The filarial parasite *Onchocerca volvulus* is the causative agent of river blindness. The adult worms produce microfilariae (mf), which are responsible for the disease pathogenesis; mf activate the complement system, but the activation stops before the formation of terminal complement complexes. Because of the arrest of complement activation, this study analyzed binding of the main alternative pathway regulator, factor H (fH), to the mf. The mf bound fH after incubation in nonimmune human serum or with purified radiolabeled fH. In the presence of factor I, mf-bound fH promoted the cleavage of complement 3 molecule b (C3b) to iC3b. An analysis with recombinant constructs of fH showed that the C-terminal short consensus repeats (SCRs) 8–20 of fH bound to mf, whereas the N-terminal SCRs 1–7 containing the complement-regulatory domains in SCRs 1–5 did not. Thus, mf of the nematode *O. volvulus* may evade human complement by binding fH and by promoting inactivation of C3b into iC3b.

The filarial parasite *Onchocerca volvulus* (discovered by Leukart in 1893), the causative agent of river blindness, infects ~17 million people in Africa, Central America, Saudi Arabia, and Yemen [1]. The infective L3 stage larvae are transmitted to the human host by female *Simulium* blackflies and develop into adult worms that reside in fibrous subcutaneous nodules for up to 15 years. The female worm gives birth to a large number of microfilariae (mf) that persist in the human host for up to 2 years. Mf migrate into the skin, subcutaneous tissues, and eyes [2] and are considered to be responsible for the pathogenesis of onchocerciasis. A prolonged persistence of mf causes blindness and severe skin disorders. Ivermectin is the drug of choice and, when given as a single dose once a year, is effective in the eradication of mf. However, the only current treatment that destroys adult worms is surgical removal of the nodules [3].

The alternative pathway (AP) of complement (C) is an important part of the innate immune defense system against microbes [4]. Covalently bound activation products of C3 molecules (C3b and iC3b) on microbial surfaces act as ligands for C3 receptors on neutrophils, macrophages, and eosinophils. C3b deposition amplifies complement activation to allow generation of AP C3 convertases (C3bBb), and, eventually, cytolytic membrane attack complexes. It is not known whether mf of *O. volvulus* activate the human complement system or whether mf are able to evade complement attack during their prolonged survival in the human host. Nevertheless, the complement system is likely to be involved in the human defense against mf because the addition of fresh nonimmune human serum as a source of complement to immune serum increases eosinophil-mediated killing of *O. volvulus* mf in vitro [5]. Complement, together with specific antibodies, is required for opsonization and phagocytosis of other pathogenic mf species (e.g., *Brugia malayi* and *Dipetalonema vitaea*) [6–8]. Studies of the pathogenic *O. volvulus* nematode and its mf have been hampered because the parasite cannot be cultivated in vitro. Thus, all studies with *O. volvulus* mf must be done with material obtained from fresh adult worms taken from patients.

Factor H (fH) is the main fluid-phase regulator of the alternative complement pathway. It consists of 20 domains, called short consensus repeats (SCR) or complement control protein modules (CCP). Each SCR domain has ~60 amino acids. fH promotes the cleavage of surface-bound C3b to inactive iC3b by acting as a cofactor for the plasma serine protease factor I (fI) [9, 10]. In addition, fH accelerates the decay of the AP C3 convertase, C3bBb, and competes with factor B for binding to C3b. Through all of these regulatory activities, fH restricts AP amplification and depletion of AP components in the fluid phase.

Some pathogenic bacteria such as *Streptococcus pyogenes* [11], *Neisseria gonorrhoeae* [12–14], and *Borrelia burgdorferi* [15–17] bind fH to their surfaces to acquire resistance against complement attack. When bound to bacteria, fH protects them...
from phagocytosis and direct complement killing. So far, only 1 parasitic helminth cestode, *Echinococcus granulosus*, has been shown to utilize host fH for the inactivation of C3b by fI in the bovine system [18]. In the present study, we assessed whether *O. volvulus* mf activate complement AP. In addition, we analyzed the ability of mf to utilize fH in C regulation.

**Materials and Methods**

**Isolation of *O. volvulus* mf.** Onchocercomas (i.e., nodules containing adult *O. volvulus* worms) were extirpated from patients with onchocerciasis (Guinea, West Africa), as described elsewhere [19]. To obtain mf, female worms were isolated from freshly collected nodules [20]. The isolated mf (~150 mm long) were prepared directly from the adult females; transferred into RPMI 1640 medium supplemented with l-glutamine (2 mM), penicillin (10 IU/mL), and streptomycin (100 mg/mL; Gibco); and immediately frozen with liquid nitrogen. For each assay, we thawed and washed with RPMI 1640 medium or an appropriate buffer the required amount of mf, depending on the assay.

**Serum samples, antibodies, and purified complement proteins.** Normal human serum (NHS) samples were obtained from healthy laboratory personnel with no history of travel to areas endemic for onchocerciasis. The serum samples were stored at ~70°C. Normal mouse serum was obtained from healthy laboratory mice (BALB/c) and kept at ~20°C. For immunofluorescence detection of human C1q, a rabbit polyclonal antibody (Dako) was used. iC3b was detected with a mouse monoclonal antibody (MAb) against an iC3b neoeptipeptide (Quidel). For detection of fH, the 196X MAb was produced and purified as described elsewhere [21]. A neoeptipeptide on C5b-9 was detected with the WU13/15 antibody (provided by R. Würzner, University of Innsbruck, Innsbruck, Austria). As a control, we used an unrelated B cell lymphoma idiotype-specific AF1 antibody. We used a polyclonal fluorescein isothiocyanate (FITC)–conjugated goat anti–mouse IgG antibody (Alexa 488; Molecular Probes) and a polyclonal FITC-conjugated goat anti–rabbit IgG (Dako). Antibodies used in the terminal complement component (TCC) ELISA were mouse anti–SC5b-9 and goat anti-C5 and -C6 (Quidel) and horseradish peroxidase-conjugated donkey anti–goat IgG (Santa Cruz Biotechnology). We used a polyclonal goat anti–βH2-globulin (DiaSorin) as an fH-neutralizing antibody in the cofactor assays.

**Complement components fH and C3 were purified from human plasma, and C3b was generated with factors B and D in the presence of Ni2+ ions, as described elsewhere [22, 23]. Recombinant constructs of fH (SCR 1–7 and SCR 8–20) and human fH-related protein (fHR)–C were cloned, expressed in the baculovirus system, and purified as described elsewhere [21, 24–26]. fH was purchased from Calbiochem. C3b and the purified constructs were labeled with 125I by using the Iodogen method (Pierce Chemical) [27].**

**ELISA for C3a and the TCC.** We used a C3a EIA kit (Quidel) to measure the amount of C3a generated by *O. volvulus* mf. The mf were pretreated with either trypsin (3 mg/mL; Sigma Chemical) or PBS at 37°C for 45 min. The activity of trypsin was stopped by incubation (37°C, 5 min) with soybean trypsin inhibitor (6 mg/mL; Sigma). We washed the mf 5 times with RPMI 1640 medium before incubation in 50% NHS (~1000 mf/75 μL NHS/treatment) for 15 min at 37°C on a shaker. Complement activation was stopped by adding EDTA to a final concentration of 10 mM. All samples were centrifuged for 2 min at 7700 g, and the supernatants were frozen to ~20°C. The supernatants were diluted 1:1000 into the manufacturer’s sample buffer and subjected to ELISAs. NHS activated with inulin (0.1 mg/mL, 15 min, 37°C; Sigma) was used as a positive control. After the ELISA, results were calculated according to the manufacturer’s instructions.

For the TCC ELISA, MaxiSorp microtiter plates (Nalge Nunc) were coated overnight with a MAb specific for the SC5b-9 neoeptipeptide (5 mg/mL; Quidel) in a coating buffer (15 mM Na2CO3 and 35 mM NaHCO3, pH 9.6) at 4°C. The wells were washed 5 times with PBS containing 0.05% Tween20 (PBS-T). The samples were diluted 1:200 or 1:1000 in PBS-T, and 100-mL aliquots were placed in the wells. After a 60-min incubation at 22°C, the wells were washed 5 times with PBS-T. A mixture of polyclonal goat–anti-C5 and –anti-C6 antibodies, diluted 1:1000 in PBS-T, was added (100 mL/well), and the plates were incubated for 1 h at 22°C. After the wells were washed 5 times with PBS-T, a peroxidase-conjugated secondary donkey anti–goat IgG antibody, diluted in PBS-T and supplemented with 1% normal mouse serum (100 mL/well), was incubated in the wells for 60 min at 22°C. After further washings, the substrate o-phenylenediamine (Dako), diluted in H2O and supplemented with 0.04% H2O2, was added. After a 15-min incubation at 22°C, the reaction was stopped by addition of 50 mL of 2 M H2SO4 per well. Absorbances were read with an ELISA reader with a 492-nm filter. Purified SC5b-9 [28] was used to set up the standard curve.

**Immunofluorescence microscopy.** We incubated mf (~1000/mf) in 40 μL of nonimmune NHS or RPMI 1640 medium for 45 min at 37°C on a shaker and washed them 3 times with RPMI 1640 medium. The primary antibodies (anti-C1q, anti-iC3b, anti-fH, anti-C5b-9, or the control, MAb AF1) were added at a concentration of 10 μg/mL and incubated with the mf for 30 min at 37°C. After the samples were washed 3 times with RPMI 1640 medium, we added the secondary FITC-conjugated goat anti–mouse IgG or goat anti-rabbit at a 1:200 dilution, and the samples were further incubated for 45 min at 37°C. The stained samples were mounted with Mowiol [29] and examined by an Olympus BX50 microscope equipped with a filter specific for FITC fluorescence. The samples were photographed with a Spot RT slider digital camera and processed with Spot RT software (version 3.0; Diagnostic Instruments).

**Protein binding assays.** fH, the recombinant forms of fH representing SCRs 1–7 and 8–20, and FHR-4 were radiolabeled by using the Iodogen method (Pierce Chemical). mf (~1000 in each assay) were washed once with 1:3 veronal-buffered saline (VBS; 50 mM NaCl and 3.3 mM diethyl barbiturate [pH 7.5]) and incubated with the radiolabeled proteins (60,000 cpm/assay) in a total volume of 100 μL in 1/3 GVB (VBS containing 0.1% gelatin) for 25 min at 37°C on a shaker (550 rpm). Particle-associated and free-radioactive proteins were separated by centrifuging the samples through a 200-μL column of 20% sucrose in GVB (3 min, 15,000 g). Radioactivities in both pellets and supernatants were counted, and the binding of proteins was calculated as a percentage of the total protein input. fH incubated with the mf served as a background control. The background was not subtracted from the values. All experiments were performed in duplicate.
Effect of heparin on fH binding.  The effect of heparin on the binding of fH to O. volvulus was assayed by incubating mf with labeled fH in the presence of heparin (100 IU/mL). Binding of fH was detected as described above. Heparin (5000 IU/mL) was from Lövens Kemiske Fabrik.

Assay for cofactor activity  Cofactor activity for cleavage of 125I-labeled C3b in the presence of purified fH was determined as described elsewhere [30]. mf were preincubated for 30 min at 37°C in either NHS or with purified fH (200 mg/mL) in RPMI 1640 medium and washed 5 times with RPMI 1640 medium. Thereafter, the mf were incubated (120 min at 37°C) with 125I-labeled C3b (~50,000 cpm/reaction) and fI (30 ng/reaction). The total reaction volume was 40 mL. 125I-labeled C3b incubated with fH (5 mg/reaction) and fI served as a positive control. To detect possible intrinsic cofactor activity of the mf, they were incubated with 125I-labeled C3b and fI.

In an inhibition assay, a polyclonal goat–anti-fH antibody was equilibrated for 5 min with the mf (1/3 of reaction volume) before addition of fH or NHS. After the incubations, the samples were centrifuged and the supernatants were treated with 2.5% β-mercaptoethanol and analyzed by SDS-PAGE [31]. The gels were fixed by use of 10% acetic acid for 10 min, dried, and subjected to autoradiography.

Results

Complement activation by O. volvulus mf.  To learn whether O. volvulus mf activate the complement system, they were incubated in nonimmune NHS. The amount of C3a generated was measured by use of ELISA after a 15-min incubation at 37°C. mf incubated in NHS (1000 mf/20 μL of NHS) generated 134 ± 3.2 ng/mL (mean ± SD) of C3a, whereas incubation of NHS with buffer alone yielded 47 ± 1.5 ng/mL C3a (figure 1). When NHS was activated with inulin (0.1 mg/mL), the amount of C3a was 245 ± 13.3 ng/mL. Thus, after incubation in human serum, the mf induced a low level of cleavage of C3 to C3a and C3b. To test whether surface proteins of the mf affected the extent of complement activation, mf were pretreated with trypsin, which cleaves polypeptides from the surface of mf. Trypsin treatment reduced the amount of C3a (80 ± 11.5 ng/mL), compared with mf incubated in NHS (figure 1).

To study whether complement activation by the mf leads to formation of TCC, TCC was measured in the above samples by ELISA. After a 15-min incubation in serum, the mf generated similar amounts of TCC (3.5 ± 0.5 mg/mL) as those spontaneously generated in NHS (3.0 ± 0.4 mg/mL) (figure 2). Trypsin treatment of mf, however, increased the generation of TCC about 2-fold (3.5 ± 0.5 mg/mL to 6.9 ± 0.7 mg/mL). When the mf were incubated in serum for a longer period (45 min), the amounts of TCC were higher, but the trend was similar: 18 mg/mL for NHS alone, 18.8 mg/mL for mf incubated in NHS, 22.7 mg/mL for trypsin-treated mf, and 60 mg/mL for inulin-activated NHS. From these experiments, we conclude that mf cause some C3 cleavage, but further complement activation seems to be prevented because no increase in the formation of the TCC complex occurs.

To analyze possible complement activation on the surface of mf, we incubated live mf in NHS (or in RPMI 1640 medium as a control) and examined deposition of different complement components by immunofluorescence microscopy. To detect activation of the classical pathway (CP) of the complement, we assayed deposition of C1q, the first component of the CP, to the surface of the mf, but no deposition was seen (data not shown) when mf were incubated in nonimmune NHS.

By using immunofluorescence microscopy, we were able to test directly the generation of iC3b, an inactive cleavage product of C3b, and the terminal complex C5b-9 on the surfaces of mf. Appearance of the iC3b neoepitope occurred on the surfaces of all the mf (figure 3A), but staining for C5b-9 was negligible. Only some distinct patches on the surface of mf became positively stained for C5b-9 (figure 3B). These results suggested that the mf activated C3 to C3a and C3b in NHS to some extent, but the C3b that was deposited on the mf had become converted to inactive iC3b and no longer supported complement activation.

Because it appeared that complement activation was arrested (at or beyond) the C3 stage, we tested whether the mf were able to acquire plasma complement regulators at their surfaces. By use of immunofluorescence microscopy, we observed binding of fH to the surface of mf after incubation in NHS (figure 3C). Staining with a control antibody (AF1) was negative (figure 3D), and no staining was seen with the conjugated secondary antibody alone (figure 3E). During the serum incubation, deposition of C3b may contribute to the secondary binding of fH. Thus, we next assessed direct binding of fH to the mf surface.

Binding of fH to mf and location of the mf binding region on fH.  By using radiolabeled fH, we tested whether O. volvulus
mf bound purified fH, the main fluid-phase inhibitor of the alternative complement pathway, also in the absence of serum. After a 15-min incubation of 125I-labeled fH with 1000 mf, ~1.1% of the offered fH bound to the surfaces of mf; the background was ~0.3% (figure 4).

To determine the region on fH responsible for the binding, radiolabeled recombinant constructs of fH encompassing either the N-terminal SCRs 1–7 or the C-terminal SCRs 8–20 were analyzed in the binding assay. As shown in figure 4, the SCRs 8–20 of fH bound better (1.6%) to the mf than did the SCRs 1–7 (0.56%). Because the fH-related protein FHR-4 has 2 C-terminal SCRs that are almost identical to domains of fH (SCRs 19 and 20 of fH), we also tested its binding to mf but observed that it did not bind any better than the fH SCR 1–7 construct (0.6%). From these results, we concluded that fH can bind directly to O. volvulus mf and that binding is mediated via SCRs 8–20.

To analyze whether the binding of fH is charge dependent, we assayed whether heparin directly inhibits surface deposition of fH to O. volvulus. As shown in figure 4, heparin (100 IU/mL) did not inhibit binding of fH. This result suggests that the binding is not due to only a charge interaction between fH and the O. volvulus cuticle.

Functional consequences of fH binding. fH acts as an inhibitor of the AP amplification cascade partly by acting as a cofactor for fI in cleaving C3b into iC3b. To test whether the fH molecules that have been bound to the O. volvulus mf are functionally active, we assayed their cofactor activity. The mf were first incubated either in NHS or with purified fH, followed by careful washing and an incubation with 125I-labeled C3b in the presence of fI. Cleavage of C3b to iC3b was detected as a conversion of the C3b α-chain into fragments of 68 and 46/43 kDa by SDS-PAGE and autoradiography. As shown in figure 5, mf incubated in

![Figure 2. Generation of terminal complement complexes (TCC) by trypsin-treated and native Onchocerca volvulus microfilariae (mf). About 1000 mf were incubated in normal human serum (NHS) for 15 min at 37°C, and TCC levels (mg/mL) were quantified by ELISA. As a negative control, no mf were added (NHS only); as a positive control, NHS was activated with 0.1 mg/mL inulin. Data are mean ± SD.](image)

![Figure 3. Immunofluorescence microscopy analysis of iC3b neoepitope generation, terminal complement component formation, and factor H (fH) binding to the surface of Onchocerca volvulus microfilariae (mf). Live mf were incubated in normal human serum (NHS) or RPMI 1640 medium (45 min, 37°C). After washing monoclonal antibodies against the complement component iC3b neoepitope, the terminal C5b-9 complex or the regulator fH was added. As a control, an unrelated antibody (AF1) or no primary antibody was used. Note generation of iC3b and binding of fH to the mf surface. Only limited amounts of C5b-9 formed on the parasite surface.](image)
NHS (lane 3) were clearly able to promote the cleavage of C3b in the presence of fI.

To examine possible intrinsic cofactor activity on the mf, they were incubated with fI alone, but no cleavage of 125I-labeled C3b was observed (figure 5, lane 1). This also ruled out the presence of any microfilarial proteases that would cleave C3b. In addition, when the mf were preincubated in serum, washed, and incubated with both 125I-labeled C3b and fH in the absence of fI, no cleavage of C3b occurred (figure 5, lane 2). This showed that fI was not absorbed from serum to the mf surface. In the presence of a polyclonal anti-fH antibody, the cleavage of 125I-C3b was prevented (figure 6), showing that the cofactor activity was specific for fH.

Discussion

In this study, we observed that O. volvulus mf can bind the human complement regulator fH to their surfaces in a functionally active form. Thereby, the mf form of this pathogenic nematode can promote the cleavage of C3b into inactive iC3b and potentially evade the destructive consequences of complement activation and subsequent phagocyte attack, particularly from eosinophil. To our knowledge, this is the first time such a mechanism has been identified for any nematode. In general, the binding of fH is an effective way of protecting against the AP amplification cascade, phagocytosis, and direct complement killing.

O. volvulus mf cause the pathologic manifestations of the disease onchocerciasis, river blindness and skin eruptions. Thus, the immunologic reactions that mf elicit in the human host are of great interest. For eradication of the disease, the microfilarial stage of the parasite provides the target for vaccination because the mf are transmitted back to black flies, which eventually transfer the infection to a new host. However, the efficiency of any vaccine is at least partly dependent on the immune resistance mechanisms of the target organisms.

Parasites use several different mechanisms to control complement activation on their surfaces [32]. For example, Schistosoma mansoni trematodes secrete proteolytic enzymes that can cleave complement components C3 and C9 [33], express the putative CD55-like molecule schistosoma complement inhibiting protein–1 [34], and acquire glycosylphosphatidylinositol-anchored inhibitors like CD55 to their surfaces [35]. They also express the complement receptor schistosoma trispanning orphan receptor, which binds C2 and thereby inhibits CP activation [36]. It is apparent that, for prolonged survival in human tissues, the O. volvulus mf must have developed means to escape complement and phagocyte attack, and multiple mechanisms may be needed.

Lack of C1q binding and cleavage of the C3 protein by O. volvulus mf in nonimmune human serum suggest that mf moderately activate the AP. However, the observed cleavage of C3 into C3a and C3b (figure 1) could, in part, have been caused by a protease, especially as free enzymes have been found on the surface of mf [37]. Since the C3b molecules that were formed on the surface of mf became inactivated into iC3b, the activation did not progress to the TCC level. This suggests that the amplification cascade of complement activation operated only to a limited extent. Because only negligible amounts of C5b-9...
O. volvulus mf Avoid Complement Attack

Figure 6. Inhibitory effect of a polyclonal anti–factor H (fH) antibody on the C3b cleavage promoting activity of fH on Onchocerca volvulus microfilariae (mf). About 1000 mf were mixed first with fH alone (lane 1) or with goat anti–human fH and fH (lane 2). After a wash, mf were incubated in the presence of factor I (fI) and 125I-labeled C3b. Mf-bound fH remains active in promoting 2 fI-induced cleavages of the α’-chain of C3b. By inhibiting fH, the polyclonal anti-fH antibody prevents C3b cleavage.

In conclusion, O. volvulus mf bind the human complement regulator fH to their surfaces in a functionally active form. This is a potential immune resistance mechanism for O. volvulus mf. Neutralization of an immune evasion mechanism (e.g., by vac-
cination) could assist in the eradication of this parasite worm that still is an important cause of blindness and other morbidity in developing areas.

References

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