein, its native substrate. The farnesylated pentapeptide (product, P) was separated from S2 by capillary electrophoresis and fluorescent detection was used to quantitate the amounts of P and S2. Farnesyltransferase inhibitors (FTIs) tested in this work were: commercial FTI-276 and FTI-277 previously shown to inhibit *Eh*FT,[5] and human FT,[8] respectively, as well as new compounds, FTI-343, FTI-391, FTI-651, and FTI-656, recently proven to inhibit mammalian FT,[9] but never tested for *Eh*FT.

First, we studied TDLFP mixing of the 4 reaction components using computer simulation (the computer program can be found at www.chem.yorku.ca/profs/krylov, in the Research section). An algorithm for the optimization of the plug order in TDLFP mixing has not been developed yet; therefore, strictly speaking, we could not optimize the plug order. We could, however, numerically simulate the concentration profiles of the mixed reaction components along the capillary for any given order of plugs (**Fig. 2A**). Using such a simulation we tested several plug orders that seemed reasonable based on two simple criteria: the number of plugs had to be small while the spatial overlap of the components after mixing had to be

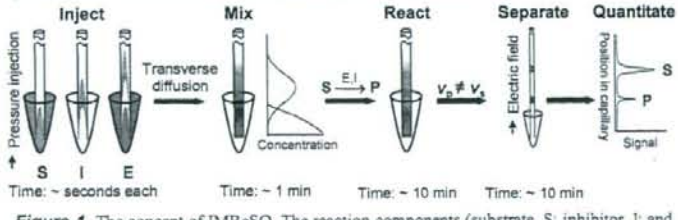

Figure 1. The concept of IMReSQ. The reaction components (substrate, S; inhibitor, I; and enzyme, E) are injected into the capillary as separate plugs, mixed by TDLFP, reacted to form the product (P), and separated. S and P are quantitated after separation.

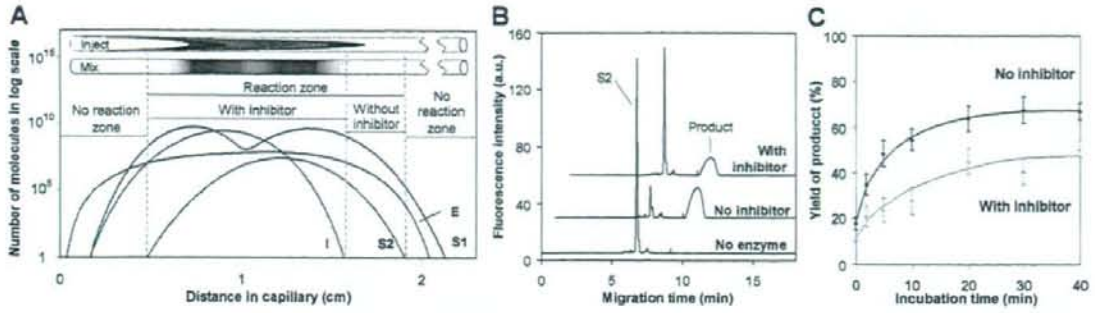

Figure 2. IMReSQ for inhibition of *Eh*FT enzyme. **(A)** Simulated distribution of reaction components in the capillary after injection (**top**) and after mixing (**bottom**) for the following plug order: enzymatic buffer (white), FPP (S1, blue), fluorescent peptapeptide substrate (S2, green), *Eh*FT enzyme (E, brown), inhibitor (I, red), FPP again, and enzymatic buffer again. **(B)** Experimental result of IMReSQ analysis of *Eh*FT inhibition by FTI-276 after 40-min incubation following injection and mixing of reaction components depicted in panel A. The pre-injection concentrations were: 75 μM for S1, 0.2 μM for S2, 3 μM for E, and 50 μM for I. **(C)** Kinetics of product formation with and without inhibitor (50 μM FTI-276).

significant. The plug order chosen for further work was: S1, S2, enzyme (E), inhibitor (I), and S1 again (**Fig. 2A**). Plugs of an enzymatic buffer were injected before and after injecting the reaction components to: (i) isolate the reaction mixture from an electrophoresis buffer containing a surfactant and (ii) improve the quality of mixing. The simulated after-mixing concentration profiles of the 4 components did not overlap perfectly, however, they revealed a significant reaction zone with all 4 components present (**Fig. 2A**). There was also a reaction zone with S1, S2, and E, but without I, suggesting that complete inhibition with this mixing scenario was not achievable.

In TDLFP, the required mixing time is defined by the time of transverse diffusion of the largest molecule, *Eh*FT. Our computer simulation showed that for the experimental conditions used, the sufficient mixing time was less than 1 min. The reaction time was longer than the mixing time, which suggested that only a negligible amount of the product was formed during mixing.

Second, we experimentally demonstrated that TDLFP indeed mixed the 4 reaction components and that the product formation could be observed along with the inhibition. The absence of either of the components meant that the concentration of this component in the injected plug was zero (an enzyme buffer was injected). In this part of our study, we used the FTI-276 inhibitor that had been proven to inhibit *Eh*FT.[5] When the concentration of E was zero, no P was formed and a single peak of S2 was detected (**Fig. 2B**, lower trace). In the presence of E but without I, S2 was converted in to P and, accordingly, two peaks were observed after P was separated from the remaining S2 (**Fig. 2B**, middle trace). Finally, in the presence of I, the reaction rate was lower and the amount of P formed during the same incubation time was smaller. The peak of P was smaller while the peak of the remaining S2 was higher (**Fig. 2B**, upper trace) than those in the absence of I. When the incubation time varied, the reaction kinetics could be measured. The kinetics in the absence and presence of I were classical Michaelis kinetics (**Fig. 2C**). Thus, all 4 components were mixed to the level at which the rate of P formation depended on I.

Third, we tested if the IMReSQ method could be used to quantitatively rank the potencies of inhibitors. Conventionally, IC_{50} values (inhibitor concentrations that cause 50% reduction of the reaction rate) are used to rank inhibitors. IC_{50} depends on enzyme and substrate concentrations and, thus, inapplicable to IMReSQ, in which the solutions are not ideally mixed. To quantitatively rank inhibitors by IMReSQ, we suggest PIC_{50}, which is a pre-injection inhibitor concentration that causes 50% reduction in the reaction rate. We compared ranking of several potential inhibitors of *Eh*FT by a 'traditional' method using IC_{50} and by the IMReSQ method using PIC_{50}. In the traditional method, microliter volumes of S1, S2, I, and E were mixed in a vial and incubated to form P. A nanoliter volume of the reaction mixture was injected into the capillary and P was separated from remaining S2 and their amounts were quantitated. The PIC_{50} to IC_{50} ratio was identical within the error limits (**Table 1**).

Table 1. IC_{50} and PIC_{50} for inhibition of *Eh*FT determined by traditional (in-vial reaction) and IMReSQ methods, respectively.

Candidate inhibitor	Traditional IC_{50} (μM)	IMReSQ PIC_{50} (μM)	PIC_{50}/IC_{50}
FTI-276	1.1 ± 0.3	11.2 ± 3.2	10.2 ± 2.9
FTI-277	2.1 ± 0.4	18.9 ± 3.8	9.0 ± 1.8
FTI-343	Not measurable	Not measurable	
FTI-591	Not measurable	Not measurable	
FTI-651	70.5 ± 16.6	800.2 ± 73.2	11.3 ± 2.7
FTI-656	60.6 ± 18.2	620.1 ± 95.7	10.2 ± 3.1

The constant ratio suggests that IMReSQ can be used for quantitatively ranking potencies of the inhibitors. Four inhibitors of mammalian farnesyltransferase (FTI-276, FTI-277, FTI-651, and FTI-656) were found to be potent for *Eh*FT and can, therefore, be used as hit compounds for drug development. To conclude, IMReSQ is the first method that consumes nanoliter volumes of the reaction components per analysis and is applicable to regulatory enzymes.

Acknowledgment. The work was funded by NSERC Canada.

Supporting Information Available: Supporting materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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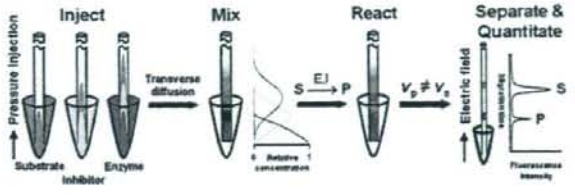

Many regulatory enzymes are considered attractive therapeutic targets and their inhibitors are potential drug candidates. Screening of large combinatorial libraries for enzyme inhibitors is pivotal to identifying large numbers of hit compounds for the development of drugs targeting regulatory enzymes. Here, we introduce the first inhibitor screening method that consumes only nanoliters of the reactant solutions and is applicable to regulatory enzymes. The method is termed Inject-Mix-React-Separate-and-Quantitate (IMReSQ) and includes five steps. First, nanoliter volumes of substrate, candidate inhibitor, and enzyme solutions are injected by pressure into a capillary as separate plugs. Second, the plugs are mixed inside the capillary by transverse diffusion of laminar flow profiles. Third, the reaction mixture is incubated to form the enzymatic product. Fourth, the product is separated from the substrate inside the capillary by electrophoresis. Fifth, the amounts of the product and substrate are quantitated. In this proof-of-principle work, we applied IMReSQ to study inhibition of recently-cloned protein farnesyltransferase from parasite *Entamoeba histolytica*. This enzyme is a potential therapeutic target for anti-parasitic drugs. We identified three previously unknown inhibitors of this enzyme and proved that IMReSQ could be used for quantitatively ranking the potencies of inhibitors.

Parasitology International 57 (2008) 386–389

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Presence and gradual disappearance of filaria-specific urinary IgG4 in babies born to antibody-positive mothers: A 2-year follow-up study

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ARTICLE INFO

Article history:
Received 6 July 2007
Received in revised form 25 March 2008
Accepted 26 March 2008
Available online 10 April 2008

Keywords:
Filariasis
IgG4
ELISA
Urine
Sri Lanka

ABSTRACT

A total of 14 Sri Lankan pregnant women, who were anti-*Brugia pahangi* urinary IgG4 positive, and their 14 newborn babies were followed up for the urinary antibody for 2 years by enzyme-linked immunosorbent assay. Eight babies showed positive IgG4 reaction, at least once within 4 months after birth. Urinary antibody titers of mothers and their babies measured around the perinatal period showed a significant positive correlation, suggesting that baby's IgG4 was transferred from the mother through the placenta. The IgG4 decreased gradually and became negative in all positive babies by day 339.3 after birth. The present result provides a basis to judge if a positive urine ELISA test among babies is due to a new filarial infection.

1. Introduction

We reported an enzyme-linked immunosorbent assay (ELISA) that uses urine as samples to detect filaria-specific IgG4. The ELISA showed high sensitivity of 95.6% with *Wuchereria bancrofti*-infected people in Sri Lanka and specificity of 99.0% with non-endemic controls in Laos, Thailand and Japan [1]. Collection of urine samples is non-invasive and thus easily acceptable for most people. It is particularly useful in a study including young children. Using a specially devised plastic bag, mothers can collect urine from their babies, who do not show fear or uneasiness. In Sri Lanka, we studied filaria-specific urinary IgG4 in children aged less than 5 years old, and reported several positive babies born within 58 days [2]. Transfer of the antibody from mothers to their babies is the most likely explanation, and if so, it is important to determine how long the antibody will persist in urine, so that transferred IgG4 and newly generated one after filarial infection can be distinguished. In this paper, we report the result of a long-term follow-up study on the specific IgG4 levels in urine obtained from pregnant women and their newborn babies.

2. Materials and methods

Fourteen pregnant women were registered for the study. Twelve of them were urinary IgG4 positives before delivery and 2 were found positive after delivery. Except for one mother, urine samples were collected from them once or twice before delivery, and after delivery, both from the mothers and their 14 babies (8 males and 6 females) at 1, 2, 3, 4, 5, 6 and 12 months, and then at 2 years, as a rule. For various reasons, the dates of collecting samples could not be accurate. The treatment for filariasis was withheld during the study. For babies, a special plastic sampling bag with an adhesive collar (ATOM Pediatric Urine Collector; ATOM Medical, Tokyo) was utilized. The collar was to fix the bag on the skin around the urethral opening. Five milliliters of urine was transferred to a plastic bottle, mixed with sodium azide at 0.1%, and kept at 4 °C until the antibody was measured at Aichi Medical University School of Medicine, Japan.

Filaria-specific urinary IgG4 was measured by ELISA with a slight modification from our previous report [1,2]. In brief, crude antigens were prepared from adult *Brugia pahangi* females. A 96-well microtiter plate was coated with the antigens (5 μg/mL), and blocked with 1% casein buffer. Urine samples, without concentration, were applied to the plate (100 μL per well) and incubated overnight at 25 °C. After washing, 100 μL peroxidase-conjugated mouse monoclonal antibody to human IgG4, purchased from Southern Biotechnology Associates, Inc., AL, was reacted. The coloration was with ABTS peroxidase substrate (KPL Inc., Gaithersburg, MD). With the ELISA

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doi:10.1016/j.parint.2008.03.006

system, antibody levels were expressed as arbitrary units (U), ranging from 0 to 7290 U, and the cutoff value was 54.7 U [1]. To standardize the measurement of antibody titers, pooled and serially diluted sera from bancroftian filariasis cases were applied to each ELISA plate to construct a standard curve. Each urine sample was measured in duplicate and the average titer was used. A pair of mother and child urine was examined in the same plate.

The study proposal was reviewed by the ethical committees of University of Ruhuna, Sri Lanka and Aichi Medical University School of Medicine, Japan, and cleared. The mothers were explained on the purpose and methods of the study, and all participants gave their consent.

3. Results

The urinary IgG4 antibody of mothers fluctuated considerably during the 2-year period, and only 5 of 14 positive mothers showed consistently positive results (Fig. 1). Three borderline-positive mothers (mother–baby pair Nos. 5, 9, and 12 in Fig. 1) became positive only 1–3 times out of 8 measurements each after delivery. In an extreme case of fluctuation (pair No. 6), the mother's titers changed from 791 U to 14 U, and then to 1783 U, respectively, 35 days before, and 27 and 59 days after delivery. As a whole, however, ELISA results were highly consistent in terms of producing a positive reaction among mothers: by 6 months after delivery, a total of 84 samples were collected from 11 mothers (3 borderline positives excluded), and 80 of them (95.2%) gave positive reactions.

Eight of 14 babies (57%) showed a positive urine reaction, at least once within 4 months after birth. In 11 babies, IgG4 titers were constantly lower than those of mothers; in other words, 3 babies (pair Nos. 5, 6, and 8) showed, at one point, higher titers than their mothers'. All but 2 babies showed relatively high IgG4 titers 1–6 months after birth. In one exceptional baby (pair No. 2), IgG4 units were constantly 0 U in the first 6 months (186 days) despite relatively high titers in the mother, and in the other baby (pair No. 11), the titers were only 1 U at 1 month (30 days), and 0 Us thereafter. These 2 babies are

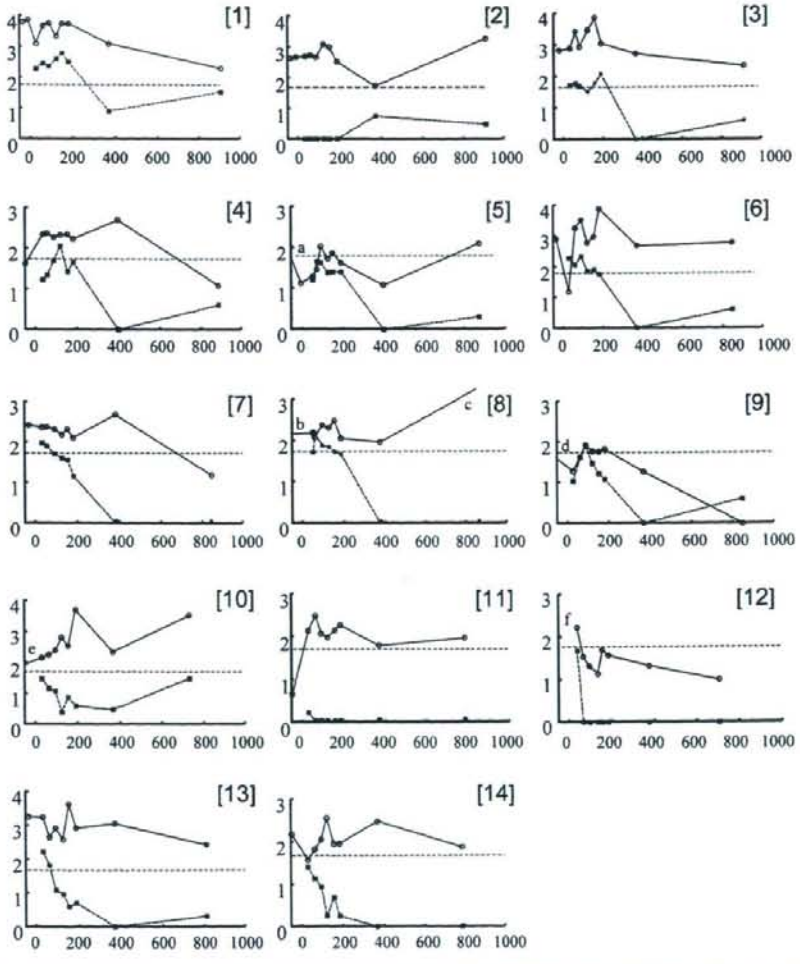

Fig. 1. Change in anti-*Brugia pahangi* urinary IgG4 titers in 14 mother–baby pairs. Pregnant women and their newborn babies were followed up for 2 years. Vertical axis: Log (IgG4 units + 1). Note that the scales for Nos. 1–3, 6, 10, and 13 range from 0–4, and those for the other Nos. from 0–3. Horizontal axis: days before or after delivery (day = 0). [ ]: mother–baby pair number, dotted line: cutoff level, ○: mother, ■: baby. [a]IgG4 titer was 73 at day −61, [b]143 U at day −83, [c]1775 U at day 850, [d]56 U at day −95, [e]80 U at day −159, and [f]IgG4 was not measured before delivery.

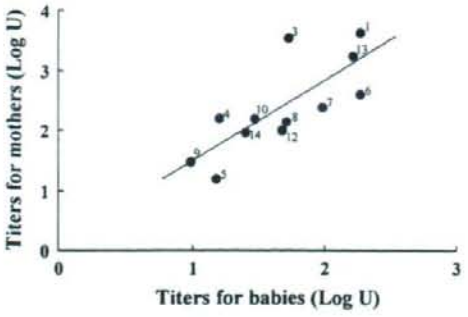

Fig. 2. Correlation of IgG4 levels in urine between mothers and their babies. An attached number to each dot corresponds to that for a mother–baby pair in Fig. 1.

considered different from the others in terms of 'completeness' of negative reaction.

In order to obtain an evidence for transplacental transfer of IgG4 antibody, perinatal IgG4 units of mothers and their babies were analyzed for correlation. In this case, the mother's unit is the average of 2 measurements nearest to the delivery day, and the baby's unit is the first measurement after birth. The analysis, excluding pair Nos. 2 and 11, revealed significant positive correlation ($r=0.770$, $P<0.004$), indicating that babies' IgG4 titers were dependent on those of mothers (Fig. 2).

After birth, babies' titers decreased gradually. All of 8 IgG4 positives became negative within a year (Fig. 1). The number of exact days required for negative conversion was estimated by simple interpolation using two data sets of IgG4 titer (U) and the number of days after birth, immediately before and after the final negative conversion. The antibody turned negative between 65.7 and 339.3 days, and the negative conversion rates of 50% and 80% were estimated at days 143.5 and 222.2 respectively (Kaplan–Meier method). The numbers of days required for negative conversion tended to be positively correlated with IgG4 levels of mothers, though this was not significant ($r=0.635$, $P<0.091$).

4. Discussion

Despite frequent samplings both from mothers and their babies for a period of 2 years, the mothers were consistently cooperative for the study. A most likely reason for the cooperation was the simplicity and non-invasiveness of urine collection. Blood collection from babies is often a troublesome and uncomfortable experience for mothers, especially in this type of study that does not have direct merit for them.

The present study confirmed a significant amount of filaria-specific IgG4 in urine among neonates born to the antibody-positive mothers. In addition, the titers of mothers and their babies correlated positively. Using serum samples, a positive correlation between antibody concentrations in mothers and their infants was considered as an irrefutable evidence for materno-fetal transfer of antibodies [3]. Our result with urine could be understood in the same way, though we did not encounter similar studies with urine only. IgG antibodies can be transferred from mothers to their fetuses by an active transport mechanism involving the neonatal Fc receptor for IgG (FcRn) expressed in the placenta [4]. The transfer of IgG subclasses was reported to be dependent on the terms of gestation [5,6], and levels of maternal IgG subclasses [6], and influenced by an infection like placental malaria [7], and birth weight of neonates [8]. As for IgG4, a cord concentration was reported to increase by gestational age, and reach the level of mother's serum by 36–37 weeks [6]. Thus, it will be possible that specific urinary IgG4 titers of babies become close to those of their mothers. In the present study, IgG4 was undetected after birth in 2 babies, even when the mothers had relatively high IgG4 titers, suggesting that appearance of IgG4 in urine involves complicated unknown mechanisms. The *in utero* sensitization of fetuses by filarial antigens would occur [9–11], but the effect on this study is unknown.

Transferred IgG disappears gradually after birth, and the speed of disappearance is reported different by species of infectious agents. For example, antibodies to mumps became undetected earlier than measles [12]. Our present study revealed that filaria-specific urinary IgG4 became negative by day 339.3. With babies born to *Onchocerca volvulus*-infected mothers, specific cord blood IgG4 at birth was reported strongly reduced 12 months after birth [11]. In applying urine-based ELISA to very young children, it is necessary to judge if a positive IgG4 reaction is due to transferred antibodies from an infected mother or a new filarial infection. Based on the present study, positive IgG4 at 1 year old and above can be regarded as filarial in Sri Lanka. All but one mother breast-fed the babies. Milk contains maternal antibodies including IgG4, but, in humans, they do not enter infant circulation [13].

In the present study, IgG4 levels of the same mothers were followed up for 2 years, and this will be an opportunity to consider the fluctuations, which could show tremendous ups and downs as observed in the pair No. 6. Possible factors causing fluctuations could be (i) time of the day to collect urine, (ii) chemicals in urine whose concentrations can be changing according to ingested foods, drinks, medicines, etc., (iii) urinary infections, and (iv) other factors, including pathology caused by filarial parasites. In relation to the factor (i), which would reflect, in part, hourly fluctuation of urine concentrations, we reported that timing of urine collection did not influence ELISA results, in terms of deciding positive or negative result [1]. With commercial urine ELISA kits for *Helicobacter pylori* infection, random single-void urine was reported suitable for IgG measurement [14]. As for the factor (ii), 28 different chemicals, including glucose, amino acids, caffeine, atropine, and acetaminophen, were tested with the same *H. pylori* ELISA, and reported to have no effect on titers [14]. However, using our IgG4 ELISA, we have observed opaque precipitation at the bottom of ELISA well in 24 of 10,409 (0.23%) urine samples collected in China [15], suggesting the presence of unknown substance in urine. Concerning (iii), the presence of microorganisms in urine and pH of urine did not influence urine *H. pylori* ELISA [16]. The pathology caused by filariae could influence urinary IgG4 titers. Chyluria and hematuria, which will indicate the leak of plasma into urine, are common symptoms of filariasis and tend to repeat remission and exacerbation. In this study with asymptomatic women, no trial was made to investigate the leak into urine. Technical variability is inevitable, but this could be minimized with utmost care: filaria-specific urinary IgG4 titers determined twice approximately 70 days apart in Thailand with 67 individuals showed a very good stability [17].

The present study confirmed the materno-fetal transfer of filaria-specific IgG4 and its disappearance from urine within a year. The information will enhance usefulness of the urine-based ELISA in the epidemiology and control of lymphatic filariasis, where young children are indispensable as a sentinel population to know filarial transmission.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research (B) No. 14406005 and No. 17406011 from the Japan Society for the Promotion of Science.

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Parasitology International 57 (2008) 390–395

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Distribution of filarial elephantiasis and hydrocele in Matara district, Sri Lanka, as reported by local leaders, and an immunological survey in areas with relatively high clinical rates

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ARTICLE INFO

Article history:
Received 3 August 2007
Received in revised form 31 March 2008
Accepted 1 April 2008
Available online 12 April 2008

Keywords:
Filariasis
Rapid assessment procedure
Sri Lanka
IgG4
Geographic information system

ABSTRACT

To eliminate lymphatic filariasis by means of mass drug administration, it is essential to have reliable data on the disease distribution and prevalence in targeted areas. In Matara district, Sri Lanka, self-administered questionnaires were mailed to 2105 local leaders questioning the presence and the numbers of elephantiasis and hydrocele cases. The information provided by them revealed that elephantiasis was clearly aggregated in the southern part of the district along the coast, while hydrocele was distributed rather evenly in the whole district, including Deniyaya region where no endemic filariasis had been known. To confirm active transmission of filariasis in Deniyaya, *Wuchereria bancrofti* antigen and filaria-specific urinary IgG4 antibody were measured with 2436 subjects. The positive rates for antigen and antibody were 0.6% and 4.3%, respectively. The titer analysis of IgG4 according to age revealed that the youngest IgG4 positive was 3 years old, and that in 10 years old or less, there were 16 positives out of 607 children examined (2.6%). It was concluded that filarial transmission at a low level was going on in the region.

1. Introduction

For a national lymphatic filariasis elimination program based on mass drug administration, it is essential to have information on the distribution and prevalence of infection. In order to obtain the information as quickly and cost-effectively as possible, a variety of methods have been tested and applied in different epidemiological settings. These methods are referred to as rapid assessment procedures (RAPs). The RAPs include, for example, filarial antigen detection using commercially available immunochromatographic tests at systematically sampled points [1,2], or with a targeted subpopulation like schoolchildren [3], detection of obvious clinical signs such as elephantiasis and hydrocele [4], and vector mosquito surveys for filarial infection [5–8]. Although scientific accuracy might be sacrificed to various extents, the clinical signs have been utilized to obtain necessary information to plan and execute a filariasis elimination program, especially in areas with limited information on the disease. A hydrocele rate based on examination by a physician was reported useful to estimate a level of endemicity, as the rate correlated well with a community microfilaria rate [9]. A more simple survey based on self-administered questionnaires was also shown to be useful, in which local leaders were questioned on the presence and the numbers of elephantiasis and hydrocele [4].

In Sri Lanka, it has been reported that bancroftian filariasis is endemic in the 'filarial belt' that lies along southwestern and southern coasts of the island country. Although the belt was described by Abdulcader and Sasa in their map as a zone of about 10 km in width [10], the boundary of the belt is vague and only a limited number of surveys were done outside the belt. Therefore, it is necessary to clarify if the infection is occurring in inland areas, and how far it extends, if any. In the present study, we mailed a questionnaire to local leaders of Matara district, and collected information on the presence and the numbers of elephantiasis (including lymphedema) and hydrocele cases in their territories of responsibility. The reliability of this method has been confirmed in our separate studies in Sri Lanka, in which the estimates of community hydrocele prevalence based on village leaders' information showed a significant positive correlation with the clinical and immunological estimates by clinicians (Yahathugoda et al., unpublished). After obtaining the information, and the data having been mapped out, a field survey was carried out in inland villages, where no filarial infection had been recorded before but the leaders reported relatively high rates of clinical cases. Schoolchildren and villagers were examined by Og4C3 ELISA for *Wuchereria bancrofti* antigen, and by ELISA for filaria-specific

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doi:10.1016/j.parint.2008.04.007

urinary IgG4 antibodies, with the purpose to determine if transmission is actually occurring in those areas.

2. Materials and methods

2.1. Study areas and subjects

The study was carried out in Matara district, located southernmost part of the country, facing the Indian Ocean. The district occupies the land area of 1290 km^2 and had the registered population of 761,236 in 2001 (Government census, 2003). The inland areas are hilly or mountainous, in general, and tea plantations cover a large part of the land. The district has 16 administrative divisions each administered by a Divisional Secretary. The 16 divisions are further divided into 650 *Grama Niladari* (GN) divisions. In each GN division, there are 3 categories of local leaders. They are *Grama Niladari*, *Samurdhi Niyamaka*, and *Govi Niyamaka*. There are 1 *Grama Niladari*, 1–3 *Samurdhi Niyamakas*, and 1 *Govi Niyamaka* in a GN division, as a rule. The *Grama Niladari* is the Village Headman and his/her duties include monitoring of census, electoral registers, housing and population data, and amenities of the community. He/she also keeps records of epidemics and accidents, and attends to general administration. The *Samurdhi Niyamaka* administers the disbursement of monthly allowances to poor families given by the government that helps rural communities to develop their economy and improve personal hygiene. The duties include activities on water supply, power supply and roads. The *Govi Niyamaka* assists communities in all matters pertaining to agriculture. The local leaders in the 3 categories are appointed government employees placed under the supervision of District Secretary (Government Agent). All local leaders joined the questionnaire-based survey, which took place in September–November, 1999.

An immunological survey was carried out in Feb., 2002 in 14 GN divisions (total population: 29,181) in Deniyaya region (Fig. 1A), which is the northernmost part of Matara district (about 40–50 km from the coast) and a tea plantation area in mountains. Fifteen schools along main roads were visited in the morning and blood and urine samples were collected from as many children as possible. They were students of grades 1 to 12, whose ages are between 6 and 18 years, as a rule. A community located close to each school was also visited in the afternoon and the residents excluding schoolchildren were examined. Both urine and blood samples were collected from a total of 2436 people.

Before the field visit, the study plan was reviewed by the ethical committees of University of Ruhuna (Sri Lanka) and Aichi Medical University (Japan), and approved.

2.2. Questionnaire distribution and data management

The questionnaire form in local language, which had been pretested for easiness in use and clarity, included several identification tags and 9 simple questions. Main questions were on the presence and the numbers of elephantiasis and hydrocele cases. Some economic indicators such as types of housing and toilet were also questioned, but not analyzed in this study. The 3 categories of local leaders were requested by District Secretary to cooperate on the questionnaire survey. A questionnaire form in an envelope was then mailed to each local leader, together with a reply paid envelope. Three weeks after posting, the second questionnaire form was mailed to all non-respondents, and the same was repeated after another 3 weeks.

Of 1730 replies received, 62 were abandoned due to unclear ID tags. With remaining 1668 replies, 647 GN divisions could be identified. As expected, numerical information given by leaders from the same GN division showed considerable inconsistency. To deal with this problem, detailed criteria were made how to compute representative figures for each GN division. For example, for the questions relating to population, housing and economic indicators, answers given by a *Grama Niladari* were used, and without his/her reply, answers by a *Samurdhi Niyamaka* or a *Govi Niyamaka* were substituted in this order. For the question on the numbers of elephantiasis and

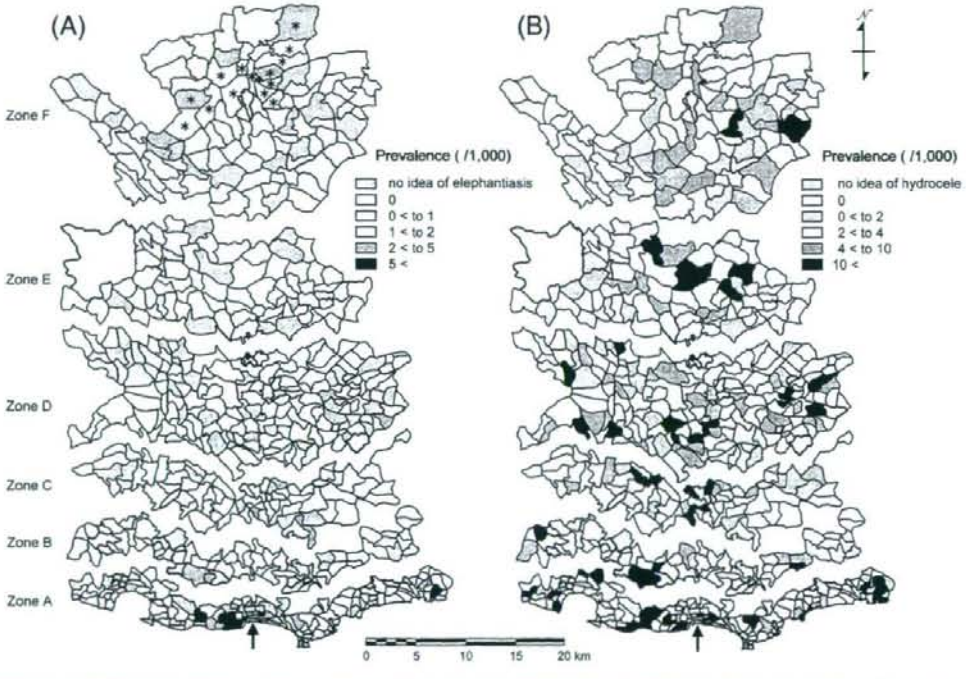

Fig. 1. Elephantiasis (A) and hydrocele (B) prevalence maps according to distance from the coastline. GN divisions with * in Fig. 1A are those selected from Deniyaya region for the immunological study. An arrow indicates the location of Matara City.

Table 1
The number of GN divisions reporting elephantiasis analyzed by prevalence level and distance from the coast

Prevalence level (/1000)	No. of GN divisions (% of all zones)						
	Zone A	Zone B	Zone C	Zone D	Zone E	Zone F	All zones
0	54 (13.9)	35 (9.0)	49 (12.6)	114 (29.3)	56 (14.4)	81 (20.8)	389 (100)
(a) 0 < to 1	27 (28.4)	11 (11.6)	8 (8.4)	28 (29.5)	8 (8.4)	13 (13.7)	95 (100)
(b) 1 < to 2	12 (37.5)	5 (15.6)	4 (12.5)	7 (21.9)	3 (9.4)	1 (3.1)	32 (100)
(c) 2 < to 5	11 (64.7)	2 (11.8)	3 (17.6)	1 (5.9)	0 (0.0)	0 (0.0)	17 (100)
(d) 5 <	10 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	10 (100)
(a)+(b)+(c)+(d)	60 [52.6]	18 [34.0]	15 [23.4]	36 [24.0]	11 [16.4]	14 [14.7]	154 [28.4]
All levels	114	53	64	150	67	95	543

Zones A, B, C, D, E, and F are, respectively, 0 ≤ to <3, 3 ≤ to <6, 6 ≤ to <10, 10 ≤ to <20, 20 ≤ to <30, and ≥30 km from the coast.
[ ] : % of GN divisions reporting elephantiasis cases, i.e., 100×{(a)+(b)+(c)+(d)}/All levels.

hydrocele, figures given by a *Grama Niladari* and a *Samurdhi Niyamaka* were averaged, and, without one of them, figures by a *Govi Niyamaka* were substituted. These and other detailed criteria were made considering different roles of leaders, and quality/reliability of information they could provide. The prevalence of clinical cases (per thousand) for each GN division was calculated as follows;

elephantiasis rate = 1000 × (reported number of cases)
÷(population of GN division provided by Grama Niladari)
hydrocele rate = 1000 × (reported number of cases)/(male population)

The male population was estimated as 50% of the total population.

2.3. Making a digitized map

A map of Matara district including all GN divisions is required to describe the distribution and prevalence of the clinical cases. A blueprint copy of hand drawn map, entitled "Matara District – Farmer Organizations and Agrarian Services Areas" and including 601 GN divisions, was provided by the District Secretariat of Matara. The scale is 1:63,360, and it was not known when the map was made.

The map was computerized with an image scanner, and then the data were imported into a geographic information system (GIS) using TNTmips (MicroImages, Inc., NE, USA). In the process of mapping, 86 GN divisions were abandoned, because their names did not match up to the GN divisions appearing on the hand drawn map. As the map was apparently old, new or renamed GN divisions could not be matched. Twelve areas in the hand drawn map were identified as forest or uninhabited land. Finally, the data from 561 GN divisions were included in the GIS map. To analyze the distribution and prevalence of elephantiasis and hydrocele according to the distance from the coastline, the map of Matara was sliced into 6 zones: zone A (0 to <3 km from the coast line), zone B (3 to <6 km), zone C (6 to <10 km), zone D (10 to <20 km), zone E (20 to <30 km), and zone F (≥30 km). In making a zoning based on a distance from coastline, many GN divisions were found covering 2 neighboring zones. In this case, the whole division was allocated to the zone further from the coastline. There were 12 GN divisions with a detached territory in a different zone. They were counted twice in analysis and thus the total number of data was inflated to 573.

2.4. Immunological survey

The antigen test was carried out using Og4C3 ELISA kit (TropBio Pty Ltd., Australia) with some modifications. Finger-prick blood was collected on a filter paper (Advantec Toyo, Japan), dried and kept at 4 °C until use. The filter paper was soaked in phosphate buffered saline and the soaking fluid was used as a sample. This filter paper ELISA showed almost identical efficacy compared with the ordinary Og4C3 ELISA using serum samples. The technical details are described elsewhere [11].

Urinary IgG4 was detected using ELISA reported previously [12] with slight modifications. In brief, a urine sample was collected in a plastic tube and mixed with sodium azide at 0.1% for preservation. Urine without concentration was added into a well of the microtiter plate pre-coated with female *Brugia pahangi* adult antigens (5 μg/ml), and incubated overnight at 25 °C. After washing, peroxidase-conjugated mouse monoclonal antibody to human IgG4 (Southern Biotechnology Associates, Inc., AL, U.S.A.) was added for 1 h at 37 °C. Coloration was with ABTS peroxidase substrate. Serially diluted positive serum samples were prepared for each microtiter plate to construct the standard curve, with which IgG4 levels were quantified using arbitrary antibody unit (U) ranging from 0 to 7290 U. The cutoff value was 54.7 U. The ELISA showed the sensitivity of 95.6% and the specificity of 99.0% [12].

3. Results

3.1. Distribution and prevalence of the clinical signs as reported by local leaders

A total of 2105 questionnaire forms were mailed to local leaders in the 1st round, and 1031 (49.0%) answers were obtained. In the 2nd round, 49.5% of previous non-respondents gave answers. The 3rd round resulted in much lower response (33.7%). In total, 1730 answers were obtained from 2105 addressees, with the final response rate of 82.2%. The population of 647 GN divisions identified in this study based on the questionnaire was 873,288. The distribution and prevalence of elephantiasis are shown in Fig. 1A, which clearly shows that

Table 2
The number of GN divisions reporting hydrocele analyzed by prevalence level and distance from the coast

Prevalence level (/1000)	No. of GN divisions (% of all zones)						
	Zone A	Zone B	Zone C	Zone D	Zone E	Zone F	All zones
0	57 (18.2)	28 (8.9)	41 (13.1)	81 (25.8)	45 (14.3)	62 (19.7)	314 (100)
(a) 0 < to 2	9 (17.6)	2 (3.9)	7 (13.7)	17 (33.3)	6 (11.8)	10 (19.6)	51 (100)
(b) 2 < to 4	9 (15.0)	10 (16.7)	6 (10.0)	22 (36.7)	7 (11.7)	6 (10.0)	60 (100)
(c) 4 < to 10	18 (25.7)	6 (8.6)	6 (8.6)	21 (30.0)	6 (8.6)	13 (18.6)	70 (100)
(d) 10 <	22 (46.8)	5 (10.6)	5 (10.6)	11 (23.4)	3 (6.4)	1 (2.1)	47 (100)
(a)+(b)+(c)+(d)	58 [50.4]	23 [45.1]	24 [36.9]	71 [46.7]	22 [32.8]	30 [32.6]	228 [42.1]
All levels	115	51	65	152	67	92	542

Zones A, B, C, D, E, and F are, respectively, 0 ≤ to <3, 3 ≤ to <6, 6 ≤ to <10, 10 ≤ to <20, 20 ≤ to <30, and ≥30 km from the coast.
[ ] : % of GN divisions reporting hydrocele cases, i.e., 100×{(a)+(b)+(c)+(d)}/All levels.

Table 3
Prevalence of *W. bancrofti* antigen and filaria-specific urinary IgG4 in selected GN divisions from Deniyaya region

GN division	No. exam.	No. Ag (+)	%Ag (+)	No. IgG4 (+)	%IgG4 (+)
Adaradeniya	125	0	0.0	4	3.2
Batandura North	184	2	1.1	5	2.7
Batandura South	85	0	0.0	0	0.0
Beliattakumbura	175	1	0.6	7	4.0
Deniyaya	215	3	1.4	22	10.2
Deniyaya West	153	0	0.0	5	3.3
Kalugalahena	238	3	1.3	16	6.7
Kotapola North	125	1	0.8	2	1.6
Morawaka	162	1	0.6	10	6.2
Pallegama North	233	2	0.9	11	4.7
Pathawita	183	0	0.0	4	2.2
Pussawela	180	0	0.0	7	3.9
Thanipita	202	0	0.0	6	3.0
Viharahena	176	2	1.1	5	2.8
Total	2436	15	0.6	104	4.3

elephantiasis is more confined to the coastal zone. Further, the number of GN divisions was analyzed according to zone and prevalence level in Table 1, in which 30 GN divisions had to be excluded because the village leaders answered that they had no idea of elephantiasis. The symptom was reported in more than a half (52.6%) of GN divisions in the zone A. The rate decreased gradually in inland zones and was 14.7% in the zone F. As for the GN divisions with 'high' prevalence, defined arbitrarily as >2 cases/1000 of population, 26 of 27 such divisions were in the zones A to C, within 10 km from the coastline. The distribution and prevalence of hydrocele are shown in Fig. 1B, and the GN divisions were analyzed by zone and prevalence level in Table 2. A total of 31 GN divisions were excluded because the leaders answered that they had no idea of hydrocele. The GN divisions with the highest prevalence (>10 cases/1000 of male population) were apparently more in the zone A than in the other zones, but the distribution of the symptom was more or less even in the whole district; the rates of GN divisions with hydrocele ranged from 50.4% in the zone A to 32.6% in the zone F, with the average of 42.1%.

3.2. Immunological study

A total of 2436 people in 14 GN divisions in Deniyaya region, where 8 elephantiasis and 49 hydrocele cases were reported, were examined for antigenemia and urinary IgG4 (Table 3). Of those, 15 (0.6%) were positive for antigen, and 104 (4.3%) were positive for antibody. By GN division, Deniyaya had the highest rates of antigen (1.4%) and antibody (10.2%), followed by Kalugalahena (1.3% and 6.7%). No correlation was found between infection rates (antigen and IgG4) and clinical rates (elephantiasis and hydrocele).

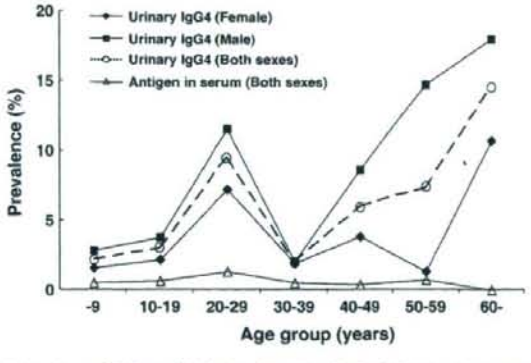

Fig. 2. Prevalence of *W. bancrofti* infection determined by Og4C3 ELISA and urinary IgG4 ELISA, and analyzed by sex and age group.

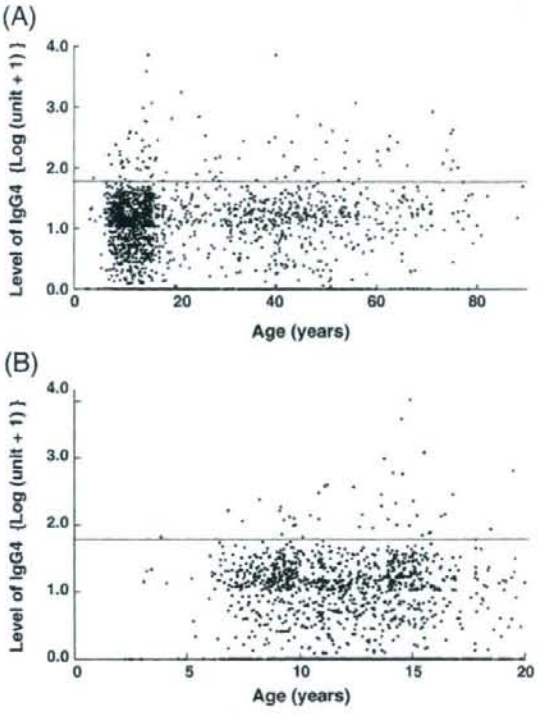

Fig. 3. Urinary IgG4 titers in the selected GN divisions from Deniyaya. (A) All people examined (n=2436), (B) People aged <20 years old (n=1611).

The prevalence of antigen and antibody was analyzed by age group and sex (Fig. 2). The prevalence of antigen was very low in all age groups, while that of urinary IgG4 showed an irregular pattern. In males, the age groups 20–29, 50–59 and 60-years showed higher prevalence than the age groups −9, 10–19 and 30–39 years ($P<0.03$ for all comparisons with χ^2 test). In females, the age group 20–29 years showed higher prevalence than the age group 10–19 years ($P<0.05$), and the age group 60- was higher than the age groups -9, 10–19 and 30–39 years ($P<0.05$). The oldest group (60-years) had the highest prevalence in both sexes (male: 18.0%, female 10.7%).

Urinary IgG4 levels are plotted against ages in Fig. 3. The youngest positive was a 3-year-old boy, with a low titer of 64.6 U. Under 11 years, all of 16 positive titers were 300 U or less, while in 11–19 years, 10 of 20 positive titers were more than 300 U ($P=0.007$, Fisher's exact test). At 14 and 15 years, 3 children had high titers over 1000 U (Fig. 3B).

4. Discussion

Two species of filarial parasite were recorded in Sri Lanka. Before 1940, filariasis was caused by *Brugia malayi*, with only exception of 2 towns, Galle and Matara, where *W. bancrofti* was endemic. Then, *B. malayi* started disappearing rapidly mainly because of deliberate removal of *Pistia*, the water plant required for breeding of *Mansonia* vectors, and had been eliminated mostly by mid 1960's. On the other hand, *W. bancrofti* gradually expanded its distribution along the coast, and after 1960's, it has become only significant species in the country [13,14]. Very old elephantiasis caused by *B. malayi* would not influence the present study, and hydrocele is a symptom of only *W. bancrofti*. *B. malayi* infection produces cross-reactive urinary IgG4, but it is unlikely that it interfered with the present IgG4 study in Deniyaya: the villages are mostly on mountain slopes, and there was no past report of endemic filariasis in the region.

Bancroftian filariasis in Sri Lanka was reported to be confined to a strip of land along the southwestern and southern coasts of the county [10]. However, a relatively recent report revealed some inland foci in Kurunegala, Gampaha and some other areas [14,15], suggesting a wider distribution of filariasis. To study the latest distribution of elephantiasis and hydrocele, we decided to mail questionnaires to local leaders, as Sri Lanka has reliable mailing services. In applying the questionnaire-based surveys, however, it is prerequisite to confirm the reliability of information given by local leaders. In order to verify this, we conducted a separate study in the neighboring district of Hambantota that has 576 GN divisions. A questionnaire was mailed to each *Grama Niladari* and collected data were analyzed as in the present study. Based on the results, GN divisions were categorized into the high-, medium- and low-endemic areas, and then, clinical surveys by doctors were carried out at 24 selected GN divisions (8 divisions/category), with a total of 1170 males of age ≥10 years. The 24 hydrocele rates obtained by doctors and the rates based on information by village leaders from corresponding GN divisions correlated positively ($r=0.55$, $P<0.05$) (Yahathugoda et al., unpublished). In yet another study in Galle district, questionnaire-based and urinary IgG4-based prevalence in 13 GN divisions resulted in a clear positive correlation ($r=0.78$, $P<0.01$) (Yahathugoda et al., unpublished). These results will indicate that local leaders can provide reliable information in terms of relative prevalence of hydrocele in Sri Lanka.

The surveys utilizing the postal services were successful in involving non-medical people as informers: 2105 questionnaires were mailed and 82.2% of addressees responded. The total population of Matara district reported by the leaders in 647 DN divisions was 873,288, which is higher than the official census result of 761,236 in 2001. This is probably caused by duplicate registration of families and individuals who were migrating, or changed residence for various reasons. For example, schooling outside the designated school area is common. This higher figure than expected would suggest, at least, that the *Grama Niladaris* did cover most of the people in the district. However, the reported figures of clinical cases seemed to be underestimates. In our previous clinical study in 3 suburban areas of Matara City (Polhena, Madihe and Walgama), the rates for elephantiasis/lymphedema and hydrocele were 3.0% and 6.2%, respectively [16]. In the present study, the GN divisions corresponding to the 3 study areas, though they are not exactly the same, showed the elephantiasis rate of 0.6% and hydrocele rate of 0.5%. The much lower prevalence is most probably due to reports of only obvious cases recognizable by people, and a tendency for a hydrocele patient to hide it. Similar gross under-reporting by key informants was reported in Ghana. However, both elephantiasis and hydrocele rates obtained through key informants and those in a clinical survey correlated well [17], indicating that people could provide reliable relative prevalence. In our present study, the information by local leaders revealed clearly an aggregated distribution of elephantiasis in the most endemic and long-standing filarial belt in Sri Lanka.

The questionnaire-based study revealed a clear contrast between the distributions of elephantiasis and hydrocele. It was a surprise to find so many reports of hydrocele cases in tea plantation areas on mountains, where no endemic filariasis had been reported. In order to confirm if filariasis transmission is actually occurring in these areas, an immunological survey was conducted in Deniyaya region, where relatively high clinical rates were reported. The antigen study with 2436 people revealed that the positive rate was very low (0.6%) and there was no difference in the rate by age group. From this result alone, active filarial transmission could not be confirmed in the region, because (i) the positives might get infection elsewhere, and (ii) false positive results were possible. The positive rate of urinary IgG4 was much higher (4.2%), and showed variation by age. The 20–29 year group had relatively high rates of 11.5% in males and 7.2% in females. Males of 50 years and above also had high rates. However, the prevalence in the 30–39 year group was very low and the reason was unknown. When urinary IgG4 titers were analyzed by age, a clear evidence of transmission was obtained, that is, (i) in 10 years old or less, there were as many as 16 IgG4 positives out of 607 examined (2.6%), and (ii) all of them had titers of ≤300 U, while 10 of 20 positives had >300 U in ages 11–19 years (Fisher's exact test, $P=0.007$), indicating the increase of titers in older children (Fig. 3B). Children are regarded as an important sentinel population to know recent transmission. Our studies in other endemic areas revealed the similar increase of IgG4 units by age among children [18,19]. We therefore concluded that filariasis transmission was going on in Deniyaya region at a low level.

Elephantiasis was more restricted in the filarial belt, while hydrocele was found widespread in the district. One possible explanation for different distribution could be as follows: In 3 suburban areas of Matara City, where microfilaria prevalence was 4.4%, we reported that the prevalence of lymphedema/elephantiasis increased lineally after 40 years of age, whereas, hydrocele increased lineally after 20 years [16]. As filariasis in Matara district was restricted inside the filarial belt until 1965 [10], the infection must have been spreading beyond the belt since then. Thus, the history of endemic filariasis in most inland areas is not longer than 40 years, which would not be enough for elephantiasis to make appearance.

It is a general recognition that RAPs based on finding out clinical cases are only effective when endemicity is high enough to produce many clinical cases [20]. In fact, the presence of a few clinical cases does not confirm the site of active transmission. In the present study, reports on a small number of clinical cases triggered an immunological survey in Deniyaya region, and revealed an active endemic focus. The combined use of questionnaire-based RAP and urine IgG4 ELISA would be a useful method to detect unnoticed foci, which could pose a problem in the final stage of the elimination program.

Acknowledgements

We would thank the District Secretary Matara, Divisional Secretaries and their staff, Zonal Education Director, Morawaka and all the school principals and their respective staff members for assistance in this study. The contribution by all the members of the Filariasis Research, Training and Service Unit in field activities is greatly appreciated. This study was supported by Grants-in-Aid for Scientific Research (B) No. 14406005 and No. 17406011 from the Japan Society for the Promotion of Science.

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Am. J. Trop. Med. Hyg., 79(4), 2008, pp. 599–604

Enzyme-linked Immunosorbent Assay to Detect Urinary Antibody Against Recombinant rKRP42 Antigen Made from *Leishmania donovani* for the Diagnosis of Visceral Leishmaniasis

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Abstract. We recently reported the production of the recombinant kinesin-related protein of *Leishmania donovani* with a molecular weight of 42 kd (rKRP42) and the value of the antigen in serum-based ELISA for the diagnosis of visceral leishmaniasis (VL). In this study, the rKRP42 antigen was validated with ELISA using urine samples (rKRP42 urine ELISA). The urine-based ELISA showed 94% sensitivity (108 positives among 115 VL samples) and 99.6% specificity (239 negatives among 240 non-VL samples). The sensitivity and specificity are almost similar to our previous results by ELISA with acetone-treated *L. donovani* promastigote antigen and direct agglutination test, both methods being done by use of urine samples. A comparison of the rKRP42 urine ELISA with the commercially available urinary antigen detection kit (KAtex) using 108 VL samples showed much higher sensitivity of the ELISA (96.3%) than KAtex (55.6%). The use of the rKRP42 antigen with urine samples will facilitate epidemiologic studies.

INTRODUCTION

Visceral leishmaniasis (VL) is caused by a protozoan parasite *Leishmania donovani* complex. In full-blown disease, the mortality rate ranges from 80% to 100% if not treated. Even with treatment, sometimes the case fatality rate reaches > 10%.[1] Recently, in a population-based study in Bangladesh, Ahlwalia and others[2] reported a VL incidence of 2% per year from 2000 to 2002, with a case fatality rate of 19% among adult women (≥ 15 years of age) and 6–8% among the other demographic groups (girls < 15 years of age and males of all ages). They also reported that the median duration of illness before receiving treatment was significantly longer in females than males. Delays in diagnosis and treatment increase the risk of complications and death.[1] VL is targeted for control under the neglected tropical diseases program.[3] The effective control of VL has been hampered because of the lack of suitable tools for early detection of infection and effective chemotherapy. Therefore, an accurate, easy, and low-cost diagnostic method is an urgent necessity.

Because of invasiveness and inadequate sensitivities, demonstration of the causative parasites in aspirates from lymph nodes, bone marrow, and the spleen has limited its use in a field survey.[4] In the last two decades, several serodiagnoses, like ELISA with crude or recombinant antigens,[5–7] and direct agglutinin test (DAT),[8,9] have been providing good diagnostic results. However, the use in endemic areas has been limited because of the expertise required for execution. In Bangladesh, DAT only has been available for clinicians at the Institute of Epidemiology, Disease Control and Research (IEDCR) situated in the capital, Dhaka. Recently, a recombinant antigen rK39, a part of *L. chagasi* kinesin-related protein, has been widely evaluated with ELISA or in dipstick format.[10,11] Although the antigen has been reported satisfactory in Indian subcontinent, the results varied considerably in different endemic areas. In India and Nepal,[12–14] the test showed the highest sensitivity of 100%, whereas it was significantly lower in Venezuela (88%),[15] in southern Europe (71.4%),[16] and in Sudan (67%).[17] The variation could be explained by differences in subspecies of *L. donovani* complexes, genetic differences in individual patients or in racial subgroups, and epidemiologic factors such as the length or severity of diseases.[18] Thus, it is desirable to develop new antigens for comparison. Recently, we reported the production of recombinant protein rKRP42, a part of *L. donovani* kinesin-related protein and a homolog to rK39, and evaluation of this antigen in ELISA with serum samples for the diagnosis of VL.[19]

Recently, the use of urine for blood has been considered valuable for the diagnosis of VL. A low-molecular-weight heat-stable leishmanial carbohydrate antigen was detected in urine by a latex agglutination test (KAtex).[20,21] We reported an ELISA with urine samples using acetone-treated *L. donovani* promastigotes antigen (acetone-treated urine ELISA),[22] and a direct agglutination test with urine samples (urine DAT)[23] for the diagnosis of VL, which showed, respectively, sensitivities of 95% and 90.7% and specificities of 95.3% and 97.3%. These figures are almost comparable to serum-based ELISA and DAT. In this study, we report the usefulness of rKRP42 antigen in a new urine ELISA and compare the results with other urine-based immunologic tests.

MATERIALS AND METHODS

Urine and serum samples. Most urine samples tested in this study were the same ones used in our previous study.[23] Additional samples from 44 VL patients and 17 Japanese healthy controls were included. A total of 115 urine samples from defined VL patients, collected from different medical college hospitals (96 from Rajshahi Medical College hospital, 11 from Mymensingh Medical College hospital, and 8 from Institute of Epidemiology, Disease Control and Research) in Bangladesh, were used to compute sensitivity of rKRP42 urine ELISA. VL was diagnosed in accordance with the WHO guideline of initiation of treatment.[24] Briefly, the diagnosis was based on well-defined clinical symptoms along with sup-

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portive hematologic features (anemia, leucopenia, reversed albumin globulin ratio, etc.) and was confirmed by parasitologic test or at least by one serologic test. The clinical symptoms included at least three of the following symptoms: intermittent chronic fever for > 1 month, splenomegaly, hepatomegaly, anemia, wasting, and lymphadenopathy. Malaria, tuberculosis, enteric fever, and other diseases that could produce similar clinical symptoms had been ruled out as much as possible. Among the 115 patients, 40 were confirmed parasitologically: Leishman-Donovan bodies were detected in smears of splenic aspirates (30 patients) or bone marrow aspirates (5 patients), and promastigotes were shown in 5 patients after inoculation of aspirate materials in Novy, MacNeal, and Nicolle medium. Of the other 75 clinically confirmed patients, 47, 9, and 19 were positive by conventional DAT, aldehyde test, and rK39 dipstick test, respectively. At the time of sample collection, patients were either in the course of treatment with sodium antimony gluconate at a WHO-recommended dose[25] or just before the initiation of treatment. A total of 240 non-VL urine samples were used for a specificity study. Fifty-nine control samples were taken from apparently healthy individuals with no past history of kala-azar from endemic areas in Bangladesh (endemic healthy control [EHC]). Eighty samples from Japanese individuals were used as non-endemic healthy controls (NEHC). Fifty-eight malaria samples collected in Solomon Islands, 13 tuberculosis samples from Bangladesh, 23 cutaneous leishmaniasis (CL) samples from Ecuador, and 7 samples from patients with other diseases were also included. Other diseases category included amebic liver abscess (2 cases), aplastic anemia (2), aplastic anemia with nephrotic syndrome (1), aortic stenosis (1), and viral fever (1). Immediately after collection of urine, NaN_3 was added to each sample at the final concentration of 0.1% as preservative, and the samples were transported to Japan at ambient temperature and kept at 4°C until immunologic tests.

The study was reviewed and approved by the Ethics Committee of Aichi Medical University School of Medicine, Japan, and the Ethical Review Committee of the Bangladesh Medical Research Council.

Effect of storage of urine samples on *L. donovani*–specific IgG titers. This study included urine samples that had been collected > 6 years before. To study the effect of storage of NaN3-added urine samples at 4°C, *L. donovani*–specific IgG titers of the same urine samples from 51 VL and 211 non-VL subjects were measured with acetone-treated urine ELISA at three different times: the first measurement with relatively fresh urine samples (collected within 1 year) was in February 2001, the second one in May 2003, and the third one in November 2007. The ELISA with acetone-treated *L. donovani* promastigotes antigen was described elsewhere.[22] The measurement (all in duplicate) was standardized using a set of serially diluted positive sera prepared for every microtiter plate. The sensitivities and specificities obtained in the three measurements were compared using the χ^2 test, and the geometric mean IgG titers were compared using paired t test.

The rKRP42 urine ELISA. The production of rKRP42 antigen was described elsewhere.[19] An ELISA was performed as follows. Flat-bottomed 96-well microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 1 μg/mL (100 μL/well) rKRP42 antigen and incubated overnight at 4°C. After blocking with casein buffer (1% casein in 0.05 mol/L Tris-HCl buffer with 0.15 mol/L NaCl, pH 7.6) for 2 hours at room temperature, the wells were loaded with 100 μL of urine without concentration and incubated at 37°C for 1 hour. After four washes with PBS(−) (pH 7.4) containing 0.05% Tween 20, peroxidase-conjugated goat anti-human IgG (Tago, Camarillo, CA), diluted 1:4,000 with casein buffer, was added and incubated at 37°C for 1 hour. After washing four times, ABTS substrate (KPL, Gaithersburg, MD) was reacted for coloration for 1 hour at room temperature. The optical density was measured at 415 and 492 nm as reference. Each sample was assayed in duplicate, in which, if the absorbance values of the duplicates differ > 40% from their average, the sample was retested. Antibody levels were expressed arbitrarily as unit (U), which was estimated from the standard curve constructed for each plate with serially diluted positive sera. The cut-off point for anti-rKRP42 IgG was first calculated as the mean plus 3 SDs of log (unit + 1) values of the NEHC, and [antilog of (the mean + 3 SDs) − 1] was regarded as the cut-off unit.

Urine latex agglutination test (KAtex). The KAtex was performed with 108 VL (7 VL samples could not be tested because of inadequate quantity) and 240 non-VL urine samples according to the manufacturer's protocol (Kalon Biological, Aldershot, UK) except that 100 μL of urine was used for pretreatment of 5-minute boiling instead of recommended 250 μL to 1 mL. Before changing the volume of urine, we tested 10 urine samples and confirmed that the use of 100 μL did not influence the results. After bringing all the test reagents to ambient temperature, 50 μL of urine sample was placed to a reaction zone in the glass slide, and a drop of test latex was added to it. The liquids were stirred to a completely homogenous mixture and rotated continuously for 2 minutes. A negative control in the reaction zone next to the test sample was run each time. Any agglutination discerned compared with the negative control was considered positive.

RESULTS

Effect of storage of urine samples. Fifty-six VL and 211 non-VL urine samples stored at 4°C were tested with the acetone-treated urine ELISA in February 2001, May 2003, and November 2007, which showed sensitivities of 91.1%, 91.1%, and 82.1% and specificities of 95.3%, 97.2%, and 98.1%, in order of measurement (Table 1). There were no differences among the sensitivities (χ^2 test, 2 df, $P > 0.24$) and the specificities (χ^2 test, 2 df, $P > 0.23$). The mean IgG units in the first, second, and third measurements with the VL samples were 103.10, 83.86, and 61.11 U, respectively, and those with the non-VL samples were 0.93, 0.94, and 0.89 U, respectively (Table 1). In the VL samples, paired t test showed significant differences between the first and second means ($P < 0.012$), the second and third means ($P < 0.002$), and the first and third means ($P < 0.001$). Because the first measurement was done with relatively fresh samples, we considered its mean unit as 100% and calculated the percentages of IgG units in the second and third measurements. They were 81.3% and 59.3%, respectively (Table 1). The percentage reduction, when plotted on a graph, showed an approximate linearity, and a yearly rate of reduction was estimated to be 6.0% (40.7% reduction in 6.8 years). In the non-VL samples, no significant differences were obtained ($P > 0.28$ in all comparisons).