Introduction

Angiostrongyliasis is caused by the rat lung worm, Angiostrongylus cantonensis, which is widely distributed in tropical and subtropical areas where rodents (the natural and definitive host) are known to be infected with this parasite. To date, this parasite has been identified throughout the island of Taiwan, where many cases of CNS disease caused by this parasite have been reported.\(^1,2\) Our previous study showed that the tissue-type plasminogen activator (uPA) and urokinase-type plasminogen activator (uPA) play an important role in eosinophilic meningitis of BALB/c mice.\(^3\) Additionally, matrix metalloproteinase-9 (MMP-9) was associated with eosinophilic meningitis and found to be a useful marker for angiostrongyliasis meningitis in BALB/c\(^4\) and ICR mice.\(^5\)

The transport of compounds from the circulating blood into the CNS is restricted by blood–CNS barriers, such as the blood–brain barrier (BBB) and blood–CSF barrier, which are formed by tight junctions connecting the cerebral endothelial and epithelial cells of the choroid plexus, respectively.\(^6\) Breakdown of the BBB is a key feature of neuroinflammatory conditions and is associated with an influx of inflammatory cells, fluid and proteins, including complement and cytokines.\(^7\) MMPs have been implicated in the pathogenesis of BBB breakdown.\(^7,8\) Additionally, the ability of the uPA to recruit leukocytes and promote blood–CNS barrier breakdown, may play an important pathophysiological role in bacterial meningitis.\(^9\)

Interleukin-5 (IL-5) is an important cytokine for the induction of A. cantonensis-induced eosinophilia.\(^10\) The CSF eosinophils of mice infected with A. cantonensis are resistant to apoptosis, and IL-5 is an important determinant in the survival and activation of eosinophils.\(^11\) The proinflammatory cytokine tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) is a potent activator of neutrophils, modulating adherence, chemotaxis and degranulation; it is responsible for the severe cachexia that occurs in chronic infections.\(^12\) MMP-9 has been shown to cleave inactive TNF-\(\alpha\), so

Keywords: parasites, angiostrongyliasis, thalidomide, proteinases, cytokines

Biochemical and pathological evaluation of albendazole/thalidomide co-therapy against eosinophilic meningitis or meningoencephalitis induced by Angiostrongylus cantonensis

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Objectives: To evaluate the curative effect of albendazole/thalidomide co-therapy on eosinophilic meningitis in BALB/c mice caused by Angiostrongylus cantonensis.

Methods: Male mice were infected with 50 A. cantonensis larvae and treated with albendazole (5, 10 or 20 mg/kg per day) alone, thalidomide (25, 50 or 100 mg/kg per day) alone, or a combination of albendazole (10 mg/kg per day) and thalidomide (50 mg/kg per day) for 7 consecutive days on days 5, 10 and 15 post-inoculation (PI), respectively.

Results: Indicators used to measure this effect included: (i) worm recovery; (ii) histopathological score of meningitis; (iii) eosinophil counts; (iv) level of pro-inflammatory cytokines, such as tumour necrosis factor-\(\alpha\), interleukin-1\(\beta\) and interleukin-5; (v) activity of enzymes, such as tissue-type plasminogen activator, urokinase-type plasminogen activator and matrix metalloproteinase-9; and (vi) CSF/serum albumin ratio. The results showed that albendazole/thalidomide co-therapy significantly decreased \((P < 0.05)\) these factors when treatment was initiated on days 5 or 10 PI compared with treatment initiated on day 15 PI.

Conclusions: The timing of medication use is important and is closely related to the anthelmintic efficacy of a drug. For a given dosage, earlier medication use is more effective. This novel approach to treating parasitic meningitis may suggest other new methods of treatment.
Albendazole/thalidomide co-therapy in angiostrongyliasis

that active TNF-α is released.13 In addition, MMP-9 gene expression by macrophages and polymorphonuclear leucocytes, and hence the release of pro-MMP, can be induced by TNF-α and interleukin-1β (IL-1β).14,15

Some effective anthelmintics have been proposed to treat A. cantonensis in humans. But dead worms in the brain may evoke a severe immune response resulting in brain damage. Though considered the drug of choice for treating angiostrongyliasis, albendazole is frequently used in combination with steroids to prevent the inflammatory reaction due to dead larvae. However, the use of steroid therapy is still controversial. The objectives of the present study were to assess the efficacy of the anthelmintic albendazole combined with the TNF-α inhibitor thalidomide as treatment in a mouse model of parasitic meningitis caused by A. cantonensis infection, and to find out whether the TNF-α inhibitor could substitute for steroid treatment.

Materials and methods

Experimental animals

Five-week-old male mice (weighing 20–25 g), BALB/c strain, were purchased from the National Laboratory Animal Center, Taipei, Taiwan. They were maintained on a regular 12:12 h light/dark cycle and provided with Purina Laboratory Chow and water ad libitum. All of the procedures involving animals and their care in this study were approved by the Institutional Animal Care and Use Committee of Chung-Shan Medical University in accordance with institutional guidelines for animal experiments.

Larval preparation

The infective larvae (L3) of A. cantonensis, originally obtained from wild giant African snails (Achatina fulica), were propagated for several months in our laboratory by cycling through rats and snails ( Biomphalaria glabrata). The larvae within tissues were recovered using a modification of the method of Parsons and Grieve.16 Briefly, the shells were crushed, and the tissues were homogenized, digested in a pepsin-HCl solution (pH 1–2, 500 IU pepsin/g tissue) and incubated with agitation in a 37°C waterbath for 2 h. Host cellular debris was removed from the digest by centrifugation at 1400 g for 10 min. The larvae in the sediment were collected by serial washing in double-distilled water and counted under a microscope.

Drugs

Albendazole (Zentel®, GlaxoSmithKline, NC, USA) is a benzimidazole derivative. The molecular formula is C₁₂H₁₅N₃O₂S and the molecular mass is 265.34.17 It is widely used in veterinary medicine and has been found to be a safe broad-spectrum anthelmintic in animals and humans. Albendazole acts by binding to the parasite’s β-tubulin, inhibiting its polymerization and impairing glucose uptake, causing death.18 Thalidomide (TTY Biopharm, Taiwan) has the empirical formula C₁₃H₁₀N₂O₄ and a molecular mass of 258.23.19 Thalidomide affects cytokine production and T-lymphocyte proliferation. Thalidomide suppresses TNF-α production by macrophages and may thereby reduce the inflammatory response.20

Treatment of animals

A total of 300 mice were randomly divided into five treated groups (60 mice/group), and subdivided into three different time points of administration (20 mice per time point). Food and water were withheld for 12 h before infection. The uninjected control mice were administered only distilled water orally on days 5, 10 and 15 post-infection (PI). All other groups were infected with 50 larvae, including the infected–untreated control mice, which were treated with only distilled water orally on days 5, 10 and 15 PI. The albendazole-treated mice were treated with albendazole only (5, 10 or 20 mg/kg per day) for 7 consecutive days on days 5, 10 and 15 PI, respectively; the thalidomide treated mice were treated with thalidomide only (25, 50 or 100 mg/kg per day) for 7 consecutive days on days 5, 10 and 15 PI, respectively; and the albendazole/thalidomide co-therapy mice received a treatment combining albendazole (10 mg/kg per day) and thalidomide (50 mg/kg per day) for 7 consecutive days on days 5, 10 and 15 PI, respectively. All groups were sacrificed on day 22 PI; the brains and CSF were collected for histopathological study and biochemical analysis.

Worm recovery and assessment of efficacy

Treated and control mice were killed by cervical dislocation on day 22 PI. Each brain was sliced into small pieces, digested in a pepsin-HCl solution (pH 1–2, 500 IU pepsin/g tissue) and incubated with agitation in a 37°C waterbath for 2 h. Host cellular debris was removed from the digest by centrifugation at 1400 g for 10 min. Larval counts were measured under 25× magnification using a dissecting microscope. The mean number of larvae was compared with that of the positive control (infected–untreated) group to assess drug efficacy.

Histopathological examinations

The mouse brains were fixed separately in 10% neutral buffered formalin for 24 h. The fixed specimens were dehydrated in a graded ethanol series (50%, 75% and 100%) and xylene, and then embedded in paraffin at 55°C for 24 h. Serial sections (5 μm thick) were cut for each brain from each mouse. Paraffin was removed by heating the sections for 5 min at 65°C. These sections were dewaxed by washing three times for 5 min each in xylene, then rehydrated through 100%, 95% and 75% ethanol for 5 min each, and finally rinsed with distilled water. After staining with haematoxylin (Muto, Tokyo, Japan) and eosin (Muto, Tokyo, Japan), pathological changes were examined under a light microscope.

Eosinophil counts in the CSF

The mice were sacrificed and their brains removed into a 35 mm dish. The cranial cavity and cerebral ventricles (lateral, third and fourth ventricles) were rinsed with 1 mL of PBS. The washing solution was collected in a centrifuge and spun at 400 g for 10 min. The resultant sediments were then gently mixed with 100 μL of Unopette buffer (Vacutainer System, Becton Dickinson, Franklin Lakes, NJ, USA) and 2 μL of acetic acid and placed in a haemocytometer cell-counting chamber (Paul Marienfeld, Lauda-Koenigshofen, Germany) to count eosinophils.

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Cytokine assay
Production of cytokines (TNF-α, IL-1β and IL-5) in the CSF was assayed using commercial enzyme-linked immunosorbent assay (ELISA) kits (Biosource, Camarillo, CA, USA) according to the manufacturer’s instructions. CSF samples for cytokine assays were pooled since the amounts of CSF harvested from each mouse were frequently insufficient for individual assays.

Gelatin and casein substrate zymography
Activity of MMP-9 was determined by gelatin zymography, and plasminogen activator (PA) activities by casein zymography. Briefly, the CSF was centrifuged at 12000 g for 10 min to remove debris. The protein contents of supernatants were analysed on SDS/polyacrylamide gels copolymerized with 0.1% gelatin (Sigma, St Louis, MO, USA) for gelatinase activities, 0.1% casein (Sigma) for plasmin activities and plasminogen (13 mg/L, Sigma) for plasminogen activator (PA) activities. Electrophoresis was performed in running buffer (25 mM Tris, 250 mM glycine and 1% SDS) at room temperature at 120 V for 1 h. The gel was washed twice at room temperature for 30 min each in 2.5% acetic acid. The final gel had a uniform background except in regions to which MMP-9, tPA or uPA had migrated and cleaved their respective substrates. Quantitative analysis of the gelatinolytic and caseinolytic enzymes was performed with a computer-assisted imaging densitometer system, UN-SCAN-IT gel Version 5.1 (Silk Scientific, Orem, UT, USA).

Table 1. Effect of albendazole on *Angiostrongylus cantonensis* larvae in BALB/c mice treated for 7 days at different dosages and on different days post-inoculation

<table>
<thead>
<tr>
<th>Medication</th>
<th>oral dosage (mg/kg per day)</th>
<th>days post-inoculation</th>
<th>days treated</th>
<th>No. of worms recovered (mean ± SD)</th>
<th>Worm reduction (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Controlb (5)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>30.0 ± 3.5</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>7</td>
<td>6.5 ± 1.8</td>
<td>78.4</td>
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</tr>
<tr>
<td>10</td>
<td>5</td>
<td>7</td>
<td>3.3 ± 1.9</td>
<td>89</td>
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<tr>
<td>20</td>
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<td>7</td>
<td>3.6 ± 2.2</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Controlb (10)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>31.4 ± 5.1</td>
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<tr>
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<td>7</td>
<td>8.9 ± 1.6</td>
<td>71.7</td>
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<tr>
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<td>7</td>
<td>6.2 ± 1.8</td>
<td>80.3</td>
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</tr>
<tr>
<td>20</td>
<td>10</td>
<td>7</td>
<td>6.7 ± 2.1</td>
<td>80.7</td>
<td></td>
</tr>
<tr>
<td>Controlb (15)</td>
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<td>—</td>
<td>29.2 ± 3.5</td>
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</tr>
<tr>
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<td>15</td>
<td>7</td>
<td>16.3 ± 2.1</td>
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<tr>
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<td>13.6 ± 2.6</td>
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<td></td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>7</td>
<td>11.2 ± 1.9</td>
<td>61.7</td>
<td></td>
</tr>
</tbody>
</table>

*Each group consists of 5 mice infected with 50 infective larvae and sacrificed on day 22 post-inoculation.

Table 2. Effect of thalidomide on *Angiostrongylus cantonensis* larvae in BALB/c mice treated for 7 days at different dosages and on different days post-inoculation

<table>
<thead>
<tr>
<th>Medication</th>
<th>oral dosage (mg/kg per day)</th>
<th>days post-inoculation</th>
<th>days treated</th>
<th>No. of worms recovered (mean ± SD)</th>
<th>Worm reduction (%)</th>
</tr>
</thead>
<tbody>
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<td></td>
</tr>
<tr>
<td>Controlb (5)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>31.0 ± 3.8</td>
<td>—</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>7</td>
<td>28.0 ± 3.6</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>7</td>
<td>27.2 ± 4.1</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>100</td>
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<td>7</td>
<td>26.6 ± 4.2</td>
<td>14.2</td>
<td></td>
</tr>
<tr>
<td>Controlb (10)</td>
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<td>—</td>
<td>—</td>
<td>32.0 ± 3.1</td>
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<tr>
<td>25</td>
<td>10</td>
<td>7</td>
<td>29.8 ± 3.7</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>7</td>
<td>27.3 ± 3.5</td>
<td>14.7</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>7</td>
<td>26.5 ± 4.1</td>
<td>17.2</td>
<td></td>
</tr>
<tr>
<td>Controlb (15)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>28.0 ± 2.8</td>
<td>—</td>
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<tr>
<td>25</td>
<td>15</td>
<td>7</td>
<td>26.4 ± 3.9</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>15</td>
<td>7</td>
<td>26.2 ± 3.7</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>15</td>
<td>7</td>
<td>25.8 ± 3.5</td>
<td>7.9</td>
<td></td>
</tr>
</tbody>
</table>

*Each group consists of 5 mice infected with 50 infective larvae and sacrificed on day 22 post-inoculation.

Table 3. Effect of albendazole/thalidomide co-therapy on *Angiostrongylus cantonensis* larvae in BALB/c mice treated for 7 days on different dosages and different days post-inoculation

<table>
<thead>
<tr>
<th>Medication</th>
<th>oral dosage (mg/kg per day)</th>
<th>days post-inoculation</th>
<th>days treated</th>
<th>No. of worms recovered (mean ± SD)</th>
<th>Worm reduction (%)</th>
</tr>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controlc</td>
<td>—</td>
<td>31.0 ± 4.5</td>
<td>—</td>
<td>96.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>1.2 ± 1.0</td>
<td>86.2</td>
<td></td>
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</tr>
<tr>
<td>10</td>
<td>7</td>
<td>4.3 ± 1.9</td>
<td>86.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>7</td>
<td>11.6 ± 1.8</td>
<td>62.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Each group consists of 5 mice infected with 50 infective larvae and sacrificed on day 22 post-inoculation.

Measurement of albumin in CSF and serum
The CSF and serum were centrifuged at 12000 g at 4°C for 10 min, and the concentration of albumin in CSF and serum was measured by the Dade Behring assay system using the automated Dade Behring Nephelometer (BN ProSpec, Newark, NJ, USA).

Statistical analysis
Results in the different groups of mice were compared using the non-parametric Kruskal–Wallis test followed by post-testing.
Albendazole/thalidomide co-therapy in angiostrongyliasis

Using Dunn’s multiple comparison of means. All results are presented as mean ± standard deviation (SD). P values of <0.05 were considered statistically significant.

Results

Influence of treatment on larvae recovery

The larvicidal effect of treatment with three doses of albendazole beginning on days 5, 10 and 15 PI was lower on day 15 PI than on days 5 or 10 PI (Table 1). There was no significant difference (P > 0.05) in worm recovery between the non-treated group and the thalidomide-treated group (Table 2). Larvae recovered from brain tissue were virtually absent in infected mice treated with albendazole/thalidomide beginning on days 5 and 10 PI (Table 3). The numbers of recovered larvae were less after albendazole alone or albendazole/thalidomide than no treatment.

Influence of treatment on pathological changes

Optical microscopic examination of haematoxylin and eosin stained tissues revealed the mechanical damage caused by...
migrating larvae and meningeal inflammation characterized by eosinophilic infiltration. In the subarachnoid space, large-scale infiltration by leucocytes was evident 22 days after the inoculation of the larvae. Treatment with albendazole alone or albendazole/thalidomide beginning on days 5 and 10 PI significantly decreased the number of infiltrating leucocytes ($P < 0.05$) and resulted in larval degeneration (Figure 1), but thalidomide alone caused only mild reduction in leucocyte number. Treatment on day 15 PI: leucocytes were only mildly reduced by albendazole alone or albendazole/thalidomide and not significantly affected by thalidomide alone. Evidence of brain parenchyma and ventricle infection included haemorrhage, perivascular cuffing, nodular lesions, reactive proliferation of glial cells, diffuse infiltration by inflammatory cells, and a spongy

**Figure 2.** Influence of treatment on infection of the brain parenchyma and the fourth ventricle. (a) No larvae or leucocytes are found in the fourth ventricle (4V) of the uninfected brain. C, choroid plexus of the fourth ventricle; G, granular cells of the cerebellum; ML, molecular layer of the cerebellum. (b) The ventricles of mice infected with *Angiostrongylus cantonensis* contain larvae (arrowheads) and evidence of haemorrhage (H). (c) Many larvae (arrowheads) are found in the medulla (M) of the cerebellum. Treatment with albendazole beginning on day 5 PI (d) significantly decreased the number of larvae (arrowhead) and the amount of haemorrhage (H). Treatment with albendazole beginning on day 10 PI (e) significantly decreased the number of larvae (arrowheads) and mildly reduced the amount of haemorrhage (H). Treatment with albendazole beginning on day 15 PI (f) mildly decreased the number of larvae (arrowheads), amount of haemorrhage (H) and leucocyte numbers. Treatment with thalidomide beginning on day 5 PI (g) could not kill the larvae (arrowheads) and only mildly decreased leucocyte numbers (L). Treatment with thalidomide beginning on day 10 PI (h) could not kill the larvae (arrowheads) and only mildly decreased leucocyte number. Treatment with thalidomide beginning on day 15 PI (i) could not kill the larvae (arrowheads) and permitted significant leucocyte (L) accumulation. Albendazole/thalidomide co-therapy beginning on day 5 PI (j) significantly decreased the numbers of larvae and leucocytes (L) in the ventricle. Albendazole/thalidomide co-therapy beginning on day 10 PI (k) significantly decreased leucocyte numbers (L) in the ventricle. Albendazole/thalidomide co-therapy beginning on day 15 PI (l) caused significant larval degeneration (arrowheads) and mildly decreased leucocyte numbers (L) in the ventricle.
**Figure 3.** Influence of treatment on eosinophil counts. (a) The CSF eosinophils were significantly increased (*P < 0.05) in *Angiostrongylus cantonensis*-infected mice compared with uninfected control mice. Albendazole (ABZ) alone, thalidomide (TM) alone or albendazole/thalidomide (ABZ + TM) co-therapy beginning on day 5 PI (b) and day 10 PI (c) significantly reduced the number of eosinophils (*P < 0.05); and beginning on day 15 PI (d), co-therapy mildly reduced leucocyte numbers (*P < 0.05), but albendazole alone or thalidomide alone had no significant effect (*P > 0.05).

**Figure 4.** Influence of treatment on TNF-α concentrations. (a) TNF-α concentration was significantly increased (*P < 0.05) in *Angiostrongylus cantonensis*-infected mice compared with uninfected controls. Albendazole (ABZ) alone, thalidomide (TM) alone or albendazole/thalidomide (ABZ + TM) co-therapy beginning on day 5 PI (b), 10 PI (c) and 15 PI (d) significantly lowered TNF-α concentration (*P < 0.05).
appearance of parenchyma in all areas of the brain. Treatment on
days 5 and 10 PI: albendazole alone or albendazole/thalidomide
co-therapy significantly decreased \( (P < 0.05) \) haemorrhage and
infiltrating leucocytes, whereas these were only mildly reduced
by thalidomide. Treatment on day 15 PI: albendazole alone or
albendazole/thalidomide mildly reduced haemorrhage and infil-
trating leucocytes, and thalidomide alone had no significant
effect. Additionally, albendazole treatment alone or albendazole/
thalidomide caused larval degeneration (Figure 2).

**Influence of treatment on eosinophil counts**

The CSF eosinophils were significantly increased \( (P < 0.05) \) in
mice infected with *A. cantonensis* compared with uninfected
controls. Treatment on days 5 and 10 PI: albendazole alone or
albendazole/thalidomide significantly reduced eosinophil counts
\( (P < 0.05) \), whereas thalidomide treatment mildly reduced these
counts \( (P < 0.05) \). Treatment on day 15 PI: albendazole/thalido-
mide mildly reduced eosinophils \( (P < 0.05) \), but albendazole
alone or thalidomide alone had no significant effect \( (P > 0.05) \)
(Figure 3).

**Influence of treatment on cytokine production**

To determine the kinetics of cytokine production and its possible
role in mouse resistance to *A. cantonensis*, sandwich ELISAs
were used to assay TNF-\( \alpha \), IL-1\( \beta \) and IL-5 in CSF. The concen-
trations of TNF-\( \alpha \), IL-1\( \beta \) and IL-5 were significantly higher
\( (P < 0.05) \) in infected mice than in uninfected mice, which had
low levels of these. When compared with the corresponding
cytokine concentrations in infected–untreated mice, TNF-\( \alpha \)
concentration was significantly lowered \( (P < 0.05) \) in infected
mice by albendazole alone, thalidomide alone or albendazole/
thalidomide (Figure 4), IL-1\( \beta \) concentration was significantly
lowered \( (P < 0.05) \) by albendazole alone or albendazole/
thalidomide but not by thalidomide alone, which had no effect
(Figure 5), and IL-5 concentration was significantly lowered
\( (P < 0.05) \) by albendazole alone, thalidomide alone or albenda-
zole/thalidomide beginning on day 5 PI. For mice treated with
albendazole alone or albendazole/thalidomide beginning on days
10 and 15 PI, IL-5 concentration was significantly lowered
\( (P < 0.05) \), but thalidomide alone had no effect \( (P > 0.05) \)
(Figure 6).

**Influence of treatment on tPA and uPA activity**

Casein zymography measures the activity of enzymes such as
tPAs and uPAs, which migrate electrophoretically as 70 kDa
and 55 kDa species, respectively. These enzymes were signifi-
cantly increased \( (P < 0.05) \) in the CSF of infected mice. For
mice treated beginning on days 5 and 10 PI, albendazole
alone, thalidomide alone or albendazole/thalidomide signifi-
cantly reduced tPA and uPA activities \( (P < 0.05) \). Treatment on
day 15 PI: albendazole alone or albendazole/thalidomide
co-therapy mildly reduced these activities \( (P < 0.05) \), but
thalidomide alone had no significant effect \( (P > 0.05) \)
(Figures 7 and 8).

![Figure 5](https://academic.oup.com/jac/article-abstract/59/2/264/727224/bjyv)

**Figure 5.** Influence of treatment on interleukin (IL)-1\( \beta \) concentrations. (a) IL-1\( \beta \) concentration was significantly increased \( (*P < 0.05) \) in *Angiostrongylus
cantonensis*-infected mice compared with uninfected control mice. (b) Albendazole (ABZ) alone or albendazole/thalidomide (ABZ + TM) co-therapy
beginning on day 5 PI significantly lowered IL-1\( \beta \) concentrations \( (*P < 0.05) \), whereas thalidomide (TM) alone had no significant effect \( (P > 0.05) \).
Albendazole alone or albendazole/thalidomide beginning on day 10 PI (c) and day 15 PI (d) also significantly lowered IL-1\( \beta \) concentrations \( (*P < 0.05) \).
Influence of treatment on MMP-9 activity

Gelatin zymography measures the activity of enzymes such as MMP-9, which migrates electrophoretically as a 94 kDa species. This metalloproteinase activity was significantly increased \( (P < 0.05) \) in the CSF of mice infected with *A. cantonensis*. For mice whose treatment began on day 5 PI, albendazole alone, thalidomide alone or albendazole/thalidomide \((ABZ + TM)\) co-therapy beginning on day 5 PI significantly lowered the MMP-9 activity \( (P < 0.05) \). Albendazole alone or albendazole/thalidomide beginning on day 10 PI \((c)\) and day 15 PI \((d)\) also significantly lowered MMP-9 concentrations \( (P < 0.05) \), but thalidomide alone had no significant effect \( (P > 0.05) \) (Figure 9).

Influence of treatment on CSF/serum albumin ratio

The CSF/serum albumin ratio was significantly increased \( (P < 0.05) \) in infected mice and when albendazole alone, thalidomide alone or albendazole/thalidomide therapy began on day 5 PI, it was significantly decreased \( (P < 0.05) \) in infected mice. Treatment on days 10 and 15 PI: albendazole alone or albendazole/thalidomide mildly reduced the ratio \( (P < 0.05) \), and thalidomide alone had no significant effect \( (P > 0.05) \) (Figure 10).

Discussion

Albendazole is reported to be effective against most intestinal nematodes, with a single oral dose being adequate in most cases.\(^{22}\) Animal studies with albendazole showed that at least 50% of an orally administered single dose is absorbed.\(^{22}\) In this study, oral albendazole at 10 mg/kg per day for 7 consecutive days beginning on days 5 and 10 PI had remarkable larvicidal effect. The *A. cantonensis* larvae in the brains of mice were almost eliminated. However, less satisfactory effects were observed when medication was begun on day 15 PI. This may be explained by the fact that albendazole acts against the third or fourth but not fifth stage larvae of *A. cantonensis*. After the infective L₃ larvae of *A. cantonensis* are ingested, they migrate from the gastrointestinal tract to the CNS via the circulatory system. Most of the larvae complete their third moult on day 5 PI and the fourth moult around day 10 PI. On day 15 PI, most are fifth stage larvae (young adult worms) that migrate to the surface of the brain\(^{23}\) and into meningeal spaces. Treatment at this time is more difficult than on days 5 and 10 PI. These data confirm the previous finding\(^{17}\) that the timing of drug administration affects anthelminthic efficacy and larvicidal activity in *A. cantonensis*-infected mice.

In the early stage of *A. cantonensis* infection, treatment is primarily supportive. Repeated lumbar punctures provide relief for patients with persistent headaches from increased intracranial pressure.\(^{24}\) Anthelminthics are not effective and often worsen symptoms, possibly because of the inflammatory reaction to antigens released by dying worms.\(^{25}\) In the present study, the degree of brain inflammation correlated with the number of larvae found in the CNS. We initiated treatment before (on day...
and after (on days 10 and 15 PI) the appearance of inflammatory signs in the experimental animals. The treatment reduced the number of worms, reducing the pathology associated with infection. Though albendazole alone could increase neurological damage by causing simultaneous death of a large number of *A. cantonensis* larvae in the early stage (5 to 10 PI) (data not shown), in fact the severity of meningitis was lessened owing to the lower number of worms.

The blood–CNS barrier breakdown is regarded as an important pathophysiological event in meningitis, since it allows extravasation of leucocytes into the subarachnoid space. MMPs are implicated in the disruption of the BBB during leucocyte diapedesis, and uPA promotes blood–CNS barrier breakdown. Excessive proteolytic activities of PAs and MMP-9 can be detrimental, leading to disruption of the blood–CNS barrier. Measuring ratios of CSF to serum albumin may be a good index to monitor blood-to-CNS-barrier integrity without the use of invasive methods. Yii reported that CSF protein levels were higher in patients with *A. cantonensis*-induced eosinophilic meningitis than in normal individuals. Our previous studies have suggested that the MMP-9 inhibitor GM6001 used as an adjunct to the anthelmintic albendazole significantly inhibited the proteolytic enzyme MMP-9 and decreased eosinophilia in angiostrongyliasis. In the present study, the CSF/serum albumin ratio

![Graphs showing influence of treatment on tPA activity.](https://academic.oup.com/jac/article-abstract/59/2/264/727224)

Figure 7. Influence of treatment on tPA activity. (a) tPA was significantly increased (*P* < 0.05) in the CSF of mice infected with *Angiostrongylus cantonensis* compared with uninfected control mice. (b) Albendazole (ABZ) alone, thalidomide (TM) alone or albendazole/thalidomide (ABZ + TM) co-therapy beginning on day 5 PI (b) and day 10 PI (c) significantly reduced tPA activity (*P* < 0.05). Albendazole alone or albendazole/thalidomide co-therapy beginning on day 15 PI (d) mildly reduced tPA activity (*P* < 0.05), whereas thalidomide alone had no significant effect (*P* > 0.05). Quantitative analysis of tPA activity was performed using a computer-assisted imaging densitometer system.
and eosinophilia were significantly decreased when mice were treated with albendazole/thalidomide co-therapy. However, thalidomide alone only partially reduced CSF/serum albumin ratio and eosinophilia. These results suggest that CSF/serum albumin ratio might be an important indicator in eosinophilic meningitis and imply that treatment improves blood–CNS barrier damage by reducing activity of PAs/MMP-9 proteolytic enzymes and reducing the CSF/serum albumin ratio.

The anti-inflammatory and immunomodulatory effects of thalidomide have led scientists to assess its therapeutic value clinically. The efficacy of thalidomide is thought to be based on its ability to alter the phenotype of circulating immunological cells, inhibit TNF-α synthesis by macrophages, alter the level of cytokine and lymphokine synthesis and release, alter lymphocyte trafficking and neutrophil migration, and induce alterations in cellular adhesion molecules, consequently changing the interactions of leucocytes with the endothelial cell layer and causing the modulation of inflammation and immunity. Thalidomide is safe and well tolerated in children with stage 2 tuberculous meningitis. Thalidomide is now being considered as an adjuvant treatment for angiostrongyliasis meningitis. However, thalidomide alone reduces cytokine synthesis, has no larvicidal effect, and only mildly reduces the number of CSF eosinophils. The mild reduction of inflammation might be explained by the persistence of larvae that stimulate the immune response. 

Figure 8. Influence of treatment on uPA activity. (a) The uPA was significantly increased (*P < 0.05) in the CSF of mice infected with *Angiostrongylus cantonensis* compared with uninfected control mice. Albendazole (ABZ) alone, thalidomide (TM) alone or albendazole/thalidomide (ABZ + TM) co-therapy starting on day 5 PI (b) and on day 10 PI (c) significantly reduced uPA activity (*P < 0.05). Albendazole alone or albendazole/thalidomide co-therapy starting on day 15 PI (d) mildly reduced uPA activity (*P < 0.05), whereas thalidomide alone had no significant effect (P > 0.05). Quantitative analysis of the uPA activity was performed using a computer-assisted imaging densitometer system.
Th2-type cytokine production, especially of IL-5, is prevalent during *A. cantonensis* infection in mice and is probably involved in the induction of CSF and peripheral eosinophilia. Thalidomide, by interfering with the production of TNF-α, can reduce the inflammatory response. Though basal levels are essential for normal growth and development, TNF-α increases during *A. cantonensis* infection causing pathology. Co-therapy with albendazole and thalidomide significantly lowered the IL-5 level and thereby strongly depressed CSF eosinophilia. The severity of meningitis was lessened to a large degree by reduction in the number of worms. The number of dead worms in the brain capable of stimulating inflammation may be reduced by alendazole/thalidomide co-therapy targeting cytokines (TNF-α, IL-1β and IL-5) may offer an effective treatment for eosinophilic meningitis.

In conclusion, the present study was designed to estimate the efficacy of combining the anthelmintic albendazole with the TNF-α inhibitor thalidomide to treat eosinophilic meningitis in a mouse model. Albendazole/thalidomide co-therapy as early as 5 or 10 days after infection significantly reduced indicators of disease, such as worm recovery, eosinophil counts, cytokines, proteolytic enzymes and CSF/serum albumin ratio. The severity of meningitis was lessened to a large degree by reduction in the number of worms. The number of dead worms in the brain capable of stimulating inflammation may be reduced by alendazole/thalidomide co-therapy.

**Figure 9.** Influence of treatment on MMP-9 activity. (a) The MMP-9 was significantly increased (*P* < 0.05) in the CSF of mice infected with *Angiostrongylus cantonensis* compared with uninfected control mice. Albendazole (ABZ) alone, thalidomide (TM) alone or albendazole/thalidomide (ABZ + TM) co-therapy beginning on day 5 PI (b) significantly reduced MMP-9 activity (*P* < 0.05). Albendazole or albendazole/thalidomide co-therapy beginning on day 10 PI (c) and beginning on day 15 PI (d) mildly reduced MMP-9 activity (*P* < 0.05), but thalidomide alone had no significant effect (*P* > 0.05). Quantitative analysis of the MMP-9 activity was performed using a computer-assisted imaging densitometer system.
thalidomide. This combination of drugs probably exploits the potential synergistic effects of lowering cytokines and PAs/MMP-9 proteolytic enzymes on eosinophilic meningitis.

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Transparency declarations

None to declare.

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