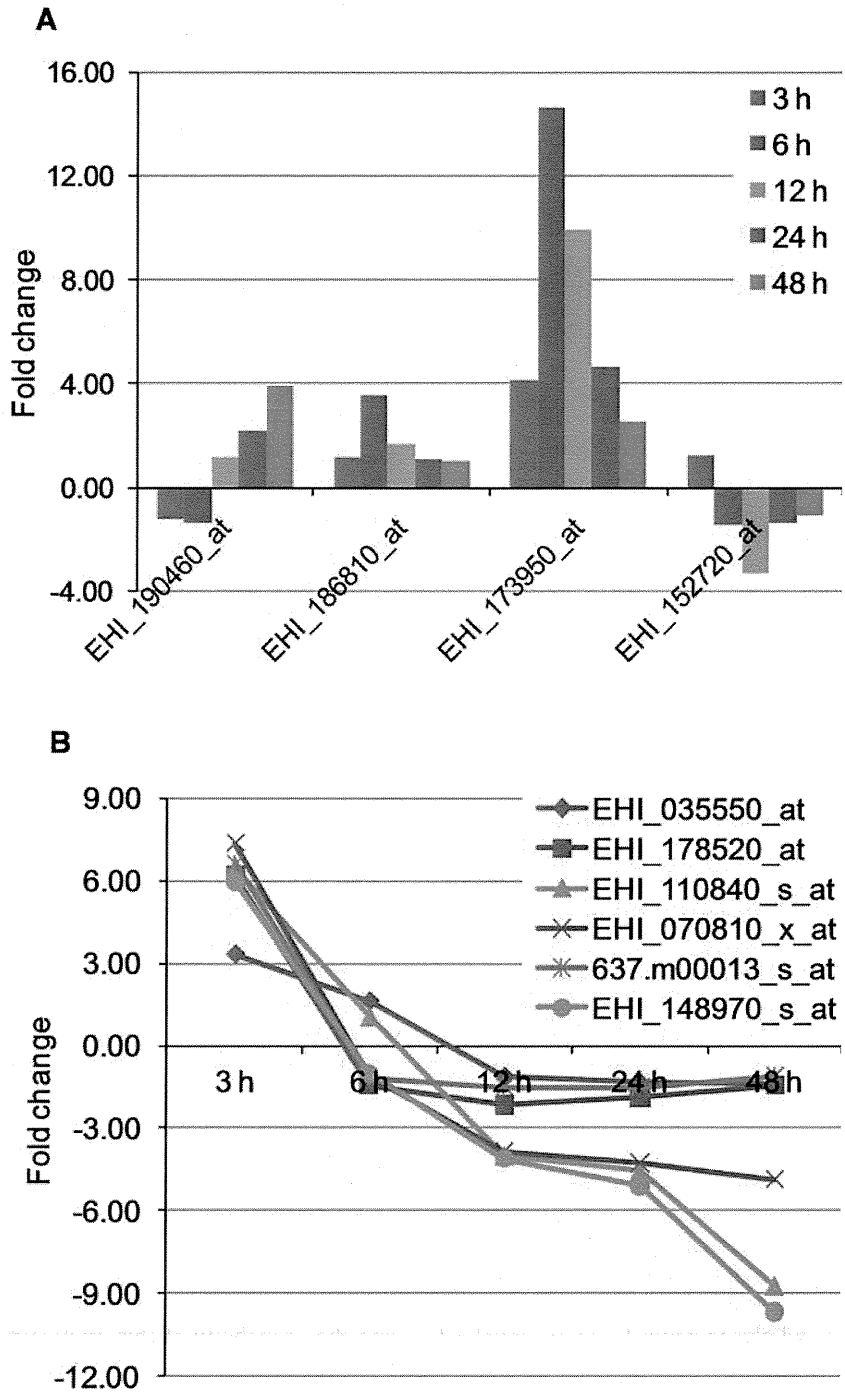

Figure 5 Changes in the expression of the genes encoding membrane transporters and regulator of nonsense transcripts upon L-cysteine deprivation. Values are expressed as fold changes in the expression of the transcripts relative to the trophozoites culture in the presence of normal concentration of L-cysteine. ***A***) Changes in the expression of the genes encoding putative transporter proteins. ***B***) Time-dependent changes in the expression of the genes encoding putative regulators of nonsense transcripts.

ammonia lyase will, in theory, lead to the decreased catabolism of these amino acids, and will prevent further accumulation of malate and fumarate. Aspartate aminotransferase in various organisms is known possess L-cysteine aminotransferase activity, which leads to the formation of mercaptopyruvate from L-cysteine [36]. Like EhNO2, this enzyme might also be regulated by the availability of its alternative substrate (L-cysteine), which is highly decreased upon L-cysteine deprivation. We also noticed time-dependent modulation in the expression of genes encoding putative fatty acid elongases. It has been shown that L-cysteine depletion decreases PtdEtn, and thus affects PtdCho/PtdEtn ratio, which potentially alters membrane fluidity, integrity, protein translocation across membranes, and membrane fusion events [12,37-39]. Changes in the expression of fatty acid elongases may be associated with the modulation of fatty acid chains in the phospholipids to compensate for the physical changes induced by the decrement in PtdEtn upon L-cysteine deprivation.

Effect of L-cysteine deprivation on nucleic acid metabolism

Expression of several genes encoding proteins with functions in DNA/RNA metabolism was significantly modulated upon L-cysteine deprivation. They include several genes encoding regulator of nonsense transcripts (RENT), which participate in the nonsense mediated decay (NMD) of mRNAs containing a frameshift or a nonsense mutation (Figure 5B). This surveillance system protects cells from the production of non-functional proteins by eliminating mutant mRNAs. In addition to RNA surveillance, NMD is also involved regulating the abundance of hundreds of naturally occurring mRNAs [40].

The *E. histolytica* genome database at AmoebaDB [6,41] contains 8 different entries that showed similarity to the RENTs from other higher eukaryotes. Based on the fact that some of these entries showed very high mutual sequence identities (85-95%), there are only 4 independent RENT genes in *E. histolytica*. All of the probe sets representing amebic RENTs showed a common pattern of expression during L-cysteine deprivation. They were induced during early time points of L-cysteine deprivation, and then down-regulated during the later time points (Figure 5B). Thus, like *Giardia lamblia*, the components of NMD pathway seem to be present and functional in *E. histolytica*. In *G. lamblia*, a large number of naturally occurring transcripts have been shown to be under the control of NMD [42]. However, the functionality, its targets, and role of NMD in the gene regulation of *E. histolytica* have not yet been demonstrated. Changes in the expression of RENTs suggest that some of the observed changes in the gene expression upon L-cysteine deprivation might be resulted from the corresponding changes in the NMD pathway of mRNA degradation.

Beside RENTs, several other genes with functions in nucleic acid metabolism were also modulated upon L-cysteine deprivation (Additional files 2 and 3). A gene encoding a putative zinc finger protein was induced at the early time points, and then repressed at the later time points. Other genes, such as DNA/RNA helicase, a myb-like transcription factor, and a putative DNA repair protein were down-regulated upon L-cysteine deprivation. In addition, two genes encoding putative high mobility group (HMG) box proteins were slightly down-regulated upon L-cysteine deprivation. These proteins are associated with chromatin, and are involved in various processes including transcription, replication, recombination, and DNA repair [43]. Recently, expression of a large number of genes has been demonstrated to be modulated by the over-expression of a HMGB1 protein in *E. histolytica* [44]. Other genes encoding a putative La ribonucleoprotein and a ribosomal protein S30 were also up-regulated on L-cysteine deprivation. These results showed that L-cysteine modulates several genes involved in transcriptional and posttranscriptional regulation of the gene expression.

Effect of L-cysteine deprivation on signal transduction

A significant number of genes (27) encoding signalling proteins were modulated in response to L-cysteine deprivation. Of these modulated genes, 11 were up-regulated and 16 were down-regulated (Figure 1). They include several genes encoding key signalling proteins such as protein kinases, phosphatases, guanine nucleotide exchange factors (Ras-GEF), GTPases, and GTPase activating proteins (GAPs). Alterations in mRNA abundance of these key signalling genes upon L-cysteine deprivation suggest a significant cellular re-programming to cope up with the consequences of L-cysteine deprivation or to help trophozoites get adapted to low cysteine environment. Deprivation of amino acids, including L-cysteine, is known to activates an amino acid response (AAR) that alters cellular functions by regulating the expression of various genes using transcriptional and post-transcriptional mechanisms [18,45]. Activation of AAR leads to increased protein abundance of activating transcription factors, which in turn modulate the expression of genes containing AAR element (AARE) [18,45]. However, such an AAR was not induced in *E. histolytica*, as it appears to lack activating transcription factors. These results suggest that *E. histolytica* does not employ canonical pathways to cope with the amino acid deprivation, but may employ other novel strategies.

Effect of L-cysteine deprivation on vesicular trafficking, cytoskeleton, and secretion

Response to changing environmental conditions by eukaryotic cells also includes modulation of protein degradation, targeting, transport to specific organelles, and secretion. Amino acid deprivation has been shown to regulate vesicular trafficking, secretion, exocytosis, and autophagy [46]. L-Cysteine limitation also modulates several proteins associated with these processes in *E. histolytica*. For example, four genes encoding putative cysteine proteases (EHI_123950, EHI_121160, EHI_160330, EHI_182260) were down-regulated in a time-dependent manner during L-cysteine deprivation (Table 2; Additional file 3). A gene encoding vacuolar protein sorting 26 (Vps26) was up-regulated during L-cysteine deprivation. In addition, several genes encoding guanine nucleotide exchange factors (Ras-GEF), GTPases, and GTPase activating proteins (GAPs) were also modulated in response to L-cysteine deprivation. Modulation of the genes encoding putative ankyrin and actin binding protein suggested that L-cysteine deprivation may affect cytoskeleton re-organization, mobility and vesicular trafficking.

Miscellaneous

In addition to the modulation of above mentioned genes expression of several other transcripts was also changed upon L-cysteine deprivation. For example, a transcript for a putative heat shock protein 20 was induced 4-5 fold, and two WD40 domain-containing proteins were down-regulated 3-4 fold upon L-cysteine deprivation (Additional files 2 and 3). WD-repeat proteins are a large family found in almost all eukaryotes and implicated in a variety of cellular functions ranging from signal transduction and transcription regulation to cell cycle control. One of the common functions of most of the WD-repeat proteins is to coordinate multi-protein complex assemblies [47]. Several genes encoding leucine-rich repeat proteins were down-regulated 3-6 fold at early time points upon L-cysteine deprivation (Additional file 3). Leucine-rich repeats serve as recognition motifs for surface proteins in bacteria and eukaryotes.

Repression of genes encoding ISF causes growth defects

In order to further characterize the functional role of the genes induced upon L-cysteine deprivation, we utilized the epigenetic silencing in *E. histolytica* G3 strain to repress genes of interest [48,49]. Using this epigenetic silencing strategy, we were able to repress (≥90%) two genes encoding ISFs (ISF1, EHI_138480 and ISF2, EHI_025710) that were highly induced gene upon L-cysteine deprivation (Figure 6A). However, we could not repress the third highly induced gene (MFS; EHI_173950). Repression of ISF2, but not of ISF1, showed slight growth deflects when cultured in normal medium. However, a severe growth defect in ISF2-repressed, and relatively mild growth defect in ISF1-repressed G3 trophozoites were observed in L-cysteine-deprived medium (Figure 6B). We also checked if repression of ISF1 or 2 also affects the tolerance of trophozoites to H_2O_2 mediated cytotoxicity. However, no significant difference in the tolerance to H_2O_2 cytotoxicity was observed (Figure 6C). L-Cysteine deprivation induced growth defects in ISF1- and 2-repressed G3 trophozoites suggest that in addition to their proposed roles in combating oxidative stress, ISF1 and 2 proteins may also play important roles under L-cysteine deprivation. These ISF are very similar to bacterial NADPH-dependent FMN reductases, which are induced upon sulfate or L-cysteine starvation [50]. In *Escherichia coli*, this enzyme, called a two-component alkanesulfonate monooxygenase, allows utilization of alkanesulfonates as sulfur sources under sulfate or cysteine starvation [29]. However, it still remains unclear whether ISFs in *Entamoeba* are also involved in similar processes.

Conclusions

This study represents the first genome-wide analysis of transcriptional changes induced by L-cysteine deprivation in protozoan parasites, and in eukaryotic organisms where L-cysteine represents the major intracellular thiol. We showed global changes in the expression of genes implicated in metabolism, signalling, oxidative defence, DNA/RNA regulation, and transport. Although a large number of genes were modulated upon L-cysteine deprivation, significant transcriptional changes in genes involved in SAA metabolism were not observed, which confirmed that changes in the metabolic flux across SAA metabolism are not caused by the changes in the expression of corresponding genes. Similarly, we also showed that the changes in the gene expression induced by L-cysteine deprivation are not shared by those induced by oxidative or nitrosative stress. The most important changes that occurred upon L-cysteine deprivation were the induction of iron sulfur flavoproteins and major facilitator super-family transporter. Repression of ISF1 and 2 genes caused growth defects under L-cysteine-deprived conditions. Further studies on the kinetic and biochemical analysis of ISFs and MFS transporter, and their regulation should help to better understand the physiological role of these proteins in the biology of *E. histolytica*. L-Cysteine depletion mediated time-dependent changes in the expression of RENTs suggest that similar to other eukaryotic cells, NMD may also be functional in *E. histolytica*. This study also confirmed that most of the L-cysteine deprivation-mediated metabolomic changes in amino acid, central energy, and phospholipid metabolism are not associated with the

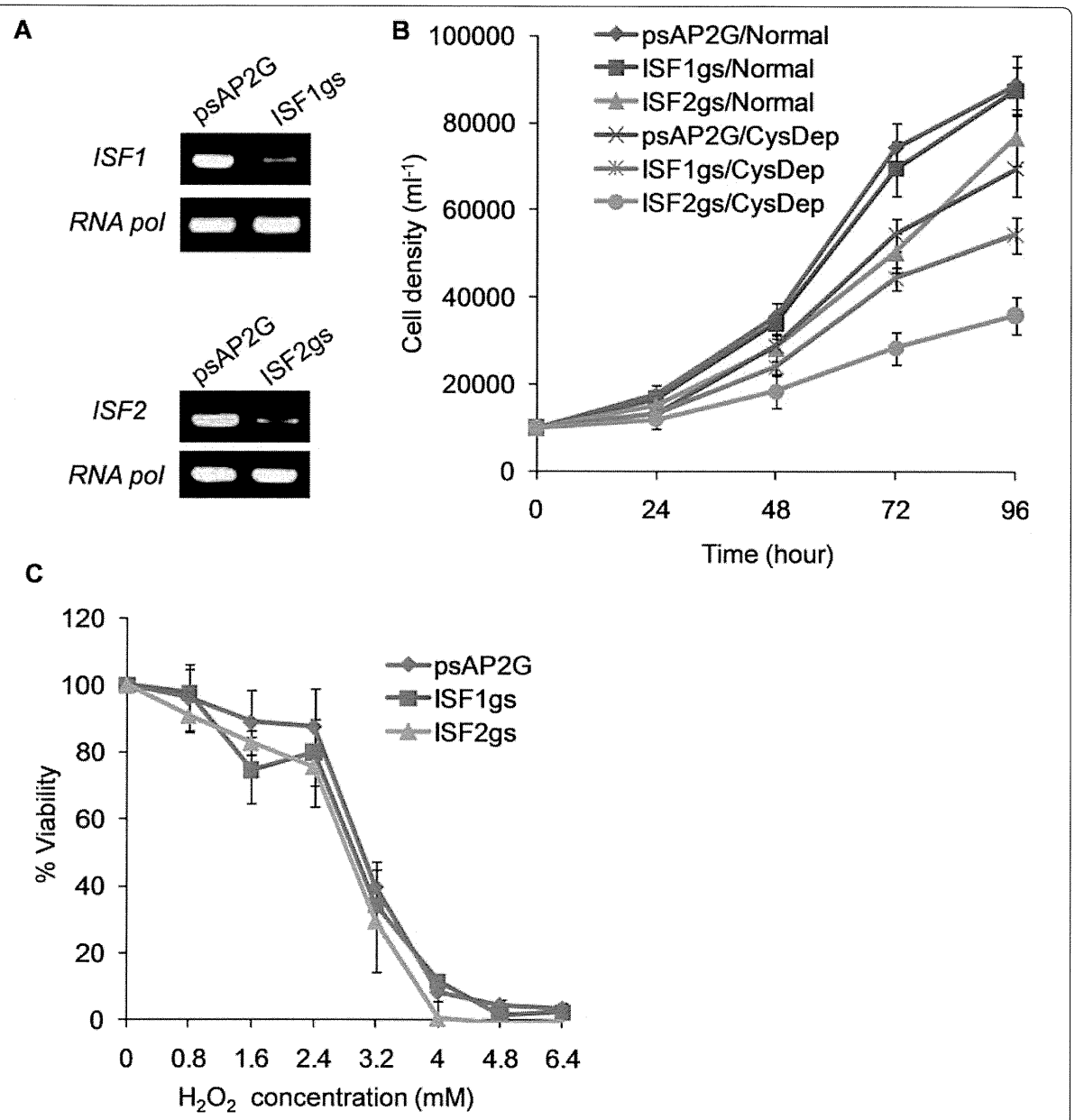

Figure 6 Epigenetic repression of iron sulfur flavoproteins in *E. histolytica* G3 strain. ***A***) Semi-quantitative RT-PCR analysis of *ISF1* and *ISF2* expression levels in G3 parasites transfected with either empty vector (psAP2Gunma) or gene silencing plasmids (psAP2G-*ISF1* and psAP2G-*ISF2*). cDNA from these cell lines (psAP2G, *ISF1*gs, *ISF2*gs) were subjected to 30 cycles of PCR using specific primers for *ISF1* or *ISF2*. RNA polymerase was used as a control. PCR from samples without RT served as controls to exclude the possibility of genomic DNA contamination. ***B***) Effect of *ISF1* or *ISF2* repression on the growth of trophozoites cultured under normal ("Normal") or L-cysteine-depleted ("CysDep") conditions. ***C***) Effect of *ISF1 or ISF2* repression on the tolerance of amoebic trophozoites to oxidative stress.

changes in the expression of the corresponding genes. This general lack of correlation between metabolome, proteome, and transcriptome appears to be a general characteristic in various organisms including *E. histolytica*, indicating that they have more complex mechanisms of expression regulation.

Methods

Microorganism and cultivation

Trophozoites of the *E. histolytica* clonal strain HM1: IMSS cl 6 and G3 strain, kindly provided by David Mirelman, Weisman Institute, Israel [48,49], were maintained axenically in Diamond's BI-S-33 medium at 35.5°

C as described previously [51,52]. Trophozoites were harvested in the late-logarithmic growth phase for 2-3 days after inoculation of one-thirtieth to one-twelfth of the total culture volume. After the cultures were chilled on ice for 5 min, trophozoites were collected by centrifugation at 500 × g for 10 min at 4°C and washed twice with ice-cold PBS, pH 7.4.

RNA isolation and Affymetrix microarray hybridization

Trophozoites were first grown in normal culture medium containing a high concentration of L-cysteine (8 mM) for approximately 24 hrs. After culture medium was replaced with the one containing no exogenous L-cysteine, culture was continued for the next 3, 6, 12, 24, or 48 h. Total RNA was isolated from harvested trophozoites using Trizol reagent (Invitrogen, Carlsbad, CA, U. S.A.) according to the manufacturer's protocol. The RNA was quantified and checked for purity by comparison of absorbance at 260 and 280 nm in the NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Integrity of isolated RNA was verified by using Bio-Rad's automated electrophoresis system Experion (RNA StdSens analysis kit). All reagents and protocols followed those described in the Affymetrix manuals. Briefly, total RNA (5 μg) was reverse transcribed using T7-Oligo (dT) primer in the first strand cDNA synthesis. After second strand synthesis, the double-stranded cDNA template was used for in vitro transcription, in the presence of biotinylated nucleotides to produce labelled cRNA. The cRNA was purified, quantified, fragmented, and hybridized for 16 h at 45°C to custom-generated Affymetrix platform microarray (49-7875) with probe sets consisting of 11 probe pairs representing 9,327 *E. histolytica* (Eh_Eia520620F_Eh) and 12,385 *E. invadens* open reading frames (Eh_Eia520620F_Ei). After hybridization, the arrays were washed and stained with streptavidin-phycoerythrin using a GeneChip® Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA), according to the recommendations of the manufacturer. After washing and staining, the GeneChip® arrays were then scanned using the Hewlett-Packard Affymetrix Scanner 3000 (Affymetrix, Santa Clara, CA, USA), and the probe intensities were extracted using Affymetrix® GeneChip® Command Console™(Affymetrix, Santa Clara, CA, USA).

Analysis of microarray data

A minimum of two arrays were used for each condition and each time point. Raw Mas5 gene expression data were imported into the GeneSpring GX 10.0.2 program and normalized expression values for each probe set were obtained from raw probe intensities in R 2.7.0 (downloaded from the BioConductor project http://www.bioconductor.org) using robust multiarray averaging with correction for oligosequence (gcRMA). Standard correlation coefficients were calculated using GeneSpring GX 10.0.2. One way ANOVA analysis with Tukey's post hoc test was performed to extract differentially expressed genes. The p-values were calculated using Welch's test, and were corrected by Benjamini-Hochberg method.

Quantitative real-time PCR

Total RNA from the trophozoites cultured in either normal or L-cysteine-deprived medium was extracted as described above. cDNA was synthesized from 5 μg of total RNA using Superscript III First-Strand Synthesis System, and oligo$(dT)_{20}$ primer (Invitrogen). PCR was performed with the resulting cDNA as a template and specific oligonucleotide primers using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). A list of primers for qRT-PCR is shown in Additional file 6. Parameters for PCR were: an initial step of denaturation at 95°C for 9 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 65°C for 1 min. A final step at 95°C for 9 s, 60°C for 9 s and 95°C for 9 s was used to remove primer dimers [34].

Creation of *E. histolytica* transformants where expression of the genes induced upon L-cysteine deprivation were repressed

In order to construct plasmids for epigenetic silencing of *ISF1*, *ISF2*, and *MFS*, a fragment corresponding to a 420-bp long 5' end of open reading frame of *ISF1*, *ISF2* and *MFS* genes was amplified by PCR from cDNA using sense and antisense oligonucleotides containing *StuI* and *SacI* restriction sites, respectively. A list of these primers is provided in Additional file 7. These PCR amplified products were digested with *StuI* and *SacI*, and ligated into the *StuI*- and *SacI*-double digested psAP-2-Gunma shuttle vector.

psAP-2-Gunma was constructed as follows. 5'ap-a fragment were amplified from psAP-2 [48,49], as a template, using sense and antisense oligonucleotides containing appropriate restriction sites at the 5' end, with 5'-AGCTCTAGA**ccgcgg**CGGCTTGCTGCACCCTTTG-3' primer and 5'-CTCT*gagctc*GAGCTCGTTTAAaggcct-CATGATTGTTTGTAAGATAT G-3' primers (*SacII, SacI, and StuI* restriction sites are shown by bold-, italicized-, or underlined-text). PCR product and psAP-2 vector were digested by *SacI* and *SacII*. Digested PCR product was ligated into psAP-2 to yield psAP-2-Gunma vector (psAP2G).

StuI- and *SacI*-digested PCR products corresponding to a 420-bp long 5' end of open reading frame of *ISF1*, *ISF2* and *MFS* genes were ligated into psAP-2-Gunma to construct gene silencing plasmids of target genes

(psAP2G-*ISF1*, psAP2G-*ISF2*, and psAP2G-*MFS*). The trophozoites of *G3* strain were transformed with either empty vector or silencing plasmids by liposome-mediated transfection as previously described [11]. Transformants were initially selected in the presence of 1 μg/ml geneticin (Invitrogen), and the geneticin concentrations were gradually increased to 7 μg/mL during the subsequent two weeks prior to subjecting the transformants to analyses. The expression of the respective genes was confirmed by semi-quantitative RT-PCR as described previously [23]. These transformants were named as psAP2G (control) or -ISF1gs, ISF2gs, and MFSgs.

Growth assay of *E. histolytica* trophozoites

Approximately 6 × 10^4 exponentially growing trophozoites of *E. histolytica* G3 strain transformed with psAP2G-*ISF1*, psAP2G-*ISF2*, or psAP2G (control) plasmid were inoculated in 6 ml of normal or L-cysteine-deprived BI-S-33 medium containing 7 μg/mL geneticin, and the parasites were counted every 24 h on a haemocytometer.

Assay of hydrogen peroxide sensitivity

To examine sensitivity to H_2O_2, *E. histolytica* G3 trophozoites harbouring psAP2G-*ISF1*, psAP2G-*ISF2*, or psAP2G were seeded into a 96-well plate (10^4 trophozoites per well) in BI-S-33 medium containing 7 μg/mL geneticin and incubated for 12-16 h at 35.5°C. The cells were then exposed to varying concentrations (0.8-6.4 mM) of H_2O_2 for 1 h in the same culture medium. Following incubation, medium was removed and 100 μL pre-warmed Opti-MEM® I (Invitrogen) containing 10% (v/v) Cell Proliferation Reagent WST-1 (Roche Diagnostics, Mannheim, Germany) was added. After 1 h of incubation at 35.5°C, the optical density at A_{450} was measured with that at A_{595} as a reference using a microplate reader (Model 550, Bio-Rad, Tokyo, Japan). The initial density and incubation period of the cultures were chosen to maintain the control trophozoites in the late-logarithmic growth phase throughout the experiment, and also to allow the measurement of optical density in the linear portion of the curves. The assays were performed 3 times in triplicate.

Additional material

Additional file 1: All transcriptomic data analyzed in this study. Normalized average raw data (signal intensity), their converted data (in log_2), and present call (P, present; M, marginal; A, absent) of the duplicates of all the probe sets at 0, 3, 6, 12, 24, and 48 h of L-cysteine deprivation are shown. Fold changes of expression relative to 0 h, and up/down-regulation of expression, as well as p-value and corrected p-value of ANOVA are also shown.

Additional file 2: List of genes induced ≥3 fold at one or more time points upon L-cysteine deprivation. Probe ID, corrected p-value by ANOVA, fold change and up/down-regulation, and normalized expression levels in log_2 scale at each time point are shown. Locus ID, accession numbers, annotations, and other information related to GO term, InterProScan domains are shown.

Additional file 3: List of genes down-regulated ≥3 fold at one or more time points upon L-cysteine deprivation. Probe ID, corrected p-value by ANOVA, fold change and up/down-regulation, and normalized expression levels in log_2 scale at each time point are shown. Locus ID, accession numbers, annotations, and other information related to GO term, InterProScan domains are shown.

Additional file 4: List of changes in expression of genes that are involved in sulfur-containing amino acid metabolism upon L-cysteine deprivation. Normalized average raw data (signal intensity), their converted data (in log_2), and present call (P, present; M, marginal; A, absent) of the duplicates of all the probe sets at 0, 3, 6, 12, 24, and 48 h of L-cysteine deprivation are shown. Fold changes of expression relative to 0 h, and up/down-regulation of expression, as well as p-value and corrected p-value of ANOVA are also shown.

Additional file 5: List of 41 genes modulated ≥3 fold by L-cysteine deprivation and also modulated ≥3 fold by oxidative (1 mM of H_2O_2 for 1 h) and/or nitrosative stress (200 μM of DPTA-NONOate for 1 h). The list contains genes shown in Figure 3B.

Additional file 6: List of primers used for qRT-PCR.

Additional file 7: List of primers used for the construction of plasmids for the repression of genes that were induced upon L-cysteine deprivation.

Abbreviations

ISF: Iron sulfur flavoprotein; MFS: major facilitator super-family; SAA: Sulfur-containing amino acid.

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Authors' contributions

Conceived and designed the experiments: AH, GJ, DS, TN. Performed the experiments: AH, GJ, DS. Analyzed the data: AH, GJ, DS. Contributed reagents/materials/analysis tools: TN. Wrote the paper: AH, TN. All authors read and approved the final manuscript.

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Review

The Global Programme to Eliminate Lymphatic Filariasis: History and achievements with special reference to annual single-dose treatment with diethylcarbamazine in Samoa and Fiji

Eisaku Kimura

Abstract: Diethylcarbamazine (DEC), first introduced in 1947, was shown to have strong efficacy and safety for treatment of human lymphatic filariasis, which is caused mostly by a species *Wuchereria bancrofti*. Many studies to optimize the dosage and treatment schedule of DEC followed, and, based on the results, control programs with various regimens were implemented in different endemic areas/countries. By the mid 1970s, with endorsement by the WHO Expert Committee on Filariasis (3rd report, 1974), the standard DEC regimen for *W. bancrofti* infection in mass treatment had been established in principle: a total dose of 72 mg/kg of body weight given in 12 divided doses, once weekly or monthly, at 6 mg/kg each. Not long after the committee report, the efficacy of annual single-dose treatment at 6 mg/kg, which is only one twelfth of the WHO-recommended dose in a year, was reported effective in French Polynesia (study period: 1973-78), and later in Samoa (study period: 1979-81). These results were published between 1978 and 1985 in the Bulletin of WHO but received little attention. In the mid 1980s, the efficacy of ivermectin, the first-choice drug for onchocerciasis, against lymphatic filariae came to light. Since the effect at a single dose was remarkable, and often better than DEC, it was predicted that the newly introduced drug would replace DEC. Treatment experiments with ivermectin increased quickly in number. Meanwhile, annual single-dose mass drug administration (MDA) with DEC at 6 mg/kg was under scrutiny in Samoa and Fiji. In the early 1990s, the Samoan study, which covered the entire population of 160,000 with 3 annual MDAs, reported a significant reduction in microfilaria (mf) prevalence and mean mf density, while in Fiji, the efficacy of 5 rounds of annual MDA (total dose, 30 mg/kg) was shown to be as effective as 28 multi-dose MDA spread over 2 years (6 weekly plus 22 monthly treatments at 5 mg/kg; total dose, 140 mg/kg). Several additional studies carried out in Samoa in relation to the annual single-dose MDAs revealed that low density mf carriers, who have a very low mf count of 1-20/ml of venous blood, could not play a significant role in filariasis transmission.

From around 1990, studies on spaced low-dose DEC treatments and various types of combination chemotherapy with DEC and ivermectin increased. Albendazole, a well-known anti-intestinal helminths agent, was later added to the combination. The main findings of these studies with *W. bancrofti* are: (i) a single dose of DEC at 6 mg/kg reduced mean mf density by ca. 90% 1 year after treatment; (ii) the same dose could damage/kill adult worms; (iii) a single dose of ivermectin at ca. 400 μg/kg was more effective than DEC in reducing mf density during the first year and was similarly or less effective in the second year; (iv) ivermectin probably could not kill adult worms; (v) a single combined dose of albendazole (400 mg) and DEC (6 mg/kg) was effective to reduce mf density by 85 to nearly 100% 12-24 months after treatment; and (vi) ivermectin or albendazole included in the combination chemotherapy produced "beyond-filariasis" benefits: clearance/reduction of intestinal helminths, and, additionally, in the case of ivermectin, skin-dwelling ectoparasites.

The Global Programme to Eliminate Lymphatic Filariasis (GPELF) started its worldwide activities in 2000, with the target of elimination by 2020. The basic strategy is to conduct annual single-dose MDAs for 4-6 years. In 2000-2007, a minimum of 570 million individuals were treated in 48 of 83 endemic countries. The drugs used are DEC 6 mg/kg plus albendazole 400 mg in most countries, or ivermectin 200-400 μg/kg plus albendazole 400 mg particularly in onchocerciasis endemic countries in Africa. (MDAs with DEC alone had been used in India.)

The GPELF achieved impressive results in terms of parasitological cure/improvement, clinical benefits, social and economic impacts, etc. However, the most impressive result of all was the programme's success in mobilizing hundreds of millions of local people, who not only took drugs but many of them actively supported MDAs as drug distributors and volunteers. Beyond filariasis, the role people can play in supplementing rural health services is now a topic of discussion and a source of hope for a new sustainable system.

Keywords: Lymphatic filariasis, global programme for elimination, diethylcarbamazine, albendazole, ivermectin, annual single-dose, mass drug administration, Samoa, Fiji

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A. Introduction

a-1. *Parasite and disease*

Human lymphatic filariae, which are characterized by the parasitism of their adult worms in the lymphatic system, include 3 species, *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. Female adults reproduce offspring or microfilariae (mf) which are carried into blood circulation by the lymph flow and accumulate in the lungs. Mf are released from the lungs into the circulation at night (nocturnal periodicity of mf), synchronizing with the circadian biting cycle of mosquitoes that transmit the parasite between humans. In some Pacific islands, where *Aedes* mosquitoes bite humans in the daytime, the release from the lungs is mainly in the afternoon (diurnally subperiodicity). The detection of mf in blood is a basic method of diagnosis. After being ingested by mosquito vectors, mf develop to the infective stage of larvae in about 10-14 days. When mosquitoes with infective larvae bite humans, filarial parasites have an opportunity to enter the host. Infective larvae, males and females, penetrate the human skin, migrate in the body and reach the lymphatic system where they mature, mate and reproduce mf in about 6-12 months after skin invasion. Adult worms will damage and dilate lymphatic vessels, and cause lymphostasis often in the lower limb and around the testes and kidney. This is the basic pathology of chronic filariasis characterized by lymphedema, hydrocele, and chyluria. Lymphedema often triggers secondary bacterial infections resulting in acute fever attacks (acute dermatolymphangioadenitis [1]), which aggravate lymphedema/inflammation. In some people, the edematous skin gradually thickens, hardens and may grow to wart-like lesions. The word 'elephantiasis' refers to this condition with the often serious deformities that have caused enormous suffering among affected people worldwide for thousands of years (Fig. 1) [2].

a-2. *Epidemiology and global efforts to eliminate lymphatic filariasis*

The total number of lymphatic filariasis cases in the world, as estimated in 1996 [3], was 120 million, about 90% of which were caused by *W. bancrofti*. The total figure includes 16 million lymphedema (including elephantiasis) and 27 million hydrocele cases, the rest being cases with microfilaremia only. The infection was more prevalent among males, and adults. By region/country, India and sub-Saharan Africa had more than 40 million cases each, followed by other Asia and Islands (ca. 20 mil.) and China (ca. 10 mil.). With the swollen leg and/or scrotum, lymphatic filariasis was ranked as the 4th leading cause of permanent and long-term disability [4]. Most of the patients have been neglected and suffer from mental distress, social isolation, and economic misery due to the stigma of the disfiguring disease [5]. The estimated disability-adjusted life years (DALYs) lost in 1999 was 4.92 million [6]. As for economic loss, in India alone, the cost of treatment for acute fever attacks and chronic symptoms reached an estimated US$ 31.1 million per year, and the loss of productivity US$ 811 million per year [7].

These gloomy statistics have changed rapidly for the better since the Global Programme to Eliminate Lymphatic Filariasis (GPELF) started in 2000 (details in Section F). The Global Alliance to Eliminate Lymphatic Filariasis (GAELF) was formed the same year to support the unprecedented global program. The alliance includes health ministries of endemic countries, UN agencies (especially WHO as the secretariat), the private sector, NGDOs, academia, and government bodies (including JICA). Particularly noteworthy are the contributions of two pharmaceutical companies: GlaxoSmithKline donates albendazole free of charge and Merck & C., Inc. ivermectin. Both drugs are essential for the Mass Drug Administration (MDA) carried out annually in endemic countries. In 2007, 48 out of 81 endemic countries conducted MDAs, and 546 million people in the world were treated for lymphatic filariasis. The same year, China declared the elimination of filariasis, which was followed by Korea's declaration in 2008 [8].

B. The "new" anti-filarial drug diethylcarbamazine (DEC): Early studies to find the optimal dosage

b-1. *Trials with multi-dose treatment*

The anti-filarial effect of 1-diethylcarbamyl-4-methylpiperazine hydrochloride (DEC hydrochloride) was first reported in 1947 by Hewitt et al. [9] using naturally acquired filarial parasites in cotton rats (*Litomosoides carinii*) and dogs (*Dirofilaria immitis*). The same drug was tried for human bancroftian filariasis and its microfilaricidal and possible adulticidal effects were confirmed the following year [10]. A series of experimental treatments was conducted using DEC citrate for DEC hydrochloride to determine suitable dosage and treatment schedule. Many important studies were carried out in the South Pacific islands, where more than 10,000 American soldiers suffered clinical filariasis due to diurnally subperiodic *W. bancrofti* during World War II [11].

In American Samoa, 5 different multi-dose trials with DEC (3-9 mg/kg of body weight per day for 7-30 days, total dosage 21-270 mg/kg) confirmed rapid microfilaricidal effects, but the treatments could not prevent the reappearance of mf in 2-year follow-up studies (reported in 1953 [12]). Using 111-175 mf positives, Mahoney & Kessel [13] reported in 1971 that DEC given at 6 mg/kg daily for 6 days

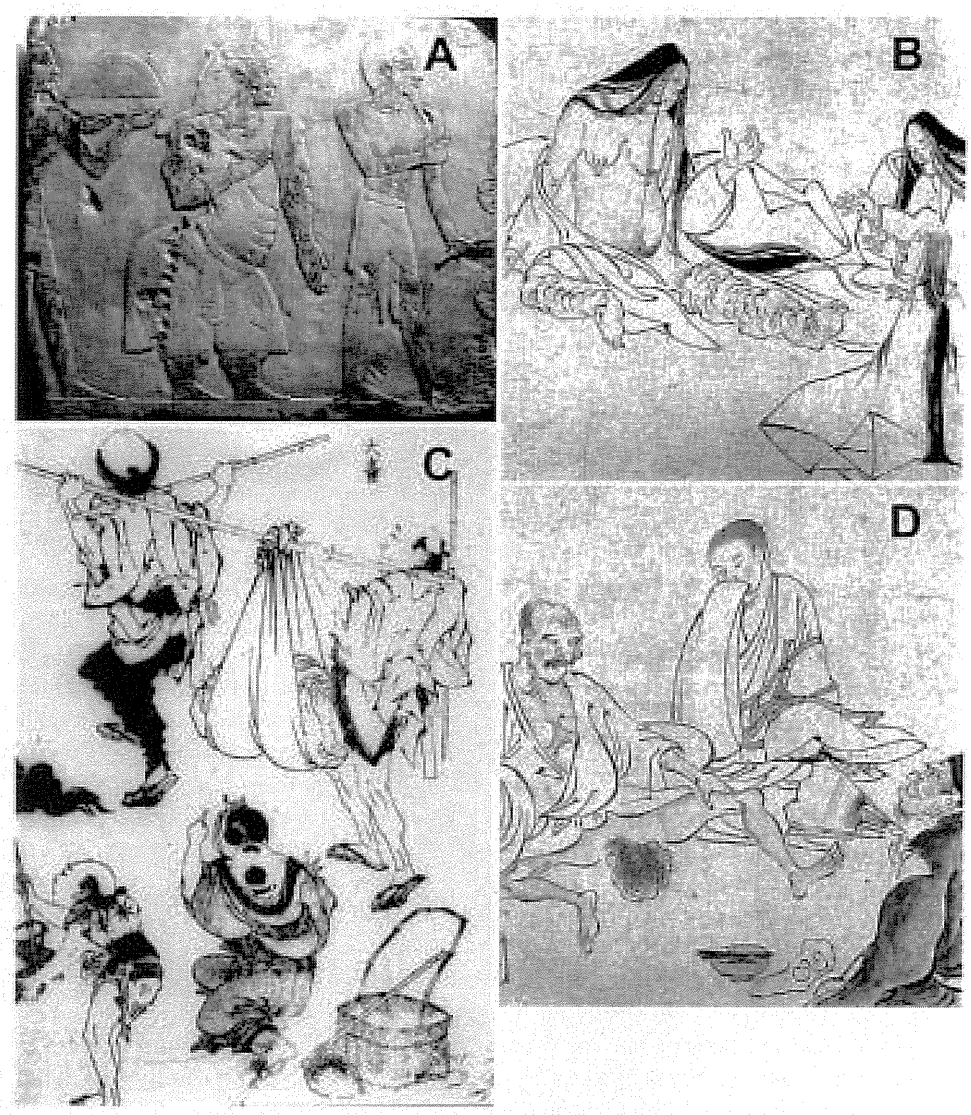

Fig. 1 Chronic symptoms of lymphatic filariasis
A: An Egyptian relief from Queen Hatshepsut's temple, Luxor, depicting the Princess of Punt, a possible elephantiasis case. (The Queen's reign: 1503-1482 B.C.); B and D: Elephantiasis of the legs and scrotum, described some 1,000 years ago in "Strange Diseases Picture Scroll." (Kyoto National Museum, Japan); C: Huge elephantiasis of the scrotum, painted by Hokusai Katsushika (1760-1849). Manga in the Edo era of Japan. {From Ref. [2], courtesy of Prof. Yoshihito Otsuji}

resulted in 32% persistence (rate of mf positive 1 week after treatment) and that the recurrence rate was 24% (rate of mf reappearance within 1 year of treatment). In this study, the diagnosis of infection was made by the detection of mf in 20 μl capillary blood obtained by the finger-prick (F-P) method.

In Fiji, Manson-Bahr (1952) [14] conducted treatment experiments with Hetrazan (DEC) at 100-300 mg daily for 15 to over 70 days and concluded that treatment must be continuous for at least two months. This conclusion was based on a finding that, even after multi-dose treatments cleared mf in 20 μl of F-P blood, mf were still positive when 1 ml of venous blood was examined by Knott's method. Burnet & Mataika (1961) [15] administered 6 weekly doses of DEC at 400 mg (ca. 5-8 mg/kg) each and repeated the same regimen half a year later (total dosage 4,800 mg). The treatment reduced mf rate, determined with 60 μl of F-P blood, from 12.2% to 2.7%. However, they noted that over 40% of mf negatives (who had been positive before treatment) were in fact positive by Knott's method

using 1 ml of venous blood.

In French Polynesia, Kessel (1957) [16] compared 3 different dosages and reported that 6 mg/kg once a month for 24 months showed the best result in terms of mf prevalence and density (mf/20 µl of blood) reductions. In Japan, among various dosage schemes tested by different workers, Sasa (1976) [17] stressed the importance of the size of total dosage given, rather than the schedule of daily, weekly or monthly treatment, and recommended a total dosage of 72 mg/kg at 6 mg/kg daily for 12 days.

The WHO Expert Committee on Filariasis analyzed the accumulated data on DEC dosage and reported in 1967 [18] that "an adequate amount seems to be a total dose of about 72 mg of diethylcarbamazine citrate per kg body weight". Spaced doses of 6 mg/kg once a week or once a month (12 times) were preferred to daily doses to reduce adverse reactions. The next WHO Expert Committee Report (1974) [19] confirmed the same total dose of 72 mg/kg for *W. bancrofti* infections and 30-40 mg/kg for *B. malayi* infections. Daily treatment was considered impractical for mass treatment. In 1984, the 4th Committee Report [20] reiterated the same total dosage for *W. bancrofti*.

b-2. ***Effect of low-dose treatments: results from a "minority" group***

Several studies with *W. bancrofti* reported the remarkable effectiveness of DEC at low dosages. Rachou & Scaff (1958) in Brazil (quoted by Hawking, 1962 [21]) reported that only one dose at 6 mg/kg reduced the mf prevalence rate from 100% (pretreatment level) to 62%, and mf density by 91.4% when assessed 12 months after treatment. The authors recommended annual or biannual mass treatments without prior blood tests for mf. In Gambia, McGregor & Gilles (1960) [22] observed that a total dose of 12.5 mg/kg (2.5 mg/kg daily for 5 days) reduced microfilarial load by 90-98% 43 months after treatments and left the noteworthy comment that "mass-treatment campaigns aimed at dosing all inhabitants at spaced intervals (2-4 years) might in the long run prove to be the most effective and economical way." Nearly 2 decades later, in French Polynesia, Laigret et al. (1978) [23] reported that DEC 6 mg/kg (400 mg for males and 300 mg for females) given once per year for 3 years successfully reduced the mf rate from 100% (pretreatment level) to 12% and the average mf density from 15 per 20 µl of blood to 0.3. The annual single-dose treatment was applied to ca. 50,000 people for 4 years and succeeded in reducing the mf prevalence from 4.4% to 1.9%. In practice, not all of the people took 4 doses; the result was obtained with the average of 2.76 doses in 4 years [24].

It is surprising to find that the dosages recommended for DEC treatment differed so widely in range. Understandably, researchers seemed to focus more on the cure of infection in the early stage of dosing trials. Due to the reappearance of mf after treatments, adulticidal effect was considered a key issue in judging the efficacy of a drug. Thus, the necessity of multi-dose treatment with a high total dosage must be stressed. On the other hand, it seems that a small number of researchers, especially those working in less-developed settings, paid more attention to the applicability of a treatment scheme. Also, the experience of difficulty in conducting multi-dose treatments and the recognition of adverse effects in such treatments must have convinced researchers to accept regimens not ideally effective but operationally feasible. The realization that very light infections had been missed previously when conventional blood tests (with 20-60 µl of F-P blood) were used for diagnosis called for a more suitable means of large-scale mass treatment. In 1984, the WHO Expert Committee on Filariasis, for the first time, mentioned the effectiveness of yearly treatments at 6 mg/kg [20].

C. Filariasis control in Samoa and Fiji with annual single-dose MDA using diethylcarbamazine

c-1. ***Countries and their filariasis situations***

Samoa, an independent country in the South Pacific, had a population of 160,000 (1990) in the 2 main islands of Upolu and Savaii. Diurnally subperiodic *W. bancrofti*, transmitted by *Aedes polynesiensis* and *Aedes samoanus*, is endemic. The prevalence study in 1965 revealed a mf rate of 19.1% (n = 10,129) by the 20 µl F-P blood smear method. The first nationwide MDA in 1965/66 using DEC (5 mg/kg once a week for 6 weeks, followed by the same dosage once a month for 12 months) reduced the mf rate to 1.63% (n = 42,697) in 1967, and the second MDA in 1971 (6 mg/kg once a month for 12 months) further reduced the rate – assessed by the 60 µl F-P method in 1972 – to 0.24% (n = 6,361). Despite continuing treatment of known mf positives, the prevalence increased gradually, and in 1979, reached 3.8% (n = 8,385) by 60 µl blood smear and 4.5% (n = 8,385) by the nuclepore filtration method using 1 ml of venous blood (1 ml NP method). The situation was alarming, because mf rate (by 1 ml NP method) of adult males aged ≧30 years had already reached the 20% level [25].

Fiji is the largest island country in the region, with a total population of 726,400 (1990) scattered over 100 islands. The two main islands are Viti Levu and Vanua Levu. Diurnally subperiodic type *W. bancrofti*, transmitted by *Ae. polynesiensis*, *Ae. pseudoscutellaris* and several other mosquito species, is endemic. In 1958, the mf prevalence determined by 60 µl F-P method in the delta area of the Rewa River, Viti Levu, was 12.2% (n = 1,200), which decreased

to 2.7% (n = 1,123) in 1959 after 2 rounds of MDA with DEC (400 mg once, 6 times weekly). However, by 1963, the rate increased to a level of 5% [26]. The 1968-69 studies in Taveuni and Koro islands, and Vanua Levu revealed a mf prevalence of 23% (n = 947) and 13% (n = 3,538) by the 60 µl F-P method, respectively (computed from the data by Mataika et al., 1971 [27]). A nationwide MDA campaign was commenced in 1969 using DEC at 5 mg/kg weekly for 6 weeks, followed by 22 monthly treatments (totally 28 doses, 140 mg/kg). The whole country was covered in 5 stages, and the campaign, which reached completion in 1975, successfully reduced the mf prevalence to 1% or less, but subsequent blood surveys suggested a gradual increase in infection [28]. The surveys in 1983-84 in the 2 remote islands of Lau and Rotuma revealed mf rates of 7.9% (n = 2,329) and 21.2% (n = 1,689) by the 60 µl F-P method, respectively [29]. In a 1985 survey in Kadavu island, the mf rate was 6.9% by the same method (n = 4,686) [30].

c-2. WHO/Samoa Filariasis Research Project: Confirmation of efficacy of annual single-dose treatment

In 1976, when the WHO/Samoa Filariasis Research Project started, it had become standard practice in the treatment of bancroftian filariasis to give a total DEC dose of 72 mg/kg in 12 treatments at 6 mg/kg each. However, difficulties in multi-dose treatment had been encountered in many endemic countries. Especially in Samoa, where a year-long multi-dose MDA was conducted twice in 1965/66 and 1971 utilizing limited health resources, the government was reluctant to repeat the procedure. In the midst of this situation, a report from French Polynesia that annually spaced single-dose DEC at 6 mg/kg was effective in reducing mf rate and density [23] brought encouraging news. Although the regimen was not popular in those days, the Research Project in Samoa decided to evaluate the efficacy of DEC single dose 12 months after treatment.

In the study in 1979-81, a single DEC dose of 4 mg/kg, 6 mg/kg or 8 mg/kg was administered to mf positive persons and the change in mf was assessed at 12 months by the 1 ml NP method (Table 1). The cure rate (% mf negative after treatment) was 29.4%, 53.7% and 40.0%, and the % decrease in geometric mean mf count was 81.5%, 94.4% and 93.5%, respectively for 4 mg/kg, 6 mg/kg and 8 mg/kg regimens. There was no significant difference among the cure rates, but the % decrease obtained with 4 mg/kg regimen was less than that of the 6 mg/kg or 8 mg/kg regimen ($P < 0.01$). Side reactions were studied by questioning people between 5 and 15 days after treatment. The occurrence of reactions (all types combined) was significantly higher in the 8 mg/kg regimen (77.9%) than in the other regimens (57-59%). It was concluded that annual single-dose DEC treatment at 6 mg/kg was suitable for the nationwide treatment for filariasis [31]. In 1981, upon completion of the study, the government of Samoa in collaboration with the Western Pacific Regional Office of WHO decided to implement a national MDA program based on this treatment.

Table 1. Comparison of the effects of 3 different DEC dosages given as a single dose and assessed 12 months after treatment

	Dosages		
	4 mg/kg	6 mg/kg	8 mg/kg
No. examined (mf carriers)	51	41	45
No. mf negative after treatment	15	22	18
(% cure rate)	(29.4)	(53.7)	(40.0)
Decrease in mf count, expressed as mean of log (mf +1)			
Pre-treatment (A)	2.117	2.003	2.198
Post-treatment (B)	1.384	0.751	1.010
Change	0.733	1.251	1.188
% decrease*	81.5	94.4	93.5

* Calculated as 100 x [antilog (A) - antilog (B)]/antilog (A).
{Source: Partially adopted from Ref. [31]}

c-3. Nationwide MDA in Samoa with once-a-year treatment: Long-term efficacy

A national MDA using a single dose of DEC at 6 mg/kg was started in 1981. All Samoans, except infants under the age of 1 year, pregnant women, sick people, and the very old, were the targets. After completion of the census in every village and town in the country with assistance of village Women's Committees, 3 MDAs were carried out under the supervision of medical staff by members of Women's Committees in 1982, 1983, and 1986, with a treatment coverage of 86.3%, 83.8% and 82.6%, respectively. The total population in 1986 was 159,199. The evaluation blood surveys were carried out 4 times, before and after each MDA, using 60 µl blood smears from some 9,600-13,700 people in 26-34 villages on each occasion. The MDAs reduced the mf prevalence gradually from 8.0% to 3.8% (52% reduction) in males, and from 3.2% to 1.3% (59% reduction) in females. The mf densities (geometric mean of positive counts per 60 µl) decreased from 23.1 to 9.1 (61% reduction) in males and from 14.6 to 9.4 (36% reduction) in females. The change in mf prevalence is summarized in Fig. 2, before and after 3 MDAs according to sex and age [32].

The transmission potential or infectivity index (%) of total population (IIT), which is an estimated mosquito in-

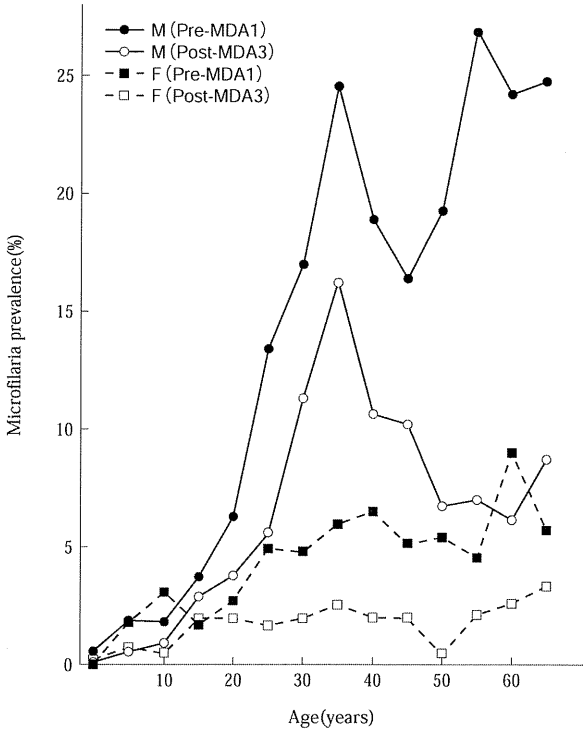

Fig. 2 Change in microfilaria prevalence before and after 3 annual single-dose MDAs with DEC at 6 mg/kg, analyzed by sex and age group (study in Samoa) {Figure redrawn from Ref. [32]}

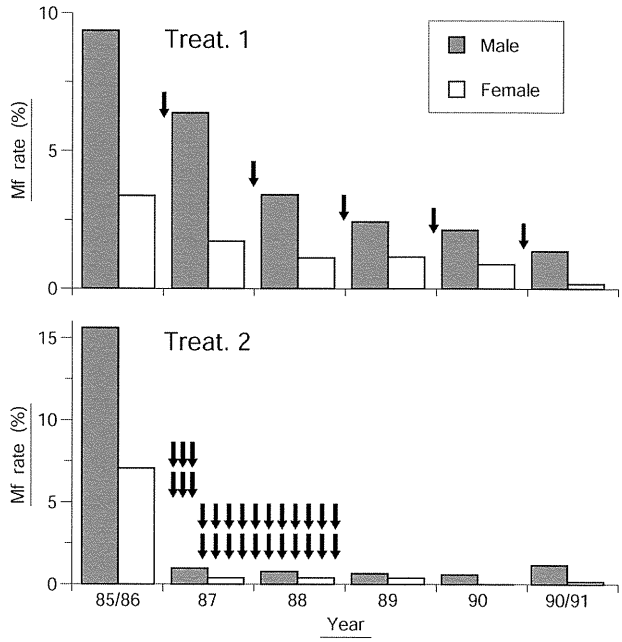

Fig. 3 Comparison of 2 MDA schemes with DEC: 5 rounds of annual single-dose treatment at 6 mg/kg (Treat. 1) and 28-dose treatment at 5 mg/kg given weekly for 6 weeks and monthly for 22 months (Treat. 2). Each arrow indicates a single treatment. {Figure redrawn from Ref. [35]}

fection rate [33], was reduced from 2.18 before MDA to 0.67 (70% reduction) after 3 MDAs. Entomological studies were also conducted at Vailu'utai village on Upolu Island. A total of 1,758 *Ae. polynesiensis* were dissected before MDA and 5,206 after the 2nd MDA. The results revealed a decrease in mosquito infection rate from 0.97% to 0.06% and the infective rate (% of mosquitoes having the infective stage of larvae) from 0.28% to 0.02% [32].

These findings indicated the remarkable long-term efficacy of annually spaced single-dose MDAs, given in fact 3 times in 6 years, and at the same time, the feasibility of a nationwide control program in which people are the major players.

c-4. Fiji study for confirmation of efficacy of 5 rounds of annual single-dose treatment with DEC: comparison with 28 multi-dose MDA

Mataika et al. (1993) [34] carried out an extensive study comparing DEC efficacy between 5 annual single-dose MDAs at 6 mg/kg (total 30 mg/kg) and very intensive 28-dose MDA (5 mg/kg once a week for 6 weeks, then monthly for 22 months; total 140 mg/kg). The results are shown in Fig. 3 [35]. The annual scheme reduced the mf rate year by year from 6.5% before treatment (average of males and females) to 0.9% after 5 treatments (87% reduction), while in the multi-dose scheme, the rate dropped sharply from 11.6% to 0.8% in 1987, and to 0.9% at 5 years (93% reduction). However, without treatment for more than 2 years after completion of the intensive 2-year regimen, a slight but significant increase in mf rate was observed in 1990/91 compared with the previous year. This indicates the advantage of continued annual doses rather than a concentrated multi-dose treatment. This study reconfirmed the effectiveness of annual single-dose MDAs. It was obvious that the annual scheme was much easier and more practical than the multi-dose scheme.

D. Low-density microfilaremia in Samoa and its significance in filarial transmission

d-1. What is low-density microfilaremia?

There are mf densities that are too low to be detected by conventional blood smears with 20-60 μl of F-P blood. The employment of nuclepore/millipore filtration of 1 ml venous blood has facilitated the detection of low density mf carriers. Low mf density was defined variously by re-

searchers. A mf range of 1-10 mf/ml was cited but often proved inconvenient for analytical studies as not many cases fell into this category. Comparing the 60 µl F-P method and 1 ml nuclepore filtration method, 1-20 mf/ml was defined as low-density microfilaremia (l.d.m.) for studies in Samoa [36]. The prevalence of l.d.m. in all mf positives was 23.6% (90/381), which led to an estimate of 1,700 low-density mf carriers in 1979 throughout the country. L.d.m was proportionally more frequent in villages with lower mf prevalences and in people < 20 years of age. By sex, there was no difference.

Since low-density mf carriers were recognized to occupy a substantial proportion of all mf carriers in Samoa, and *Ae. polynesiensis* were known to ingest more mf than estimated (up to 4.7 times) while having a blood meal on low-density mf carriers [37], the significance of l.d.m. as a source of transmission was seriously discussed, especially because it was suspected that annual single-dose DEC would not exert a sufficient adulticidal effect and therefore produce more low-density carriers than multi-dose treatment.

d-2. Significance of low-density mf carriers in filariasis transmission: Quantitative assessment

Based on the mosquito "infectivity index" concept proposed by Sasa [33], the significance of l.d.m. in transmission can be determined by estimating the proportion of mosquito infectivity produced by the l.d.m. group as compared to the total mosquito infectivity produced by all levels of mf carriers in an endemic community. Assuming that all people are exposed evenly to mosquito bites, the latter can be computed as follows:

Total mosquito infectivity = Σ (infection rate when mosquitoes feed on a carrier with mf density of k) x (No. of carriers with mf density of k in a community)

where k is from 1 to the maximum mf density (/1 ml of venous blood) observed in the community. Mosquito infectivity produced by low-density mf carriers will be obtained using the same formula with k value from 1 to 20.

To study the rate of mosquito infection, Samarawickrema et al. (1985) [37] conducted a detailed transmission experiment using *Ae. polynesiensis*, a main vector in Samoa, and 14 mf carriers with different mf densities ranging from 0 to 5,290 mf/ml. The results showed that the percentage of mosquitoes infected and the average number of larvae found in each infected mosquito were directly proportional to the mf densities in the carrier at the time of feeding. Based on the data from this transmission experiment, it was possible to obtain theoretical mosquito infection rates when mf carriers with different mf densities were blood sucked by mosquitoes [36]. To estimated the numbers of mf carriers with certain mf densities, a negative binomial distribution was fitted following the method of Pichon et al. (1980) [38] to the 1979 mf data from Samoa (n = 358 mf positives) [25].

The computation of mosquito infectivity is shown in Table 2. The infectivity produced by l.d.m. was 251.2 (B) and that by all levels of mf density was 11645.8 (A), and the contribution of l.d.m. to the total (B)/(A) was 0.0216 or only 2.16%. This would suggest a minor role of l.d.m. in the transmission of filariasis in Samoa [36].

E. Re-evaluation of low-dose DEC treatment, introduction of new drugs and their combination therapy

e-1. New evidences supporting the efficacy of low-dose DEC treatments

After around 1990, a variety of low-dose DEC treatments were tested in various countries where *W. bancrofti* is endemic. In Tahiti, a single dose of 3 mg/kg was reported effective in reducing geometric mean mf count by 95% when assessed 180 days after treatment [39]; in Papua New Guinea, 2 annual single doses at 6 mg/kg reduced the mf rate from 41% to 17%, and mf density from 71 mf/20 µl to 20 [40]; in Brazil, the efficacy of 6 mg/kg given only once was reported equally effective to 12 daily doses at 6 mg/kg when measured 12 months after treatment, although the single dose was significantly less effective than the multi doses during the first 6 months [41]; and in Tanzania, two 6-monthly treatments at 6 mg/kg reduced the geometric mean mf intensity by 92.2%, while 12 daily doses at 6 mg/kg reduced it by 98.6%, when assessed 1 year after the start of treatment. The former regimen was considered more suitable for MDAs than the standard 12-dose treatment [42]. The single dose treatment with DEC at 6 mg/kg was also applied to *Brugia malayi* infections. In Kerala, India, 2 annual mass treatments reduced the mf prevalence from 4.9% to 1.2%, and the mean mf count by 81%. In addition, clinical benefits such as a reduction in acute manifestations and recent edema cases were reported [43].

A single dose DEC at 6 mg/kg reduced not only the mf level by 90.5% but circulating filarial antigen by 39.7% 18 months after treatment, suggesting that the treatment was effective against adult parasites [44]. Brazilian researchers successfully studied the adulticidal effect of low-dose DEC treatment by direct observation of live adults using ultrasonography. The adults live in a dilated lymph vessel in the scrotal area, making a "nest" and moving actively. Amaral et al. (1994) [45] named the movement "filaria dance sign" ([46] for video image). Norões et al. (1997) [47] reported

Table 2. Mosquito infectivity produced by the low-density microfilaria (mf) carriers (B) and all levels of mf carriers (A)

Microfilaria density (mf/ml)	Theoretical % of infected fed mosquitoes (I)*	Theoretical No. of mf positive persons in each mf density group (II)	(I) x (II)	
1	0.492	13.214	6.5	
2	1.152	8.584	9.9	
-4	2.102	11.999	25.2	
-6	2.842	8.774	24.9	
-8	3.473	7.074	24.6	
-10	4.032	6.000	24.2	
-20	6.254	21.729	135.9	
			Subtotal 251.2	...(B)
-30	7.985	15.076	120.4	
-40	9.458	11.854	112.1	
-50	10.766	9.892	106.5	
-60	11.954	8.550	102.2	
-70	13.052	7.565	98.7	
-80	14.078	6.805	95.8	
-90	15.045	6.198	93.2	
-100	15.963	5.700	91.0	
-200	23.456	41.077	963.5	
-300	29.292	26.579	778.6	
-400	34.259	19.642	672.9	
-500	38.666	15.443	597.1	
-600	42.673	12.589	537.2	
-700	46.374	10.511	487.4	
-800	49.834	8.925	444.8	
-900	53.094	7.675	407.5	
-1000	56.187	6.666	374.5	
-2000	81.449	36.479	2971.2	
-3000	100 (101.123)	13.242	1324.2	
-4000	100 (117.869)	5.524	552.4	
≥4001	100 (--)	4.634	463.4	
			Subtotal 11394.6	
Total		358.000	11645.8	...(A)

* Estimated from $\log (Y + 1) = 0.5278 \log X + 0.1739$, where Y = % infected, X = mf density of a carrier.
{Source: Adopted from Ref. [36]}

that within a week after treatment at 6 mg/kg single dose, the dance sign became undetectable in 7 of 14 nests, and scrotal nodules became palpable at each site of the 7 nests. Biopsy specimens from these nodules revealed "nests" of degenerating adults, confirming the adulticidal effect. A separate histopathological study with DEC-induced nodules reported that even a single dose of 1 mg/kg could damage adult worms. However, it should be noted that, even after repeated high dose DEC treatments, some worms in the same nest remained intact [47, 48].

Fortunately, no drug resistance has been reported so far with DEC.

e-2. Ivermectin as a new drug against lymphatic filariae

On the other hand, ivermectin, the drug of choice for onchocerciasis that is also effective against intestinal helminths and ectoparasites such as lice, was reported in 1988 [49] to be effective against *W. bancrofti*. Since it is effective with a single oral dose at 25-200 μg/kg, ivermectin was cited as a candidate to replace DEC. The efficacy was further confirmed: when assessed at 6 months, a single dose at 21.3 μg/kg and 126 μg/kg reduced mf to 18.3% and 19.5% of the original levels, respectively, while 13 daily DEC treatments (one 3 mg/kg dose, followed by 12 daily doses at 6 mg/kg) reduced the level to 6.0% [50]. With a

higher dosage of 420 μg/kg (20 μg/kg at day 1 plus 400 μg/kg at day 5), the geometric mean mf density was reduced to 0.9% of the pretreatment level 1 year after treatment, while DEC at 6 or 7 mg/kg reduced the mean to 9.3% ($P < 0.006$) [51]. Another study with 420 μg/kg of ivermectin reported a mean mf reduction of 86.3% after 18 months, while it was 90.5% with a single dose of DEC at 6 mg/kg ($P > 0.05$). In this study, the efficacy of ivermectin was much stronger than DEC in the first 30 days after treatment, but by 18 months, the latter took over the lead and resulted in a slightly higher reduction [44]. The stronger effect of DEC as opposed to ivermectin in the second year was also reported in Brazil [52]. To study adulticidal effect of ivermectin, Dreyer et al. (1995) [53] treated 15 *W. bancrofti*-infected Brazilian men with a single dose of ivermectin at 400 μg/kg and observed the filaria dance sign by ultrasound for 3-9 months. Contrary to all expectations, there was no observable change in the dance sign, and it was concluded that ivermectin had no effect on adult worms.

e-3. Combination chemotherapies

Having two potent anti-filarial drugs, DEC and ivermectin, researchers tested the efficacy of their use in combination. More recently, the effectiveness of albendazole, an established antiparasitic agent, against filarial parasites was reported, and various combinations of these 3 drugs have been evaluated.

A possible additive or synergistic effect of DEC and ivermectin was reported in Haiti based on a finding that 20 μg/kg ivermectin given as a clearing dose at day 1, followed by a single 6 mg/kg dose of DEC at day 5 resulted in higher efficacy in reducing mf density than DEC alone 1 year after treatment [50]. The same regimen tested in Brazil produced the best results among different combinations of the 2 drugs: reduction of microfilaremia to 2.4% of the pretreatment level (100%) at 2 years [51]. In Tahiti, 2 annual single dose MDAs were conducted using 4 different regimens: (i) ivermectin 400 μg/kg plus DEC 6 mg/kg, (ii) ivermectin 400 μg/kg alone, (iii) DEC 6 mg/kg alone, and (iv) ivermectin 400 μg/kg plus DEC 3 mg/kg [54]. After 1 year, regimens (i) and (iv) resulted in the same 32% reduction in mf prevalence, while the reduction was only 11-14% using regimens (ii) and (iii). As for mf density, the former 2 regimens brought about a 95-96% reduction from the pretreatment level, and the latter 2 regimens 80-82%. To clarify the effect of combination therapy on adult worms, a single dose of DEC at 6 mg/kg was co-administered with either 200 μg/kg or 400 μg/kg of ivermectin, and filaria dance sign was observed with ultrasonography [55]. Interestingly, the dance sign was evident in all of the 30 nests studied, suggesting that the co-administration interfered with the already established adulticidal effect of DEC.

Other studies have looked at the combination of albendazole and DEC or ivermectin. A review by Ottesen et al. (1999) [56] summarized the role of albendazole for lymphatic filariasis elimination. Ismail et al. (1998) [57], working with *W. bancrofti*, showed more reduction in geometric mean mf density with a combined single dose of albendazole (600 mg) plus ivermectin (400 μg/kg) than with a combination of albendazole (600 mg) plus DEC (6 mg/kg) up to 12 months after treatment. At 15 months, however, there was no significant difference between the two (reduction of > 98% in both regimens). As for circulating filarial antigen, the latter combination resulted in significantly more reduction (77%) than the former at 15 months. A combined single dose of albendazole (400 mg) plus DEC (6 mg/kg) reduced mean mf density by 85.7-99.6% 12-24 months after treatment [58-61]. On the other hand, several studies could not confirm the benefit of albendazole in various combinations with ivermectin/DEC [62-65]. Dreyer et al. (2006) [66] compared the adulticidal effect of DEC (6 mg/kg) alone and DEC (6 mg/kg) plus albendazole (400 mg), and reported that the combination resulted in a much lower effect on filaria dance sign. The authors concluded that co-administration appeared to reduce the adulticidal effect of DEC. Further studies are necessary to confirm or refute anti-filarial effects of albendazole in combined use with the other anti-filarials [67].

The role of albendazole in the global program for filariasis elimination has to be emphasized for its "beyond-filariasis" benefits [55]. The drug is very effective against intestinal helminths such as *Ascaris*, *Trichuris* and hookworm, which have inflicted a tremendous burden on the health of poor people in developing countries. In Haiti, 2 annual single-dose MDAs with DEC (6 mg/kg) and albendazole (400 mg) reduced *Ascaris*, *Trichuris* and hookworm prevalences from 20.9% to 14.1%, 34.0% to 14.6%, and 11.2% to 2.0%, respectively, 9 months after the second MDA [68]. In India, the same treatment reduced the prevalence of *Ascaris* by 83%, *Trichuris* by 63% and hookworm by 69%, 11 months after the second MDA [69]. These "beyond-filariasis" benefits will not only give the anti-filariasis campaign a broader public health significance but also help to improve compliance among people and sustain the elimination program. The combination of drugs is also said to be effective in preventing the acquirement of drug resistance by filarial parasites.

F. Global Programme to Eliminate Lymphatic Filariasis (GPELF)

f-1. Strategies/activities

The World Health Assembly made a resolution in 1997 to eliminate lymphatic filariasis from the world as a public health problem by 2020. To execute the resolution, the GPELF was organized with a main strategy of conducting annual single-dose MDA for 4-6 years. Under MDA, all people living in endemic areas with or without filarial infection are expected to be treated, meaning that the number in 83 endemic countries will reach some 13 billion people. The drugs used for MDA are the combination of DEC (6 mg/kg) and albendazole (400 mg) in onchocerciasis-free areas, and ivermectin (200-400 μg/kg) and albendazole (400 mg) in onchocerciasis-endemic areas of Africa. The reason for the use of 2 separate regimens is that DEC could cause severe reactions if administered to *Onchocerca*-infected individuals. The biggest barrier to financing the drug supply was removed by the donation of albendazole and ivermectin by the 2 pharmaceutical giants. The GPELF has also put particular emphasis on the care of existing clinical cases of lymphedema/elephantiasis and hydrocele. Simple procedures for lymphedema management have been established [70], in which the cure and prevention of bacterial/fungal infections by maintaining good hygiene of the affected skin is the basic concept. Daily washing with soap and water, together with exercise and elevation of the affected limb to drain accumulated lymph fluid, is the most essential part of the "care" for which family members and volunteers have been trained [71].

In the beginning, many researchers/clinicians were suspicious about the success of such a huge program. Some rejected the idea outright because they considered filariasis a low-priority disease. However, a tectonic shift was already underway: the idea of DALY had changed disease priority in favor of filariasis that produces permanent or long-lasting disability, and non-profit activities for the underprivileged by a variety of voluntary groups/citizens had matured. People suffering from lymphatic filariasis, one of the world's most neglected diseases, have gained global attention for the first time in history.

f-2. Achievements

Ottesen et al. (2008) [72] described in detail the results of 8 years of global effort (2000-2007). More than 740 million albendazole tablets and 590 million ivermectin tablets were donated by the partner drug companies of the GAELF, while 4.7 billion DEC tablets were purchased by endemic countries. A minimum of 570 million individuals were treated in 48 of the 83 endemic countries. In 68 pre-fixed sentinel sites to monitor treatment effects, 5 rounds of MDA reduced mf prevalence by ca. 85% and cleared mf in 63% of the sites. The WHO report in 2008 [8] listed 5 countries which no longer have active transmission foci, and 2 countries (China and Korea) where the elimination of filariasis was declared.

The benefits of the MDAs conducted in 2000-2007 include the following: 6.6 million newborns were protected from filarial infection, of whom 1.4 million and 0.8 million individuals will escape hydrocele and lymphedema, respectively, in their lifetimes; 9.5 million asymptomatic parasite carriers were protected from developing hydrocele (6.0 mil.) or lymphedema (3.5 mil.). The DALYs averted in 8 years were estimated to reach 32 million, for which US$ 190 million was spent to cover MDA-related costs. Thus, the cost per DALY averted was US$ 5.90 (excluding donated drugs), which is one of the most cost effective programs in the world [73].

In addition to the benefits of the 2000-07 MDAs relating to filariasis, 56.6 million children and 44.5 million women of childbearing age were treated with albendazole for intestinal parasitic infections, and in onchocerciasis endemic countries of Africa, more than 45 million were treated with ivermectin for various skin diseases caused by *Onchocerca volvulus*, scabies mites and lice, although DALYs averted by these treatments were not quantified. It can readily be said that the global filariasis program has already established new precedents: collaboration in combating neglected diseases, single-dose treatment for different diseases, and confidence of local people in maintaining a public health program, all on a global scale.

f-3. The future

The progress made by the GPELF has been remarkable so far. However, the elimination program is not necessarily proceeding satisfactorily in all endemic countries. Problems arise when compliance to MDAs is not sufficient, pretreatment endemicity levels are high, the species of vector mosquito is an efficient transmitter, MDA drug dosage is not sufficient (particularly with ivermectin), etc. [74]. A more serious question will be the endpoint for the elimination program in each endemic country. The variability of biological, human-behavioral and socio-economic factors make it difficult to clarify a threshold at which filariasis transmission disappears spontaneously. With strong continuous global cooperation as a precondition, each endemic country needs to carry out well-organized and effective MDAs. The drug administration may have to be repeated if necessary. Vector control measures may become an essential part of the program in some areas [75]. And it is expected that mathematical models will play a more important

role in planning future operations [76].

G. Expansion of intervention activities by people: Community-directed treatment (ComDT)

In many endemic areas where health manpower is running short, it is difficult to carry out a large scale MDA. In Okinawa, Japan, in the 1960s, senior high school graduates living in the endemic areas were trained as "ad hoc" laboratory technicians [77]. In Sri Lanka, the MDA in 2003 achieved 80% drug coverage of 9.8 million endemic population, and more than half of the drug coverage (55.2%) was executed by volunteers making door-to-door visits [78].

In West Africa, when the Onchocerciasis Control Programme started employing annual mass treatment with ivermectin, they faced the same problem of manpower shortage. Then in 1995, WHO/TDR conducted a landmark study in 5 African countries to clarify how well local people can plan and execute the distribution of ivermectin by themselves [79]. In the study, 2 different treatment schemes were compared: community-designed treatment and program-designed treatment. In the former, after a minimal essential health education/information session, the community was invited to decide who in the community would be drug distributers and when and how the drugs would be distributed, while in the latter, experienced program staff pre-designed the criteria for selecting drug distributers and detailed procedures for drug distribution. The results were rather unexpected: the former achieved as good a treatment coverage as the latter. This was a clear indication that local people can be a reliable player in public health activities. A similar study with lymphatic filariasis followed in Ghana and Kenya [80], where community-directed treatment with some input from health services (ComDT/HS) was compared with the treatment planned and executed by the regular care system (HST). The results: ComDT/HS achieved a high treatment coverage of 75.7% - 88.0%, whereas HST obtained only 43.6% - 46.5%.

The African Programme for Onchocerciasis Control (APOC), which was set up in 1995 covering 16 countries, adopted the idea of community-designed treatment from the above 1995 TDR study and implemented the community-directed treatment with ivermectin (CDTI). The program was so successful in reducing the burden of onchocerciasis that, in the year 2007, close to 1 million DALYs could be averted [81]. For CDTI, "community drug distributers" (CDDs) play an essential role. After training, they take a census, distribute drugs, monitor adverse reactions and keep records. In 2006, there were 429,385 trained CDDs in APOC countries. These batteries of manpower with health care knowledge have become involved as a matter of course in other intervention programs. In Nigeria, successful integration of insecticide-treated bed net distribution for malaria control with lymphatic filariasis/onchocerciasis MDA was reported [82]. WHO/TDR, in 2005, launched a study to investigate whether the concept and experience of CDTI can be applied to other interventions such as delivering vitamin A supplement, insecticide treated nets, DOTS treatment for tuberculosis, and home management of malaria. The results showed that all 5 interventions (including CDTI) could be done simultaneously by the community [83].

Community-directed treatment, which was invented as a measure necessary to conduct a large scale MDA for onchocerciasis in areas where health infrastructures were poor, has been transformed into a new sustainable way of delivering therapeutic and preventive measures for rural people suffering from a variety of neglected diseases. The MDAs for lymphatic filariasis, which have been conducted side by side with APOC, can be expected to strengthen the new development synergistically.

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