Heterogeneity in *Onchocerca ochengi* antigen recognition by IgG_2 antibodies in cattle showing two divergent infestation patterns

M.D. ACHUKWI[1,2*].; W. HARNETT [2]; J. BRADLEY[3] and A. RENZ [4]

[1]Institute of Agricultural Research for Development, Wakwa Centre, P. O. Box 65 Ngaoundere, Cameroon. [2]Strathclyde University, Dept. of Immunology, 31 Taylor street, Glasgow, G4 ONR. [3]Dept. of Biological Science Salford University, Salford M5 4WT, UK. [4]University of Hohenheim, Dept. of Parasitology, Email-Wolff-Strabe 34, D-70599 Stuttgart, Germany. *Corresponding author.

ABSTRACT

Considerable individual variations in infection levels are common in parasitic infestation even when the risk of challenge is similar and these variations may be reflected in the parasite antigens recognized by the different hosts. As part of an experiment meant to longitudinally study *Onchocerca ochengi* parasite acquisition in naïve cattle over a four-year period, two animals were found to have contrasting parasite buderns (2 and 101 nodules) under similar vector challenge. The animal with only two nodules was thought to have developed a sustained ability to limit parasite abundance (a steady state) despite prolonged continuous exposure to infection like the highly infested one. Their IgG2 antibody profile as detected by ELISA, from 18 to 49 months of natural exposure to the vectors of the disease, were not similar for the two distinct patterns of infestation. Similarly, the animals showed a considerable heterogeneity in the antigens they recognized in immunoblots following separation of the *O. ochengi* proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The animal with the least parasite burden apparently recognized a number of bands between 17 KDa and less than 27 KDa which were not detected by the highly infested animal. Furthermore, other animals with very low parasite burden prominently detected the 29 Kda, which was only faintly recognized by the high parasite burden animal. The increased level of the IgG_2 antibody isotype may be related to the steady state infestation pattern. The specific antigens detected during the steady state condition need to be identified in a larger sample size and studied further as they may be of value in understanding the immunity developed during the infestation.

Key words: Cattle, *Onchocerca ochengi*, concomitant immunity, IgG2 antibodies

RÉSUMÉ

Dans les cas des infestations parasitaires, des variations considérables du taux d'infections individuels sont constamment observées. Une étude a été menées dans le but de suivre l'acquisition du parasite *Onchocerca Ochengi* par les bovins naïfs sur une période de près de 4 années. Les résultats obtenues ont montré que 2 animaux soumis dans les mêmes conditions d'attaque du vecteur, présentaient des différences des niveaux de la charge parasitaire (2 et 101 nodules). L'animal présentant seulement 2 nodules, aurait développé un état de stabilité permanent qui lui aura permis de limiter la multiplication des parasites dans son corps malgré l'attaque continu du vecteur. Le cas contraire a été observé chez l'animal présentant. Une haute charge parasitaire. Le profile de leurs anticorps IgG2 détecté par la méthode ELISA entre 18 an 49 mois d'exposition naturelle aux vecteurs de l'infection ont montré des différences pour les 2 types d'infestations. De même, ces animaux ont montré une hétérogénéité considérable des antigènes reconnus après séparation des protéines de O. Ochengi par SDS-PAGE. L'animal présentant la plus faible charge parasitaire a reconnu sur le gel des bandes protéique 17 KDa et 27 Kda. Ces bandes étaient absentes chez l'animal à forte charge parasitaire. Le taux élevé des anticorps IgG2 serait dû à l'état constant de l'infestation. Les antigènes spécifiques détectés pendant la phase constante de l'infestation méritent une étude plus approfondie avec un nombre plus élevé des animaux. Car ces antigènes pourraient aider à comprendre le mécanisme du système immunitaire développé pendant l'infestation.

Mots clés: bovins, *Onchocerca ochengi*, système Immunitaire Anticorps IgG2

INTRODUCTION

Most epizootiological investigations often neglect the abundance or density of infection despite frequent reports of considerable individual variations in infection levels. Variations in parasite burden may correlate with particular infestation patterns and individual hosts may cope differently with the different parasite burdens. Longitudinal studies using natural host systems of *Onchocerca* parasite could permit a better understanding of the mechanism (s) regulating this worm's density in its definitive host. The search for an animal model system, which naturally supports the parasitism of nodule-forming *Onchocerca* species and closely resemble the human parasite (*O. volvulus*), has been on for some time. The variations in infestation levels may correlate with particular infestation patterns and individual hosts may cope differently with the different parasite burdens. Detailed longitudinal studies using natural host systems of *Onchocerca* parasite could permit a better understanding of the mechanism (s) regulating this worm's density in the its definitive host. Although bovine *Onchocerca* species are ubiquitous in Africa, these parasites received little attention until recently. In the search for animal models which naturally support parasitism of nodule-forming *Onchocerca* species and closely resemble the human parasite *O.volvulus*, *Onchocerca ochengi* in African cattle has recently attracted much interest (1,28,35,37). Cattle in North Cameroon are highly infested with *O. ochengi* and often associated with concurrent (multiple-*Onchocerca* species) infestations (36). However, little is known about their host-parasite inter-relationship and the immunoepidemiology of the disease in young exposed animals is still obscure as very few young animals have adult worm infestation (1). *O. ochengi* is phylogenetically very closely related to *O. volvulus* (4), and indeed shares the same natural vector, *S. damnosum s.str.* and *S. squamosum* (25,37).

The present study focused on two animals that were examined for *O. ochengi* nodule and microfilariae acquisition over a four-year period and were found to show two distinct patterns of *O. ochengi* infestation. The two patterns consist of an abundant parasite acquisition in one and a steady state low parasite load despite continuous exposure to a high risk of new infections from *Simulium* bites in the other. The antibody (IgG_1 and IgG_2) profile in response (a marker of infection) to a cocktail of two *O. volvulus* recombinant antigens (Ov10/Ov11) and the antigens recognized by the IgG_1 antibody in crude *O. ochengi* adult worm PBS soluble extracts were also evaluated for the two animals.

MATERIALS AND METHODS

The study site

The studies were carried out in a private ranch that is located about 12 Km south of Ngaoundere, near Galim village (7° 12' N, 13° 35' E, 1100 m altitude). Galim has a population of about 150 while the cattle (local Gudali breed) population in the ranch and its neighbourhood was about 9000. The ranch lies along the banks of the Vina du Sud river -a big and fast-flowing perennial river. The lake in the ranch, an inland watering spot for the animals, is located about 2Km uphill from the riverbank. In this area, the dry season starts from November to March while the rainy season is from April to October.

The animals and experimental set up

The two animals (one male and the other female) were part of seven male and eight female cattle (the oldest was 11 months at start of study), calved in February and March 1992, that were randomly picked up from several herds. They were grazed together as male and female herds with other animals of the same sex and age in the same farm. Bi-monthly nodule count, skin biopsy and serum collection were carried out from January 1993 until March 1996 when the studies were terminated; implying that the animals were about 4 years old at the end of the study. For the purpose of clarity, this longitudinal study will be named the 'animals studied after the minimum patent age'.

Counting and mapping of O. ochengi nodules.

During evaluation, each animal was restrained on lateral recumbency and all palpable nodules of *O. ochengi* were counted by inspecting both sides of the animal. All newly acquired nodules were then coded on a map allocated to the animal.

Skin microfilariae (mf)

From the shaved skin, three superficial skin biopsies were taken with a scalpel blade from along the *linea alba*: one just posterior to the umbilicus, one mid-way between umbilicus and udder and one just anterior to the udder/scrotum. The skin biopsies (range was 20-50 mg for all three skin snips) were processed and mf morphologically identified and distinguished according to the method of Wahl *et al*, (36). Microfilariae of *O. gutturosa* and other filarial species such as the occasionally detected *O. dukei*, *O. armillata* and *Setaria species* were also counted. The total number of mf counted were divided by the skin biopsy weight to obtain the mf density per mg of skin.

Transmission dynamics

The monthly biting rates of *Simulium* flies, for the last year of the study, were assessed by catching flies on a

fly collector at the banks of the river or the inland watering point. After dissection of these flies data on annual transmission potential and other critical values necessary for the understanding of the epizootiology of the infestation were derived (Achukwi, in preparation). Relative attractiveness of *Simulium* flies to cattle (Achukwi, unpublished data) showing different parasite abundance in the same herd was similar.

Preparation of recombinant Ov10/Ov11 antigens
Ov11 has also been referred to as OvMBP20/11 (5). Ov10 (6) is an analogue of Ov7 (21). Ov10/Ov11antigens (a marker of infection) obtained as fusion proteins with maltose binding protein (MBP) were used as a 2 lg/mL (1 lg each of Ov10 and Ov11) cocktail in sodium carbonate buffer pH 9.7. In practice, half of each plate was coated with Ov10/Ov11 MBP and the other parallel half coated with MBP alone (New England Biolabs, USA)-referred to as MBP ELISA control. MBP responses were subtracted from the anti-Ov10/Ov11MBP results.

Preparation of PBS extract of whole adult O. ochengi
Nodules were extirpated from hides of slaughtered cattle and the worms isolated from them were extensively washed in PBS pH 7.4. Prior to processing, adult worms isolated from nodule capsules by collagenase (Sigma) digestion (31), were washed four times in PBS pH 7.4 and then extensively homogenised in a tissue homogeniser in PBS pH 7.4 containing proteinase inhibitors: N-a-P-tosyl-L-lysine-chloromethyl ketone (TLCK)-50lg/mL, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-50lg/mL, phenylmethylsulphonyl fluoride (PMSF)-50 lg/mL (Cabrera and Parkhouse, 1986). The homogenized tissue was cooled on ice for 30 minutes, centrifuged at 20,000g for 45 minutes and the supernatant, the PBS extract, recovered. The amount of protein in the sample was determined using the Biorad micro assay method protocol as described by the manufacturer.

ELISA for bovine Ig G subclass-specific measurement.
Polystyrene 96-well micro-ELISA plates were sensitized overnight (at least 16 hours) at 4°C with 100lL of "antigen" solution constituted as described above. The wells were emptied and remaining non-specific binding sites blocked with 125lL of 5% marvel^R in PBS containing 0.05% (v/v) Tween 20 (PBST) at room temperature for 30 minutes. After 4 washes in PBST, 100 lL of plasma samples diluted 1:100 in PBS containing 0.2% marvel^R was applied to the wells in triplicate. Test negative control samples were derived from 5 fly-proof house-raised animals while positive control samples were from a pool of 5 naturally infested animals. After two hours incubation on the bench, the plates were washed four times with PBST and monoclonal mouse anti-bovine IgG_1 (Mab1-SEROTEC Ltd, Kidlington, Oxford, U.K) or monoclonal anti-bovine IgG_2 (Mab2-SIGMA) diluted 1:2000 and 1:1000 respectively in PBST were applied. The PBST contained 0.2% (w/v) or 0.1% (w/v) marvel^R respectively for Mab1 and Mab2. After another two hours incubation on the bench, the plates were washed 4 times with PBST and anti-mouse IgG (Fc specific) peroxidase conjugate (Sigma) was applied. Anti-mouse IgG peroxidase conjugate was used at a dilution of 1:10,000 in PBS containing 0.5% (w/v) marvel^R and 0.05% Tween 20 (for IgG_1 response to all 'antigens'). For the IgG_2 response to the MBP and recombinant antigens the dilution was 1:10,000 in PBS containing 0.5% (w/v) marvel^R. After 2 hours incubation at room temperature the wells were emptied and washed as above. Freshly prepared (100 lL) tetramethylbenzidine (TMB) substrate solution: 1mg TMB (Sigma) dissolved in 1ml DMSO was added to 9 ml 0.05M phosphate citrate buffer pH 5.0. This substrate solution was added and plates incubated in the dark for 25 minutes at room temperature. The reaction was stopped with 50 lL of 10% sulphuric acid. The absorbance of the peroxidase reaction product in the ELISA was read on an automated microplate reader (Multiskan MCC/340p version 2.20) at 450 nm. Mean values for the triplicate readings were calculated.

Separation of O. ochengi proteins SDS- PAGE
Polyacrylamide electrophoresis in the presence of sodium dodecyl sulphate (SDS -PAGE) was performed with 10% (v/v) separation gels and 4.5% stacking gels using an ATTO apparatus (Tokyo, Japan) according to the manufacturer's instructions. As the gels were destined for immunodetection by chemiluminescence, Biotinylated SDS molecular weight standard markers (Sigma) were loaded.

Western blotting, probing with antibodies and immunodetection by enhanced chemiluminescence
The gels were arranged in a Biorad Trans-blot^R semi-dry transfer cell and blotting carried out according to the manufacturer's instructions. The transfer membrane used was Hybond-C (Amersham International, Buckinghamshire, UK). The tertiary antibody was anti-mouse Ig, horseradish peroxidase-linked species specific antibody (from sheep) [Amersham] diluted 1:1000 in Tris-buffered saline (TBS) pH 8.5 with 5% (w/v) marvel^R or 2% BSA when detecting bands of biotin-labelled SDS molecular weight standard mixture markers (Sigma). After protein transfer onto the nitrocellulose membrane, it was rinsed in TBS and blocking of free binding sites undertaken using 10% marvel^R in TBS for at least 14 hours at 4°C. The membranes were cut

into strips, rinsed in TBS and incubated in cattle plasma samples at 1:100 in 5%(w/v) marvel[R] on a rotating table in TBS overnight at 4°C. The strips were washed 8 times (lasting 30 minutes in all) with TBS containing 1% Tween 20. Monoclonal anti-bovine IgG_2 (mab2, Sigma) at a dilution of 1:500 was applied and then incubation at room temperature on a rotating table for 2 hours undertaken. The strips were again washed for 30 minutes as indicated earlier and sheep anti-mouse IgG, peroxidase-linked species-specific whole antibody conjugate (Amersham) applied for two hours on the rotating table at room temperature. A final wash lasting 45 minutes was effected and the protein bands detected by chemiluminescence using the SuperSignal™ chemiluminescent substrate (Pierce Chemical Co.) and ECL X-ray film (Amersham) and an AGFA Curix 60 processor, all according to the manufacturers' instructions. Polypeptide molecular weights in kilodaltons were calculated using values of known standards in log graphs.

RESULTS

Parasite infestation pattern

The prepatent period for acquired nodules was 10 to 24 months (both sexes combined). The first palpable nodules to appear were observed only from about 10 months of age. *O. ochengi* adult worms formed nodules that were located intradermally mainly in the region around the umbilicus, the udder/scrotum and flank. When located very close to the udder/scrotum the nodules were often in the subcutaneous tissue. In intense infestation of the peri-genital subcutaneous tissue some small and large *O. ochengi* nodules conglomerated, forming a bundle of individual nodules embedded in the subcutaneous tissue and this was clearly seen when similar clinical cases were examined in abattoir-slaughtered cattle. During four years, the 11 animals studied after the minimum patent age (Figure 1) acquired a total of 465 *O. ochengi* nodules (104, average of 17.3/female and 361, average of 72.2/male). When the parasite burden and time of acquisition of parasite after birth are jointly considered, the two animals 1256F and 1030M could be regrouped into two distinctive infestation patterns: animal with very low (low susceptibility) or high parasite load (high susceptibility). The highly susceptible animal, number 1030M, acquired 101 *O. ochengi* nodules meanwhile the low parasite load animal, 1256F, acquired only 2 nodules during the same period of exposure to *Simulium* biting risk. Both animals had the same age. The *O. ochengi* nodule (adult worm) load increased steadily with the age of animal in animal number 1030M showing a boom of nodule acquisition as from three years of age (the age of maturity in bulls).

Both animals were concurrently infested by *O. gutturosa* mf. The peak mf density for the animal (1030M) with the highest number of *O. ochengi* nodules was 9.75 mf/mg (*O. ochengi*) and 0.57 mf/mg (*O. gutturosa*) and this was during its fourth year of life. Meanwhile the animal 1256F had extremely low levels of both parasites throughout the study period and frequently oscillated between zero and 0.5 mf/mg (*O. ochengi*) and zero to 3 mf/mg (*O. gutturosa*) after the first appearance of dermal mf. The mf density was significantly higher during the dry season than during the rainy season.

IgG_1 and IgG_2 antibody response to Ov10/Ov11 MBP recombinant O. volvulus antigens.

The antibody responses were predominantly IgG_1 relative to IgG_2 (Figure 1 (IgG_1) and 2 (IgG_2)). IgG_1 responses were detected in both animals throughout the

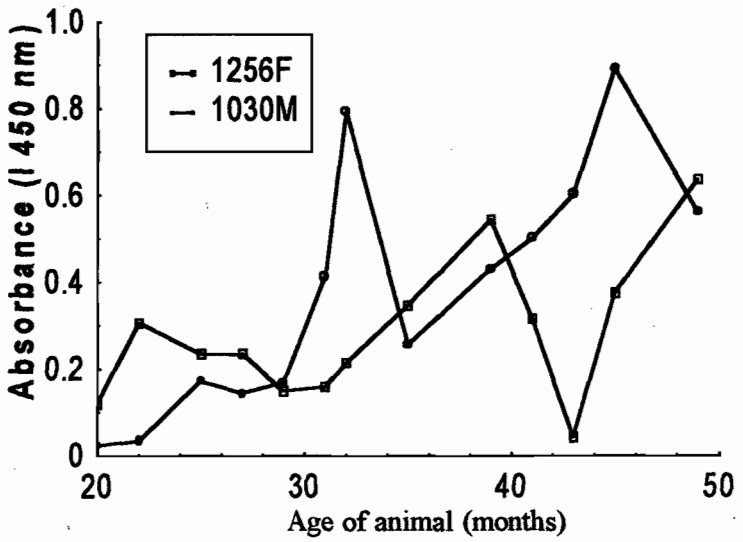

Figure 1 : IgG1 response to Ov10/Ov11 MBP recombinant antigens in animals exposed to *Simulium* fly bites

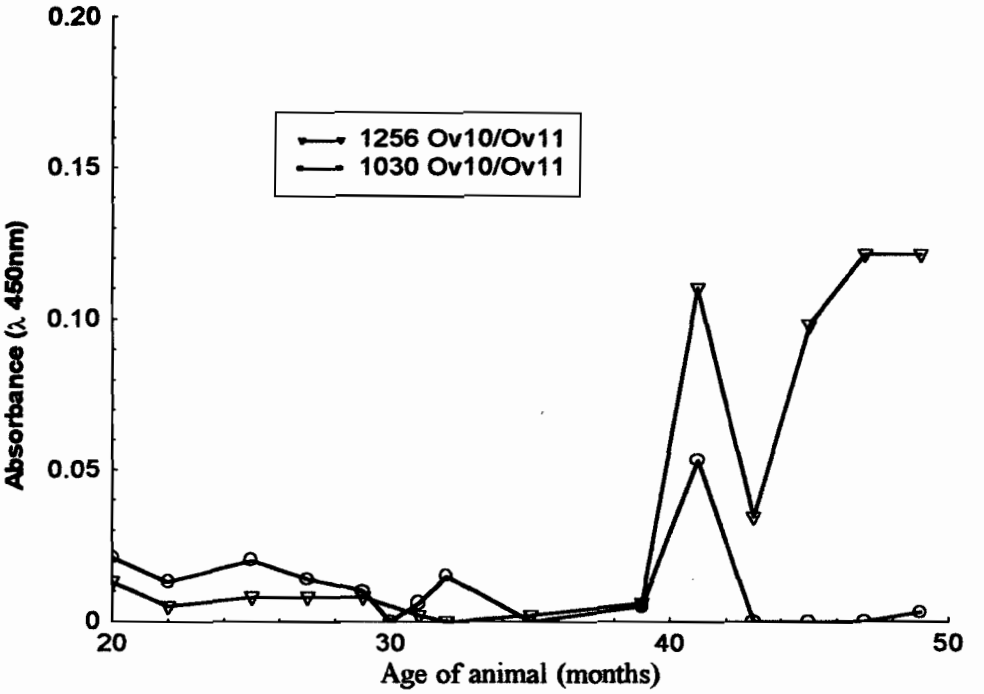

Figure 2: IgG2 antibody response to Ov10/Ov11 MBP recombinant antigens in animals exposed to *Simulium* fly bites

study while elevated IgG_2 responses were detected more frequently in animal number 1256F. Accumulated *O. ochengi* nodules count and IgG_2 response to recombinant *O. volvulus* antigens were positively correlated but IgG_1 responses were not. The mf density did not correlate with either IgG_1 or IgG_2 responses.

O. ochengi antigens detected by Western blotting using IgG_2-specific monoclonal antibody

Circulating antibodies of the two infected microfilariadermic animals recognized a multitude of *O. ochengi* high and low molecular weight polypeptides (results not illustrated). Most of the other animals examined (data not shown) recognized *O. ochengi* antigens of about 49 KDa 30, 28 and 27 KDa although some were only faintly detected. The animal (1256F) with the least parasite burden (Figure 3: lane a) apparently recognized a number of bands between 17 KDa and less than 27 KDa that were not detected by the highly infested animal (lane b). Other animals with very low parasite burdens prominently detected the 29 Kda, which was only faintly recognized by the high parasite burden animal.

DISCUSSIONS

The observed heterogeneity in antigens recognized by the definitive host and its association with specific antibody isotype responses during parasite infestation, suggests that a single factor may not dictate the two distinguished susceptibility patterns (very low and high parasite burden). This is also an indication that the host immune responses to infestation by this parasite show a heterogeneous pattern. Other factors such as the host age, sex, season and the intensity of challenge or the varying monthly transmission pressure (Achukwi, in preparation) over the years are likely factors which appear to exert strong mediating influences to regulate worm burden. The presence of a season effect on mf density may contribute immensely to the transmission pattern of the parasite and may also explain why the transmission (which is also linked to the seasonal breeding of *Simulium*), was noted to be seasonal (Achukwi, in preparation). This suggests that a strategic treatment of animals on a regional basis at the start of the period of peak mf density will cut down transmission. The analysis of data on relative attractiveness of animals to *Simulium* flies however failed to link the observed individual differences in parasite acquisition to different *Simulium* biting rates (Achukwi, unpublished data). The animal 1030M acquired few parasites, during the first three years of its life and this was followed by an acerbated infestation in the fourth year. This may indicate a breakdown of 'resistance' to further infestation, probably due to increase in transmission intensity. It has already been speculated that resistance to infestation in bancroftian filariasis may not be absolute when attained and with sufficient pressure of infection, it might be overcome (30). The animal, 1256F, that demonstrated a more sustained or steady state ('resistance') to the establishment of further adult parasites in the presence of the very first few living adult worms may be considered to indicate concomitant immunity. Concomitant

immunity is also known to have been experimentally demonstrated in rhesus monkeys (*Macaca mulatta*) infected with *Schistosoma mansoni* (33). The steady state level of infestation described in this study despite long term continuous exposure to new infections was also associated with the elevation of IgG_2 levels. Such animals that limit the acquisition of further adult worms need to be continuously exposed to natural vector challenge for more years to see whether the condition is temporal or not. No putatively immune animals were identified during this prolonged study period since all animals continuously exposed to the vectors of the disease for up to four years became infested with both *O. ochengi* and *O. gutturosa*. The attribution of a putatively immune state to the absence of *O. ochengi* infestation to a 21 months old calf by Tchakouté *et al* (32) in the same endemic area may therefore be an overstatement considering the totality of the data presented here and unpublished.

Furthermore, the use of the recombinant antigens permitted the diagnosis of prepatent infestations, which was not the case when crude worm extract of *O. ochengi* or *O. gibsoni* ES was used (data not shown). Unlike the IgG_1 response, which dominated the kinetics of the antibody response, the IgG_2 response levels varied greatly between the two animals. The predominance of IgG_1 responses relative to those of IgG_2 observed is similar to that recently reported with respect to cattle experimentally infected with *Onchocerca lienalis* in the United Kingdom (18). Bovine IgG_1 isotype has also been reported to be the dominant isotype in infections with *Dictyocaulus viviparus* (34) and *Ostertagia ostertagi* (23). In a study with *Loa loa* in Gabon (3), similarly high levels of IgG_1 were reported to block the establishment or development of newly invading infective larvae thereby limiting the level of human infection. Bovine IgG_2 has been shown to be more efficient than IgG_1 at catalyzing antibody-dependent cell cytotoxicity (ADCC) reactions by polymorphonuclear leucocytes in ruminants (15, 24). ADCC reactions are also the most important means of defense against filarial nematodes (12,22). By implication therefore, the elevated levels of IgG_2 identified in the animals cited, probably indicate the development of T_H2 responses partly due to chronic presence of mf. A speculated general feature of helminth infections holds that first infections initially induce a strong T_H1 response while repeated infections (e.g chronic exposure to mf) may lead to the development of T_H2 cells; but a critical threshold must be reached for this to happen (19). However, data on cytokine responses need to be gathered in subsequent studies to enable a complete appreciation of these findings.

The heterogenous recognition of antigens by IgG_2 anitbodies of the two animals studied here is similar to reports that individuals within a host species show considerable heterogeneity in the specificity of antibodies produced in response to parasitic infections (7, 16, 14). Great variations in the antibody repertoire of individuals infected by some other nematodes have also been attributed to genetic control (16). In the present study, the two animals which were microfilaridermic (microfilaria density very high in 1030M but extremely low in 1256F) had elevated levels of IgG_2 at certain time points and preferentially recognized low molecular weight antigens in western blots. The increased intensity of the bands of the polypeptide molecules in Western blots, when later as opposed to earlier plasma was used (data not shown), match the results of the ELISA and suggest increased antibody production as infestation or exposure intensity increased. This spectrum suggests that the possible resistance to new infections may be due to a set of specific parasite antigens and not a single polypeptide. Also individual differences in filarial infestations with respect to *O. volvulus* in humans due to sex and genetic factors have been reported by Ward *et al* (38). In West Africa (9, 17) and North Cameroon (27) males were reported to be significantly more infected with human onchocerciasis than females. In a background of no obvious difference in exposure to the vectors in the hyper-endemic area of Ecuador, Elson *et al* (11) however reported more parasite-free females than males in humans. Meanwhile Prost and Paris (26) attributed sex differences observed in humans in Upper Volta of similar age groups to differences in production systems and social distribution of jobs. The reports of Anderson *et al* (2) and Renz *et al*. (27) also indicate that old women had similar or even higher microfilarial densities than males at the same age. Apart from daily life patterns of humans particularly boys culminating in higher exposure to infection of boys than girls, (27) it was thought that hormonal differences might account for the observed differences. Male Gudali cattle mature into good reproductive bulls between the age of 3 and 4 years, the period in this study when the male animal was observed to distinctly acquire more parasites than the female. Perhaps the rapid endocrine changes during this period could, like suggested for humans, account for the observed sex differences in susceptibility.

The 'boom' of nodules in the animals studied after the minimum patent age tended to coincide with a period of about 8 to 10 months (which is similar to the minimum prepatent period) after peak inoculation of L_3 (or the peak in transmission pressure). This may suggest a relationship between the vector inoculation intensity,

and the pattern of patent infection (Achukwi *et al*, in preparation). Studies related to human filariasis (20, 29) and in *Acanthocheilonema viteae* using jirds (10) have considered the molting infective larva as an important target for the host immune response; and this is consistent with earlier suggestions in this study. Antigens uniquely detected when this event occurs are consequently important elements for studies in immunity.

CONCLUSION

This study has in addition, demonstrated that the cocktail of Ov10/Ov11 MBP recombinant *O. volvulus* antigens provides a useful tool in the evaluation and screening of cattle *O. ochengi* infestation. Graham et al. (1999) have similarly reported that an extensive panel of other recombinant *O. volvulus* antigens was recognized by sera from cattle experimentally infested with *O. ochengi*. This immunological cross-reactivity between the two species could be due to the fact that they are phylogenetically very closely related. The significance of elevated IgG_2 levels over a sustained period in the animal showing extremely low parasite load necessitate further evaluation. Furthermore, the identification of the antigens exclusively recognized by more animals showing such steady state infestation patterns, despite continuous exposure to the risk of *Simulium* bites, will throw more insights into individual host-parasite interactions in the cattle *O. ochengi* system in endemic areas.

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