FcRIIa (CD32) Polymorphism and Onchocercal Skin Disease: Implications for the Development of Severe Reactive Onchodermatitis (ROD) Magdi M. M. Ali,† Gehad ElGhazali,† Scott M. Montgomery, Salah E. Farouk, Amre Nasr, Suzan I. A. Noori, Mahdi M. Shamad, Omar E. Fadlelseed, and Klavs Berzins* Tropical Medicine Research Institute, National Centre for Research, Khartoum, Sudan; Department of Immunology, The Wenner–Gren Institute, Stockholm University, Stockholm, Sweden; Department of Clinical Epidemiology Karolinska Hospital, Karolinska Institute, Stockholm, Sweden; Clinical Research Centre, Örebro University Hospital, Sweden; Department of Neurology, Khartoum Teaching Hospital, Sudan; Department of Neurology Rashid Hospital, Dubai, UAE; Departments of Dermatology and of Biochemistry, Faculty of Medicine, University of Juba, Sudan; Department of Microbiology and Immunology, Faculty of Medicine, University of Khartoum; Department of Clinical Immunology, Faculty of Medicine, King Fahad Medical City, Riyadh, Saudia Arabia Abstract. The pathologic manifestations of Onchocerca volvulus infection depend on the interplay between the host and the parasite. A genetic single nucleotide polymorphism in the FcRIIa gene, resulting in arginine (R) or histidine (H) at position 131, affects the binding to the different IgG subclasses and may influence the clinical variations seen in onchocerciasis. This study investigated the relationship between this polymorphism and disease outcome. FcRIIa genotyping was performed on clinically characterized onchocerciasis patients (N = 100) and healthy controls (N = 74). FcRIIa genotype R/R131 frequencies were significantly higher among patients with severe dermatopathology (P0.001). Increased risk of developing this form was mostly associated with one tribe (Masalit) (OR = 3.2, 95% CI 1–9.9, P = 0.042). The H131 allele was found to be significantly associated with a reduced risk of having the severe form of the disease (adjusted OR = 0.26, 95% CI = 0.13–0.46, P0.001). Our findings suggest that the polymorphism influences the clinical outcome of onchocerciasis. INTRODUCTION Onchocerca volvulus is the causative agent of one of the major filarial diseases. More than 18 million people are af- fected throughout the world, 90% of them from the African continent.1 Onchocerciasis is the second leading cause of pre- ventable blindness; more than one million people suffer visual impairment, with at least 340,000 cases of blindness attribut- able to the disease.2 Some of the clinically symptomatic pa- tients have severe dermatological lesions. The mechanism un- derlying the propensity of some patients to develop an ex- tremely debilitating skin condition, known as reactive onchodermatitis (ROD) or sowda, has been a matter of con- troversy.3–6 The factors that predispose patients to develop- ing ROD are not clearly defined. O. volvulus can survive in humans for over 12 years, despite ongoing cellular immune responses and high titers of parasite-specific IgG and IgE antibodies.7 The consequent pathology represents the cumu- lative tissue and functional outcomes of a long-standing in- terplay between host and parasite. It has been shown in an experimental O. volvulus infection in mice that an induced keratitis depends both on antibodies8 as well as on FcR.9 FcRIIa were identified over 35 years ago as glycoproteins found on the surface of haematopoietic cells.10 These recep- tors provide a link between the cellular and humoral arms of the immune system, allowing immunoglobulins to trigger ef- fector responses from cells, such as macrophages (phagocy- tosis), NK cells (antibody–dependent cellular cytotoxicity, ADCC), neutrophils (activation), and B cells (antigen pre- sentation). In humans, there are 3 biochemically and struc- turally distinct classes of FcR: FcRI (CD64), a high affinity

receptor that binds monomeric IgG1/3/4 subclasses,11 Fc□RII * Address correspondence to Klavs Berzins, Wenner–Gren Institute, Department of Immunology, Stockholm University, Svante Arrhe– nius väg 16, SE–106 91 Stockholm, Sweden. E–mail: klavs@imun.su.se † The first two authors contributed equally to this study. 1074 (CD32) and Fc□RIII (CD16), which are of lower affinity and interact only with complexed or aggregated forms of IgG.10 Fc□R isoforms exist and, despite the structural similarities, their functional properties seem to be distinct.12 Indeed ge– netic variation in Fc□Rs constitutes an important determinant for host defense capabilities.13 The Fc□RIIa subtype, being the most widely distributed, is expressed on neutrophils and monocytes/macrophages and initiates phagocytosis, ADCC, and cellular activation. Notably, the major receptor for C– reactive protein (CRP) on monocytic cells is Fc□RIIa.14 A polymorphism in the gene encoding Fc□RIIa has been shown to alter the ability of the receptor to bind the different IgG subclasses.15 This polymorphism is a point mutation (G to A) that results in an amino acid substitution of arginine (R) to histidine (H) at position 131, in the region specifying the ligand–binding domain of the receptor.13,16 The relative fre– quency of the R131and H131 allotypes varies among different ethnic groups and influences the release of certain cytokines (e.g., IL–2, IL–6, IFN□, and TNFa).16 Host defenses against pathogens, including O. volvulus, is likely to depend on cel– lular activities that could in turn be influenced by the Fc□RIIa polymorphism. This study investigated the association of Fc– □RIIa polymorphism with disease severity in O. volvulus– infected patients, in a hypo–endemic area in Eastern Sudan. PATIENTS AND METHODS Study population. The individuals included in this study re– side in villages in the Sundus area of Gedarif State, Eastern Sudan near Atbara River along the Ethiopian border, 650 km from Khartoum, where there is a high prevalence of the se– verest form of onchodermatitis (ROD or sowda).17 The study area has a population of around 20,000, and is an area re– garded by international official control programs as hypo– endemic for onchocerciasis infection, therefore not involved in major mass treatment activities. The major ethnic groups present in this area were included in the study (i.e., Fallata [Fulani], Haussa, Masalit, and Fur). Authorization for the study was given by the Gedarif State, by the Ministry of Health, and by the local community leaders. The Khartoum University human experimentation guidelines for the conduct of clinical research were followed, and informed consent was obtained from all participants. Clinical examination and group definition. Details of the individual's clinical presentation were recorded, with a focus on those dermatological, ophthalmologic, and parasitological aspects related to onchocerciasis (onchocercal dermatitis, lymphadenopathy, and the palpable presence of nodules), and using the scoring system previously developed for dermal onchocerciasis.18,19 Microfilarial skin loads were estimated by the standard skin snip assay, which measures emerging para– sites. Standard onchocercal skin biopsies (snips) were taken, using a Walser corneoscleral punch, from the iliac crest for detection of microfilariae (mf). Furthermore, negative control skin snips were taken for histopathologic examination. The snips were fixed in formalin, prepared for paraffin embedded sectioning, and then stained with hematoxylin and eosin or Giemsa. The slides were examined by the pathologist using bright–field microscopy. Ophthalmologic examination was carried out including visual acuity with an illiterate E chart, direct and indirect ophthalmoscopy, and examination with a Haag–Streit 900 slit–lamp, after dilation of the pupils. There were two major groups: Group A—those with severe onchodermatitis, including ROD

or sowda (N = 51), and group B—individuals with mild cutaneous disease (N = 49). A control group was composed of individuals living in the endemic area, who had never been diagnosed as having on- chocerciasis and who did not have any signs associated with the disease (N = 74). All known patients with the severe reactive form of the disease within the different villages were recruited, and a corresponding number of individuals with the mild form of the disease and endemic controls were also re- cruited from the same locality. Consecutive patients with the relevant diagnosis attending field clinics were enrolled in the study. Consecutive visitors to the field clinic, without on- chocerciasis, were enrolled as controls. A questionnaire form that included various demographic details was completed, and this added to data on clinical status. Blood sample collection. Venous blood samples (5 mL), collected using vacuum tubes containing EDTA, were al- lowed to stand for 2 hours. The buffy coat was harvested in cryotubes and then kept frozen until processed for DNA ex- traction and analysis. The samples were later all shipped to Stockholm, Sweden in dry ice. DNA extraction. Genomic DNA was isolated from each buffy coat sample using a QIAmp DNA blood mini kit (QIAGEN, Hilden, Germany). FcçRIIA-H/R131 genotyping. Genotypes were examined by using a polymerase chain reaction (PCR), utilizing ge- nomic DNA and allele-specific primers as described previ- ously.20 Briefly, PCR conditions were modified as follows: one cycle at 96°C for 5 min., 30 cycles at 94°C for 30 sec. and 56°C for 30 sec., and one cycle at 72°C for 45 sec. and final extension for 6 min. at 72°C. The product was digested by the allele- specific restriction enzyme BstU1 (Fermentas Inc., Hanover, MD) by incubation for 2 hours at 37°C according to the manufacturer's recommendation, and then detected by gel electrophoresis on a 2% agarose gel containing ethidium bromide and visualized using UV light. FcRIIa-specific primers (CyberGene AB, Sweden) were used for amplification: Forward: 5'-GGA AAA TCC CAG AAA TTC TCG C-3' Reverse: 5'-CAA CAG CCT GAC TAC CTA TTA CGC GGG-3' A 343-bp fragment represents the FcRIIa-H/H 131 geno- type, whereas the R/R131 genotype produces a 322-bp frag- ment. Heterozygotes H/R produce both fragments.20 Statistical analysis. Data were analyzed using SPSS (version 10.0) software for Windows (SPSS, Inc., Chicago, IL). Logis- tic regression, with disease severity of onchocerciasis (mild or severe) as the dependent variable, was used to investigate its association with FcRIIa genotype, modeled as a series of binary dummy variables. H/R 131 was used as the reference value as this genotype is more prevalent in the human popu- lation.21 To investigate the association of allelic frequency with the clinical form of the disease, the FcRIIa alleles were analyzed using the same software. We performed an overall compari- son of allele frequency using a 2X2 y2 test and logistic regres- sion. Both logistic regression models were adjusted for tribe and age, modeled as binary dummy variables. A logistic re- gression model was used to compare all those with the disease (mild and severe forms combined) with the disease-free con- trols. The model was adjusted for sex, tribe, age, and geno- type (categorized as shown by Table 1) as appropriate. Odds ratios (OR) and their 95% confidence intervals (CI) were used to describe the associations. The statistical significance was defined as confidence intervals that did not include 1.0. RESULTS The distribution of FcRIIa genotypes and allele frequen- cies in 100 onchocerciasis patients and 74 healthy controls were analyzed and related to clinical presentation, ethnicity, and age (Table 1). The genotype frequencies differed mark- edly among the varied clinical forms, ethnic groups, and age groups. There was a

significant over-representation of the R/R131 genotype among the group of patients with the severe dermato-pathologic form of the disease compared with those with mild disease (OR = 60.9, 95% CI: 7.6–85.7, P0.001) (Table 1). This association was enhanced after adjustment for ethnicity (tribe) and age in multivariate logistic regression analyses, in which FcRIIa-H/R131, the more prevalent geno- type in the study population, was used as the reference cat- egory21 (Table 1). When taking into account differences in ethnicity (Fulani was defined as the reference category due to their higher number), a statistically significant increased risk of develop- ing the severe form of the disease was found to be associated with the Masalit tribe (OR = 3.2, 95% CI: 1–9.9, P = 0.042). Interestingly, adjustment for the genotype eroded this asso- ciation (Table 1). Severe dermatopathology was found to be more frequent among the youngest group (0–15 years), P = 0.007 and in- termediate group of patients (16–30 years), P = 0.035, when compared with the oldest group. No statistically significant association was seen with sex (data not shown). Analysis of allelic frequencies revealed that the presence of the H allele was significantly associated with a reduced risk of having the severe form of the disease (OR = 0.26, 95% CI: 0.13–0.46, P0.001), suggesting a protective role for the H allele. This association remains statistically significant after TABLE 1 Multivariate analysis of the effects of FcRIIa genotypes, ethnicity (tribe), and age of the risk of developing severe dermatopathology in onchocerciasis patients Un-adjusted Adjusted* Mild count (%) Severe count (%) OR (95% CI) P value OR (95% CI) P value Genotype RR 1 (2) 28 (54.9) 60.9 (7.6–85.7) < 0.001 119.7 (8.4–632.2) < 0.001 HH 11 (22.4) 6 (11.8) 1.2 (0.4–3.7) 0.77 1.3 (0.4–4.5) 0.5 HR 37 (75.4) 17 (33.3) 1 – 1 – Allele frequency† H131 60% 28% 0.26 (0.13–0.46) < 0.001 0.26 (0.14–0.50) < 0.001 R131 40% 72% Tribe Masalit 7 (14.3) 15 (29.4) 3.2 (1–9.9) 0.042 2.8 (0.7–10.7) 0.133 Others 21 (42.9) 22 (43.1) 1.6 (0.6–3.9) 0.326 0.9 (0.3–3) 0.953 Fulani 21 (42.9) 14 (27.5) 1 – 1 – Age 0–15 2 (4.1) 11 (21.6) 9.2 (1.8–45.4) 0.007 14 (2.5–76.6) 0.002 16–30 12 (24.5) 19 (37.3) 2.6 (1.1–6.5) 0.035 4.5 (1.5–13.2) 0.007 Š 31 35 (71.4) 21 (41.1) 1 – 1 – * Data were adjusted for sex, age, tribes, and genotype where appropriate. † Relative frequency of alleles in the study population. adjustment for age and ethnicity (Table 1). Noteworthy, how- ever, is that the frequency of the polymorphism did not differ between the patient groups (mild and severe forms com- bined) and disease-free controls (Table 2). DISCUSSION The pathogenesis of onchocerciasis involves both acute and chronic inflammation. The clinical variations observed are believed to parallel variations in the immune response against Mf4,5 and the endosymbiontic bacterium Wolbachia.22,23 Be- cause this disease is found in populations with diverse genetic backgrounds, it is possible that host genetic factors may me- diate these immune variations. Genetic polymorphisms in- volve factors that govern mechanisms underlying susceptibil- ity or clinical outcomes. FcR gene polymorphisms have been indicated as factors that may influence susceptibility to infec- tious and autoimmune diseases.24,25 Our data provide evi- dence for the FcRIIa genotype as an inherited risk factor in the pathogenesis of onchocerciasis, the relative frequency of the R131 and H131 alleles being determinant in susceptibility to ROD, a particularly severe form of onchocerciasis. In the present study, the R/R131 genotype was associated with an increased risk of developing the severe form of onchocercia- sis, and allelic frequency analysis suggests that this is due to the negative association, and possibly protective effects, of the H131 allele in this context. Although an association be- tween genotype and disease severity was

identified, no such association was found with the risk of contracting the infec- tion. We also found an association between different ethnic groups and the disease severity; this may be explained by a variation in the genotypes for this polymorphism among the different ethnic groups, providing more evidence of a poten- tially causal role for this genotype. While the FcγRIIa of both alleles interact efficiently with IgG1 and IgG3, the H131 receptor binds IgG2 efficiently, in contrast with the poor binding of this subclass to the R131 receptors.24,25 As IgG2 is a poor activator of the classic complement pathway, the H131 receptors might be essential for the disposal of IgG2 immune complexes (IC). The clinical consequences of this differential IgG2 binding could be pro- found, with those who are homozygous for R131 being at higher risk of serious infection with encapsulated organisms, gram- negative bacteria, and for the impaired IC re- moval.24,26,27 Cross linking of the FcR on monocytes and neu- trophils by the Fc region of antibodies initiates signal trans- duction that leads to phagocytosis. Because the H131 allele is associated with the mild form of dermatitis, it may be inferred in protection from the antibody–dependent enhanced immu- nopathological consequences seen in the group with the se- vere form of the disease, including ROD or sowda. The bind- ing of IC to FcγR on the neutrophil surface stimulates pro- duction of chemotactic and immunoregulatory cytokines such as IL–12 and MIP. 1a.26 FcγR interactions with IgG may mediate various immuno- pathological reactions (e.g., ocular inflammation affecting vi- sion).9 Our findings indicate that these receptors may also mediate immunopathological reactions in the skin. Patients TABLE 2 Distribution of FcγRIIa genotypes in the onchocerciasis–infected and healthy controls Un–adjusted Adjusted* Disease count (%) Healthy count (%) OR (95% CI) P value OR (95% CI) P value RR 29 (60.4) 19 (39.6) 1.1 (0.5–2.2) 0.788 1.1 (0.4–2.8) 0.903 HH 17 (51.1) 16 (8.5) 0.8 (0.3–1.7) 0.515 1.5 (0.5–4.4) 0.431 HR 54 (58.1) 39 (41.9) 1 – 1 – * Data were adjusted for sex, age, and tribes. with the severe form of the disease typically demonstrate on- going mf destruction, an action that could cause enhanced release of gram–negative bacterial (Wolbachia) products me- diating the inflammatory responses seen in these patients. Also, as part of an immunoregulatory network, IL–10 may be required to suppress the severe inflammatory consequences seen in the severe form of the disease. This was indicated by the hypo–responsive effect mediated by the Th3–type cyto- kines IL 10 and TGFβ seen in onchocerciasis patients with the mild form of the disease.28 Indeed, in our parallel study, IC were found to induce IL–10, which could then downregulate the pro–inflammatory effects of the TNF–a and IL–1β that were found to be at elevated levels in our patients (Ali and others, submitted). Previous findings in Sudan demonstrated that IgG1 and IgG3 are associated with microfilarial destruction in patients, thus lowering microfilarial loads.29 In our study, however, this anti–parasitic activity might be inhibited in those with mild pathology, who mostly express the H/H 131 genotype, which is associated with the efficient binding of IgG2 to FcγRIIa, thus blocking IgG1/3 mediated opsonization and phagocyto- sis. Such a mechanism would result in higher mf loads due to their impaired ability to kill mf in vivo, and diminished in- flammatory responses, leading to the phenotypically mild form of the disease. The data described here are in accordance with a report by Shi and others,24 showing that the FcγRIIa, which also has a high affinity for CRP, is associated with protection against high parasitemia in P. falciparum infection in Kenya. Of note, in our parallel study we also found higher levels of CRP in those with severe dermatopathology (Ali et al, submitted). These

collective findings suggest that the binding of CRP to FcRIIa might also play a role in the development of pathol- ogy in onchocerciasis. It is tempting to speculate that selective pressures resulted in the Onchocerca worm decorating itself with CRP, thus ensuring ligation of FcRIIb (inhibitory) in its vicinity. This could allow the parasite to protect itself from local immune responses. Alternatively, pressures on the host might have resulted in selection of an R131 variant of the FcRIIa, which could bind the CRP coating and induce an inflammatory response. The propensity to develop ROD appears to also have an ethnic basis, as certain tribes (Masalit) appear to be more susceptible than others. This particular tribe emigrated re- cently from a non–endemic area in western Sudan (Darfur) and the eastern part of Chad only 40–50 years ago, whereas the Fulani tribe originally came more than 70 years ago from West Africa, an area where infection with onchocerciasis is very common. The present study indicates that age also has an impact on the likelihood of developing the severe or ROD form of the disease, confirming the higher prevalence of this form among younger patients.5 Differences in disease patterns in different age groups reflect a rich mix of maturation, environmental factors (influencing the development of functional immunity with age), and may also reflect population changes in genetic factors, particularly where migration occurrence has been high. There was no notable variation in genotype when those with and without the disease were compared, suggesting that the studied polymorphism influences disease severity and not susceptibility to infection or rapid clearance of the organism. Thus, other selective pressures are more likely to have oper- ated through the influence of disease severity rather than initial infection. In conclusion, for patients with onchocerciasis, the R/R131 genotype appears to be associated with severe dermatopa- thology, whereas the presence of the H131 allele of the Fc- RIIa appears to be associated with protection from severe dermatopathology, including the reactive form. Received March 13, 2007. Accepted for publication July 2, 2007. Acknowledgments: We are indebted to all the villagers who willingly participated in this study and to the health personnel in the medical centers in the villages in Sundus area. We are also thankful to ad- ministrative staff at Doka locality, Gedarif state. Our thanks also extend to the staff at the National Onchocerciasis Control Program, Tropical Medicine Research Institute, Khartoum, Sudan. Special thanks go to Alex Szalai, The University of Alabama, USA, for the fruitful discussion and comments. Our thanks extend to Tom Nut- man, Laboratory of Parasitic Diseases, National Institute of Health, USA, for the critical reading of the manuscript. Financial support: This work was supported by the Tropical Medicine Research Institute- National, Centre for Research, Academy of Medi- cal Sciences and Technology, Sudan and by grants from the Swedish Medical Research Council (VR) and SIDA/SAREC. This work is part of the activities of the BioMalPar European Network of Excel- lence supported by a European grant (LSHP-CT-2004-503578) from the Priority 1 “Life Sciences, Genomics and Biotechnology for Health” in the 6th Framework Programme. Disclosure: The authors have no conflict of interest concerning the work reported in this article. Authors’ addresses: Magdi M.M. Ali, Tropical Medicine Research Institute, National Centre for Research, P.O. Box 1304, Khartoum, Sudan. Magdi M.M. Ali, Salah E. Farouk, Amre Nasr, and Klavs Berzins, Department of Immunology, The Wenner-Gren Institute, Stockholm University, SE-10691 Stockholm, Sweden. Telephone: 46-8-164170, Fax: 46-8-157356, E-mail: klavs@imun.su.se. Gehad ElGhazali, Department of Microbiology and Immunology, Faculty of Medicine,

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