obtained MZ in mice infected with the Japanese strain of *S. japonicum*. Therefore, this experimental study gives extra support to previously reported negative evaluation about the effectiveness of this drug in the treatment of schistosomiasis against many other published positive results. Based on the findings of this work, we cannot recommend the use of Mirazid in schistosomiasis patients.

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CD36-related protein in *Schistosoma japonicum*: candidate mediator of selective cholesteryl ester uptake from high-density lipoprotein for egg maturation

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ABSTRACT **Familial cholesteryl ester transfer protein (CETP) deficiency is more common in some East Asian populations than elsewhere, suggesting the possibility of a selective advantage of this genetic defect against regional infectious diseases. Historically, infection with the Asian blood fluke *Schistosoma japonicum* has been endemic in these regions, including Japan. We previously reported that eggs of *S. japonicum* require cholesteryl ester uptake from normal high-density lipoprotein (HDL) but not from CETP-deficient HDL for their maturation to miracidia, a critical step of the hepatic pathogenesis of schistosomiasis. Herein we show that cholesteryl ester uptake is selective from HDL, and identified CD36-related protein (CD36RP) as a candidate to mediate the reaction. CD36RP was cloned from the adult and the egg developmental stages of *S. japonicum*, with 1880 bp encoding 506 amino acid residues exhibiting the CD36 domains and two transmembrane regions. Using antibodies against recombinant peptides representing the potential extracellular domains of CD36RP, Western blotting detected a protein with a molecular mass of 82 kDa in the particulate fraction of the adult parasite cells, which was reduced to 62 kDa after *N*-glycanase treatment. The extracellular domain peptide bound human HDL, as established by immunoblots following nondenaturing gel electrophoresis. Antibodies against the extracellular domain suppressed HDL cholesteryl ester uptake and maturation of the eggs *in vitro*. CD36RP is a candidate receptor on eggs of *S. japonicum* that facilitates uptake of HDL cholesteryl ester necessary for egg embryonation and maturation.—Okumura-Noji, K., Miura, Y., Lu, R., Asai, K., Ohta, N., Brindley, P. J., Yokoyama, S. CD36-related protein in *Schistosoma japonicum*: candidate mediator of selective cholesteryl ester uptake from high-density lipoprotein for egg maturation. *FASEB J.* 27, 1236–1244 (2013). www.fasebj.org**

Key Words: CETP deficiency · miracidium · CETP · embryonation · hepatic granulomatosis · HDL

THE MAJOR AND FATAL PATHOGENESIS of schistosomiasis due to infection with *Schistosoma japonicum* or *Schistosoma mansoni* is ectopic implant of the eggs in the liver *via* the portal blood flow and their intrahepatic maturation to miracidia to cause hepatic granulomatogenesis and, accordingly, hepatic cirrhosis (1–5). Schistosomes take up lipids as their nutrient sources from the host blood plasma lipoproteins, and the receptors for low-density and very low density lipoproteins (LDLs and VLDLs) that mediate this interaction have been identified on their surfaces (6–9). On the other hand, it is not yet clear whether or not schistosomes use high-density lipoprotein (HDL) lipids as a nutrient source. In this regard, we previously showed that eggs of *S. japonicum* in culture require the presence of HDL to grow and develop to miracidia. Notably, maturation of schistosome eggs was significantly retarded when they were incubated with HDL from homozygous cholesteryl ester transfer protein (CETP)-deficient patients (10). In addition, expression of the CETP transgene significantly enhanced this process in mice that lack endog-

Abbreviations: apoA-I, apolipoprotein A-I; apoB-LP, apoB-containing lipoprotein; CD36RP, CD36-related protein; CETP, cholesteryl ester transfer protein; EndoH, endoglycosidase H; GSH, reduced glutathione; GST, glutathione *S*-transferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; *N*-acetylglucosaminidase, *N*-acetyl-β-D-glycosaminide-*N*-acetylglucosamino-hydrolase; *N*-glycanase, peptide-*N*-glycosidase F; PBS, phosphate buffered saline; SR-BI, scavenger receptor BI; VLDL, very low density lipoprotein

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enous CETP activity in plasma (10). These findings indicate that there is a specific pathway available to eggs of *S. japonicum* to utilize mammalian HDL lipid, including cholesterol or cholesteryl ester, to grow to miracidia. The abnormal HDL generated in the CETP-deficient plasma, large and cholesteryl ester-rich HDL, may not be a good substrate for such reactions. Thus, CETP deficiency may render humans resistant to hepatic maturation of eggs of *S. japonicum*, a phenomenon central to liver pathology characteristic of schistosomiasis japonica. We proposed that this could be a background behind the high prevalence of CETP deficiency in Far Eastern Asia (10–14).

Accordingly, we have undertaken an investigation to locate specific mediators of the schistosome parasite involved in interaction with human HDL lipids. We cloned a cDNA composed of 1880 bp encoding 506 aa that includes the CD36 domains and two transmembrane domains. Whereas functional expression of this protein remains to be accomplished, we now demonstrate binding of the proposed extracellular domain of a schistosome CD36-like glycoprotein to HDL, and suppression of cholesteryl uptake and maturation of the eggs by the antibody against this domain.

MATERIALS AND METHODS

Reagents commercially purchased or previously established

Anti-scavenger receptor BI (SR-BI) rabbit antisera (NB 400-101) was purchased from Novus Biologicals, Inc. (Littleton, CO, USA). Anti-apolipoprotein A-I (apoA-I) antibody was raised with rabbit against human apoA-I. Peptide-*N*-glycosidase F (*N*-glycanase) was purchased from PROzyme, Inc. (Hayward, CA, USA),endoglycosidase H (EndoH) was from Seikagaku Corp. (Tokyo, Japan), and *N*-acetyl-β-D-glycosaminide-*N*-acetylglucosamino-hydrolase (*N*-acetylglucosaminidase) was from Calbiochem (Berlin, Germany). Anti-glutathione *S*-transferase (GST) antibodies were purchased from GE Healthcare (Piscataway, NJ, USA), and anti-RGS-His antibodies were from Qiagen (Valencia, CA, USA).

Parasites and egg embryonation *in vitro*

S. japonicum (Yamanashi strain) was maintained by passage through *Oncomelania nosophora* and BALB/C mice (15,16). The pairs of adult worms were recovered from the portal vein of the infected mice and cultured as 1 pair/well of 12-well culture plates in RPMI 1640 medium supplemented with 5 or 10% human serum in 5% CO_2 atmosphere, as described previously (10). C57Black/6J mouse serum or lipoprotein-depleted serum was also used as a supplement in some experiments. For egg culture, the adult worms were removed from the wells after 2 d; eggs left in the wells were incubated further for 8 d in the same medium with or without 10% serum. After incubation for 10 d, the eggs were collected, the numbers of miracidia were counted microscopically, and the percentage of maturation was estimated as a maturation/embryonation rate.

Cholesterol uptake from [^{3}H], [^{14}C] double-labeled HDL and binding of [^{3}H]- or [^{125}I]-HDL by *S. japonicum* eggs

HDL and apoB-containing lipoprotein (apoB-LP, including LDL and VLDL) fractions were prepared from fresh human serum as density fractions at 1.063–1.21 g/ml and <1.063 g/ml, respectively, by sequential ultracentrifugation and labeled differentially with [1α,2α-^{3}H]cholesteryl ester and [4-^{14}C]cholesterol (Amersham, Piscataway, NJ, USA) as described previously (10). No [^{14}C] cholesteryl ester was detected in lipoproteins after labeling. ^{125}I-labeled HDL was prepared as described previously (17), using ^{125}I (Amersham) and iodine chloride. Total and free cholesterol in the labeled lipoproteins were measured by Determiner L reagents (Kyowa Medex Co. Ltd., Tokyo, Japan). *S. japonicum* eggs were collected from the homogenates of the liver and the intestine of the *S. japonicum*-infected BALB/C mice by the digestion method (18), and incubated with [^{3}H], [^{14}C] double-labeled lipoproteins (70–280 μg cholesterol/ml) in 0.5 ml RPMI 1640 in 12-well plates for 24 h at 37 or 4°C in 5% CO_2 atmosphere. The eggs were collected by centrifugation and washed with phosphate buffered saline (PBS), and the radioactivity of ^{14}C and ^{3}H in the egg pellet was analyzed. Uptake of free and esterified cholesterol was estimated by counting uptake of ^{14}C and ^{3}H radioactivity, respectively (10). Active uptake was determined by the difference between the results at 37 and 4°C. To observe selectivity of the lipid uptake, 10 vol of cold HDL or apoB-LP was added before adding the labeled HDL. HDL binding was also studied in parallel with [^{3}H] cholesteryl ester-labeled HDL and ^{125}I-labeled HDL. Use of human plasma lipoprotein was justified by institutional guidelines and approval of Nagoya City University.

RNA isolation, cDNA synthesis, and PCR amplification

Total RNA was extracted from the adult *S. japonicum* by using Isogen (Nippon Gene, Toyama, Japan). From 1 or 2 μg of total RNA, first-strand cDNA was synthesized by a SuperScriptII RT-PCR system (Invitrogen, Carlsbad, CA, USA) with random hexamer primers, according to the manufacturer's instruction. To search a new CD36 family protein in *S. japonicum*, the sequence of coding region of Sj-Ts2 protein consisting of 671 bp (Genbank AF291715), which appeared to have one of the CD36 domains defined by Prodom (release 2001.3; http://prodom.prabi.fr) analysis of the U.S. National Center for Biotechnology Information (NCBI) database, was used for preparation of the hybridization probes for Northern blot analysis and for screening of the *S. japonicum* adult cDNA library. First-strand cDNA was amplified by PCR using the specific primers for Sj-Ts2 protein: sense, 5′-TAATGAAATG-AATACAGTC-3′; antisense, 5′-AACAAACATATAATGACAAT-3′; and for GAPDH: sense, 5′-TGTACTCCGTGCAGCTTTTC-3′; antisense, 5′-AATGGATCCCTCTCGCAGTA-3′ (synthesized by Hokkaido System Science, Sapporo, Japan). The PCR products (488 and 198 bp) were purified by gel extraction and ligated in pGEM-T Easy vector (Promega, Madison, WI, USA). Ligation products were transformed into DH10B competent cells, and the sequences of inserts of positive clones were analyzed with T7 primer by using Applied Biosystems 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The 488-bp PCR product corresponded to nucleotide positions 5–492, consisting of coding region of Sj-Ts2 protein mRNA. For screening of the *S. japonicum* egg cDNA library, another probe was obtained by PCR using the specific primers for CD36-related protein (CD36RP): sense, 5′-CCGT-GAAAAACGTTTGAAGC-3′; antisense, 5′-AACATCATTGGATT-GATGGCTA-3′. The resulting PCR product size was 1177 bp. To analyze the size of the coding region of egg CD36RP, first-strand cDNA from *S. japonicum* adults or eggs was amplified by PCR using the 5′ primer with *Kpn*I site addition (5′-GCGTGGTACCTCTTGTACACGATGATATCTCG-3′) and GSP2 primer (below). PCR analysis with the center-region primer was also carried out: sense (L1), 5′-CCGTGAAAAACGT-

TTGAAGC-3′; antisense (R3), 5′-GTGCACCAGGTTGACATGA-3′. The PCR product corresponds to positions 321–1060 of CD36RP. Quantitative RT-PCR analysis was performed in a 7300 Realtime PCR System (Applied Bioscience) by using probe sets of 5′-primer GSP2 and L1-GSP2.

Northern blot analysis

Total RNA (10 μg) from mixed-sex adult *S. japonicum* worms was electrophoresed in 1.0% agarose-formaldehyde gel and transferred to nylon membranes. The 489-bp fragment of Sj-Ts2 cDNA and 198-bp fragment of *S. japonicum* GAPDH cDNA were purified by SigmaSpin column (Sigma-Aldrich, St. Louis, MO, USA) and labeled with [α-^{32}P]dCTP by using Klenow fragment, *Escherichia coli* DNA polymerase I (Takara Bio, Otsu, Japan), and hybridized to the membrane in a 5× SSPE hybridization solution containing 20% formamide at 42°C for 16 h. After washing, hybridization signals were detected by autoradiography on X-Omat film (Eastman Kodak, Rochester, NY, USA).

Screening and sequencing of CD36-related protein

Two cDNA-libraries (2×10^4 pfu) derived from *S. japonicum* adult (China) and eggs (Philippine) (19) were amplified using *E. coli* XL1-blue host cells (Stratagene, La Jolla, CA, USA) followed by plating and incubating at 37°C overnight. Plates were transferred to nylon membrane (Hybond-N+; Amersham), after which membranes were hybridized to the ^{32}P-labeled 488-bp probe (for *S. japonicum* adult) or 1177-bp probe (for *S. japonicum* eggs) in similar fashion as for the Northern blot analysis (above), except that 6× SSC solution containing 15% formamide was employed. Secondary screening was carried out after selection of positive phage plaques, and the final positive plaques were excised into SOLR cells by using the pBluescript II phagemid vector kit (Stratagene). Sequence analysis of the inserts was performed by using T3, T7, and the specific primer Sj-Ts2. For determination of 5′ end sequencing, 5′ RACE was carried out by the Gibco 5′RACE system, ver.2 kit (Gibco, Carlsbad, CA, USA), using two primers: GSP1, 5′-ATTGAATCCATGCGTTGACA-3′; GSP2, 5′-AGAAACCATGGCATTGAATTG-3′. The nucleotide sequence (1892 bp) and amino acid sequence (506 aa) of the product have been assigned Genbank accession no. AY496973 and termed CD36RP of *S. japonicum* (Supplemental Fig. S1).

Recombinant CD36RP

To prepare the antigens for anti-CD36RP antibodies, cDNA from *S. japonicum* adults was amplified by PCR with specific primers: sense, 5′-ATGGTAGTGATGGAACATT-3′; antisense, 5′-ATTGGTAGAAGAGTAGTTGA-3′. This PCR product, corresponding to the positions 797–1280 and coding the predicted extracellular half region, Ex160 (aa G249-P408, 160 residues) of CD36RP (see **Fig. 7**), was ligated first into pGEM-T Easy and then into the bacterial expression vector, pQE30 (Qiagen). For analysis of lipoprotein binding, a shorter discrete extracellular fragment of CD36RP, Ex121 (aa G249-Y369, 121 residues; see Fig. 7) was expressed as a GST-fusion protein in *E. coli*, BL21 with pGEX-6p vector (Amersham). GST-fusion proteins of full size CD36RP and Ex121 in the lysates of BL21 cells were adsorbed to reduced glutathione (GSH)-Sepharose gels, and the GST-free proteins were obtained in the supernatant in 50 mM TBS, 1 mM DTT, and 1 mM EDTA from GSH-Sepharose gels by treatment with PreScission Protease (GE Healthcare), according to the protocol of the kit.

Raising antibodies against CD36RP

The expression and purification of this His-tagged protein product (Ex160), as well as immunization of rabbits with Ex160, were carried out by Medical and Biological Laboratory. Co. Ltd. (MBL; Nagoya, Japan). An IgG fraction was affinity purified from polyclonal antiserum against Ex160 for Western blotting. Peptides 331-348, CQPGAPIVVSQPHFLNAN, amino acid residues 331-348 of CD36RP were synthesized and used by MBL to immunize rabbits.

Homology search

Homology and structural analysis of predicted polypeptides was carried out by using BLAST-X (20), BLOCKS (21), PROSITE motif analysis (22), and PHD searching (23) from the NCBI database.

Western blotting analysis

S. japonicum adult worms and mouse liver were sonicated for 5 s in hypotonic 50 mM phosphate buffer (pH7.4), containing protease inhibitor cocktail (Sigma-Aldrich). For preparation of the particulate fraction of *S. japonicum* eggs, freeze-thawing and sonication were repeated 10 times. After removing cell debris and nuclei by centrifugation at 1000 rpm for 5 min, the supernatant was further treated by centrifugation at 90,000 rpm for 30 min (Himac model CS120GX centrifuge; Hitachi, Tokyo, Japan). Both the pelleted particulate fraction and the supernatant cytosol fraction were solubilized in 1% SDS sample buffer and subjected to 10% SDS-PAGE, after which gel contents were transferred to polyvinylidene fluoride membrane (Bio-Rad). Western blotting analysis was carried out using 1:1,000 dilution of the rabbit anti-Ex160 IgG or anti-SR-BI antiserum and 1:10,000 horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. For detection of recombinant protein Ex121, a 1:500 dilution of rabbit anti-peptide 331-348 (anti-P) antibody was used. A 1:200,000 dilution of the anti-human apoA-I rabbit serum was used for detection of apoA-I in HDL.

Deglycosylation of particulate fractions

The particulate fractions of *S. japonicum* adults (48 μg protein) and mouse liver particulate fractions (100 μg protein) were suspended and heated at 100°C in the denaturing solution containing 0.1% SDS and 50 mM β-mercaptoethanol, and after addition of 0.75% Nonidet P-40 detergent, reacted with 10 mU *N*-glycanase in 50 μl for overnight at 37°C or room temperature, according to the manufacturer's protocol. In some cases, 35 mU *N*-acetyl-glucosaminidase was added to the reaction mixtures. The particulate fraction of *S. japonicum* eggs was incubated with 10 mU EndoH at 37°C for 1 h. Subsequently, reaction mixtures were solubilized with SDS sample buffer and subjected to SDS-PAGE for Western blot analysis.

Lipoprotein binding of recombinant Ex121

Ex121 preparation (1 or 2 μl) was incubated with 5–30 μg (protein) of human HDL or LDL in 8–12 μl PBS at room temperature for 30 min, and then mixed with native sample buffer (62 mM Tris-HCl, pH 6.8; 10% sucrose; and 0.1% bromphenol blue), followed by application to nondenaturing PAGE with 4%–20% gradient Tris-Gly gel (Invitrogen). Two parallel samples were run; one was analyzed with the anti-P antibody and the other with the anti-apoA-I antibody. The

reference positions of lipoproteins were identified by staining with Coomassie Brilliant Blue and prestaining with Sudan black.

RESULTS

Dependence on HDL of *S. japonicum* egg embryonation

Maturation and embryonation of *S. japonicum* eggs was investigated during the culture by counting formation of miracidia. The eggs were cultured with or without 10% pooled human serum for 8 d after separation from the parent adults precultured for 2 d in medium containing 5 or 10% serum. Embryonation significantly decreased without serum supplementation when the eggs were laid by parents precultured in 5% serum, while the maturation proceeded even without serum when the parent flukes were precultured in 10% serum (15.8 *vs.* 38.0%; **Fig. 1***A*). When the apoB-LP-deficient serum (bottom fraction of d=1.063 g/ml) was used for culture of the eggs, the eggs from the 5% serum-treated parents achieved the similar level of embryonation (Fig. 1*B*). Egg embryonation was estimated in the medium after pairs of *S. japonicum* worms were incubated for 10 d with and without HDL fraction ($1.063<d<1.21$) in addition to the lipoprotein-deficient serum ($d>1.21$) at the equivalent concentration of 10% serum. Egg embryonation by HDL reached a similar level to that by 10% serum, but lipoprotein deficient serum alone showed little or no maturation (Fig. 1*C*).

The results indicated that the egg maturation requires serum nutrients. Preconditioning of the parents or the eggs in the early stage by exposure to well-supplemented nourishment overcomes insufficient nourishment in the later stage. Nutritional support for the adults is perhaps primarily essential to deposit egg yolk/vitelline materials for embryonation, but the eggs also are able to take up nutrients from serum to grow even when they were laid in an insufficiently nourished condition. ApoB-LPs were shown not to be functional, but a normal HDL fraction is essential for egg maturation. Interestingly, minimal embryonation of the eggs was seen in culture medium containing 10% serum from wild-type mice, which is deficient in CETP, whereas the maturation proceeded well in medium containing serum from CETP-transgenic mice or wild-type mouse serum presupplemented with human CETP (ref. 24 and Fig. 1*D*), supporting our previous findings (10).

Uptake of cholesterol and cholesteryl ester from HDL *S. japonicum* eggs

To characterize association of HDL with the *S. japonicum* eggs, HDL was labeled with [^{3}H]cholesteryl ester or ^{125}I on the HDL protein, and association of their radioactivity with *S. japonicum* eggs was determined. Apparent association of HDL was estimated as its protein based on specific radioactivity of HDL. As shown in **Fig. 2***A*, [^{3}H]-based association, assessed as increment from 4 to 37°C, was markedly higher than that of ^{125}I binding, which showed no difference between 37 and 4°C, indicating that no active HDL protein processing was involved, suggesting that cholesteryl ester is selectively taken up by the eggs. As the concentration of HDL increased, the ^{125}I binding seemed saturated at lower concentrations of HDL (100 μg/ml) than the [^{3}H]-based association (≫600 μg/ml). The egg takes up cholesteryl ester also from apoB-LP, but the rate seems saturated at lower concentrations (150 μg/ml) with less maximum uptake than HDL, based on cholesteryl ester (Fig. 2*A*, right panel). Uptake by schistosome eggs of free and esterified cholesterol was examined by using double-labeled HDL and apoB-LP with [^{14}C]cholesterol and [^{3}H]cholesteryl ester. Uptake of [^{14}C]cholesterol was impeded by excess apoB-LP and HDL, but the uptake of [^{3}H]cholesteryl ester was suppressed only by HDL and not by apoB-LP (Fig. 2*B*). This profile indicated a specific pathways for selective uptake of cholesteryl ester from HDL, at least different from a pathway for the uptake of apoB-LP cholesteryl ester (25), while free cholesterol uptake is nonspecific from LDL and HDL, including its exchange among lipoproteins and cell membranes.

Northern blot analysis

Assuming that selective uptake is mediated by SR-BI-like protein or CD36 family protein, expression of

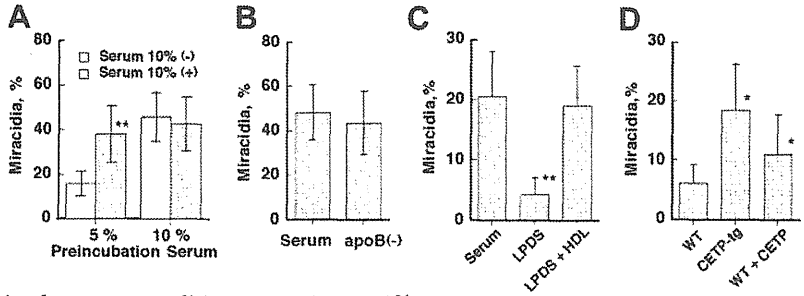

Figure 1. Embryonation and maturation of the *S. japonicum* eggs in culture. The percentage of miracidia in total eggs per pair of adult parasites cultured for 10 d was counted and estimated as efficiency of embryonation. *A*) Eggs were separated from the adult worms after 2 d of culture in RPMI 1640 medium supplemented with 5 or 10% human pooled serum, and cultured further in fresh medium supplemented with or without 10% serum for 8 d. *B*) Eggs were separated from the parents cultured in the same condition as in *A* with 5% serum, and the eggs were further cultured in 5% CO_2 atmosphere with 10% of whole serum and its d = 1.063 bottom fraction (apoB(−)). *C*) A pair of parent adults was cultured for 10 d in the medium with lipoprotein-depleted serum (LPDS; 4 mg protein/ml) with or without isolated HDL fraction (150 μg cholesterol/ml). *D*) A pair of *S. japonicum* adults was cultured with mouse sera (4 mg protein/ml) of wild-type, CETP transgenic, and wild-type presupplemented with purified human CETP (24) to make it equivalent activity in human serum. Numbers of adult pairs assayed were: 6 (*A*), 8 (*B, C*), and 5 (*D*). Data represent average and SE. $*P < 0.05$, $**P < 0.005$.

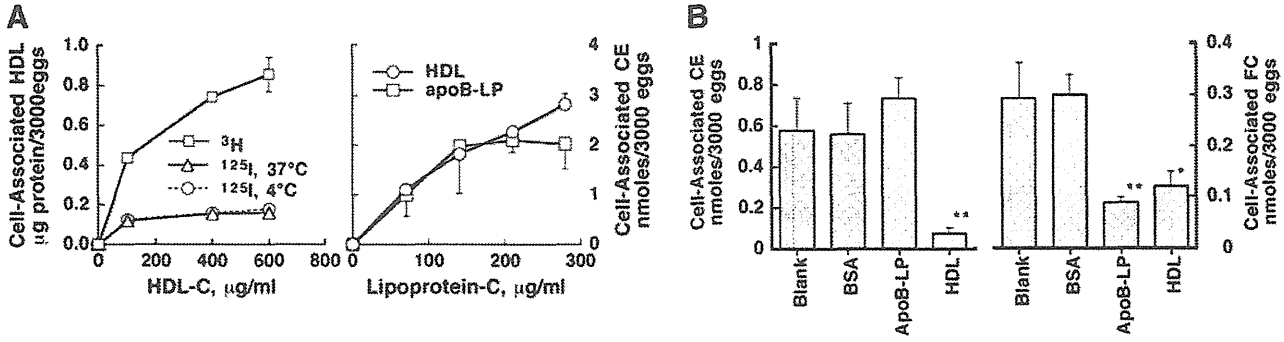

Figure 2. Specific cholesterol uptake from lipoproteins by *S. japonicum* eggs. *A*) Left panel: human HDL was labeled with [^{3}H]cholesteryl ester ([^{3}H]-CE) or ^{125}I. *S. japonicum* eggs (8400/well) were incubated with indicated concentrations of [^{3}H]-CE-HDL (^{3}H; squares) or ^{125}I-HDL (^{125}I; triangles and circles) at 37°C and 4°C, in 0.5 ml/well RPMI 1640 at 37°C for 20 h. Association of HDL with the eggs was estimated as HDL protein calculated from each specific activity (694.2 dpm/μg protein for [^{3}H], 53,380 cpm/μg protein for ^{125}I). Specific association was estimated by displacement with a 10× amount of nonlabeled HDL. Specific association of [^{3}H] was assessed as difference between the results at 37 and 4°C. Right panel: human HDL and apoB-lipoprotein (apoB-LP, as d>1.063) were double-labeled with [^{3}H]-CE [^{14}C]cholesterol as described in Materials and Methods. *S. japonicum* eggs (3000/well) were incubated as in left panel, with the labeled HDL (circles) or apoB-LP (squares). Specific active uptake of CE (cell-associated CE) was determined as difference between the specific uptake values at 37 and 4°C. Horizontal axes indicate lipoprotein cholesterol (C). *B*) Selective uptake of cholesterol by *S .japonicum* eggs from HDL. HDL was double-labeled with [^{3}H]-CE and [^{14}C]cholesterol, and incubated with the *S. japonicum* eggs (58 μg HDL cholesterol for 3000 eggs/ml medium/well) in the presence of 10 vol of nonlabeled HDL, nonlabeled LDL, or 1 μg/ml BSA. Uptake of CE (cell-associated CE, right panel) or cholesterol (cell-associated FC, right panel) was calculated as the difference between the specific values at 37 and 4°C. Data represent average and SE of the triplicate assay. *$P < 0.05$, **$P < 0.01$ *vs.* blank.

mRNA was searched by using the 489-bp probe derived from the cDNA of Sj-Ts2 protein that has one of the CD36 domains (671 bp was submitted to Genbank; see above). The mRNA representing a CD36 family protein was identified in *S. japonicum* adult (**Fig. 3***A*). The approximate size was 1.8 kb, longer than that previously reported for Sj-Ts2 (671 bp). GAPDH mRNA was detected as a 1.2-kb band, consistent with the size of 1148 bp reported for *S. japonicum.*

Screening of a CD36-related gene from the cDNA libraries of *S. japonicum* adults and eggs

From the *S. japonicum* adult cDNA library, Sj-Ts2-containing cDNAs of variable sizes longer than 1 kb were obtained as inserts of $^{32}P^{+}$ clones. These cDNA fragments all seemed to be derived by single transcription, including the sequence of the reported Sj-Ts2 protein. Inserts of 8 clones showed >1068 bp elongated from the 5′ terminus, and one showed ~100 bp elongated from the 3′ terminus of Sj-Ts2 protein. By 5′-RACE analysis, the start codon downstream of a stop codon was detected, and the sequence of the final full-length (1880-bp) original mRNA was determined and deduced to 506 amino acid residues (Supplemental Fig. S1). On the other hand, more than half of 26 inserts of positive clones derived from the egg cDNA library had a deletion of 68-bp nucleotides at positions 98–165 within the coding region. However, the inserts of remaining clones had no deletion and identical sequences with the nucleotide, beginning at position 14 of adult mRNA. These findings indicate that alternative splicing of this mRNA may occur. The size of the PCR product of 5′ fragment with 5′ primer and GSP2 with the first-strand cDNA derived from total RNA as template was similar (467 bp) between eggs and adults (Fig. 3*B*), so that the CD36RP mRNA is likely to be expressed in eggs, though the level seems lower (Fig. 3*C*).

The amino acid sequence indicated that the protein sequence belongs to the CD36 family. It had two transmembrane regions by PHD analysis (23), 15 *N*-glycosylation sites by PROSITE motif analysis, and CD36 domains by conserved domain (CD) search analysis (Supplemental Figs. S1 and S2). Blastp analysis revealed identity with high score to SRC1_RAT (SR-B1) (48%; *E* score 8e-51) and with CD36_RAT (49%; *E* score 5e-45) and SRC_HUMAN (CLA-1) (49%; *E* score 2e-52. By multialignment (pfam01130) of 32 CD36 family proteins from a variety of organisms by CDD, the highly conserved 4 Cys and 4 Pro residues were demonstrated in domains from IPB002159D to IPB002159F in CD36 family proteins, including this CD36RP (Supplemental Fig. S2, boxed). Three *N*-glycosylation sites (aa 97, 205, and 248) identified among the 15 candidate glutamines in CD36RP were conserved in mammalian SR-BI and CD36 (not shown). It had 15 nucleotide polymorphism sites in the coding region identified during screening, resulting in 11 amino acid substitutions.

Expression of recombinant CD36RP

Recombinant GST-fused full size CD36RP (rCD36RP) expressed in *E. coli* and isolated as GSH-Sepharose gel-bound protein was shown as an 82-kDa band by Western blotting, by using anti-GST and anti-Ex160 antibodies (**Fig. 4**). His-tagged extracellular half size CD36RP (Ex160) was expressed and shown as a 25 kDa polypeptide by Western blotting with anti-RGS-His antibody and antibody was raised against this peptide. After treatment of the GSH-gel-bound rCD36RP with protease, the anti-Ex160^{+} band of the apparent molec-

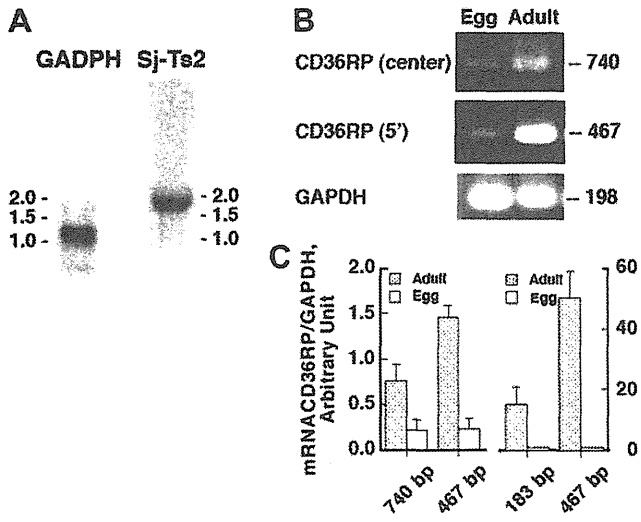

Figure 3. Expression of a CD36-like gene in *S. japonicum*. *A*) Northern blot hybridization of total RNA isolated from adult *S. japonicum*. Total RNA was subjected to electrophoresis in 1.0% agarose-formaldehyde gel, transferred to nylon membrane, and hybridized with the ^{32}P-labeled 489- and 198-bp oligonucleotide probes corresponding to Sj-Ts2 and *S. japonicum* GAPDH cDNA, respectively, as described in Materials and Methods. *B*) Expression of CD36RP mRNA in the eggs and adults of *S. japonicum*. RT-PCR of total RNA was performed for both in the same concentration by using the 5′ primer and GSP2 primer for CD36RP (5′), 467 bp; the center primers (L1 and R3) for CD36RP (center), 740 bp; and primers for glyceroaldehyde-3-phosphate dehydrogenase (GAPDH). Size of the PCR products is shown. *C*) Quantitative data of conventional RT-PCR (left panel) and results of quantitative RT-PCR (right panel) performed by using primers L1 and GSP2 (183 bp) and the same primers for CD36RP (5′) (467 bp).

ular mass of ~60 kDa (Fig. 4, arrow) was detected in the supernatant, which had no reactivity to anti-GST antibody, indicating the cleavage of GST of 22 kDa. The anti-Ex160 antibody showed stronger reactivity for recognition of CD36RP in *S. japonicum* adults and *S. japonicum* eggs than anti-P 331–348. Ex160 peptide was, however, hardly soluble in aqueous solution without detergents to perform lipoprotein-binding experiments in nondenaturing PAGE. Therefore, a shorter recombinant peptide Ex121, trimmed at the C terminus of Ex160, was expressed in *E. coli* as a GST-fusion protein. Free Ex121 released from GSH Sepharose gel after cleavage of GST (Fig. 4) was able to enter into the gel in nondenaturing PAGE. This solution was used for binding to lipoproteins.

Characterization of *S. japonicum* CD36RP

The particulate and cytosol fractions were prepared from *S. japonicum* adults and mouse liver, and Western blotting analysis was carried out in 10% SDS-PAGE. CD36RP and SR-BI were detected as 82- and 85-kDa bands, respectively, only in the particulate fraction (**Fig. 5**) and not in the cytosol fraction. After treatment with *N*-glycanase, the size of CD36RP in the *S. japonicum* adult particulate fractions decreased from 82 to 62 kDa, probed by anti-Ex160 antibody, indicating CD36RP glycosylation (Fig. 5). By similar treatment of the mouse liver particulate fractions, deglycosylated SR-BI appeared as a 60-kDa band by anti-SR-BI antibody. Murine SR-BI was reported to have 11 *N*-linked glycosylation sites, 2 of which were important for expression in plasma membrane (26). Additional treatment with *N*-acetylglucosaminidase resulted in no further reduction of the size of CD36RP. The EndoH treatment showed similar results. The main band in the *S. japonicum* egg particulate fractions was broader around 82 kDa, with a minor band of 62 kDa. Treatment with EndoH seemed to cause no apparent change of the bands, giving inconclusive results as to whether CD36RP is glycosylated and the experimental condition is good for deglycosylation in the eggs.

Lipoprotein binding of the recombinant peptide, Ex121

The water-soluble extracellular domain peptide Ex121 was incubated with HDL and LDL and subsequently analyzed by density-gradient nondenaturing PAGE. The Ex121 peptide was detected by immunoblotting with anti-P 331–348. The position of Ex121 was shifted from that of free Ex121 to the position corresponding to HDL, assured by anti-apoA-I antibody and by HDL samples prestained with Sudan black B (not shown and **Fig. 6*A***). Binding of Ex121 to HDL increased and free Ex121 decreased as the concentration of HDL increased. HDL isolated from the CETP-deficient human serum showed less binding of Ex121 than normal HDL (Fig. 6*B*, left panel). Ex121 also seemed to bind to LDL but to a much lesser extent, and the Ex121$^+$ band was at the position a little smaller than regular LDL parti-

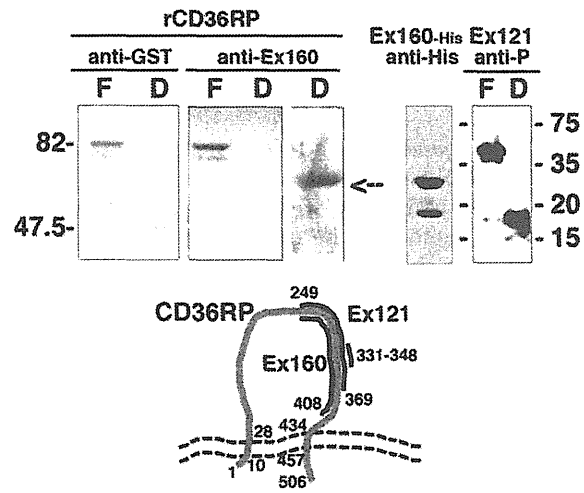

Figure 4. SDS-PAGE of recombinant CD36RP. Full-length CD36RP (rCD36RP) and the extracellular region peptide, Ex121, Gly249-Tyr369, were expressed in *E. coli* as the GST-fusion proteins. Digested product (D) was obtained after treatment of the GST-fusion protein (F) by precision protease, as described in Materials and Methods (arrow). Anti-Ex160 and anti-GST antibodies were used for detection of GST-free rCD36RP (D) and GST-fusion protein (F), and anti-peptide 331-348 (anti-P) for Ex121. Ex160-His peptide was detected by anti-His antibody. Bottom panel: predicted topology of CD36RP and positions of the peptides used.

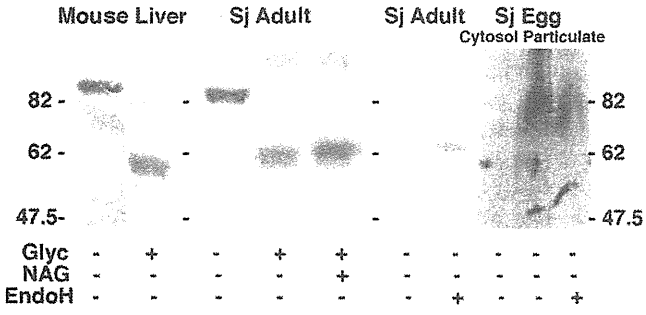

Figure 5. Deglycosylation of Sj CD36RP in the particulate fractions from *S. japonicum* adults. Particulate fractions (48 μg protein of *S. japonicum* or 100 μg protein of mouse liver) were treated with or without 10 mU *N*-glycanase in 50 μl reaction mixture overnight at 37°C, as described in Materials and Methods. Reaction mixtures without enzyme (−), with glycanase (Glyc), or with glycanase plus 35 mU *N*-acetyl glucosaminidase (Glyc, NAG) were analyzed in SDS-PAGE and Western blotting carried out by using anti-Ex160 or anti-SR-BI antibody. Particulate fraction of *S. japonicum* eggs was similarly treated with (+) or without (−) 10 mU EndoH, and CD36RP was detected with anti-P.

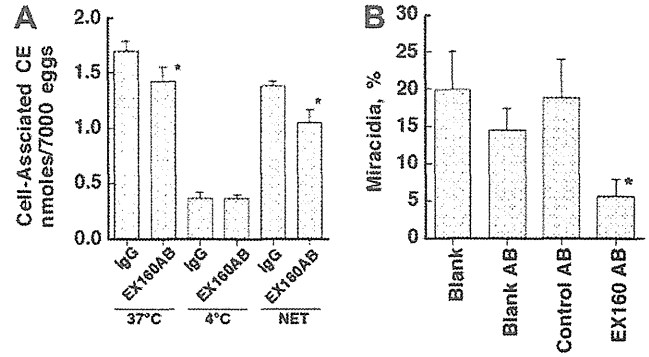

Figure 7. Suppression of HDL-cholesteryl ester (CE) uptake and maturation of the eggs by the antibody against EX160. *A*) Uptake of HDL-CE was measured in the same system as used in Fig. 2, in the presence of the antibody. *B*) Maturation of *S. japonicum* eggs was estimated in the same condition as Fig. 1, except for using 5% serum, in the presence of the antibodies. IgG, nonimmune rabbit IgG; EX160AB, antibody against Ex160; blank, with no additional antibody/antisera; blank AB, with 1:100 vol of nonimmune rabbit serum; control AB, with 1:100 vol of rabbit antisera against the intracellular domain peptide of CD36RP (anti-P 331–348). Titer of Ex160 was adjusted to <1/25 of the anti-P antisera. Data represent means ± SE of $n = 6$/group. $*P < 0.05$ *vs.* IgG (*A*) or all other treatments (*B*).

cles. When HDL is added, EX121 interacted with LDL is likely to be transferred to HDL, but the Ex121-HDL complex seemed shifted to a position of higher molecular mass (Fig. 6*B*, right panel). These results suggest that an extracellular fragment containing the conserved Cys and Prorich domain of *S. japonicum* CD36RP binds to HDL particles (27). This association seems to be selective.

Effect of the anti-Ex160 antibody on HDL-cholesteryl ester uptake and maturation of the eggs

The antibody against the extracellular domain peptide Ex160 was examined for CD36RP activity for HDL cholesteryl ester uptake and egg maturation (Fig. 7). The effect of the antibody was observed on HDL-cholesteryl ester uptake by the eggs. The antibody suppressed the cholesteryl ester uptake at 37°C but not at 4°C, so that significant suppression was for the active uptake shown as NET uptake in Fig. 7*A*. The antibody was added to the egg maturation assay system of Fig. 1, except that a pair of the parent adults was cultured for 10 d in the presence of 10% serum in the presence of the antibody. Maturation of the eggs to miracidia was significantly reduced in the presence of the anti-Ex160 antibody in comparison to control antibody, anti-P 331–348, or blank (Fig. 7*B*).

DISCUSSION

Schistosomes are the parasites that finally reside in the blood vessels of the host patients, and in the case of *S. japonicum*, the adult flukes locate to the portal vein and its draining venules. Chronic schistosomiasis causes various pathological problems in the body, not only from the parasites themselves but also the eggs laid in the body. The adult worms lay eggs in the portal vein to be released to the intestinal tract, but many are flushed back to the liver, where they embolize and develop into miracidia, a phenomenon that deals with the morbidity and mortality of hepatic granulomatosis (1–5).

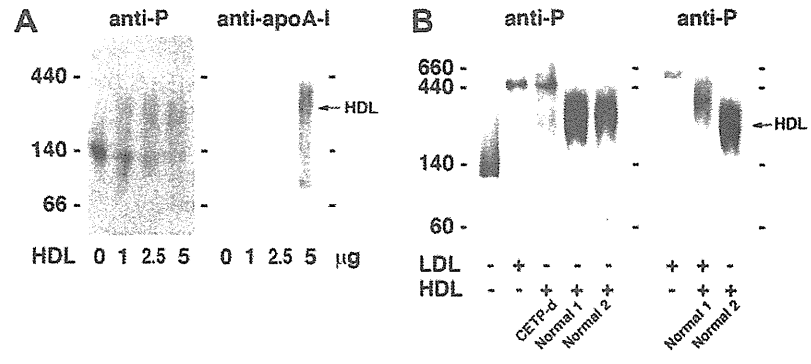

Figure 6. Association of recombinant protein Ex121 with HDL. *A*) Ex121 (35 ng) was incubated with 1–5 μg protein of HDL in 8 μl PBS at room temperature for 30 min and at 4°C for 3 h, and then mixed with 6 μl native sample buffer (62 mM Tris-HCl, pH 6.8; 10% sucrose; and 0.1% BPB). The double samples were subjected to nondenaturing PAGE with 4–20% gradient Tris-Gly gel and detected with anti-P parallel with anti-apoA-I. *B*) Ex121 (70 ng) was incubated with 30 μg protein of HDLs obtained from normal subjects (normal 1, normal 2) or CETP-deficient subject (CETP-d), or LDL in 7 μl PBS (left panel), or with 15 μg protein of HDL or LDL or mixture of HDL and LDL (right panel) in 12 μl PBS, at room temperature for 30 min. Western blotting was carried out using anti-P. Arrows indicate positions of HDL prestained by Sudan black.

We previously reported that maturation of *S. japonicum* eggs to miracidia requires normal HDL, presumably as a nutrient supplier, but it is insufficient in culture in the presence of the HDL recovered from CETP-deficient patients (10). We confirmed this process in more detail, as presented in Fig. 1. This was consistent with the *in vivo* findings that the egg maturation in the liver and hepatic granulomatosis were less in wild-type mice, which lack endogenous CETP activity, than in the CETP transgenic mice (10). We therefore investigated potential candidate factors to catalyze this reaction. Both schistosome eggs and adults were found to selectively take up cholesteryl ester from HDL particles. As this type of reaction is known to be carried out by CD36-like proteins, including SR-BI, in many animals (25–27), we searched for schistosome genes homologous to CD36. We used probes with the sequence consisting of coding region of Sj-Ts2 protein (Genbank AF291715), which appeared to have one of the CD36 domains by Prodom analysis, for Northern blot analysis screening of the *S. japonicum* adult cDNA library, and an identified positive band of mRNA of ~1.8 kb in length. Based on this information, we cloned from the *S. japonicum* adult cDNA library a cDNA of 1880 bp that was deduced to encode 506 aa, and have termed this CD36RP. The transcript encoding this protein was also expressed by eggs of *S. japonicum*. Sequence and structural analyses showed clear relatedness to other CD36 family proteins. The extracellular domain peptide of CD36RP demonstrated selective binding to normal HDL but markedly reduced interaction with HDL from CETP-deficient patients. Finally, the antibody against the extracellular domain of CD36RP suppressed HDL-cholesteryl ester uptake and maturation of the eggs *in vitro*.

Based on these new findings, we now propose that CD36RP is a lead candidate for a mediator of selective uptake of cholesteryl ester from HDL by *S. japonicum* necessary for egg maturation to miracidia. It is instructive that preexposure of the adult schistosomes to standard (wild-type) HDL is sufficient for the eggs to mature, perhaps because the vitelline of the egg were preformed adequately in such a condition. In contrast, even the eggs with inadequate vitelline (yolk) provisions may mature provided that normal HDL is supplied after the eggs are laid (*i.e.*, released from the female schistosome into the culture medium). The data we present here support the view that absence of normal HDL retards maturation of the *S. japonicum* eggs in the host liver and, accordingly, prevents hepatic granulomatosis, in a situation such as CETP deficiency where abnormal large HDL does not efficiently bind CD36RP (Fig. 6*B*). This may be one of the reasons why the prevalence of CETP deficiency is so high in the Far East (11–14) where schistosomiasis japonica has been and/or remains common in rural or underdeveloped areas. If this hypothesis were valid, CETP inhibitors could be useful to prevent hepatic granulomatosis in schistosomiasis. This would be reminiscent of the selective advantage that hemoglobinopathies confer against malaria (*e.g.*, see ref. 28 and references therein).

The question may remain whether cholesterol is a specific nutrient for this reaction. Although triglyceride is a minor component of HDL core, it may still contribute to maturation of the *S. japonicum* eggs and HDL in CETP deficiency is characterized as low content of triglyceride (29,30). The particular CETP-deficient HDL used in the experiment, however, contained triglyceride as 1.6% of cholesteryl ester and 3.3% of phospholipid as mass, while normal HDL contained it as 0.5–4% and 1–6%, respectively, depending on plasma VLDL concentration. Therefore, contribution of triglyceride is less likely.

We have attempted at length to demonstrate functional alteration of cholesteryl ester uptake by adults or eggs of *S. japonicum* by manipulating expression of the whole CD36RP protein. However, neither functional expression by transfection of full-length CD36RP nor knockdown/knockout of the gene in the parasite cells was successful so far, seemingly because of various profound technical problems, including expressing the parasite genes in cells or cell lines from other species. Therefore, we do not have direct evidence that CD36RP mediates the selective uptake of cholesteryl ester by schistosome adults or eggs. However, structural similarity of CD36RP to CLA1 or SR-BI that mediate cholesteryl ester uptake from HDL in the cells of human and rodents, respectively, can be extrapolated to the functional similarity. Furthermore, the extracellular domain of CD36RP indeed demonstrated selective binding to HDL, and its antibody suppressed HDL cholesteryl uptake and maturation of the eggs in culture. Thus, it is not unreasonable to speculate that CD36RP is a strong candidate for a mediator of HDL cholesteryl ester uptake by the adults and eggs of *S. japonicum*, and therefore a key molecule for maturation of the egg to the miracidium. This means that CD36RP, as well as host plasma HDL, is a key protein for hepatic granulomatosis in *S. japonicum* infection that can represent a fatal pathological process in infected persons.

Clearly it will be necessary to demonstrate more direct evidence for CD36RP to catalyze selective cholesteryl ester uptake before this schistosome glycoprotein can be definitively ascribed a role as the mediator of the reaction. Technical difficulties remain to be overcome in order to accomplish the necessary manipulations, such as functional transfection and expression of the gene or knockdown of the gene to down-regulate the reaction. However, given recent advances with transgenesis approaches in schistosomes and other parasitic helminths (31–33), it is feasible that informative functional genetics approaches may soon allow definitive assignment or not of a physiological role for schistosome CD36RP in selective uptake of cholesteryl ester from host HDL. FJ

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Schistosomicidal and antifecundity effects of oral treatment of synthetic endoperoxide N-89

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ABSTRACT

1,2,6,7-Tetraoxaspiro[7.11]nonadecane (N-89) is a chemically synthesized compound with good efficacy against malaria parasites. We observed strong anti-schistosomal activities of N-89 both *in vitro* and *in vivo*. In a murine model with experimental infection of *Schistosoma mansoni*, orally administered N-89 at the dose of 300 mg/kg resulted in a significant reduction in worm burden (63%) when mice were treated at 2-weeks postinfection. Strong larvicidal effects of N-89 were confirmed *in vitro*; schistosomula of *S. mansoni* were killed by N-89 at an EC50 of 16 nM. In contrast, no significant reduction in worm burden was observed when N-89 was administered at 5 weeks postinfection *in vivo*. However, egg production was markedly suppressed by N-89 treatment at that time point. On microscopic observation, the intestine of N-89-treated female worms seemed to be empty compared with the control group, and the mean body length was significantly shorter than that of controls. Nutritional impairment in the parasite due to N-89 treatment was possible, and therefore quantification of hemozoin was compared between parasites with or without N-89 treatment. We found that the hemozoin content was significantly reduced in N-89 treated parasites compared with controls ($P<0.001$). The surface of adult worms was observed by scanning and transmission electron microscopy, but there were no apparent changes. Taken together, these observations suggested that N-89 has strong antischistosomal effects, probably through a unique mode of drug efficacy. As N-89 is less toxic to mammalian host animals, it is a possible drug candidate against schistosomiasis.

1. Introduction

Schistosomiasis is a parasitic disease caused by trematode flatworms of the genus Schistosoma that is common in many tropical countries and affects more than 200 million people living in conditions of poor sanitation and/or with less developed social infrastructure [1–3]. The World Health Organization (WHO) is leading the global strategy of schistosomiasis control, with a focus on morbidity control through chemotherapy. Praziquantel (PZQ) is a safe and effective drug for schistosomiasis and has been the drug of choice since the late 1970s. This has raised concerns about the development of drug resistance, and suggestive cases of PZQ-resistant parasites have been reported in *Schistosoma mansoni* from African countries [4–6]. Therefore, the development of new antischistosomal drugs is a matter of priority, and new candidate compounds have been reported [7–9].

Artemisinin-derivatives (ADs) are compounds extracted from the plant *Artemisia annua* used in traditional Chinese herbal medicine, which have strong malaricidal effects [10–13]. Recent studies clearly showed that these compounds also had strong effects against schistosome parasites [14,15]. The most notable difference between PZQ and ADs is the developmental stages of the parasite at which the drugs show efficacy [16,17]. Adult worms are highly sensitive to PZQ, while the larval stages are less sensitive to the drug [18,19]. On the other hand, ADs are effective mainly against the larval stage parasites, while adult worms are less sensitive to treatment with these drugs. In this sense, PZQ is a therapeutic drug, while ADs are drugs for prophylaxis [20]. Therefore, it is recommended to use a combination of the two drugs [21,22].

Although the mechanism of the efficacy has not yet fully been elucidated, peroxide bridge is necessary for antimalarial activities of ADs [10]. Previously, we reported that synthetic endoperoxide (1,2,6,7-tetraoxaspiro[7.11]nonadecane: N-89) [23] has high antimalarial activity against *Plasmodium falciparum in vitro* and *Plasmodium berghei in vivo*, and it shows low levels of cytotoxicity in mice and rats (LD50: >2000 mg/kg) [23–25]. ADs are structurally complicated and their chemical synthesis is

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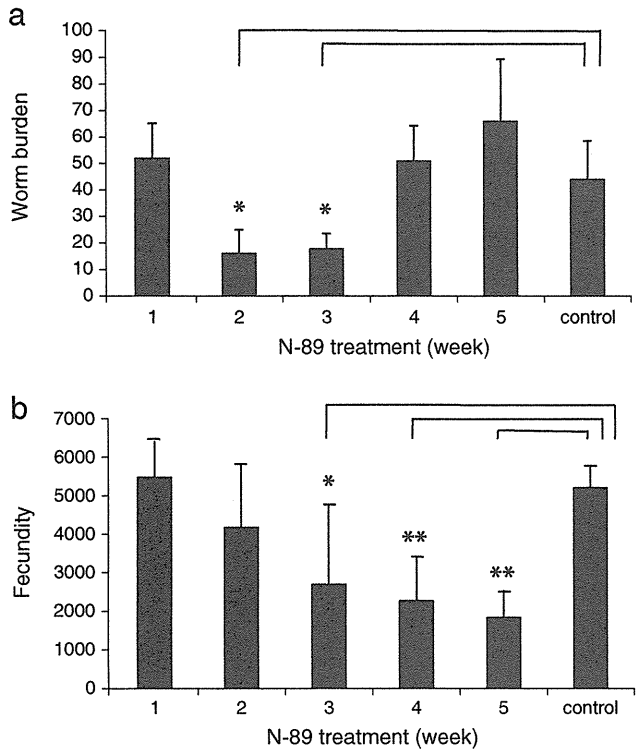

Fig. 1. *In vivo* effects of N-89 to *S.mansoni*. *S. mansoni*-infected mice were orally treated with N-89 from week 1 through week 5 postinfection. (a) *Y*-axis shows the number of worms that were collected by perfusion 9 weeks postinfection (**P*<0.001). (b) *Y*-axis shows the number of eggs produced per female worm. (**P*<0.05, ***P*<0.001).

not easy. On the other hand, N-89 is a compound with a relatively simple structure and is inexpensive to mass produce [23–25]. If N-89 also has strong effects against schistosome parasites, this will allow a new strategy of schistosomiasis control using a lower cost agent.

In this study, we found strong effects of N-89 against *S. mansoni* both *in vitro* and *in vivo*. The efficacies of N-89 were almost comparable to those of ADs. However, N-89 had additional effects that were not reported in the case of ADs, suggesting that N-89 may be a novel compound with unique antischistosomal activities.

2. Materials and methods

2.1. Parasites and animals

Puerto Rican strain *S. mansoni*, which was kept in our laboratory, was used for the present study. Female 5-week-old BALB/c mice were purchased from CLEA (Tokyo, Japan).

2.1.1. In vivo treatment of S. mansoni-infected mice with N-89

For *in vivo* study, mice were infected with 180 cercariae by the standard method in which mice were percutaneously exposed *via* the tail to cercariae for 1 h at room temperature [14]. BALB/c mice infected with *S. mansoni* were orally treated with N-89 suspended in olive oil at a dose of 300 mg/kg twice a day for two consecutive days. Mice were divided into 6 groups and treated with N-89 at various time points, *i.e.*, from week 1 through week 5 postinfection. To analyze parasite egg burden, eggs were recovered from the liver and intestine by the method reported previously [26]. Briefly, chopped liver and intestine were digested in 4% KOH at 37 °C for 1 h. After incubation, the digested samples were centrifuged at 1500 rpm for 5 min at room temperature, and pellets were resuspended in distilled water. Eggs were counted under a light microscope. Effects on pathological lesions after N-89 treatment were determined by observation of egg granulomas formed in the liver. Liver sections of Azan staining were prepared, and granuloma size was measured by using Image J image processing software (NIH). The mean size of 100 granulomas formed around a single egg in N-89 treated mice was compared to that in control (olive oil-treated mice). In addition, we calculated the body length of the worms using Image J. All *in vivo* experiments were approved by the Committee of Animal Rights and Ethics, Tokyo Medical and Dental University.

2.1.2. In vitro treatment of S. mansoni with N-89

As N-89 seemed to be effective against larval stage parasites, we prepared schistosomula from the lungs of mice and incubated them in

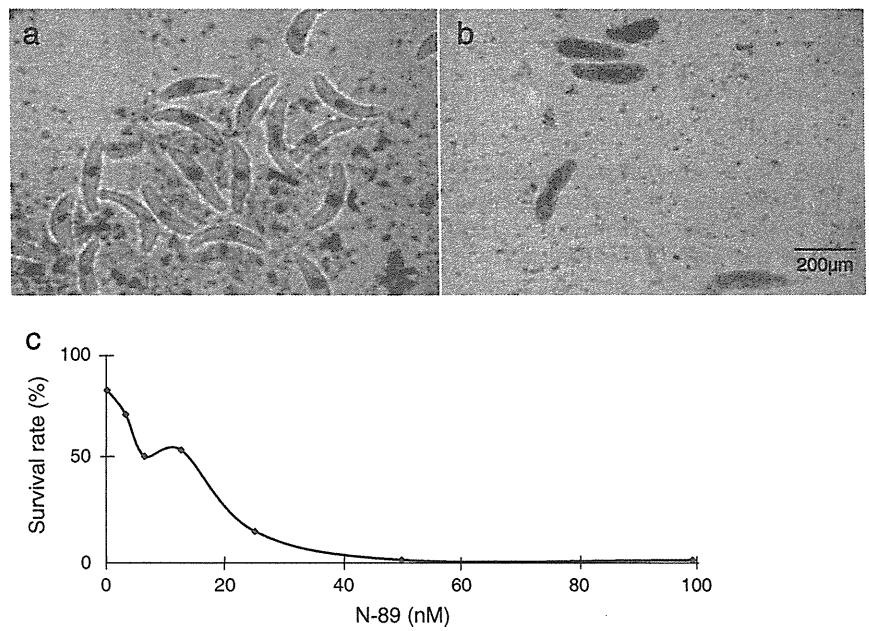

Fig. 2. Schistosomicidal effects of N-89 *in vitro*. (a) 14-day schistosomula were round-shaped and in a state of continuous contraction and extension when they are alive in the medium containing DMSO (2.5%) alone. (b) Schistosomula treated with 50 nM of N-89 were stiff and easily stained with trypan-blue. (c) Y-axis indicates the survival rate of 14-day schistosomula after treatment with serial dilutions of N-89.

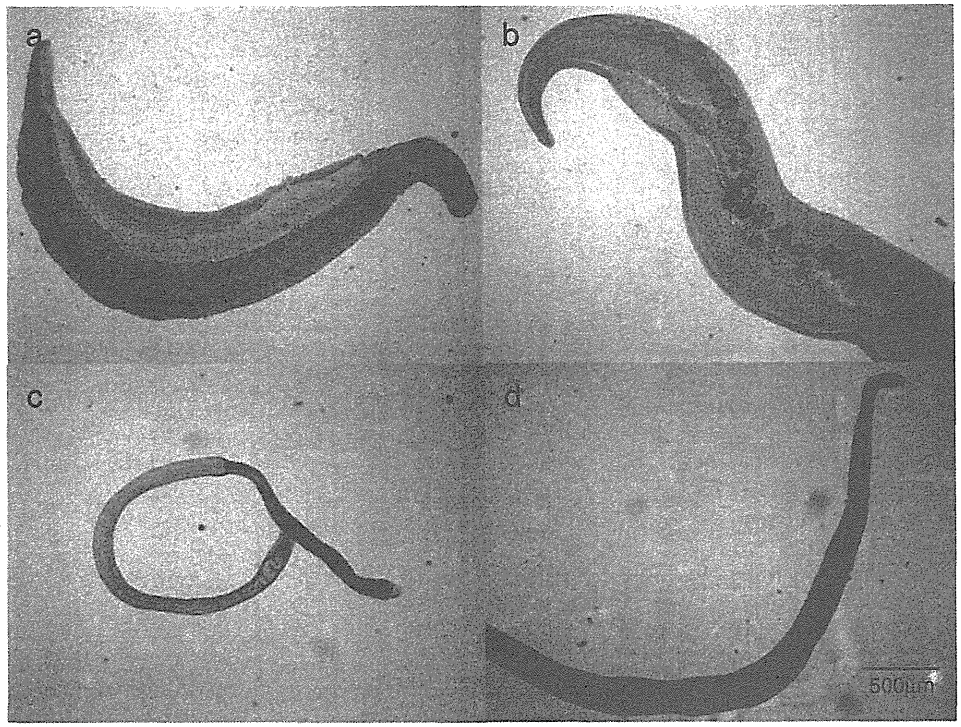

Fig. 3. Light microscopic observation of adult parasites after *in vivo* treatment with N-89. *S. mansoni*-infected mice were treated with or without N-89 5-weeks postinfection. Worms were collected 2 weeks after the treatment. 7-week *S. mansoni* worms were stained with hematoxylin–carmine solution. A male worm from mice treated with N-89 (a), a male worm from control mice (b), a female worm from mice treated with N-89 (c), a female worm from control mice (d).

RPMI-1640 (Wako, Osaka, Japan) supplemented with 10% FBS (JRH Biosciences, Kansas, MO), 150 U/ml of penicillin, and 150 μg/ml of streptomycin (Gibco, Gaithersburg, MD) in 24-well plates (Greiner, Ulm, Germany). N-89 was dissolved in dimethylsulfoxide (DMSO) and added 25 μl to the plates which contains 1 ml of RPMI at various concentrations from 3.12 to 100 nM. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 7 days. Survival of the treated schistosomula was determined by trypan blue dye-exclusion test. Based on the observations, we calculated the EC_{50} of N-89 against schistosomula of *S. mansoni in vitro.*

2.2. Morphological observation of adult parasites after treatment with N-89 in vivo

To observe the morphological changes after N-89 treatment, infected BALB/c mice were administered orally with N-89 at 5 weeks post-infection at a dose of 300 mg/kg, and 2 weeks later adult worms were recovered by portal perfusion. Recovered parasites were washed thoroughly with 0.85% NaCl and 0.45% Na-citrate in distilled water, and paired worms were fixed in 70% ethanol and stained with hematoxylin–carmine solution for light microscopic observation. Parasites were observed by scanning electron microscopy and transmission electron microscopy (Hitachi, Tokyo, Japan) according to the method reported previously [27,28].

2.3. Quantification of hemozoin contents of S. mansoni

Hemozoin was extracted from *S. mansoni* and quantified by the method reported previously [29–31]. Protein contents of worm homogenates were measured using a protein assay kit (Bio-Rad, Hercules, CA). Infected mice were administered orally with N-89 (300 mg/kg) at 5 weeks postinfection, and 2 weeks later adult parasites were tested for hemozoin contents. The worms used for the tests were paired to compare worms in the same/similar developmental stages. For each experiment, 15 to 30 worms were used from each mouse. Worms were homogenized in 1 ml of PBS (pH 7.2), and centrifuged for 10 min at 10,000×*g*. Insoluble pellets were washed with 0.1 M sodium hydrogen carbonate, and then dissolved in 0.1 N NaOH. Hemozoin was converted to heme in this treatment, and we then measured the converted heme as hemozoin in accordance with the reagent manufacturer's protocol (Hemin, Sigma-Aldrich, St. Louis, MO). Heme was quantified spectrophotometrically by measuring absorbance at 405 nm. Hemozoin content in the parasite was expressed as ng heme/mg protein.

2.4. Statistical analysis

Statistical analyses were performed by Student's *t* test. In all analyses, $P<0.05$ was taken to indicate statistical significance.

3. Results

3.1. Schistosomicidal effects of N-89 in vivo

Reduction of worm burden was observed when mice were treated 2 or 3 weeks postinfection, and the maximum effect of N-89 driven reduction in worm burden was observed at 2 weeks postinfection compared with the olive oil control group (Fig. 1a). Schistosomicidal effects became less apparent at 3 weeks postinfection, and there was no detectable reduction in worm burden when mice were treated at 5 weeks postinfection. However, egg production per paired female worm was significantly reduced when mice were treated with N-89 at 5 weeks postinfection. Reduction in egg production per female worm in the N-89-treated group was statistically significant in comparison to the olive oil control group (Fig. 1b). These observations indicated that the larval stage is the target for the killing effect of N-89, while this agent showed inhibitory effects on fecundity of adult worms without killing the parasite.

3.2. In vitro effects of N-89 for schistosomula of S. mansoni

To confirm the direct effects of N-89 against the larval stage of *S. mansoni*, schistosomula were treated with serial dilutions of N-89 and

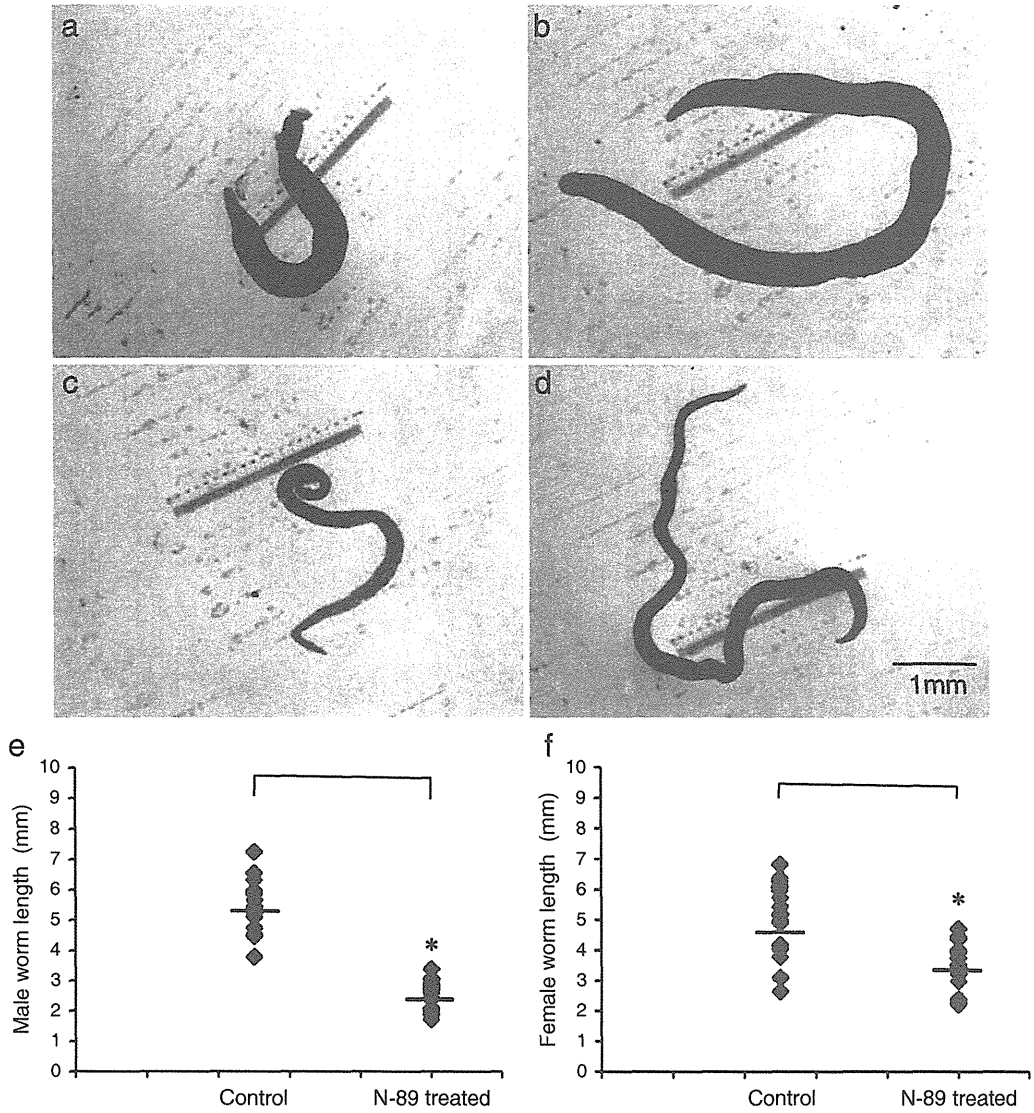

Fig. 4. The mean body length of the worms. All worms used were obtained in the same manner as described in Fig. 3. *Y*-axis indicates the length of male worms (a) (*$P<0.01$) and female worms (b) (*$P<0.01$).

cultured for 7 days *in vitro*. The schistosomicidal effects of N-89 were dose-dependent, and the EC_{50} against *S. mansoni* larvae was calculated as 16 nM (Fig. 2a–c). During the observation period, all schistosomula were alive and active under culture conditions containing DMSO alone (data not shown).

3.3. Pathological changes in the liver in infected mice treated with N-89

The sizes of granulomas formed around single schistosome eggs in N-89 treated mice was compared to that in control animals. The liver pathology of the mice treated at 5 weeks postinfection showed significantly smaller granulomas compared with controls ($P<0.001$) (data not shown).

3.4. Morphological changes of N-89 treated adult worms

To observe morphological changes of the parasite after N-89 treatment *in vivo*, we compared morphological profiles of the adult worms with or without N-89 treatment. The most obvious difference was noted in the intestine of female worms on light microscopic observation. Briefly, the dense substances, probably hemozoin, disappeared in N-89-treated worms (Fig. 3a–d). Furthermore, the mean body length of the treated worms was smaller than that of untreated controls (Fig. 4a–f). On TEM observation, the tegument morphology was compared between parasites with and without N-89 treatment. In both males and females, there were no marked differences between N-89-treated worms and control worms (Fig. 5a–d). In the SEM profiles, we found small surface changes, such as the disappearance of tubercles on the surfaces of males and shortened spines on females, but these changes were not as severe as the findings of previous studies for PZQ and ADs [28,32] (Fig. 5e–h).

3.5. Heme contents of adult parasites with and without N-89 treatment

As hemoglobin is the main source of nutrition for adult female worms, we measured hemozoin contents of parasites with and without N-89 treatment to examine whether nutritional impairment occurred in N-89-treated parasites. In the N-89-treated group, the mean heme content was 15 nmol heme/mg protein, while it was 89 nmol heme/mg protein in the untreated controls; this difference in heme content was statistically significant ($P<0.001$) (Fig. 6).

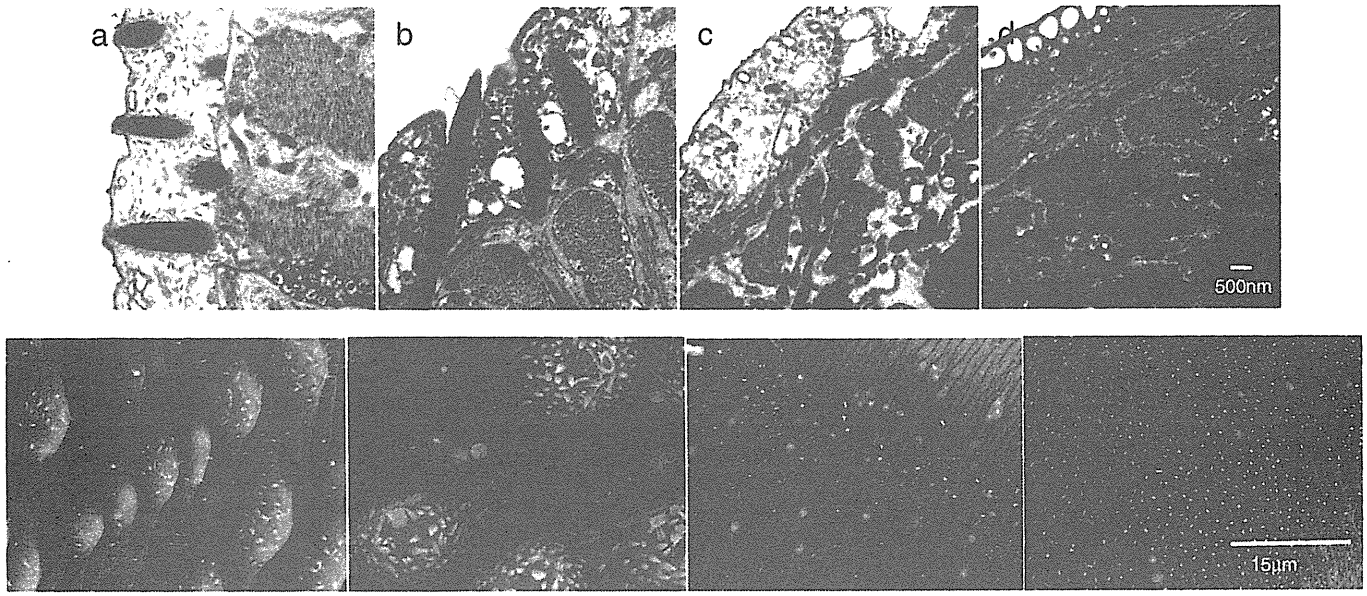

Fig. 5. EM observation of *S. mansoni* adult worms. All worms used were obtained in the same manner as described in Fig. 3. TEM observation of a male worm from mice treated with N-89 (a), a male worm from control mice (b), a female worm from mice treated with N-89 (c), and a female worm from control mice (d). SEM observation of a male worm from mice treated with N-89 (e), a male worm from control mice (f), a female worm from mice treated with N-89 (g), and a female worm from control mice (h).

4. Discussion

Rational drug design should be applied to develop new agents for use against schistosomiasis. As PZQ is the only drug available for controlling disease activity, the appearance of drug-resistant strains is a non-negligible concern. New drug candidates must be developed to address this concern, and ADs are promising candidates for this purpose. However, it should be noted that ADs are used for malaria therapy because of the recent WHO recommendation for use of artemisinin-based combination therapy (ACT). ADs are drugs prepared from plant materials. Due to their structural complexity, these compounds are not easy to chemically synthesize, and the distribution of the product depends on the supply of herbal plant materials. On the other hand, mass production of N-89 is not difficult, and it can be prepared at a much lower cost than ADs. No serious toxicity has been noted for N-89 in animal [23–25]. As N-89 is effective for reducing egg fecundity but not worm burden when it is administered 5 weeks post infection, it can supplement the effect of praziquantel that is effective for reducing worm burden.

The results of the present study suggest that N-89 is a novel antischistosomal compound with a unique mechanism of action compared to other drugs used to combat schistosomiasis, such as PZQ and ADs. Due to the structural similarity, we postulated that N-89 would have both antimalarial and antischistosomal effects in the same manner as observed for ADs. However, reference to previous publications regarding ADs indicated that there were marked differences in its anti-schistosomal effects. That is, N-89 showed two modes of antischisto-somal effect – larvicidal effects and antifecundity effects. Previous reports have indicated no such dual modes of drug efficacy for ADs [17]. Thus, it is possible that N-89 has functions distinct from those of ADs.

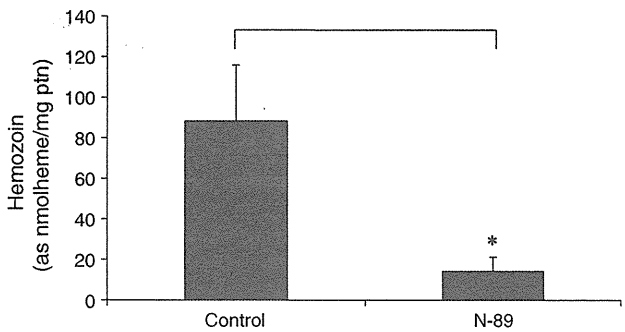

Fig. 6. Heme contents of adult parasites obtained after *in vivo* treatment with N-89.7-week worms collected in the same manner described in Fig. 3 were examined for quantification of hemozoin contents. *Y*-axis indicates the hemozoin contents (as nmol heme/mg protein) (*$P<0.001$).

It is still necessary to elucidate the detailed mechanisms of action for the two different effects of N-89. Considering the presence of endoperoxide structures in N-89, it is possible that oxygen stress generated by N-89 may be a factor involved in the schistosomicidal effects. Recent studies demonstrated the importance of the redox system for parasite survival [33,34]. However, no direct evidence in support of this possibility is available, nor killing effect of the worms was observed when *Sm*-infected mice were treated with N-89 at 5 week postinfection. In spite of this situation, we observed the reduction of egg fecundity. Morphological observations in the present study suggested that N-89 treatment induce nutritional deficits in the worms, as heme contents in N-89-treated female worms were significantly reduced compared to controls. This may be related to the antifecundity effect of the drug against female worms. It is well discussed that host hemoglobin derived from the host blood is essential for growth, development and reproduction of schistosomes [35,36]. It is possible that N-89 inhibits a process for hemoglobin usage in female worms, and more direct evidence may be obtained by testing the effects of N-89 on the biological pathways involved in hemoglobin uptake. It has been suggested that proteolysis of hemoglobin was important for worm development in male and female, and production of yolk protein in developing egg was also important for female worm [37]. The two modes of drug efficacy in N-89 raise questions regarding why the larval stages were destroyed, while the adult stage was resistant to this drug. In other cases, such as vaccine efficacy, lung stage parasites are the targets for the killing effects [7], although these are immune-mediated mechanisms. Analysis of the direct target molecules for N-89 could provide valuable information for the development of therapeutic strategies. Studies to elucidate these points using other approaches, such as proteomic analysis, are currently underway in our laboratory.

In conclusion, N-89 is a promising compound for use as an antischistosomal drug, which may supplement the effects of PZQ

through mutually different modes of efficacy. Strategies using N-89 as supplemental effect for praziquantel or ADs would be helpful to avoid the development of drug-resistance. Therefore, N-89 is a good candidate partner for its efficacy, safety, and its low cost of mass production.

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住血吸虫症に対するプラジカンテル投与法に関する考察 —1回投与か分割複数回投与か—

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Key Words: 日本住血吸虫症，メコン住血吸虫症，プラジカンテル，分割投与，薬剤耐性

はじめに

住血吸虫症の治療薬として開発されたプラジカンテルは，吸虫のみならず消化管に寄生する条虫にも効果がある．また，副作用が問題となることも殆どないので，住血吸虫症対策をはじめ，世界中の寄生蠕虫症対策で，流行地の住民を対象にした集団治療に広く使われてきた[1)]．色々な投与量や分服法が試されてきたが，住血吸虫症の集団治療の場合は，いずれの住血吸虫種に対しても，コンプライアンスの良さから40mg/kgの1回内服法が選択されることが多い．アフリカでのマンソン住血吸虫症治療例では，以前から，標準的なプラジカンテル治療を行っても，完全に治療できない例もあることが指摘されていたが，最近，耐性を疑わせる例が報告される国や地域が増える傾向が指摘されている[2)3)4)]．また，カンボジアでのメコン住血吸虫症対策でも，プラジカンテルによる集団治療後が毎年行われている4村落で，2005～2006年は，虫卵陽性者が認められなかったのに，2007年以降は，3村落で虫卵陽性率が3～5%に戻るなど，プラジカンテルによる治療効果の低下が疑われている（図1）．

そこで，2009年6月，カンボジアのメコン住血吸虫症対策の現場で，プラジカンテル集団治療後にも虫卵検査が陽性であった例を中心に，再度プ

How to use praziquantel to treat *Schistosoma japonicum* or *S. mekongi* infections ?

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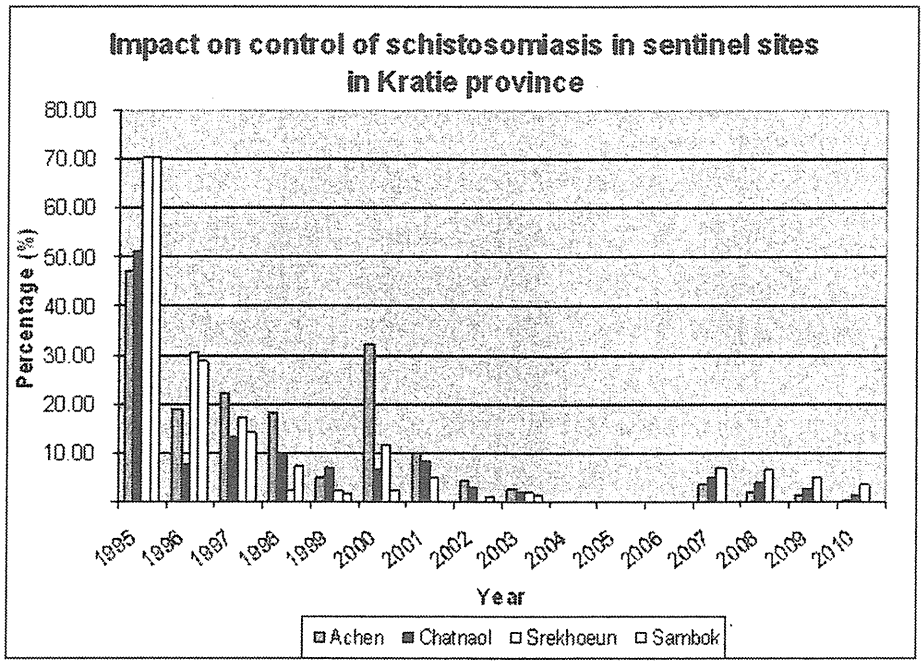

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図 1　カンボジア、クラチエ省におけるメコン住血吸虫卵陽性率の推移
ー年 1 回のプラジカンテル集団治療の成果と最近の停滞ー

ラジカンテルの効果を確認した．また，日本住血吸虫症について，中国とフィリピンにおけるプラジカンテル治療例を中心に，文献での報告例や日本国内での推奨使用例も含めて考察を行った．

方法

2009 年 3〜5 月にかけて，カンボジア王国，クラチエ省のメコン住血吸虫症の浸淫地では，全住民を対象とした寄生蠕虫症対策として，プラジカンテル（用量 40mg/kg の 1 回投与）とメベンダゾール（用量 500mg の 1 回投与）による集団治療が行われた．その効果判定のために，集団治療が行われた地域にある 6 つの小学校で，治療後 4〜6 週間で，計 939 人の児童を対象とした Kato-katz 法（3 日間連続）による糞便検査を行った．その検査で陽性を示した例に対しては，集団治療時の他の薬剤の服用状況について確認するとともに，総量 60mg/kg/日で 2 分割投与とする方法でプラジカンテル再投与を行った．そして，やはり 4〜6 週間後に，治療効果の判定を Kato-katz 法（3 日間連続）にて行った．

結果

939 人のうち，プラジカンテル投与（用量 40mg/kg の 1 回投与）後も，各々3 人の児童からメコン住血吸虫とタイ肝吸虫の虫卵が検出された．また，メベンダゾール 500mg を 1 回投与したにも関わらず，2 人から鞭虫卵が，85 人から鉤虫卵が検出された（表 1）．また，リファンピシンやクロロキン，或いはシメチジンといったプラジカンテルと相互作用を示す薬剤については，いずれの例も内服歴はなかった．

メコン住血吸虫とタイ肝吸虫については，同一村落で複数の感染者が見つかったが，別の児童が感染しており，特に感染者が同一人物に集積する傾向はみられなかった．最も感染者が多かった鉤

表1　カンボジア クラチエ省のメコン住血吸虫症有病地における集団治療（プラジカンテル+メベンダゾール）1ヶ月後の糞便検査　−2008年5月−

陽性者数（%）

村　落 学校名	検査検体数 被検者数	メコン 住血吸虫	タイ 肝吸虫	回　虫	鞭　虫	鉤　虫
Rokakandal	134	0	0	0	0	11　(8.2)
Sambox	137	0	0	0	0	8　(5.8)
Sre Khoeun	112	2 (1.8)	2 (1.8)	0	0	10　(8.9)
Chartnol	149	0	0	0	0	11　(7.4)
Sambo	165	0	1 (0.6)	0	1 (0.6)	17　(10.3)
K. Krabei	121	0	0	0	1 (0.8)	16　(13.2)
Achen	121	1 (0.8)	0	0	0	12　(9.9)
合計	939	3 (0.3)	3 (0.3)	0	2 (0.2)	85　(9.1)

虫についても，メコン住血吸虫，タイ肝吸虫など他の寄生吸虫卵検査で陽性を示した例は少なく，混合感染例はタイ肝吸虫との混合感染1例にとどまった（表2）．一方，鞭虫卵の陽性者は，鉤虫卵の陽性者と陰性者で1人ずつみられた．

また，プラジカンテル再投与（用量 60mg/kg/日で2分割投与）後の検査では，メコン住血吸虫卵，タイ肝吸虫卵とも陰性であった．

表2　鉤虫卵陽性者における他の寄生蠕虫卵の検出状況

	鉤虫陽性例 (85例)	鉤虫陰性例 (854例)	合計 (939例)
メコン住血吸虫	0	3	3
タイ肝吸虫	1	2	3
鞭　虫	1	1	2
回　虫	0	0	1
寄生蠕虫卵陰性例	83	848	930

考察

プラジカンテルは，日本国内ではビルトリシドとして市販されており，添付文書では，肝吸虫症と肺吸虫症で，総量 80mg/kg（1回 20mg/kg を1日2回2日間），横川吸虫症で，総量 20〜40 mg/kg（1回 20mg/kg を1日1〜2回）によるする内服法が，適当な例としてあげられている[5]．本来，住血吸虫症治療薬として開発されたプラジカンテルが，国内での感染例がないことより，住血吸虫症が保険適応疾患として記載されていないのは，奇異な印象を受けるが，国内で入手しやすい治療指針では，1日に複数回の投与で，2日以上の投与期間を推奨するものが多い[6]．一方，欧米の標準的な内科学書や熱帯医学のテキストでは，単回投与か1日で投与をやめる方法が記載されるのが一般的である[6]．

プラジカンテルによる治療効果の減弱は，1990年代からアフリカのマンソン住血吸虫症でよく知られており，一つはセネガル北部にある伝播の高い高度浸淫地で，もう一つはエジプトである[2)3)]．ところが，最近は，ケニアといった東アフリカの国でも，プラジカンテルの感受性低下が疑われる例が報告されるようになった[4]．これらの地域では，プラジカンテルによる治療を 2〜3 回繰り返した後でも，1〜2%の患者は治癒しなかった[2]．

今回のカンボジアのメコン住血吸虫症有病地での調査では，プラジカンテルを 40mg/kg の用量でする集団治療後，メコン住血吸虫，タイ肝吸虫とも 0.3%の例で，糞便検査で虫卵が陽性となったが，プラジカンテルを総量 60mg/kg/日で2分割という内服法で再投与したところ，全例で治癒が

確認された．一方，最近の中国での日本住血吸虫症治療例では，プラジカンテルを総量 80 mg/kg/日で 2 分割投与したにもかかわらず，185 例のうち 1 例で，治癒が確認されなかった[7]．もっとも，中国の別の報告では，用量 40mg/kg の 1 回投与で治療を受けた 584 人のうち，6 週間後の糞便検査で治癒が確認されなかった 19 例について，再度同じ用量でのプラジカンテル投与により，6 週後全例で治癒が確認されている[8]．住血吸虫の高度流行地にあっては，薬剤の感受性低下と再感染を厳密に区別することは難しい．特に，乾季に感染リスクが急速に増し新感染もその時期に集中するメコン住血吸虫については，プラジカンテルによる治療困難例と再感染例を区別することは，再治療の効果判定も含め，乾季の終わり頃でなければ困難である．少なくとも現時点では，アジア地域における住血吸虫に対するプラジカンテルの耐性出現は，はっきり確認されたとは言えず，臨床的にもまだ大きな問題とはなっていない．

ただ，プラジカンテルの投与量については，使用当初より，用量 40mg/kg の 1 回投与法よりも，用量 40～60mg/kg/日の用量で 分割する投与法で，高い治療効果が得られていた可能性もある．最近になるまで，用量 40mg/kg での 1 回投与をプラジカンテル治療の標準としてきた中国では，住血吸虫による中枢神経症状は難治とされてきたが，病院受診例を中心に，用量 60mg/kg/日の用量で 1～3 分服する投与法が選択されることが多かったフィリピンでは，中枢神経症状は，後遺症を残すことなく改善することが殆どであった[8)9)]．プラジカンテルには，Ca チャンネルを介して成虫の筋麻痺を起こし虫体の排泄を促す，よく知られた作用以外に，成熟卵に対して生体内で孵化させる働きもある[1)10)]．プラジカンテル投与後に肝線維化や門脈壁肥厚の改善傾向がみられるのは，プラジカンテルによって，肝線維化の主因である虫卵結節から，成熟虫卵が排泄されることで説明できるが，同様な作用は，中枢神経症状の原因となっている脳内の虫卵結節に対してもおきていると思われる．この際，脳－血液関門を通ってプラジカンテルの濃度が中枢神経内でも増加するには，分割投与したほうが望ましいのかもしれない．さらに，成虫が体内から排出された初回治療後にも体内に残存する虫卵排泄を目指し，虫卵の成熟を待って初回治療の 2～3 週後に，再度プラジカンテルの服用を推奨する報告もある[6]．

マラリア原虫をはじめ，単細胞の原虫では，従来から薬剤耐性株の出現と拡散が，問題となっていたが，多細胞で一般に寿命が長い寄生蠕虫では，薬剤耐性が臨床的に問題となることは従来殆どなかった．しかし，ヒトよりも駆虫薬の使用頻度がはるかに多かった家畜では，既にベンズイミダゾール系薬剤に対する耐性が，各地で報告され，ヒツジの線虫 *Haemonchus contortus* のベンズイミダゾール耐性に関する研究では，薬剤耐性と関連した遺伝子変異も明らかになっている[11)]．現在，寄生蠕虫症対策の為にプラジカンテルやメベンダゾールといったベンズイミダゾール系薬剤の使用が急速に拡大していることから考えて，これらの抗寄生蠕虫薬に対する耐性は，アジアも含め地球規模で，経時的・組織的なモニタリングをしていかねばならない．

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