Diethylcarbamazine dependent, complement mediated, adherence and cytotoxicity of cells on microfilariae of "Litomosoides carinii"

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Diethylcarbamazine dependent, complement mediated, adherence and cytotoxicity of cells on microfilariae of *Litomosoides carinii*

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Summary

Diethylcarbamazine (DEC) induced dose dependent adherence of normal spleen cells from *Mastomys natalensis* to microfilariae of *L. carinii* in the presence of serum from *Mastomys* infected with *L. carinii* or normal serum. The effect could be also induced, using fresh, normal human serum and human blood leucocytes. The responsible serum factor was heat labile (56° C, 30 min) and was eliminated from the sera by pretreatment with Inulin. The activity could be reconstituted by normal serum. Cell adhesion was inhibited by 0.004 M EDTA while 0.004 M EGTA had only a weak inhibitory effect. The addition of Mg^{++} led to high adherence rates in the presence of EGTA. The data indicate that DEC activates complement on the surface of the larval sheath by the alternate pathway. Adherent *Mastomys* cells or human cells had a cytotoxic effect on the larvae. The death of the microfilariae did not depend on the loss of the larval sheath.

Key words: Diethylcarbamazine; microfilariae; *L. carinii*; cytotoxicity; complement.

Introduction

Diethylcarbamazine (DEC) has been used as an effective microfilaricidal drug for some 30 years but its mode of action is still unclear. Some workers have reported that DEC may act directly on the microfilariae of some filarial species, as in the case of *Foleyella delichoptera* (Hewitt et al., 1947), *Breinlia sergenti* (Natarajan et al., 1973) and *Onchocerca volvulus* (Lanham and Kramer, 1980),

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but in spite of a marked in vivo efficacy, no in vitro effect could be observed on microfilariae of *L. carinii* (Hawking et al., 1950).

However, recent observations have indicated that DEC may enhance in vitro antibody mediated adherence of human blood cells to *Brugia malayi* microfilariae (Piessens and Beldekas, 1979) and of cat blood cells to *B. pahangi* microfilariae (Mackenzie, 1980). These authors have discussed their data in relation to the communications of Kobayashi et al. (1969) and Tanaka et al. (1977), in which it was reported that the in vivo effect of DEC against microfilariae of *L. carinii* in cotton rats depended on the specific immune status of the host.

In contrast to this, our data have shown that DEC acts in vivo independent of specific immunity. *L. carinii* microfilariae were eliminated after DEC treatment from the peripheral blood of *Mastomys natalensis* with normal infections, as well as from the blood of animals in which *L. carinii* microfilariae or adults had been transplanted days or hours before drug application (Zahner et al., 1977). The elimination of microfilariae from the peripheral blood of transplanted animals was accompanied by the adherence of cells and the liberation of lysosomal enzymes, similar to the situation which had been observed after treatment of normally infected animals (Zahner et al., 1976, 1978). Further investigations have shown that such cell adhesions were significantly reduced in animals which had been treated in vivo by cobra venom factor to reduce complement levels (Zahner et al., 1983).

The present in vitro studies were undertaken to determine the role of complement in DEC-dependent cell adherence and cytotoxicity in the case of *L. carinii* microfilariae.

Material and Methods

M. natalensis (Strain GRA Giessen) were used as experimental animals. They were maintained under constant climatic conditions and fed on a diet developed for Syrian Hamsters. Drinking water was available ad libitum.

The animals were infected with *L. carinii* by exposing them to infected mites *Bdellonyssus bacoti*, according to Lämmler et al. (1968).

Blood was collected from *Mastomys* by puncture of the retroorbital venous plexus. Human blood was obtained from healthy donors. Serum was isolated after clotting at room temperature (1 h) and centrifugation (5 min, 7,500 $\times g$) and was used immediately.

Mastomys cells were obtained from the spleen of uninfected animals. The spleen was removed after decapitation of the donor, cut in pieces and squeezes carefully into medium PIS 04 (Seiler et al., 1972) through a 125 μm meshed nylon sieve. Cells were washed three times in this medium (200 $\times g$; 10 min; 4° C) and adjusted to a concentration of 10^6/ml. They were used as suspensions from individual animals. Viability was tested by the dye exclusion test. Suspensions in which viability was less than 80% were discarded.

Human white blood cells were isolated from heparinized blood (300 U Heparin-Na/ml) by sedimentation with dextran (MG 200,000). 10 ml blood were mixed with 3 ml of a 5% (w/v) dextran solution and sedimentated at 37° C for 30 min. The cell-containing supernatant was used. Cells were handled in the same manner as *Mastomys* cells. Suspensions with a viability below 95% were discarded.

Table 1. Adherence of normal spleen cells of *Mastomys natalensis* to microfilariae of *Litomosoides carinii* after incubation[a] with normal serum (NS), serum from infected *Mastomys* (IS: 150 days p.i.) or medium and diethylcarbamazine (DEC) in different concentrations ($\bar{x} \pm s$ of 3 experiments with cells and sera from different animals)

DEC-concentrations[b] µg/ml	% microfilariae with adherent cells when incubated with		
	IS	NS	medium
1,000	17.0 ± 6.2	10.3 ± 2.3	2.6 ± 1.0
500	23.0 ± 2.3	18.0 ± 8.9	1.4 ± 0.6
100	24.1 ± 1.4	15.6 ± 10.7	1.1 ± 0.7
50	19.2 ± 2.2	18.1 ± 4.1	0.9 ± 0.7
10	18.3 ± 3.3	16.4 ± 3.6	2.5 ± 1.8
1	12.1 ± 1.6	7.9 ± 1.3	0.5 ± 0.8
0.1	5.5 ± 5.3	10.0 ± 9.1	0.3 ± 0.6
0	5.3 ± 3.8	6.9 ± 5.3	1.7 ± 0.9

[a] incubation of 5×10^3 microfilariae (10 µl), 50 µl serum (pool of 10 animals each), 10^6 spleen cells (40 µl), 50 µl DEC solution; 37° C; 2 h
[b] calculated for the added solution

Table 2. Adherence of normal spleen cells of *Mastomys natalensis* to microfilariae of *Litomosoides carinii* after incubation for different times with normal serum (NS) or serum from infected *Mastomys* (IS: 150 days p.i.) and diethylcarbamazine (DEC: 100 µg/ml) ($\bar{x} \pm s$ of 3 experiments with cells and sera from different animals)

Incubation[a] of microfilariae and spleen cells with	% microfilariae with adherent cells after incubation for (Hours)			
	0.5	1	2	3
IS + DEC	6.9 ± 3.6	16.8 ± 5.1	18.7 ± 7.4	20.5 ± 2.7
IS + medium	3.4 ± 0.6	3.7 ± 0.9	5.5 ± 3.1	8.8 ± 1.5
NS + DEC	3.5 ± 2.1	14.0 ± 4.0	19.3 ± 7.6	16.3 ± 10.5
NS + medium ...	1.0 ± 1.4	1.5 ± 0.6	3.7 ± 2.9	8.6 ± 5.0

[a] see Table 1 (apart from incubation time)

Microfilariae were collected from heparinized blood of infected animals, using a 25% (w/v) Ficoll (Deutsche Pharmacia, Freiburg) gradient in PBS (Kimmig and Braun, 1980). The larvae were isolated from the interface after centrifugation ($10{,}000 \times g$; 1 h), washed three times in medium ($200 \times g$; 10 min) and adjusted to a concentration of 10^6/ml medium.

DEC was diluted in the desired concentration in PBS and adjusted to pH 7.2 by 0.1 M NaOH.

Incubations were performed at 37° C at 5% CO_2 concentration. The total volume was 150 µl, containing 10 µl microfilarial suspension, 40 µl cell suspension, 50 µl serum and 50 µl DEC solution. In controls, various reagents were replaced by medium. Exceptions and details are given in the tables. When incubations lasted longer than 3 h, 100 U penicillin and 100 µg streptomycin were added per ml.

Table 3. Adherence of normal spleen cells of *Mastomys natalensis* to microfilariae of *Litomosoides carinii* after incubation with individual untreated or heated (30 min, 56° C) normal sera (NS) or sera from infected Mastomys (IS: 150 days p.i.) and diethylcarbamazine (DEC: 100 μg/ml) ($\bar{x} \pm s$ for n = 6)

Incubation[a] of microfilariae and spleen cells with	% microfilariae with adherent cells
IS (untreated) + DEC	22.2 ± 4.2
IS (untreated) + medium	8.2[b] ± 4.3
IS (heated) + DEC	6.9[b] ± 5.2
IS (heated) + medium	6.3[b] ± 1.7
NS (untreated) + DEC	18.3 ± 3.3
NS (untreated) + medium	8.2[b] ± 4.3
NS (heated) + DEC	8.5[b] ± 4.7
NS (heated) + medium	6.0[b] ± 3.2
Medium + DEC	3.2 ± 2.6
Medium + medium	3.7 ± 2.0

[a] see Table 1

[b] $p < 0.05$, when compared with untreated sera + DEC (Wilcoxon-Mann-Whitney-test)

Elimination of complement activity from serum was done either by heating (56° C, 30 min) or by incubation with Inulin $(C_6H_{10}O_5)_X$ (E. Merck, Darmstadt): 0.25 ml serum were incubated with 10 mg Inulin at 37° C for 3.5 h. Inulin was separated by centrifugation (5,000 × *g*; 5 min).

The effect of Ca^{++} and Mg^{++} was controlled by adding 0.002 M or 0.004 M ethylenediaminotetraacetic acid (EDTA: Titriplex III; E. Merck, Darmstadt) and ethylen-glycol-bis(2-aminoethylether)N,N-tetraacetic acid (EGTA: Serva, Heidelberg), partly substituting Mg^{++}.

Cell adherence was determined in living preparations under a cover glass and expressed as the percentage of microfilariae showing attached cells. Nitroblue-tetrazolium (NBT) was used as an indicator of liberation of lysosomal enzymes using a technique slightly modified after Leventhal and Soulsby (1972). For cell differentiation, smears of the incubated suspensions were stained with Giemsa.

Cytotoxic effects on microfilariae were checked after a final incubation of the suspension with 0.1% trypan blue.

Results

The incubation of *L. carinii* microfilariae with spleen cells from *M. natalensis*, sera from *L. carinii* infected *Mastomys* or from uninfected *Mastomys* and DEC in concentrations from 100 μg/ml to 1 μg/ml (calculated on the starting solution), resulted in attachment of leucocytes to the motile, ensheathed larvae (Table 1). Differences due to DEC concentrations were not marked, but DEC concentrations lower than 1 μg/ml usually did not induce adherence of cells above that in DEC free controls. Low adhesion rates were observed when the incubation was performed in serum free medium.

The concentration of 100 μg DEC/ml was selected as the standard concentration for the following experiments.

Table 4. Adherence of normal spleen cells of *Mastomys natalensis* to microfilariae of *Litomosoides carinii* after incubation with untreated, heated (30 min, 56° C) or Inulin treated normal sera (NS) or sera from infected *Mastomys* (IS: 150 days p.i.) and diethylcarbamazine (DEC: 100 µg/ml) ($\bar{x} \pm s$ of 3 experiments with cells and sera from different animals)

Incubation[a] of microfilariae and spleen cells with	% microfilariae with adherent cells
IS (untreated) + DEC	12.1 ± 4.4
IS (heated) + DEC	1.9 ± 0.7
IS (Inulin)[b] + DEC	2.0 ± 0.8
IS (heated) + NS (untreated)[c] + DEC	15.1 ± 6.2
IS (untreated) + medium	4.2 ± 1.7
IS (heated) + NS (untreated)[c] + medium	2.2 ± 1.4
NS (untreated) + DEC	11.7 ± 1.0
NS (heated) + DEC	1.4 ± 0.6
NS (Inulin)[b] + DEC	2.5 ± 2.3
NS (heated) + NS (untreated)[c] + DEC	9.1 ± 3.4
NS (untreated) + medium	3.8 ± 0.9
NS (heated) + NS (untreated)[c] + medium	4.6 ± 1.4

[a] see Table 1
[b] incubation of 0.25 ml serum with 10 mg Inulin for 3.5 h at 37° C
[c] addition of 50 µl serum

Table 5. Adherence of normal spleen cells of *Mastomys natalensis* to microfilariae of *Litomosoides carinii* after incubation with normal *Mastomys* serum and diethylcarbamazine (DEC: 100 µg/ml): influence of ethylenediamino-tetraacetic acid (EDTA) and ethyleneglycol-bis(2-aminoethyl-ether)N,N-tetraacetic aminoethyleacid (EGTA) (mean of 2 experiments)

Incubation[a] of microfilariae and cells with			% microfilariae with adherent cells after incubation for (hours)	
serum	DEC	EDTA/EGTA	2	16
+	+	–	12.3	17.5
+ (heated)[b]	+	–	0	0
–	+	–	0.8	0
+	–	–	3.2	6.1
+ (heated)[b]	–	–	0.9	0
+	+	0.004 M EDTA	2.5	0
+	+	0.002 M EDTA	6.3	5.4
+	+	0.004 M EGTA	11.0	8.4
+	+	0.002 M EGTA	9.5	16.8
+	+	0.004 M EGTA + 0.002 M Mg^{++}	n.d.	14.9

[a] see Table 1 (apart from incubation time)
[b] 30 min, 56° C
n.d. = not done

Table 6. Cell adherence to microfilariae of *Litomosoides carinii* and cytotoxicity on the microfilariae after incubation of the microfilariae with diethylcarbamazine (DEC: 100 µg/ml) and either normal spleen cells of *Mastomys natalensis* (MnSC), together with normal serum (NS) and serum from infected *Mastomys* (IS: 150 days p.i.) or normal human serum (HS) and human blood leucocytes (hBL) ($\bar{x} \pm s$ of 3 experiments)

Incubation[a] of microfilariae with	% microfilariae with adherent cells after (hours)		% microfilariae dead[b] after 18 h incubation; calculation based on	
	2	18	total no of microfilariae	microfilariae with adherent cells
IS + MnSC + DEC	13.2 ± 4.3	28.6 ± 3.6	13.7 ± 2.8	58.1 ± 6.4
IS + MnSC	4.3 ± 2.1	5.6 ± 1.3	4.0 ± 1.2	70.1 ± 5.7
IS (heated)[c] + MnSC + DEC	3.5 ± 1.8	10.7 ± 4.6	6.9 ± 2.8	63.4 ± 4.7
IS (heated)[c] + MnSC	0.3 ± 0.4	5.3 ± 0.6	5.2 ± 1.6	69.1 ± 3.3
NS + MnSC + DEC	16.8 ± 4.3	25.9 ± 2.7	18.6 ± 5.2	70.6 ± 13.4
NS + MnSC	2.3 ± 1.2	10.3 ± 4.4	7.6 ± 2.0	76.2 ± 13.4
NS (heated)[c] + MnSC + DEC	5.0 ± 3.1	14.1 ± 1.2	9.4 ± 0.9	70.0 ± 4.7
NS (heated)[c] + MnSC	1.0 ± 1.0	8.2 ± 1.6	3.8 ± 1.3	45.0 ± 7.1
HS + hBL + DEC	17.5 ± 5.8	37.2 ± 19.0	20.1 ± 10.4	49.3 ± 6.4
HS + hBL	9.2 ± 4.3	8.9 ± 4.2	4.5 ± 2.9	50.6 ± 12.3
HS (heated)[c] + hBL + DEC	1.4 ± 1.2	13.8 ± 6.3	4.4 ± 3.0	31.9 ± 6.8
HS (heated)[c] + hBL	3.7 ± 1.0	3.2 ± 1.8	3.2 ± 2.1	87.5 ± 13.5

[a] see Table 1
[b] cytotoxicity measured by dye exclusion test
[c] 30 min, 56° C

DEC dependent adherence of cells started between 10 and 20 min of incubation with either normal sera (NS) and sera from infected *Mastomys* (IS). The effect became marked after 30 min and adhesion rates increased up to the first hour. Further increases up to 3 h incubation were only moderate (Table 2).

Variations due to individual sera were insignificant (Table 3). In all cases, cell attachment was reduced markedly or abolished totally when a serum was inactivated prior to use. Similarly, the activity of both normal and immune sera could be eliminated by decomplementation with Inulin (Table 4). The reactivity of inactivated sera could be fairly restored by the addition of untreated normal serum (Table 4).

DEC induced adherence of cells was almost totally inhibited or at least was markedly reduced by the addition of 0.004 M and 0.002 M EDTA while 0.002 M and 0.004 M EGTA had only a weak influence. When 0.002 M Mg^{++} were added before EGTA treatment, increased adhesion rates were observed in comparison to EGTA treatment alone (Table 5).

Adherent cells from spleen cell suspensions consisted of polymorphonuclear cells (with a relatively high percentage – usually 20% – of eosinophilic granulocytes) and of macrophages and lymphocytes. Adherence of cells was accompanied with the liberation of oxygen radicals, detectable by the deposition of formazan granules at the contact area of larvae and cells 30 min following the addition of NBT to the suspension.

Cell adherence to microfilariae could also be induced by DEC when human serum and human blood leucocytes were used. In concert with the results which were obtained for *Mastomys* sera, cell adherence could not be induced with inactivated human sera (Table 6).

The adherence of both *Mastomys* cells and human cells led to a cytotoxic effect on the larvae. However, DEC induced cell adherence did not result in stronger cytotoxic effects than when the occasional cell adherence occurred in DEC free controls (Table 6). The loss of the sheath was presumed not to be a precondition for the death of the microfilariae.

Discussion

The results show that DEC induces, in vitro, the adherence of cells to microfilariae of *L. carinii*. This is dependent on a heat labile factor, which was present in normal human and *Mastomys* sera and in the sera of *Mastomys* infected with *L. carinii*. The factor could be eliminated by Inulin and the reaction was inhibited by EDTA while EGTA had no marked effect. Cell adherence was followed by the liberation of oxygen radicals indicating a liberation of lysosomal enzymes, and cytotoxic effects on the larvae.

The data are in accordance with results of in vivo experiments, which showed that the microfilaricidal effect of DEC in the case of *L. carinii* is independent of the immune status of the host (Zahner et al., 1977), and they differ, therefore, from reports of Kobayashi et al. (1969) and Tanaka et al. (1977), which claimed that the effect of DEC is dependent on a specific immune status of the animals.

The characteristics of the serum factor responsible, i.e., the heat lability and the sensitivity to EDTA, a substance chelating Ca^{++} and Mg^{++}, suggest that the complement system is involved in a DEC-dependent cell adhesion. Earlier in vivo results had shown, that the DEC-dependent attachment of cells to microfilariae in the parenchymatous organs of the infected hosts was suppressed in animals which had been decomplemented in vivo by treatment with cobra venom factor (Zahner et al., 1983). Cobra venom factor as well as Inulin and several other polysaccharides are regarded as activators of the alternate pathway of complement activation (Götze and Müller-Eberhardt, 1971; Platts-Mills and Ishizaka, 1974; see also Lanzer, 1981). Inulin was shown in the present experiments to eliminate the factor(s) in normal and infective sera responsible for cell adhesion.

Furthermore, the fact that both normal serum and serum of infected hosts were active in vitro suggested that complement was activated by the alternate pathway. However, this does not exclude an activation via the classical pathway – conceivable by an involvement of eventually preformed immune complexes at the surface of the sheath – however, there is no clear experimental evidence at present for an occurrence of immunoglobulins on the larval sheath (Court and Storey, 1981). Clear evidence for an activation of complement via the alternate pathway had been obtained by the observation that DEC-dependent cell adhesions were influenced by EDTA in a more marked degree than by EGTA, and, in the latter case, Mg^{++} had an enhancing effect. EGTA chelates Mg^{++} to a lesser extent than Ca^{++}, whereas both can be eliminated by EDTA (Bryant and Jenkins, 1968). In contrast to the classical pathway, C3 conversion via the alternate pathway only requires Mg^{++} (Sandberg and Osler, 1971; Fine et al., 1972; Ferrone et al., 1973).

The cytotoxic effect of the cell adherence was possibly related to the liberation of lysosomal enzymes, but the cytotoxicity did not obviously depend on a special activation of cells by the drug itself. No difference was observed in the degree of cytotoxicity between the DEC induced cell adhesion and cell adhesion resulting from DEC independent reactions. A similar percentage of cells bearing larvae suffered cytotoxic effects in each case. Mackenzie (1980) had reported that DEC leads to a selective degranulation of eosinophils. If this was the case in the present study, the reaction was without effect.

Since complement-mediated adherence of cells to microfilariae of *L. carinii* could be induced by DEC when human cells and complement was used, it is evident that the effect is not limited to the *Mastomys*. However, it will be of particular interest to determine whether the effect is restricted to microfilariae of *L. carinii*. The data reported by Piessens and Beldekas (1979) for *B. malayi* microfilariae would suggest that for this species, antibody dependent mechanisms may be more involved. More detailed studies are being undertaken to evaluate the situation for different filarial species, including those with ensheathed or unsheathed microfilariae.

The molecular basis of the mechanism(s) is open to speculation. There could be an unmasking of the microfilarial sheath, but probably not in a sense that antigenic structures are exposed, as suggested by Kobayashi et al. (1969) and Tanaka et al. (1977). It is possible that the surface becomes affected in a way that molecular structures are revealed or generated, which will activate C3 by the alternate pathway. Most of the compounds known to have such activity are polysaccharides (see Lanzer, 1981) but in the absence of information about the chemical structure of the sheath of *L. carinii* microfilariae, it is not possible to surmise what might be concerned in this instance.

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