Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 38 (1981)
Heft: 3

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Autor: Piessens, W.F. / Ratiwayanto, S. / Piessens, Patricia W.

DOI: https://doi.org/10.5169/seals-312823

Acta Tropica *38*, 227–234 (1981)

Departments of Medicine, Harvard Medical School, Brigham and Women's Hospital, Boston, MA, USA,
Jakarta Detachment of the U.S. Naval Medical Research Unit No. 2, and
National Institute of Health, Research and Development of the Ministry of Health, Jakarta, Indonesia

Effect of treatment with diethylcarbamazine on immune responses to filarial antigens in patients infected with *Brugia malayi*

W. F. Piessens, S. Ratiwayanto, Patricia W. Piessens, S. Tuti, P. B. McGreevy, F. Darwis, J. R. Palmieri, I. Koiman, D. T. Dennis

Summary

We studied the effect of treatment with diethylcarbamazine (DEC) on immune responses to parasite antigens in humans infected with *Brugia malayi*. In vitro lymphocyte proliferative responses to microfilarial antigens increased in patients who became amicrofilaremic after treatment with DEC. No changes in reactivity were observed in amicrofilaremic individuals who were given DEC or in a small number of patients who remained microfilaremic after treatment. Reactions to other antigens (PPD and SKSD) were not affected by drug treatment. Serum titers of antibodies to the sheath of *B. malayi* microfilariae did not significantly change during the period of observation. These findings indicate that DEC partially reverses the state of cellular unresponsiveness to parasite antigens associated with patent filarial infections.

Key words: filariasis; *Brugia malayi*, immune responses; DEC.

Introduction

Human infections with *Brugia malayi* or with *Wuchereria bancrofti* are associated with a state of specific immune unresponsiveness to filarial antigens (Wong and Guest, 1969; Ottesen et al., 1977; Piessens et al., 1979, 1980a, b; McGreevy et al., 1980). In experimentally infected animals, serum titers of

Correspondence: Willy F. Piessens, M.D., Harvard Medical School, Department of Medicine, Division of Parasite Immunology, The Seeley G. Mudd Building, Room No. 504, 250 Longwood Avenue, Boston, Massachusetts 02115, USA

antifilarial antibodies decline with the onset of patency (Wong, 1964; Benjamin and Soulsby, 1976). This may result from the adsorption of such antibodies on circulating microfilariae, a phenomenon known to exist in man (Piessens et al., 1980b). On the other hand, cellular unresponsiveness to filarial antigens during patent infections appears to result from the activation in vivo of adherent suppressor cells by parasite materials (Piessens et al., 1980a).

The drug of choice for the treatment and control of human filariasis, diethylcarbamazine (DEC), induces a rapid decrease in the number of microfilariae circulating in the blood of patients infected with *W. bancrofti* or *B. malayi* (WHO, 1967). DEC affects microfilariae in an as yet undetermined fashion and probably also kills a proportion of adult worms. In addition, this drug inhibits a variety of immunologic reactions, both in vivo and in vitro (Orange et al., 1968; Katiyar et al., 1974).

Treated patients remain at a high risk of developing recurrent filariasis, because the drug most likely fails to eliminate all adult worms and because reinfections can be acquired from residual human or animal reservoirs (WHO, 1967). Because an individual's state of immune reactivity may influence his susceptibility to recurrent patent infections after treatment with DEC, the present study was undertaken to evaluate the long term effect of this drug on the immunologic responsiveness to filarial antigens of humans infected with *B. malayi*.

Materials and methods

Patient population and parasitologic evaluation. All 45 patients studied were adult volunteers living in an area of South Kalimantan, Indonesia, where infections with subperiodic *Brugia malayi* are endemic (Piessens et al., 1979; McGreevy et al., 1980). Patients whose clinical, parasitologic and immunologic status was known prior to treatment with diethylcarbamazine were selected for inclusion in the present study solely on the basis of their willingness to donate blood for repeat evaluations. Treatment was neither given nor withheld to meet the objectives of the present investigation; mass treatment was administered according to a preestablished schedule.

All patients had a history of recurrent filarial fevers with lymphadenitis and/or retrograde lymphangitis; two individuals also had elephantiasis. The level of microfilaremia was determined by counting the number of microfilariae trapped on a 5μm Nuclepore filter (Nuclepore Corp., Pleasanton, CA) after filtration of 1 ml of heparinized blood (Dennis and Kean, 1970).

Starting in May 1978, clinical and parasitologic evaluations were performed every 2–3 months on all patients; cellular and/or humoral immunity to filarial antigens (see below) was assessed during May 1978 and once again during May 1979. Fifteen patients studied were treated with DEC (5 mg/kg/day $\times$ 10 days) during November 1978.

Collection of specimens. Ten ml of heparinized venous blood was collected early in the morning from patients in South Kalimantan; the blood samples were maintained at ambient temperature and transported by road and air to Jakarta, where lymphocyte cultures were set up during mid afternoon. For the preparation of serum, blood was allowed to clot for 3 h at ambient temperature; aliquots of sera were stored frozen at –20° C until used.

Preparation of antigens. Crude saline extracts of *B. malayi* microfilariae or adult worms, maintained in jirds, were prepared as previously described, sterilized by filtration through 0.45 μm filters (Millipore Corp., Bedford, MA) and stored at –20° C until used (Ottesen et al., 1977). The concentration of protein in these extracts was measured by the method of Lowry et al. (1951).

Lymphocyte transformation assay. Mononuclear cells were isolated by centrifugation of heparinized whole blood on Ficoll-Hypaque gradients (6.5% Ficoll, Pharmacia Fine Chemicals, Piscataway, NJ, and 0.7% Hypaque, Winthrop Laboratories, New York, NY); the cells were then washed three times with RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and Hepes buffer (25 mM, Microbiological Associates, Bethesda, MD) and counted in a hemocytometer. Cell suspensions prepared in this manner from blood of patients with microfilaremia were found to be devoid of contaminating parasites, which sediment below the mononuclear cell layer.

The lymphocyte transformation assay was carried out in a microculture as follows: 2×10^5 mononuclear cells were cultured in 0.2 ml of medium in round bottom microtiter plates (Cooke Engineering Co., Alexandria, VA) at 37° C in tightly sealed candle jars. The culture medium consisted of RPMI 1640 supplemented with antibiotics and Hepes buffer as described above and made to contain 10% heat inactivated human AB^+ serum from a single pool of uninfected donors. All control and stimulated cultures were done in triplicate.

In other cultures, nonadherent cells were used as indicator cells for the in vitro assay. These nonadherent cells were isolated from mononuclear cell populations as previously described (Piessens et al., 1980a).

After 6 days of incubation, 20 μl of medium containing 1 μCi of 3H-thymidine (sp. act. 6.7 Ci/mmol, New England Nuclear, Boston, MA) was added to the cultures. Twelve hours later the cells were harvested with a multiple automatic sample harvester (MASH-2, Microbiological Associates, Bethesda, MD) on glass filters. The amount of radioactivity on the filters then was determined by liquid scintillation counting.

The results are expressed as a stimulation index (SI) based on mean radioactivity (as CPM) of triplicate samples, as follows:

$$\text{SI} = \frac{\text{Mean CPM antigen stimulated culture}}{\text{Mean CPM nonstimulated control culture}}$$

An index of ≥ 2.00 indicates a statistically significant response as determined in previous experiments (Piessens et al., 1979).

Indirect immunofluorescence assay. An indirect immunofluorescence assay was used to detect antibodies reacting with antigens present on the sheath of living *B. malayi* microfilariae isolated from the peritoneal cavity of jirds (McGreevy et al., 1980; Piessens et al., 1980b). Approximately 30 microfilariae in 25μl of phosphate buffered saline (PBS) were mixed with 50 μl of twofold serial dilutions of test serum in 5×60 mm glass tubes and incubated in a shaking water bath at 37° C for 30 min. The microfilariae then were washed three times with excess PBS, mixed with 25 μl of fluorescein labelled conjugate prepared from the IgG fraction of goat antiserum with polyvalent specificity for human immunoglobulins (Cappel Laboratories, Cochranville, PA) and incubated again for 30 min. At the end of this second incubation, the microfilariae were washed three times with PBS, resuspended in a drop of buffered glycerol and transferred onto a slide for examination with an American Optical Fluorelume microscope equipped with a BG12 exciting and a K530 barrier filter. Negative and positive controls were performed in parallel, as has been previously described (Piessens et al., 1980b; McGreevy et al., 1980).

Antibody titers are reported as the highest serum dilution in which 50% of the observed microfilariae fluoresce.

Results

Parasitologic observations. On the basis of their parasitologic status before and after treatment, patients receiving DEC could be divided into three groups (Table 1). Ten of 15 patients were microfilaremic before treatment. Six months

Table 1. Parasitologic observations

Patient group	Number in group	Treatment	No. microfilariae/ml blood range (geometric mean)	
			1978	1979
1	7	yes	3–190 (14)	0
2	3	yes	3–4022 (192)	2–260 (15)
3	5	yes	0	0
4	12	no	4–230 (16)	3–4022 (18)
5	18	no	0	0

Table 2. Effect of DEC on cellular reactions to microfilarial antigens

Group No.*	No. with SI≥2/No. tested		Mean stimulation index (±SEM)	
	1978	1979	1978	1979
1	2/7	6/7**	1.69±0.61	3.84±2.49***
2	1/3	0/3	1.43±0.97	1.28±0.41
3	5/5	5/5	4.06±1.92	3.95±1.97
4	0/2	0/2	1.21±0.73	1.13±0.84
5	5/7	6/7	2.61±1.26	3.12±2.18

The parasitologic status of patients in each group was defined in Table 1.

* Groups 1–3 received DEC, groups 4 and 5 were not treated (see Table 1).
The changes in group 1 are statistically significant:

** $p < 0.05$ by χ^2 test;

*** $p < 0.05$ by paired t test.

Table 3. Effect of DEC on cellular reactions to PPD and SKSD

Group No.*	No. with SI≥2/No. tested			
	PPD		SKSD	
	1978	1979	1978	1979
1	6/7	6/7	5/7	5/7
2	1/3	1/3	2/3	2/3
3	3/5	4/5	3/5	3/5
4	1/2	1/2	2/2	2/2
5	5/7	5/7	6/7	6/7

* Groups 1–3 received DEC, groups 4 and 5 were not treated (see Table 1).

later, microfilariae were absent from the blood of 7 of these individuals (group 1), but continued to be detectable in three others (group 2). The patient with the highest level of microfilaremia experienced severe reactions to DEC administration and completed only 2 days of the prescribed 10 day course; the other 2 individuals of this group completed 10 days of treatment but did not become amicrofilaremic during the 6 month period of observation. Five patients were amicrofilaremic before and after DEC treatment (group 3).

The microfilarial density was stable over the 12 month observation period in 12 untreated individuals (group 4); none of the 18 untreated amicrofilaremic subjects (group 5) developed patent infections during this period.

Cellular reactions to microfilarial and unrelated antigens. Eight of 15 individuals studied before and 11 of 15 evaluated after treatment with DEC reacted to microfilarial antigens in vitro. The only significant change in reactivity occurred in patients in whom treatment with DEC terminated detectable microfilaremia (Table 2, group 1). Of the 7 individuals in this group, 2 reacted before and 6 after treatment (χ^2 4.34, $p < 0.05$). In contrast, none of the 3 patients who remained microfilaremic (Table 2, group 2) became reactors. Likewise, no significant differences were observed either in the prevalence of positive reactions or in the mean stimulation index of the group of amicrofilaremic donors treated with DEC (Table 2, group 3) or of the untreated control groups. Cellular reactions to the unrelated antigens PPD or SKSD were unaltered by treatment with DEC. These results are shown in Table 3.

In a previous study, we found that peripheral blood of microfilaremic individuals contains adherent cells that suppress in vitro lymphocyte responses to microfilarial, but not to unrelated antigens (Piessens et al., 1980a). It was therefore of interest to determine whether this type of specific suppressor cell remained detectable in patients who become amicrofilaremic by treatment with DEC.

Table 4. Effect of removal of adherent suppressor cells on in vitro reactivity to microfilarial antigens of microfilaremic donors

Patient No.	Microfilaremia* after treatment	Stimulation index (SI)		% increase in SI
		Unfractionated mononuclear cells	Nonadherent cells	
1	absent	2.65	3.18	20
2	absent	4.47	5.52	23
3	absent	8.13	9.07	12
4	absent	2.80	3.01	8
5	present	1.76	3.02	72
6	present	0.97	1.78	84
7	present	1.12	2.18	95

* All donors were microfilaremic prior to treatment.

Table 5. Effect of DEC treatment on titers of antibodies to the sheath of microfilariae

Patient group	Antibody titer before treatment*/Antibody titer after treatment
1	0/0, 0/0, 0/16, 32/128, 0/0, 0/0, 0/32
2	32/32, 0/0, 0/0
3	16/16, 2/2, 16/32, 64/128, 4/4
4	0/0 (all patients)
5	0/0, 0/0, 0/0, 0/0, 0/0, 64/256, 32/64, 64/32, 64/32, 256/512, 64/16

* Antibody titers are expressed as the reciprocal of the highest serum dilution in which 50% of microfilariae fluoresce.
Groups 1–3 received DEC, groups 4 and 5 were not treated.

Removal of adherent cells improved in vitro reactivity to microfilarial antigens in all patients tested. However, the percent increase in stimulation index was greater in individuals who remained microfilaremic after treatment with DEC than in those who became amicrofilaremic (Table 4).

Antibodies to microfilarial sheaths. Antibodies reacting with the sheath of living microfilariae were detected in 7/15 sera obtained before treatment, and in 9/15 sera from the same donors after treatment with DEC. Antibody titers increased by 2 dilutions or more in the sera of 3 donors of group 1 and of one person of group 3. A similar increase was noted in only one untreated individual (Table 5).

Discussion

Several studies have documented that patent filarial infections are associated with a state of immune unresponsiveness to parasite antigens (Wong and Guest, 1969; Ottessen et al., 1977; Piessens et al., 1979, 1980a, b; McGreevy et al., 1980). The observations reported here indicate that elimination of detectable circulating microfilariae by treatment with diethylcarbamazine reverses the state of cellular unresponsiveness to filarial antigens.

This phenomenon most likely is due to elimination or reduction in the amount of microfilaria-associated antigens that activate specific suppressor cells, rather than being the result of a direct immuno-potentiating effect of the drug per se. Indeed, removal of adherent suppressor cells had much less effect on the in vitro reactivity to filarial antigens of patients who became amicrofilaremic after treatment than of individuals who remained microfilaremic. This suggests that either the number or the degree of activation of suppressor cells was reduced in successfully treated individuals. Further, DEC did not increase reactivity to unrelated antigens such as PPD or SKSD in any patient tested, nor did the drug alter reactivity to parasite antigens of treated amicrofilaremic subjects.

No significant rise in post treatment antibody titers was observed in the present study, although serum levels of antisheath antibody increased in 3 of 7 individuals who became amicrofilaremic after treatment with DEC. It is possible that following elimination of circulating microfilariae less antibody molecules were removed from the sera of these individuals by reaction with antigens on the microfilariae and that, as a result, antisheath antibodies became detectable in the sera of these subjects.

Ottesen et al. (1977) previously reported that cellular reaction to filarial, as well as nonfilarial, antigens are suppressed during and immediately after a course of treatment with DEC. The reasons for the apparent discrepancy between the findings reported by these investigators and the present study are unclear, but two possibilities are suggested by a comparison of the methodologies used in the two studies. The immunosuppressive effect of DEC could be transient and reversible. This hypothesis remains to be tested. Alternatively, the immunosuppressive effect noted by Ottesen two weeks after initiation of treatment with DEC may have been unrelated to the drug itself, but caused by suppressive factors present in patients' sera used by these investigators to supplement their in vitro cultures. Such putative suppressive factors would not be expected to be present in sera from the uninfected caucasian donors used by us to supplement cultures for the study presented herein. Specific and nonspecific suppressor factors are present in sera from microfilaremic as well as from amicrofilaremic individuals (Piessens et al., 1980a). In animals, DEC causes a precipitous drop in serum titers of antibodies against somatic filarial antigens, presumably due to complexing of antibody with antigens released from damaged microfilariae (Desowitz et al., 1978). Soluble immune complexes thus formed could be immunosuppressive (Fitch, 1975; Feldman, 1972). Such a chain of events could explain the transient increase of unreactivity to filarial antigens after treatment with DEC, but this hypothesis remains to be tested by appropriate experiments.

It has long been known that patients living in an endemic area who are successfully treated with DEC can remain amicrofilaremic for extended periods, despite continued exposure to infective mosquito bites. Our observations support the possibility that the development of immune responsiveness in successfully treated patients may play a role in this extended "disease free"-period.

Acknowledgments. We thank Dr. Jusuf Alivedho, Pak Utuh Rafie and Fauzi Sangaji of the Center for Disease Control, South Kalimantan, and B. Ibrahim of NAMRU-2, Jakarta, for their superb assistance with the field work.

The authors received financial support from the Filariasis Component of the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, from the Rockefeller Foundation and from the U.S. Bureau of Medicine and Surgery, Navy Department, for Work Unit No. MF58.524.009–0085.

The opinions and assertions contained herein are not to be construed as official or as representing the views of the Indonesian Ministry of Health or the United States Navy.

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