

Circulating antibodies against corneal epithelium and hookworm in patients with Mooren's ulcer from Sierra Leone

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SUMMARY A relationship between the occurrence of Mooren's ulcer and hookworm infections was suggested by Kuriakose in 1963. Sixteen patients with clinical diagnosis of Mooren's ulcer and 15 local controls from Sierra Leone were tested with respect to serum immunoglobulin levels, circulating antibody to hookworm, circulating antibodies to corneal epithelium, stool smears, and eosinophil and lymphocyte levels. Both patients and healthy controls had circulating antibodies to corneal epithelium and to hookworm. In the controls the titres of hookworm antibodies were significantly lower than in the patients, though in both groups most people had intestinal parasite infestations as detected by the stool smear. Further investigation failed to demonstrate any other significant immune alteration in the patients as compared with local controls.

Mooren's ulcer is defined clinically as a chronic, painful, peripheral ulceration that has a steep, undermined, and occasionally infiltrated leading border and that advances round the periphery towards the centre of the cornea. There appear to be 2 types of this disease. The first occurs in young adults, runs a malignant course, is frequently bilateral and responds poorly to therapy. The second type runs a milder course, is often unilateral, more responsive to therapy, and occurs in the fourth decade of life or later.¹ Mooren's ulcer of the first type seems to be the commoner in developing countries.²

The pathogenesis of Mooren's ulcer remains uncertain, though an autoimmune basis has been suggested for this disease. Circulating antibodies to corneal and conjunctival epithelium have been found in patients with Mooren's ulcer,^{3,4} and cellular immunity to corneal antigen has been described in some patients.⁵ Deposition of immunoglobulins and complement has been demonstrated by direct immunofluorescence on corneal ulcer and conjunctival biopsy specimens.⁶ These findings are suggestive of a secondary autoimmune disease, and

possible initiating factors might be: bacterial or viral infection,^{7,8} trauma,⁹ or helminthic disease.

A relationship between the occurrence of Mooren's ulcer and hookworm infection was first proposed by Kuriakose.¹⁰ He suggested that the ulcer is the result of corneal sensitivity to a toxin elaborated by hookworms. Trojan¹¹ supported this view from his observation of 34 Mooren's ulcer patients in Togo, and Majekodunmi¹² reported similar findings in Nigeria. To date no immunological studies have been performed to test this hypothesis. Our purpose in this respect was to investigate humoral immunity in 16 Mooren's ulcer patients and in 15 controls from Sierra Leone and to look for evidence of cross-reacting immunity between hookworm and corneal antigens.

Materials and methods

Sera were collected in Sierra Leone from 16 patients with clinically defined Mooren's ulcer and from 15 controls. The controls were patients who came to Lunsar Eye Hospital for refraction or minor complaints. They were included in this series only if they had no corneal abnormalities. Patients and controls were matched as near as possible for age, sex, and tribal origin.

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Both groups were examined ophthalmologically. A leucocyte count was done and the stools were screened for parasite eggs. A blood sample was drawn, allowed to clot, and the separated serum was frozen and stored at -20°C until it was shipped to Amsterdam. Immunoglobulin G, M, and A levels were determined by radial immunodiffusion assay,¹³ and immunoglobulin E levels were determined by a radioimmunosorbent assay.¹⁴

Antibodies against hookworm were detected by an indirect immunofluorescence technique on a heterologous antigen. The infective stage of *Ancylostoma caninum* (L3 stage) was obtained as described previously.¹⁵ To see positive fluorescence the metabolism of the larvae had to be arrested.¹⁶ This was done by incubating the larvae for 1 hour with 20 mM sodium azide in phosphate buffered saline

(PBS), pH 7.2, before and throughout the test. The larvae so treated were incubated with serum dilutions for one hour at 37°C . After thorough washing with PBS containing sodium azide the larvae were incubated for one hour at 37°C with a 1/20 dilution of fluorescein isothiocyanate (FITC) labelled swine anti-human immunoglobulin G (Nordic). After the excess labelled antibody had been washed off, one drop of suspension was put on a glass slide and viewed with a Leitz fluorescence microscope with epillumination. The antibody titre was expressed as the last serum dilution to show positive fluorescence.

Circulating antibodies against corneal epithelium were assayed by an indirect immunofluorescence technique on rabbit corneas. The corneas were snap frozen, sections were cut at $4\mu\text{m}$, air dried on gelatin coated slides, and fixed in acetone for 10 minutes.

Table 1 Immunoglobulin levels, *Ancylostoma caninum* antibody titres, and corneal epithelium antibodies in 16 Mooren's ulcer patients and 15 local controls from Sierra Leone, West Africa

Patients	Sex	Age	IU/ml				<i>Ancylostoma caninum</i> reciprocal antibody dilution	Corneal epithelium antibodies
			IgA	IgM	IgG	IgE		
1	M	16	ND	446	295	460	40	-
2*	F	18	130	500	360	3680	5120	+
2			91	362	273	4200	2560	+
3*	M	21	81	137	158	11 080	2560	+
4	F	23	149	264	295	6300	1280	+
5	M	24	90	190	240	1280	40	+
6*	F	26	118	583	198	2040	80	+
7	M	29	250	310	250	2220	320	+
8	M	30	90	380	230	2190	640	-
9	M	32	128	211	250	5880	40	+
10	F	35	93	209	243	260	40	+
11	M	37	162	312	303	11 730	1280	-
12*	F	38	190	211	205	2300	2560	+
13	M	38	93	185	268	6300	40	+
13			86	185	218	4700	1280	+
14	M	40	151	206	245	670	40	+
15	M	40	278	296	328	9030	320	+
16	F	60	230	256	275	270	>640	+
<i>Controls</i>								
1	F	20	180	280	240	2540	20	+
2	M	20	81	238	250	4300	160	+
3	F	21	114	251	185	180	40	+
4	F	22	135	477	183	6090	10	-
5	M	25	130	240	250	3770	20	+
6	F	25	104	193	255	2100	640	+
7	M	25	70	190	438	1400	40	+
8	M	26	109	190	550	14 000	40	+
9	M	28	100	120	240	1370	80	-
10	M	29	118	243	183	4600	>640	+
11	M	31	170	360	470	24 000	80	+
12	M	40	81	180	258	1400	40	+
13	M	45	128	119	285	16 830	80	+
14	F	66	144	243	175	450	5120	+
15	F		146	219	408	2700	80	ND
Normal Dutch donors			40-225	45-335	65-200	<100	≤10	5-6% positive

* Patients with bilateral Mooren's ulcer. ND=not detected.

After being washed with PBS the tissues were overlaid with a 1/10 dilution of serum and incubated at room temperature for 30 minutes. The slides were washed with PBS and incubated for 30 minutes in the dark with fluorescein labelled goat anti-human immunoglobulin (Nordic), diluted 1/40 with PBS. The tissues were washed thoroughly and covered with a solution of 90% glycerin and 10% PBS containing 100 mg *p*-phenylenediamine per 10 ml.¹⁷ This solution prevents fading of fluorescence when the sections are scored with a Leitz fluorescence microscope.

To determine whether there is any cross-reactivity between hookworm antigens and corneal antigens absorption studies were performed. 100 μ l serum positive for corneal epithelium and hookworm was incubated for 90 minutes at room temperature with 3×10^5 rabbit corneal epithelium cells. After centrifugation the supernatant was tested for antibodies against corneal epithelium and against hookworm.

To eliminate hookworm antibodies 200 μ l serum, positive for both antigens, was incubated for 90 minutes at 37°C with 1×10^3 *Ancylostoma caninum* L3 stage. After centrifugation the supernatant was tested for hookworm and corneal epithelium antibodies.

Statistical analysis of the data was performed by Student's *t* test and the chi-square test.

Results

Immunoglobulin levels. Immunoglobulin G and E were increased in all the patients and all the controls. Immunoglobulin M was increased in 5 patients and 2

controls, and immunoglobulin A was increased only in 3 patients. Compared with levels normal in the Netherlands, increased immunoglobulin levels were common in West Africans. There was, however, no significant difference ($p > 0.5$) between the patients and the controls for any of the immunoglobulins tested (Table 1).

Antibodies to *Ancylostoma caninum* L3 stage. With this assay a titre of 1/10 or less is considered negative for Dutch sera. All the Mooren's ulcer patients and all but one control were found to have antibodies to *Ancylostoma caninum* L3 stage. The titres, however, were higher in patients than in controls. This difference was significant ($\chi^2 = 3.87$, $p = 0.05$) when the cut off point was taken at a titre of 1/80 or less. Furthermore, 3 out of 4 patients with bilateral Mooren's ulcer had titres of 1/640 or over (Table 1).

Antibodies to corneal epithelium. Circulating antibodies to corneal epithelium were found in surprisingly high numbers in both the patient and the control groups (Table 1). Corneal epithelium antibodies were present in 13 out of 16 Mooren's ulcer patients and in 13 out of 15 controls (Table 1). The circulating antibodies were specific for corneal epithelial cells, the corneal stroma and endothelium cells showing no fluorescence above background. The finding that circulating antibodies occurred in so many normal Sierra Leoneans was very surprising. Furthermore, corneal epithelium antibodies found in some of the patients' sera and in some of the controls' sera produced a different fluorescence pattern from that found with Dutch sera containing corneal epithelium antibodies, which might be due to the fact that the sera also contain other autoantibodies (Fig. 1).

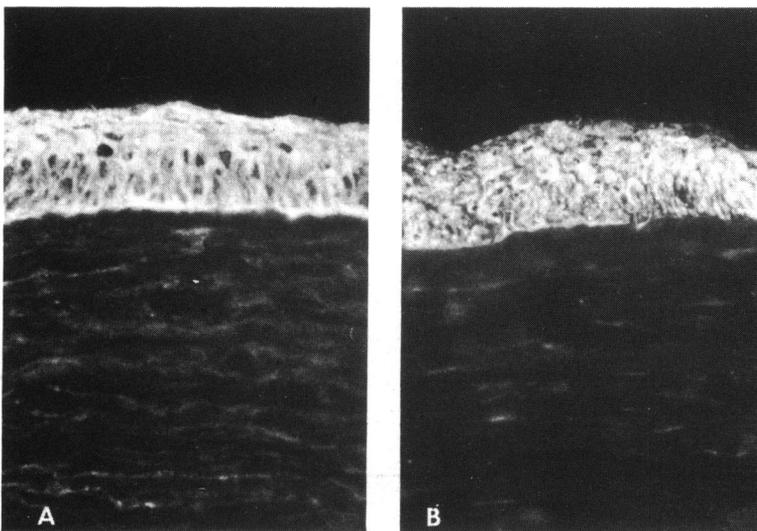


Fig. 1 (A) Antibodies to the cytoplasm of corneal epithelium as demonstrated by the indirect immunofluorescent techniques in the serum of a patient suffering from Mooren's ulcer. (B) Different fluorescence pattern found with the serum of some of Mooren's ulcer patients and some of the local controls.

Table 2 Absorption studies to determine the degree of cross-reactivity between hookworm and corneal antigens

Serum	Absorbed with:	Corneal epithelium antibodies		Ancylostoma caninum antibodies	
		Before absorpt.	After absorpt.	Before absorpt.	After absorpt.
7	Rabbit cornea epithelium (3×10 ⁵ cells)	+	-	320	320
7	Ancylostoma caninum L3 stage (1×10 ⁵)	+	+	320	0
15	"	+	+	320	0
10	"	+	+	>640	0
5	"	+	+	20	0

Absorption studies. To determine whether there is any antigenic determinant in common between hookworm antigens and corneal epithelium antigens some sera positive for both antigens were absorbed with *Ancylostoma caninum* L3 stage and one serum with scraped rabbit corneal epithelium. In both tests

the antibodies to the absorbing antigen were completely removed without affecting the reactivity to the other antigen (Table 2).

Stool examination. Hookworm eggs were commonly found both in Mooren's ulcer patients and in controls (Table 3). In fact only 2 patients and 5

Table 3 Clinical investigations in 16 Mooren's ulcer patients and 14 local controls from Sierra Leone, West Africa

Patients	Sex	Age	Ulcer, duration	Affected eye	Iris prolapse	Skin snip*	Stool examination	Differential blood count	
								% eo	% ly
1	M	16	1 (month)	R	-	-	Ascaris	4	62
2	F	18	1	R+L	-	-	Ancylostoma+	5	46
2			2	R+L	-	-	Ancylostoma	6	35
3	M	21	1	R+L	+	-	Schistosoma	14	33
4	F	23	1	L	+	-	Ascaris	15	61
5	M	24	2	L	+	-	Negative	11	38
6	F	26	12	R+L	+	-	Ancylostoma	4	25
7	M	29	1	L	+	-	Negative	17	51
8	M	30	3	L	-	-	Ancylostoma	24	48
9	M	32	2	R	-	+	Ancylostoma	11	47
10	F	35	4	L	+	-	Trichiuris	3	40
11	M	37	2	R	-	-	Ancylostoma	13	46
12	F	38	½	R+L	-	-	Ancylostoma	3	65
13	M	38	1	R	-	-	Schistosoma	6	30
13			3	R	-	-	Ancylostoma	5	54
14	M	40	3	L	-	+	Ancylostoma	2	35
15	M	40	2	R	-	+	Ancylostoma	1	49
16	F	60	1	R	-	+	Ancylostoma	2	31
Controls									
1	F	20				ND	Ancylostoma + ascaris	6	50
2	M	20				-	Ancylostoma	29	35
3	F	21				-	Schistosoma	3	35
4	F	22				-	Negative	4	51
5	M	25				ND	Ancylostoma + ascaris	10	46
6	F	25				-	Ancylostoma	25	34
7	M	25				-	Negative	6	14
8	M	26				+	Ancylostoma	7	35
9	M	28				ND	Ancylostoma	14	42
10	M	29				-	Ancylostoma	14	42
11	M	31				ND	Negative	20	48
12	M	40				-	Negative	9	50
13	M	45				-	Trichiuris	9	50
14	F	66				-	Negative	4	33

*Skin snip for oncoerca detection.
ND=not done.

controls were free of intestinal parasites, other parasites found being *Ascaris* spp., *Schistosoma* spp., and *Trichiuris* spp.

Differential leucocyte count. The percentage eosinophilic granulocytes and lymphocytes was established in patients and in controls. Approximately half the people in each group had eosinophilia and/or lymphocytosis by European standards (Table 3). However, there was no difference between values found in Mooren's ulcer patients and in controls ($p > 0.5$).

Discussion

Mooren's ulcer is a precisely defined clinical entity of unknown aetiology. There appear to be 2 types, and the cases described in this study were of the more severe form usually occurring in younger people and relatively unaffected by any kind of medical or surgical treatment. Many reports from West Africa describe similar severe cases,^{11 12 18} and a hypothesis has been put forward that there is a cause-and-effect relationship between this type of Mooren's ulcer and the presence of a hookworm infection.¹⁰

The results of our investigations show that circulating antibodies to hookworm (L3 stage) were found both in Mooren's ulcer patients and in local controls. The only difference between the 2 groups was that the antibody titres in patients were generally higher than those found in the controls, although intestinal infestation, checked by a stool smear, occurred to the same extent in both groups. The present results neither confirm nor rule out the hypothesis¹⁰ that hookworm infestation might be a causative factor of Mooren's ulcer. We cannot at present rule out the initiating role of the following factors: other-stage specific hookworm antibodies, other hookworm species, and other specific antibodies or hookworm toxins. Furthermore, we could not investigate in this series the role of hookworm antibodies belonging to immunoglobulin classes other than IgG or the involvement of cellular immunity.

Circulating antibodies to corneal epithelium have been found in many patients with Mooren's ulcer^{3 4 19} but only in 11 out of 195 (5.6%) normal healthy Dutch blood donors of blood group O, rhesus positive (H. G. M. Geertzen, personal communication). These antibodies to corneal epithelium do not cross-react with smooth muscle, parietal cells, thyroid, conjunctival epithelium, lens epithelium, or oesophagus epithelium. In the present study we found that 81% of the Mooren's ulcer patients and 87% of the local controls had circulating antibodies to rabbit corneal epithelium. An increased incidence of autoantibodies such as rheumatoid factor, cold agglutinins, anti-nuclear factor, and organ specific autoantibodies

against liver, kidney, heart, parietal cells, and thyroid has been reported in many so-called healthy individuals living in the tropics.²⁰ Epidemiological evidence^{21 22} suggests that malaria plays a role in the production of these antibodies.

There is some evidence that malaria and trypanosomiasis^{23 24} induce polyclonal activation of B lymphocytes. Malaria and *Trypanosoma gambiense* are both endemic in Sierra Leone. Therefore both the nonspecific increase of immunoglobulins G and E as well as the occurrence of corneal epithelium auto-antibodies in the healthy controls might possibly be attributed to this polyclonal B cell activation. The question remains, however, whether the observed corneal epithelium antibodies have been elicited by corneal antigens or are the result of stimulation with an unknown cross-reacting antigen. The experimental results up to now do not suggest that hookworm antigens (L3 stage of *Ancylostoma caninum*) are implicated.

Studies are still in progress further to characterise these corneal epithelium antibodies, because the immunofluorescence pattern found with some of the patients' and controls' sera is different from that found in the Dutch sera containing corneal epithelium antibodies. Another reason for suspecting that the aetiology of Mooren's ulcer in West Africa might be different from that in temperate areas is that the high immunoglobulin A levels found in many Mooren's ulcer patients^{4 18} were seen in only 3 out of 16 patients in this series.

Further clinical and experimental work in this field might reveal whether circulating antibodies to cornea epithelium, induced by an as yet unknown agent, predispose healthy individuals to Mooren's ulcer when the intact barrier of the epithelium is modified in some way.

We thank Dr H. G. M. Geertzen and Professor T. E. W. Feltkamp (Department of Autoimmune Diseases, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) for their help and suggestions concerning the detection of corneal antibodies. We thank Mrs J. C. M. Beentjes for skilled secretarial assistance.

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