Chapter 4

Antigen specific IgG4 and IgE responses in individuals infected with Oesophagostomum bifurcum and Necator americanus

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SUMMARY

Possible infections with *O. bifurcum* may not be recognised in other regions of the world, because the eggs of *O. bifurcum* and hookworm are morphologically identical. Diagnosis is mostly based on coproculture but that method has a number of serious shortcomings. First, it can be performed on fresh stool samples only and is quite fastidious and requires some practice to differentiate the larvae. Secondly, due to considerable intra-specimen and day-to-day variations in the egg output, light infections can easily be missed. Thirdly, pathology of *O. bifurcum* infections is not caused by the lumen-dwelling worm but by the immunogenic larvae encapsulated in the intestinal wall.

Diagnosis based on the detection of parasite specific antibodies would enable us to screen populations where hookworm-like eggs are commonly found in the stools. In this study, sera were collected in *O. bifurcum* endemic villages in northern Togo, and in non-endemic villages in central Togo. *O. bifurcum* specific IgG$_4$ antibodies were found in sera from northern Togo as well as from central Togo. When the serum was preabsorbed with *N. americanus* coated beads, the *O. bifurcum* specific antibodies were absent in the sera from central Togo, suggesting that there is cross-reactivity between *N. americanus*-specific IgG$_4$-antibodies and *O. bifurcum* antigen. In contrast, *O. bifurcum* specific IgE antibodies were detected in sera from individuals from northern Togo, but not in central Togo.

INTRODUCTION

In northern Togo, infections with hookworm (*Necator americanus*) and *Oesophagostomum bifurcum* are highly endemic (Polderman et al., 1991, Pit et al., 1999b). The exact life cycle of *O. bifurcum* is unknown, but accumulating evidence suggest that after oral ingestion, the L3 larvae encapsulate in the intestinal wall, where they might go into arrested larval development (ALD) for a more or less prolonged period of time, before re-entering the intestinal lumen. In the lumen the larvae rapidly mature to young adult worms, mate and start egg production (Polderman et al. 1991). In some patients later larval stages, i.e. immature juvenile worms, can be found in the bowel wall. It is not sure whether these larvae failed to return to the lumen or reinvaded the intestinal wall at a later stage. These juveniles induce a highly immunological reaction and cause pathology known as “Tumeur de Dapaong”.

41
Diagnosis based on detection of *O. bifurcum* and hookworm eggs is not parasite specific, therefore a coproculturing has been developed, such that the infective larvae of both nematodes can be differentiated through their morphological features (Blotkamp *et al.*, 1993). This method, which only detects infections with adult lumen-dwelling worms, is quite fastidious and requires some practise to differentiate the larvae. As a result possible infections with *O. bifurcum* might not be recognised in other hookworm-endemic regions of the world. In addition, due to considerable intra-specimen and day to day variations in the egg output, light infections can easily be missed (Pit *et al.*, 1999a), and fresh stool samples are required. Infections with tissue-dwelling larval stages only, as frequently seen in clinical cases, are also missed in coproculture. Diagnosis based on the detection of parasite specific antibodies would enable us to screen populations where hookworm like eggs are commonly found in the stools, and to detect clinical cases where the larvae are still encapsulated in the intestinal wall.

A recent study has shown that chronic infections with *O. bifurcum* as well as with hookworm induce a Th2-type immune response (Pit *et al.*, manuscript in preparation), generally associated with raised levels of IgE and IgG4 (Mosmann & Coffman, 1989).

A IgG4-specific enzyme linked immunosorbent assay (ELISA) was developed to diagnose human infections with *O. bifurcum* (Polderman *et al.*, 1993). Observations on small numbers of samples suggested that specificity and sensitivity were quite satisfactory but, the precise sensitivity could not be determined and possible cross-reactivity between *O. bifurcum* and *N. americanus* antibodies could not be excluded.

The aim of the present study was to evaluate the advantage of the IgG4-specific ELISA as a diagnostic tool to determine the prevalence of infection with *O. bifurcum* in two different populations in Togo: one in which both *O. bifurcum* and hookworm are transmitted and another one with endemic hookworm infections only.

The applied immunodiagnosis was less specific than expected, and there was cross-reactivity between *N. americanus* specific IgG4 and *O. bifurcum* antigen. Pritchard and Walsh (1995) reported that IgE antibodies showed minimal cross-reactivity against antigens from different nematode species. Similarly, there
was significantly less cross-reactivity with heterologous parasite antigens in the IgE antibody response to filarial infection than in the corresponding IgG antibody response (Weiss et al, 1982). We therefore studied the possibility to measure IgE antibodies against *O. bifurcum* in sera from individuals in Togo as a specific diagnostic tool.

**MATERIAL AND METHODS:**

*Study population and parasitological examination.*

Serum and stools were obtained from volunteers coming from *O. bifurcum* and *N. americanus* endemic villages around Dapaong and from volunteers from villages from Central Togo, where *N. americanus* is highly endemic but were *O. bifurcum* infections have never been found despite extensive parasitological survey's. Informed consent was obtained from the patients or their parents. The parasitological and demographic characterisation of the population is given in table 1.

<table>
<thead>
<tr>
<th>Village</th>
<th>km from Dapaong</th>
<th>n=</th>
<th>male/female</th>
<th>Median-age (range)</th>
<th>% infected with</th>
<th>O bifurcum</th>
<th>N.americanus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Gossiété</td>
<td>22</td>
<td>40</td>
<td>21/20</td>
<td>24 (7-65)</td>
<td>80</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>2 Sintodi</td>
<td>25</td>
<td>45</td>
<td>21/24</td>
<td>26 (5-69)</td>
<td>27</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>3 Tonte</td>
<td>14</td>
<td>50</td>
<td>27/23</td>
<td>28 (6-61)</td>
<td>48</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>4 Ogaro</td>
<td>35</td>
<td>38</td>
<td>21/17</td>
<td>24 (5-70)</td>
<td>46</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>5 Lotogou</td>
<td>27</td>
<td>23</td>
<td>9/14</td>
<td>23 (11-57)</td>
<td>26</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>6 Galé</td>
<td>21</td>
<td>30</td>
<td>24/6</td>
<td>38 (20-70)</td>
<td>0</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>7 Mogou</td>
<td>75</td>
<td>34</td>
<td>21/13</td>
<td>32 (10-55)</td>
<td>0</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>8 Sokodé</td>
<td>280</td>
<td>38</td>
<td>1/37</td>
<td>28 (17-43)</td>
<td>0</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>9 Sagbadai</td>
<td>300</td>
<td>46</td>
<td>32/14</td>
<td>39 (11-71)</td>
<td>0</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>10 Dutch donors</td>
<td>30</td>
<td>Unknown</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Sera from Dutch anonymous donors were used as a reference. Infections with *O. bifurcum* and *N. americanus* were detected by stool cultures as previously described (Polderman *et al.*, 1991). Briefly, three grams of stools from all individuals (except the anonymous Dutch donors) were cultured in the moist environment of a petri-dish. After seven days egg-hatched larvae were collected, identified as *O. bifurcum* or *N. americanus* and counted.

**Preparation of *O. bifurcum* (Oesag) and *N. americanus* (Necag) antigens**

Following treatment of patients with pyrantel pamoate and purgation, adult worms of *O. bifurcum* and *N. americanus* were isolated as described by Polderman *et al.* (1991). Isolated adult worms were extensively washed in PBS (Phosphate-buffered saline, pH 7.6), lyophilised, ground with a mortar and pestle and then extracted in 0.035 M PBS overnight with gentle stirring at 4°C. The protein concentration was determined with a BCA protein assay (Pierce).

**Sero logical assays.**

*O. bifurcum*- or *N. americanus*-specific IgG in patients’ sera was determined as described by Polderman *et al.* (1993).

For the determination of *O. bifurcum*- or *N. americanus*-specific IgE in patients, sera were preabsorbed with protein G (Pharmacia, Uppsala, Sweden) to eliminate IgG antibody competition, as previously described by Quinnell *et al.* (1995). Briefly, diluted sera (1:4) in 0.035M PBS (pH 7.8) were incubated on a rotor with an equal volume of Protein G at 4°C overnight. Thereafter, samples were centrifuged for 15 min and supernatants collected. Microtitration plates (Maxisorb, Nunc) were coated with *O. bifurcum*- and *N. americanus*-specific antigen at 5 μg protein/ml in 0.1M sodium carbonate buffer (pH 9.6) overnight at 4°C (*Oesag* and *Necag* respectively). Plates were blocked with PBS containing 1% bovine serum albumin for 1 h at 37°C, then washed with 0.035M PBS and preabsorbed serum (final dilution 1:40) was added in duplicates and incubated at 4°C overnight. After washing (as above), anti-human IgE mouse monoclonal antibody (Sigma) was used, followed by AP-conjugated rabbit anti-mouse antibody (Sigma) (1 h at 37°C), and after incubation with p-nitrophenyl phosphate (pNPP) for 1 h at room
temperature absorbance was read at 405 nm. To correct for assay variation, results were expressed as ratios between the absorbance values of samples and defined control sera.

**Preabsorption of the sera with N. americanus antigen (Necag)**

Beads in sepharose gel were activated with Cyanogenbromide (CnBr) after having been washed with bidest on a glass filter. After a second washing the beads were incubated with N. americanus-antigen (Necag, 1mg/ml) at room temperature overnight with gentle rocking. The coupled beads were then washed with PBS and deactivated overnight at room temperature in a solution of 0.5M 4-aminobutyric acid (C₄H₉N0₂) and 0.05 M Na₂CO₃ (pH 10). After being washed in PBS, the coupled beads were stored in the refrigerator until use.

For the absorption of N. americanus specific antibodies in the serum, the coupled beads were washed twice and incubated in overnight at 4°C with 1/40 diluted serum, while rocking. The next morning the serum and coupled beads mixture was centrifuged and the supernatant (= pre-absorbed serum) was used in an O. bifurcum-specific-IgG4-ELISA as described before (Polderman et al, 1993).

**RESULTS:**

**IgG4 antibodies against O. bifurcum and N. americanus.**

O. bifurcum specific IgG₄ levels were determined in patients from six villages in the O. bifurcum endemic area in northern Togo and in control patients from three villages in the non-endemic region of central Togo. Subjects from the Oesophagostomum-endemic area showed high levels of IgG₄ against O. bifurcum antigen in their serum. The prevalence of infections measured in northern Togo with ELISA was higher than measure with the stool cultures (respectively 73% and 40% positive). In Galé, a village where all coprocultures remained negative for Oesophagostomum, 14 out of 30 subjects tested were IgG4-positive. Low levels of reactivity were seen in two of the non-endemic villages but not in the third one, where 18 out of 44 were IgG4 positive. (figure 1a).
Fig. 1: Absorbance values in ELISA specific for IgG4 (a) and IgE (b) against O. bifurcum antigen in serum from endemic and non-endemic patients. The prevalence of infection with O. bifurcum of the patients-groupe per village is given in the central graph. The characteristics of the 10 patients groups are given in Table 1.
Fig. 2: Absorbance values in ELISA specific for IgG4 (a) and IgE (b) against *N. americanus* antigen in serum from *O. bifurcum* endemic and non-endemic patients. The prevalence of infection with *N. americanus* of the patients-group per village is given in the central graph. The characteristics of the 10 patients groups are given in Table 1.
In total 20% of the patients from central Togo showed IgG4 reactivity against *O. bifurcum* antigen. Subjects of both populations had high levels of specific anti-hookworm IgG4 (figure 2a), and prevalence of infection measured by ELISA (84% positive) was higher than by stool culture (71% positive). No reactivity was seen in the Dutch controls, neither for *Oesophagostomum* nor for hookworm.

Preabsorption of the serum with *N. americanus* antigen.

Preabsorption of the sera with *N. americanus* antigen showed that *N. americanus* specific antibodies were binding *Oesophagostomum* antigen (figure 3). Indeed, the levels of *O. bifurcum* specific IgG4 antibodies remained high in the sera from patients from northern Togo, after absorption with *N. americanus* antigen. *O. bifurcum* specific IgG4 levels from patients from central Togo, on the other hand, decreased after absorption of the sera with *N. americanus* antigen.

![IgG4 levels against Oesag in sera before and after absorption with Necag](image)

*Fig. 3: Absorbance values in ELISA specific for IgG4 against O. bifurcum in normal serum and in serum pre-absorbed with N. americanus antigen. The straight line corresponds to values found in sera from individuals from northern Togo, the dotted line to serum from individuals from central Togo.*
IgE antibodies against *O. bifurcum* and *N. americanus* antigen

*O. bifurcum* specific IgE antibodies were detected in the serum from individuals from northern Togo, but much less in central Togo (figure 1b). Prevalence of infections measured in northern Togo by ELISA (37%) was similar to the prevalence found by coproculture (40%). In central Togo IgE antibodies against *O. bifurcum* were detected in only 6% of the patients. *N. americanus* specific IgE antibodies were detected in serum of patients from both northern and central Togo (figure 2b). But on a population level prevalence of infection measured by ELISA was lower than when measured by coproculture (respectively 45% and 53% positive).

**DISCUSSION**

IgG4 is known to be a marker of (chronic) antigen exposure, in particular to chronic helminth infections (Aalberse et al., 1985). In a previous study, Polderman et al. (1993) described an ELISA to diagnose IgG4 antibodies against *O. bifurcum* in humans. Small scale testing suggested the procedure to be fairly specific and sensitive but more extensive experience was required. Therefore it has been our first choice antibody for the development of a diagnostic method based on a parasite-specific ELISA. Our current results, however, show that some patients from a neighbouring, non-endemic region also had high serum levels *O. bifurcum*-specific IgG4 antibodies. These serological results are inconsistent with our parasitological results from those patients.

The lack of specificity could be explained in different ways. First, it is likely that a nematode ill adapted to the human host, like *Oesophagostomum* species may be able to invade patients without reaching adulthood. Coproculture remains negative but serology may become positive. The high rates of false positive findings in Gale, in the *oesophagostomum* endemic region, should perhaps be explained in this way but the high rates of specific IgG4-positivity in one village in central Togo is difficult to explain.

Alternatively, cross-reactivity between antigens of different parasite species may occur, in the test system used. Indeed, such cross reactivity has been demonstrated for many parasites (Weiss et al., 1982; Correa-Olivera et al., 1988). Our absorption studies on the IgG4 antibodies also showed cross-reactivity between *N. americanus* specific IgG4 and *O. bi-
furcum antigen. Of course, all sera could be preabsorbed with N. americanus-coated beads before performing an O. bifurcum-specific ELISA, but this is time as well as antigen consuming, thus not applicable on a large scale. Sera could not be preabsorbed with N. americanus directly (data not shown). Therefore IgG4 cannot be used as a specific antibody for the diagnosis of O. bifurcum infections in a hookworm endemic area.

Greater specificity of IgE for helminth infections has been reported previously (Pritchard & Walsh, 1995; Ganguly et al., 1988; Weiss et al., 1982). Our results also indicate a lesser degree of cross-reactivity between N. americanus specific IgE antibodies and O. bifurcum antigen. Unfortunately sensitivity and specificity could not be calculated because the parasitological diagnosis is not sufficiently reliable. However, our data suggest that the IgE specific ELISA is, although more specific, not very sensitive. On an individual level parasitological and IgE levels do not correspond, but on a village level the prevalence of infection measured with both diagnostic tools are comparable. It is still remarkable and unexplained why there was no cross-reactivity in other regions (Ghana, Zaire) measured previously (Polderman et al., 1993), when there obviously was cross-reactivity in this area.

It was not possible to evaluate the value of measuring levels of IgG4 and IgE against N. americanus, because hookworm is endemic over the whole region and it’s not possible to find endemic controls.

In conclusion, the use of an IgG4 specific ELISA in hookworm endemic area’s for the diagnosis of O. bifurcum is hampered by the cross-reactivity between N. americanus specific IgG4 and O. bifurcum antigen. The IgE specific ELISA against O. bifurcum is more specific but should be used on a village level rather than on an individual level.

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