Human Autoantibody to Defensin: Disease Association with Hyperreactive Onchocerciasis (Sowda)

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Summary

Chronic hyperreactive onchodermatitis (sowda) is a severe form of onchocerciasis observed in a subset of individuals infected with the filarial nematode *Onchocerca volvulus*. SDS-PAGE and immunoblot analyses of *O. volvulus* adult worm extracts were used to characterize the antigens of the marked antibody response of sowda patients. One 2.5-kD antigen was recognized by sera from all 35 (100%) sowda patients that were studied. In comparison, only 7 of 44 (16%) patients with generalized onchocerciasis and 11 of 21 (52%) of exposed individuals with no microfilariae in skin snips and no signs of disease showed reactivity to this antigen. Microfilaricidal treatment of sowda patients with improvement of the clinical status was associated with a decrease or disappearance of antibodies to the 2.5-kD antigen. Amino acid sequencing of the antigen indicated identity to human defensins 1-3 of neutrophils. Defensin was demonstrated by immunohistochemical staining in onchocercal nodules on the surface of adult filariae and in the surrounding tissue. A similar staining pattern was observed for other proteins present in neutrophils such as myeloperoxidase, elastase, and the L-1 protein complex (MRP 8/MRP 14), indicating that neutrophils, macrophages, and their proteins predominate in the environment adjacent to the worms. These results demonstrate an association between the presence of autoantibodies to defensins and an infectious disease of known etiology. The association with a particular form of onchocerciasis, sowda, suggests a link between formation of autoantibodies to defensin and enhanced immune reactivity towards the parasite.

Human onchocerciasis affects approximately 20 million people in Africa, Central and South America, and Yemen (1). The major disease manifestations are dermal and ocular complications, including blindness. Onchocerciasis has been viewed as a disease in which the reaction of the host's immune system to the parasite, particularly to the microfilariae, is associated with pathology. According to clinical outcomes, people who have been exposed to *Onchocerca volvulus* fit into a spectrum that includes infected individuals with microfilariae and rather few clinical symptoms (generalized onchocerciasis), individuals with few microfilariae but intense dermal pathology (sowda), and individuals with no microfilariae and no clinical symptoms (endemic controls) (2). The generalized form is immunologically characterized by a hyporesponsiveness to parasite antigens (3). In contrast, sowda is considered hyperresponsive, and endemic controls are believed to reflect resistance to onchocerciasis (2). Only a minority of infected individuals develop sowda, whereas the majority are presented with the generalized form. Sowda is characterized by a severe papular dermatitis, usually localized to one limb, with darkening of the skin, as well as pachydermia and local lymph node enlargement accompanied by extremely low numbers of microfilariae in the skin (4, 5). An immunological hyperreactivity is indicated by the fact that patients have significantly higher antibody titers, including IgE and antigen-specific IgG, compared to individuals with generalized onchocerciasis (4, 6). Correspondingly, the regional lymph nodes show an extensive follicular hyperplasia (5). Skin lesions improve dramatically after long-term microfilaricidal treatment (7), indicating a relationship to the presence of microfilariae in the skin. It has been suggested that patients with sowda may possess effective mechanisms to kill microfilariae, which lead to chronic inflammation and low numbers of parasites in the skin (4, 5). It is presently unclear which factors contribute to the development of sowda. Although differences regarding the strain of the parasites (8) cannot be ruled out, differences in host reactivity appear to play a major role.
Genetic predisposition has been discussed; however, no clear association with HLA class II molecules has been found (9). Preferential reactivity of serum pools from sowda patients with *Onchocerca volvulus* 9- and 72-kD antigens by antibodies of the IgG3 subclass has been reported (10). In this study, we have analyzed antigen recognition patterns in a larger group of sowda patients that includes individuals who have been studied for up to 5 yr after microfilaricidal treatment.

Materials and Methods

**Study Subjects.** After informed consent was obtained, residents of a hyperendemic area for onchocerciasis in Liberia/West Africa were examined for the presence of onchocerciasis as previously described (7, 9). Skin snips were taken bilaterally from the iliac crest and examined microscopically for the presence of microfilariae (mf). Sera were collected and stored at -20°C for analysis. The individuals included in the present study were grouped as follows: 44 patients with generalized onchocerciasis (31 male, 13 female, 13-70 yr old, mf density, expressed as geometric mean = 31.12), 35 patients with sowda (10 male, 25 female, 5-52 yr old, mf density = 1.3), and 21 endemic controls, i.e., individuals with no clinical signs of onchocerciasis and no microfilariae in skin snips (11 male, 10 female, 12-56 yr old, mf density = 0). The patients with sowda received treatment with diethylcarbamazine (DEC) (7) or ivermectin (150-μg/kg) when this became available. 13 sowda patients were studied during a 1-5-yr period after treatment. Five individuals with no previous exposure to *O. volvulus* (nonendemic controls) and six German patients with lupus erythematosus were also studied.

*O. volvulus* Antigen Preparation (Ovag). Adult *O. volvulus* worms were obtained by collagenase digestion of surgically removed onchocercomata as previously described (11), homogenized in liquid nitrogen, and antigens were extracted in 0.0625 M Tris-Cl, 2% SDS, 5 mM EDTA, 2 mM PMSF, and 100 μg/ml tosyllysine-chloromethyl ketone. After centrifugation at 20,000 g for 90 min, the supernatant was stored at -70°C and examined microscopically for the presence of microfilariae (mf).

**SDS-PAGE and Immunoblotting.** Ovag was separated by SDS-PAGE using 5-15% and 12-17% gradient gels (12) and 12.5% Tricine gels (13). Gels were transferred to nitrocellulose membranes (Schleicher and Schuell) or to human defensin (Bachem, Germany). After washing, strips were incubated with protein G-HRP (Bio-Rad) or with goat anti-mouse IgG-HRP (Calbiochem). For the IgG3 subclass has been reported (10). Protein content was measured using the Bio-Rad protein assay kit (Bio-Rad, München, Germany).

**Statistical Analysis.** The chi-square test with continuity correction was used for comparison between groups.

Results

**Recognition of a 2.5-kD Antigen by Sowda Sera.** SDS-PAGE and immunoblots of Ovag showed preferential recognition of low molecular weight antigens by sera from sowda patients compared to sera from patients with generalized onchocerciasis and endemic controls (data not shown). Subsequent use of 12.5% Tricine gels showed recognition of an ~2.5 kD band by sera from sowda patients (Fig. 1 A). This antigen was recognized by 35 of 35 (100%) sowda patients.

**Figure 1.** Serum reactivity to the 2.5-kD band is prominent in sowda patients. Tricine gel and immunoblot of Ovag probed with individual sera from patients with sowda (A), endemic controls (B), nonendemic control (C), and patients with generalized onchocerciasis (D). All sera in *A* and two sera in *B* react with the 2.5-kD band (arrow).

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studied, by 7 of 44 (16%) patients with generalized onchocerciasis, and by 11 of 21 (52%) of endemic controls. The difference was highly significant for the sowda group compared to the other two groups (P = 7.6 × 10^-11 and P = 6.6 × 10^-3, respectively), as well as for the endemic control versus the generalized onchocerciasis groups (P = 2 × 10^-3). No reactivity with serum from nonendemic controls (Fig. 1 C) or patients with lupus erythematosus (not shown) was seen.

Differences in the intensity of the reaction to the 2.5-kD were observed. Thus, sera from sowda patients always showed strong reactivity (graded 2–3), whereas reactivity of sera from patients with generalized onchocerciasis or endemic controls was more variable (graded 1–3). Titration experiments performed on a subgroup of sera indicated that reactivity did not change at a higher serum dilution (1:100). Conversely, a lack of reactivity persisted at lower serum dilutions (1:20).

Reactivity to the 2.5-kD band correlated with the clinical status of patients with sowda. Of nine sowda patients treated repeatedly with DEC over a period of 1–5 yr, improvement of the clinical condition correlated with a gradual decrease of antibody reactivity to the 2.5-kD band. In all patients, this reactivity disappeared upon remission of the sowda manifestations. In these patients, the skin had regained almost normal appearance and no microfilariae were detected in skin snips. A representative case is shown in Fig. 2. Six sowda patients treated with a single oral dose of ivermectin showed no change in reactivity to the 2.5-kD antigen 1 yr after treatment, which correlated with the lack of improvement in the clinical status.

Identification of the 2.5-kD Band as Human Defensin. NH2-terminal amino acid sequencing of the 2.5-kD band material over 22 positions allowed identification of 18 amino acid residues (Fig. 3 A). Assuming that the four not identified residues represent cysteine residues, then the entire 22 residue sequence is identical to the first 22 residues of human defensin HNP1 (14). HNP1 contains 30 amino acid residues and has a 3.45-kD molecular mass. Approximately 30% of the 2.5-kD material lacked the NH2-terminal alanine and might therefore constitute HNP2 (14). The antigenic relationship of the 2.5-kD peptide to human defensin was indicated by its reactivity with a mouse monoclonal antibody to human defensin (Fig. 3 B, lane 2). The additional two bands reactive with the antibody may represent multimers of defensin or prodefensin.

Localization of Defensin in Onchocercal Nodules. In studied sections of two patients with sowda and four with generalized onchocerciasis, immunohistochemical analysis of onchocercal nodules showed an inflammatory cell infiltrate composed of mainly neutrophils containing defensin and macrophages surrounding adult worms. Neutrophils were concentrated around the female worms. Intense staining for defensin was observed on the worm surface and in the surrounding tissue (Fig. 4 A), suggesting extracellular release of defensin by activated neutrophils. Cellular infiltrates were associated with damage of the surrounding connective tissue (Fig. 4 A). Adjacent to larger groups of neutrophils, many macrophages were lightly stained for defensin and often phagocytized neutrophils were observed in macrophages or multinuclear giant cells. Morphologically altered dead microfilariae with attached neutrophils and staining for defensin extending to the surrounding tissue also suggested that neutrophils were activated by the worm to adhere and/or to release defensin (Fig. 4 B). Intact microfilariae without any cellular infiltrate or defensin were also observed (Fig. 4 C). The surface of adult worms showed a layer of intense staining for defensin with attached neutrophils (Fig. 4 D). This layer appeared to be shed from the surface (Fig. 4 E). No direct staining of internal structures of adult worms or morphologically intact microfilariae was detected. Staining of respective sections with antibodies to the neutrophil proteins myeloperoxidase, elastase, and the L-1 protein complex resulted in similar staining patterns, all confirming the presence of neutrophils and macrophages around the adult worms (not shown). The staining pattern most similar to that observed with antibody to defensin was observed with antiserum to myeloperoxidase, including the release and deposition of the protein on the worm surface and on microfilariae when surrounded by neutrophils. Staining with anti-L-1 protein complex gave a similar result. Staining with elastase was confined to the neutrophils, the release of the protein was not observed and fewer cells were stained than with defensin.

Discussion

The results of this study demonstrates that the sowda form of onchocerciasis is associated with the occurrence of serum antibodies to a 2.5-kD antigen present in extracts of *O. volvulus* adult worms.

The amino acid sequence suggested identity of this antigen with human defensins, a group of peptides with potent antimicrobial, cytotoxic, chemotactic, and corticosteroid properties present in the azurophil granules of neutrophils (15). Autoantibodies to neutrophil cytoplasmic constituents (ANCA) have been known to occur in autoimmune diseases (16). Antigens identified so far are some of the enzymes of the azurophil granules, such as myeloperoxidase, elastase, and proteinase 3. The most specific disease association is found between antibodies to proteinase 3 and Wegener's granulomatosis (17). Disease activity correlates with the presence of these antibodies, suggesting a functional role of these antibodies in the disease process (16). Here we found a correlation between presence of autoantibodies to defensin and active disease. Sowda shows certain parallels to Wegener's granulomatosis, in that vasculitis, granulomas in the skin, and eosinophilia occur in both diseases. The differential diagnosis of tropical pulmonary eosinophilia, which represents the sowda analogue in lymphatic filariasis, has to consider Wegener's granulomatosis (18).

The presence of other neutrophil proteins in the vicinity of the worms and on the worm surface further suggest an important role for neutrophils in the in vivo reaction to this nematode, which needs to be characterized. Differences in abundance and distribution of the individual proteins appear to exist. It will be of major interest to examine whether autoantibody formation also occurs to these proteins in onchocer-
Figure 2. Serum reactivity to the 2.5-kD band correlates to disease activity in sowda. (A) Immunoblot of Ovag probed with serum from a sowda patient before (lane 1) and at various times after long-term microfilaricidal treatment (lanes 2–6). Reactivity to the 2.5-kD band is seen before and 3 mo (lane 2), 24 mo (lane 3), 26 mo (lane 4) after treatment, and it is virtually absent at 36 mo (lane 5) and 41 mo (lane 6) after treatment. The same patient before (B) and 36 mo after (C) microfilaricidal treatment. The papular dermatitis and darkening of the skin, typical for sowda, disappeared 36 mo after treatment.

Figure 4. Distribution of defensin in onchocercal nodules. (A) Neutrophils and large amounts of defensin (red) concentrated around an adult female with deposition of defensin on the worm surface. Sowda (x243). (B) Damaged dead microfilaria in onchocercoma tissue with attached neutrophils and extracellular defensin in the surrounding area (arrow). (C) Another intact microfilaria without cellular infiltrate or defensin (arrowhead). Sowda (x618). (D) Crust-like structure on surface of adult female (arrowhead) with attached neutrophils and positively staining material for defensin. Generalized (x618). (E) Crust strongly stained by defensin antibody (arrow) in the proximity of an adult male, which is covered by a thin layer positively staining for defensin (arrowhead). Generalized (x618).
both autoantibodies and clinical manifestations. Since the etiology of most autoimmune diseases is unknown, these results may support the presumption of a link to infectious agents. The present results indicate a difference in incidence of autoantibodies to defensin in the endemic controls with low antibody titers to *O. volvulus* antigens (6, 20). A possible explanation for the autoantibody production would be that the antibodies are actually directed to parasite defensin or to other cross-reactive parasite antigens. However, our results strongly suggest that the defensin in the *O. volvulus* extract is of human origin and is deposited on the worm surface when neutrophils become activated and degranulate in the proximity of the parasite. Human neutrophils are potent effector cells in killing both microfilariae and infective larvae of *O. volvulus* (21, 22). Degranulation with deposition of defensin on the worm surface may trigger mechanisms of inactivation of defensin, such as binding of α2-macroglobulin and internalization of defensin by macrophages (15). This suggests that defensin on the worm surface may be available for antigenic presentation to T cells.

Production of autoantibodies is viewed as a T cell–regulated phenomenon. Self-reactive B cells thus need T cell help. This help could be provided by two different mechanisms. One would require that defensin-reactive T cells are activated because of a failure of peripheral control mechanisms of self tolerance, such as clonal anergy (23). Alternatively, autoreactive T cells or B cells could directly be activated by parasite antigens crossreactive with human defensin. Crossreactivity cannot be ruled out at present, particularly as *O. volvulus* antigens that are cross-reactive with other human proteins have been detected (24–26). The second mechanism of T cell help would involve activation of T cells reactive with conjugates between human defensin and onchocercal antigens. The observed parasite surface material that stained positively for defensin suggests formation of macromolecular complexes. This possibility would be consistent with the view that defensins as small peptides may not be able to induce an immune response by themselves, but that they need a carrier molecule, in this case parasite molecules.

Both these mechanisms of T cell help to autoreactive B cells require a break in T cell tolerance. Whereas the first possibility would require a break in self-tolerance, the second possibility requires a break in tolerance to onchocercal antigens. The latter is applicable to patients with generalized onchocerciasis who are tolerant to parasite antigens, reflected by lack of T cell proliferation and IL-2 production (3). As in classical tolerance, this state is reversible by addition of exogenous IL-2 (3). Tolerance appears to be related to the presence of microfilariae since microfilaricidal treatment of patients can restore the cell-mediated immune response (3, 27, 28). These findings are consistent with the fact that maintenance of tolerance depends on the continued presence of the toleragen (29), in this case microfilariae. In contrast to patients with generalized onchocerciasis, persons with sowda or with no signs of onchocerciasis (endemic controls) show a different immune reactivity to the parasite. T cell reactivity to onchocercal antigens is marked in endemic controls (20), while a heightened T cell reactivity in sowda patients is indicated by the granuloma formation in the skin (5), which is considered to reflect hypersensitivity.

The concept of tolerance to explain parasite-related unresponsiveness has first been put forward for lymphatic filari-
nosis (30), where tolerance appears to be induced by microfilarial antigens in utero (31). Our results indicate that under conditions of established tolerance to parasite antigens, as in generalized onchocerciasis, no reactivity to defensins is seen. When tolerance to parasite antigens is compromised (as in sowda) or is not established (as in endemic controls), production of defensin antibodies occurs. Thus, the observed strong association of autoantibodies to defensin with sowda and not with defensin antibodies occurs. Therefore, the observed strong association of autoantibodies to defensin with sowda and not with defensin antibodies occurs. Thus, the observed strong association of autoantibodies to defensin with sowda and not with defensin antibodies occurs. Thus, the observed strong association of autoantibodies to defensin with sowda and not with generalized onchocerciasis is highly suggestive of a link between autoantibody formation and enhanced immune reactivity to the parasite.

The exact mechanisms underlying this relationship need further study. To date, the concept of molecular mimicry has been put forward to explain a possible relationship between autoimmune diseases and infectious agents (32). Based on the present findings, we would like to propose that host antigens may become immunogenic by conjugation with parasitic molecules. According to this model, autoantibody responses would be under the control of parasite-specific T cells that are reactive with epitopes unrelated to the host antigen. This concept is supported by recent findings in studies of experimental autoimmune disease, where it was shown that autoantibody need not mirror the immunogen that initiated the immune response (33). Furthermore, this would be in accordance with the observation that in several autoimmune diseases, T cells reactive with the autoantigen are either not detectable or their presence is observed in normal individuals as well.

The role of autoantibodies to defensin in onchocerciasis has to be determined. Such antibodies may be involved in cytotoxicity towards the parasite, particularly microfilariae. Deposition of human IgG on *O. volvulus* microfilariae has been observed both in vitro (21) and in vivo in certain conditions, the latter in lymph nodes of patients with onchocerciasis after treatment with ivermectin (Knab, J., K. Darge, and D. W. Büttner, manuscript in preparation).

In view of the extremely close adaptation of *O. volvulus* to its human host, it is of considerable interest to examine the mechanisms by which *O. volvulus* down-regulates cell-mediated immunity, as well as the factors that allow autoantibody formation in this infectious disease. The study of human onchocerciasis may cast light on the relationship between autoimmunity and infection with a defined parasitic agent.

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