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# Most of the Response Elicited against *Wolbachia* Surface Protein in Filarial Nematode Infection Is Due to the Infective Larval Stage

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**Immune responses to the intracellular *Wolbachia* bacteria of filarial nematodes are thought to contribute to the pathologic process of filarial infection. Here, we compare antibody responses of subjects living in an area where lymphatic filariasis is endemic with antibody responses elicited in a murine model of filarial infection, to provide evidence that the infective larval stage (L3), not adult nematodes, are the primary inducer of responses against *Wolbachia*. In human subjects, antibody responses to *Brugia malayi Wolbachia* surface protein (WSP) are most often correlated with antibody responses to the L3 stage of *B. malayi*. Analysis of anti-WSP responses induced in mice by different stages of the rodent filariae *Litomosoides sigmodontis* shows that the strongest anti-WSP response is elicited by the L3 stage. Although adult filarial nematode death may play a role in the generation of an anti-WSP response, it is the L3 stage that is the major source of immunogenic material, and incoming L3 provide a continual boosting of the anti-WSP response. Significant exposure to the endosymbiotic bacteria may occur earlier in nematode infection than previously thought, and the level of exposure to infective insect bites may be a key determinant of disease progression.**

Filarial nematodes are the causative agents of the diseases lymphatic filariasis (elephantiasis) and onchocerchiasis (river blindness), which result in severe morbidity and considerable economic losses in >80 countries where these parasitic infections are endemic [1]. Infection involves host exposure to both a nematode and its obligate intracellular bacterium, which is most closely related to *Wolbachia pipientis* of arthropods but not formally described as a new species (hereafter referred to as "*Wolbachia*") [2]. The pathologic process of filarial disease has long been known to have an immune component, and recent studies have strongly implicated bacterial products released after parasite death as a key factor in

this process [3, 4]. As the potential significance of these bacterial parasites to the biological processes of filarial nematodes becomes more apparent [5], understanding the potential consequences of their interaction with the mammalian host becomes increasingly important. A key question is whether the immune response observed during filarial infection is directed against both organisms. We have therefore looked for evidence of immune responses to the intracellular bacteria of filarial parasites by investigating the pattern of antibody responses against the *Wolbachia* surface protein (WSP). We started the analysis by evaluating antibody responses of subjects living in an area where lymphatic filariasis is endemic. Findings from the human epidemiological study inspired us to perform an experimental investigation, using mice infected with the related rodent filaria *Litomosoides sigmodontis*. These data from human and murine studies show that antibody responses against WSP are made during natural infection and suggest that most of this response is induced by the L3 stage. This provocative finding is supported by data showing that, per gram of nematode, the WSP produced by the L3 stage of *L.*

*sigmodontis* induces the strongest immune response of all the developmental stages.

## MATERIALS AND METHODS

***Antigens.*** Extracts of *Brugia malayi* and *L. sigmodontis* were used to assay antifilarial antibody responses in human or mouse serum samples, respectively. Somatic extracts of L3-stage larvae, adult parasites, or microfilariae (Mf) were prepared by homogenization. For L3 and Mf preparations, parasites were broken down further by additional sonication in PBS. ELISAs were performed by use of soluble somatic extracts after centrifugation at 1000 *g* for 20 min.

*B. malayi* WSP (*Bm*WSP) and *L. sigmodontis* WSP (*Ls*WSP) homologs were cloned by use of polymerase chain reaction amplification of a 652-bp fragment from genomic DNA with degenerate WSP primers, as described by Bazzocchi et al. [6]. Both gene fragments were cloned into pET29b (Novagen) and expressed in *Escherichia coli* BL21(λDH3). The recombinant hexamer histidine-tagged proteins were purified by affinity chromatography and dialyzed against PBS before use. Both DNA fragments have been sequenced and deposited in GenBank (accession nos. AJ252061 and AF409112, respectively). A *Brugia pahangi* ladder protein was generated as described elsewhere [7].

***Human serum samples.*** One hundred four human serum samples from residents of 2 different areas (Rengat and Palau) of Sumatra, Indonesia, were selected for testing. These samples were divided into 3 groups on the basis of parasitological and clinical status, as described elsewhere [8–10]. European control serum samples were obtained from volunteers at Edinburgh University (Edinburgh, Scotland). Informed consent was obtained from all patients before clinical and parasitological studies and before blood samples were obtained, in accordance with the Indonesia Department of Health and Human Services. Animal experimentation guidelines of the UK Home Office were followed in the animal studies.

***Mouse infection and immunization.*** For live infection, BALB/c male mice (6–8 weeks old) were injected subcutaneously with 25 L3-stage *L. sigmodontis* in the lumbar area, as described elsewhere [11, 12]. Serum samples were collected at days 10, 20, 40, 60, and 80 after infection. To examine whether *Ls*WSP is immunogenic within nematode material, $3 \times 10$ μg of total homogenized and uncentrifuged *L. sigmodontis* material (L3-stage larvae, mixed adult parasites, or Mf) in an emulsion with Freund's adjuvant (FA) was injected subcutaneously into the lumbar area of BALB/c mice at 4-week intervals. The first immunization was given in complete FA (CFA), with subsequent doses given in incomplete FA. Serum samples were obtained before each injection and 4 weeks after the third injection. Positive control serum samples for *Ls*WSP were generated from BALB/c male mice injected 3 times with 10 μg of *Ls*WSP

at 4-week intervals. Serum samples were also collected from BALB/c mice 3 weeks after they were surgically implanted intraperitoneally with 6 live *L. sigmodontis* adult parasites removed from the peritoneal cavity of infected jirds [13]. Mice that underwent sham surgery but did not receive parasites were included for control serum samples.

***ELISA.*** ELISAs were performed as described elsewhere [14]. In brief, 96-well ELISA plates (Nunc Maxisorp) were coated with 0.5 μg of antigen resuspended in 100 μL of carbonate buffer/well. After blocking with 100 μL of 1% skim milk powder in carbonate buffer, 50 μL of serum diluted in PBS with 0.5% Tween (PBST) was plated into each well in 2-fold serial dilutions of 1:100 to 1:3200. Each sample was plated in duplicate. A dilution that included the linear range of every sample in each ELISA was chosen for analysis (see the figure legends for details). Antibodies were detected with 50 μL of peroxidase-conjugated goat anti–mouse total IgG (1:1000; Biorad) and rabbit anti–human total IgG (1:6000; Dako) diluted in PBST. Plates were developed with 50 μL of 2,2′-azinodi(ethyl-benzthiazo-line-6-sulfonate) (Kirkegaarde and Perry Laboratories) and read at 405 nm.

***Statistical analysis.*** To detect differences among the antibody responses of 3 human clinical groups against *Bm*WSP, *B. malayi* L3 and adult extracts, and *B. pahangi* ladder protein, 1-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparison tests to analyze pairwise comparisons, by use of GraphPad software (Prism). The European serum control samples were not included in this analysis. Optical density values multiplied by 100 were logarithmically transformed before these analyses to normalize the data and allow parametric analyses to be performed. This transformation was not sufficient to normalize the values against *B. pahangi* ladder protein; thus, equivalent nonparametric tests were used to analyze the transformed values against this protein (the Kruskal-Wallis test, followed by Dunn's multiple-comparison test).

Because of the small sample sizes, it was not possible to determine whether the transformed optical density values for mouse antibody conformed to the assumptions of parametric tests; thus, a nonparametric Mann-Whitney *U* test was used to analyze differences among mice. $P < .05$ was considered to be statistically significant.

General linear modeling (GLM; Minitab) was used to analyze the logarithmically transformed optical density readings indicating the human antibody responses [15]. GLM uses regression to partition the variation in an observed response between different possible variables (in this case, responses to L3 or adult *B. malayi*, age, sex, and location of the subjects' residences). Analysis of the residuals from the GLM confirmed that the transformed data accorded with the normality and homogeneity of variance assumptions of parametric tests. In each
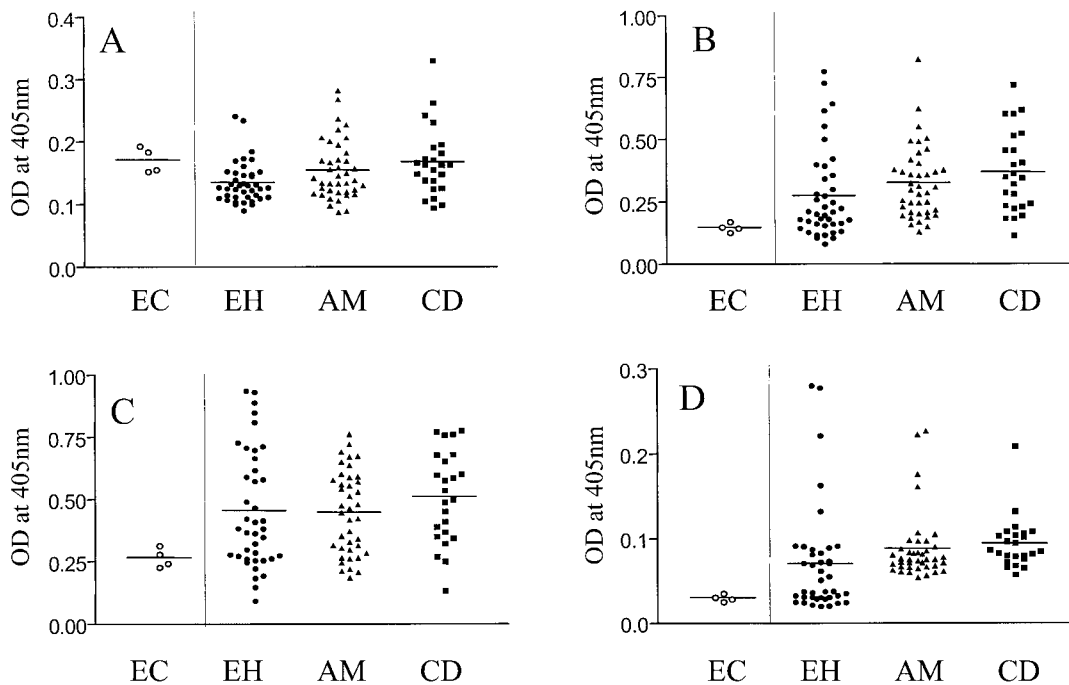
**Figure 1.** Total IgG responses to *Brugia malayi Wolbachia* surface protein *(A), B. malayi* infective larval stage (L3) extract *(B), B. malayi* adult extract *(C)*, and *B. pahangi* ladder protein *(D)* in serum (diluted 1:400) obtained from human subjects infected with *B. malayi.* Subjects were classified as being endemic healthy (EH; $n = 40$; with undetectable infection or disease), as having asymptomatic microfilaremia (AM; $n = 40$; with circulating microfilariae and no signs of disease), or as having chronic disease (CD; $n = 24$) [8–10]. Four European control (EC) serum samples were included for comparison. Each data point refers to 1 person. Bars represent the mean for each group.

model, variables that did not significantly correlate with anti-*Bm*WSP responses were removed before rerunning the model. By use of this method, GLM can untangle the noncontributing variables from the anti-L3 and anti-adult responses that serve as predictors of responses to *Bm*WSP. Significant *P* values are from the minimal model (only including significant variables).

The method used to visualize the GLM results was as follows: We investigated whether strong responses to *Bm*WSP and the L3 stage are still correlated after the contribution of anti-adult responses has been subtracted. When *Bm*WSP responses were plotted against *B. malayi* L3, few individuals responded to *Bm*WSP exactly as predicted by the best-fit line (see Results). We plotted the distance these responses fall away from the best-fit line (positive and negative residuals for strong or weak responses to *Bm*WSP for a given response to L3) against the positive and negative residuals from a plot of anti–*B. malayi* L3 against anti-adult responses. The latter residuals represent strong or weak responses to *B. malayi* L3 for a given level of anti-adult response—the equivalent of subtracting anti-adult responses from anti-L3 responses. For all plots, the logarithmically transformed optical density data were used. This method was repeated to determine whether responses to *Bm*WSP correlate with responses to adult stages after the anti-L3 responses had been removed.

## RESULTS

**Human responses to Bm*WSP* in B. malayi *infection.*** We investigated whether individuals living in an area where *B. malayi* lymphatic filariasis is endemic showed evidence of immune responses to *Wolbachia* proteins. First, we examined total IgG antibody responses to recombinant *Bm*WSP in 3 different clinical groups [10]. Serum samples were collected in the Rengat and Palau regions of Sumatra, Indonesia, from 24 individuals with chronic disease (20 in Rengat and 4 in Palau), 40 endemic healthy subjects (20 in Rengat and 4 in Palau), and 40 subjects with asymptomatic microfilaremia (20 in Rengat and 20 in Palau). The antibody response to *Bm*WSP was different in these groups ($F_{2,103} = 4.237$ and $P < .05$, 1-way ANOVA; figure 1*A*). Subjects with chronic disease had significantly greater responses to *Bm*WSP than did endemic healthy subjects ($P < .05$, Tukey's test). This trend was similar to the antibody response to the L3 stage of *B. malayi* ($F_{2,103} = 4.825$ and $P \leq .01$, 1-way ANOVA; figure 1*B*) but not the response to the adult-stage extract, which did not differ statistically among the clinical groups ($F_{2,103} = 1.069$ and $P > .1$, 1-way ANOVA; figure 1*C*). As a control, we tested responses to another recombinant protein (*B. pahangi* ladder protein) that is also nematode derived (figure 1*D*) [7]. The antibody responses

to the ladder protein exhibited a distribution similar to those of the anti-$Bm$WSP and anti-L3 responses, in that they differed statistically among the different clinical groups tested ($P < .001$, Kruskal-Wallis test). Both the subjects with chronic disease and those with asymptomatic microfilaremia had statistically significantly higher responses than the endemic healthy subjects (both $P < .05$, Dunn's multiple comparison test; figure 1$D$).

GLM was used to examine whether the within-group variation in the antibody response to $Bm$WSP could best be explained by responses to *B. malayi* larvae, *B. malayi* adults, or the age, sex, clinical status, or location of the people tested. Anti-$Bm$WSP responses were positively correlated with responses to both L3 and adult stages of *B. malayi* (anti-L3 response, $F_{1,103} = 22.88$ and $P < .001$; anti-adult response, $F_{1,103} = 17.75$ and $P < .001$; figure 2$A$ and 2$B$). However, the slope of these relationships differed among the clinical groups (anti-L3 response by clinical group

interaction, $F_{2,103} = 3.89$ and $P < .05$; anti-adult response by clinical interaction, $F_{2,103} = 2.65$ and $P > .05$). In both the chronic disease and the asymptomatic microfilaremia groups, antifilarial (L3 or adult) and anti-$Bm$WSP responses were positively correlated (both $P < .05$), with the best-fit lines being similar in the 2 groups ($P > .5$, for differences in slope and intercept). This correlation was significant even when we controlled for the potentially confounding effects of age, sex, and location of subjects ($P > .05$, for these covariates). However, in the endemic healthy subjects, responsiveness to $Bm$WSP was not significantly related to responsiveness to either *B. malayi* stage (anti-L3 response, $F_{1,39} = 0.25$ and $P > .1$; anti-adult response, $F_{1,39} = 0.04$ and $P > .1$). The variation was best explained by the geographic location of endemic healthy subjects ($F_{1,39} = 5.26$ and $P < .05$).

Responses to adult and L3 antigens were positively correlated ($F_{1,103} = 311.45$ and $P < .001$). Thus, the patterns shown in figure
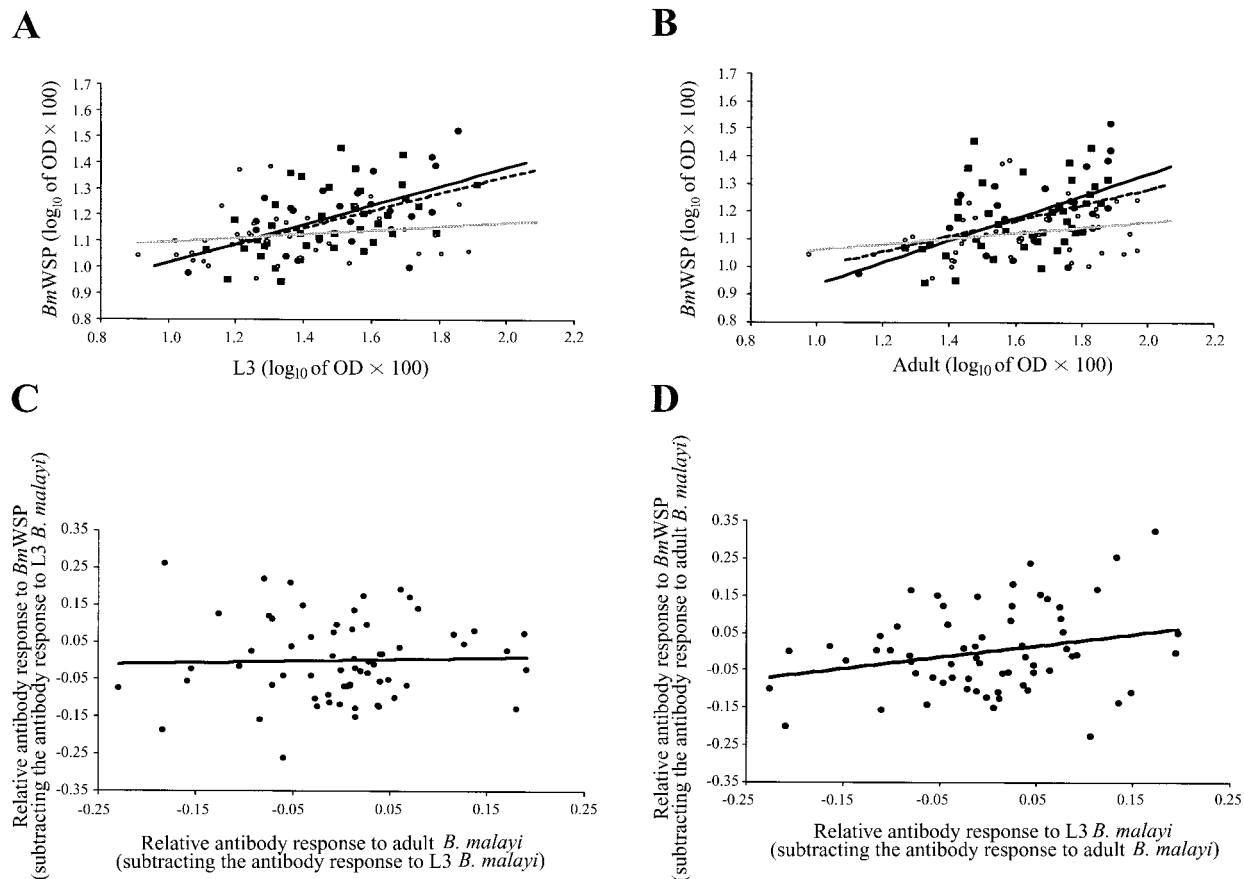


**Figure 2.** The total IgG response to *Brugia malayi Wolbachia* surface protein (*Bm*WSP) plotted against the response to infective larval stage (L3) extract *(A)* and *B. malayi* adult extract *(B)*. *White circles,* Endemic healthy subjects; *squares,* subjects with asymptomatic microfilaremia; *black circles,* subjects with chronic disease; *solid lines,* best-fit line through the data points for subjects with chronic disease; *dashed lines,* best-fit line through the data points for subjects with asymptomatic microfilaremia; and *gray lines,* best-fit line through the data points for endemic healthy subjects. Plotted values in panels *C* and *D* are the levels of disproportionate responses of individuals to adult or L3 *B. malayi,* respectively *(X*-axes), each plotted against the respective disproportionate response to *Bm*WSP *(Y*-axes). The positive and negative values represent whether there was a positive or negative response (residuals), compared with the best-fit lines through the following plots: *C, X*-axis, anti-adult responses against anti-L3 responses, and *Y*-axis, anti-*Bm*WSP responses against anti-L3 responses; *D, X*-axis, anti-L3 responses against anti-adult responses, and *Y*-axis, anti-*Bm*WSP responses against anti-adult responses. The lines in panels *C* and *D* are the best least-squares fits through the plotted points.

2A and 2B could be the result of responsiveness to BmWSP arising from exposure to L3 stages alone, to adult stages alone, or to both stages of B. malayi. To test which of these parasite stages was responsible for responsiveness to BmWSP, we asked whether anti-L3 responses and anti-adult responses are independently associated with anti-BmWSP responses. We removed the endemic healthy group from this analysis, because, in that group, there was no correlation between the anti-BmWSP responses and either the anti-L3 or anti-adult B. malayi responses. Among parasite-positive subjects, there was no evidence that the responses to adult B. malayi and BmWSP were associated when we controlled for the responses to L3 (see Methods) ($F_{1,63} = 0.08$ and $P > .1$; figure 2C). In contrast, the responses to L3 were still associated with responses to BmWSP when we controlled for the responses to adult B. malayi ($F_{1,63} = 4.29$ and $P < .05$; figure 2D). Thus, anti-BmWSP responses were correlated with responses to L3, over and above the anti-adult responses (figure 2D), but anti-BmWSP responses did not correlate with anti-adult responses independently of the L3 responses (figure 2C). Thus, these analyses reveal that anti-BmWSP responses arise through exposure to L3 stages but find no evidence that exposure to adult B. malayi independently contributes to anti-BmWSP responses. The association between anti-BmWSP and anti-adult B. malayi responses (figure 2B) exists because responses to adult and L3 stages are correlated, possibly as a result of cross-reactivity between the 2 stages.

**Mouse responses to LsWSP in L. sigmodontis infection.** GLM analysis of the human responses to BmWSP led to unexpected and provocative results with regard to the role of the L3 stage in immune responses against Wolbachia. To gain more-specific data regarding the induction and maintenance of anti-Wolbachia responses, we used a murine model of filarial infection that permits the full developmental cycle of the parasites [16]. We analyzed the total IgG response to LsWSP in BALB/c mice infected with L. sigmodontis. We found that, in a primary infection, most mice had a very low but statistically significant response to LsWSP ($P < .05$, Mann-Whitney U test) at 20 days after infection, in comparison with naive mice (figure 3A). This significant difference was observed in 2 separate experiments. The response decreased to background levels at day 40 but increased again at day 60 as the adult nematodes reached maturity and began reproducing. In contrast, there were significant responses to L. sigmodontis adult antigen at all time points from day 20 onward ($P \leq .01$, Mann-Whitney U test), which increased as the infection progressed and peaked at 60 days after infection, when adult parasites had reached patency (figure 3B). In the mouse model of L. sigmodontis infection, adult death also begins to occur at this point [16].

The responses to LsWSP induced by adult parasites in L. sigmodontis primary infection were highly variable and lower than might be expected if Wolbachia antigen is released pre-
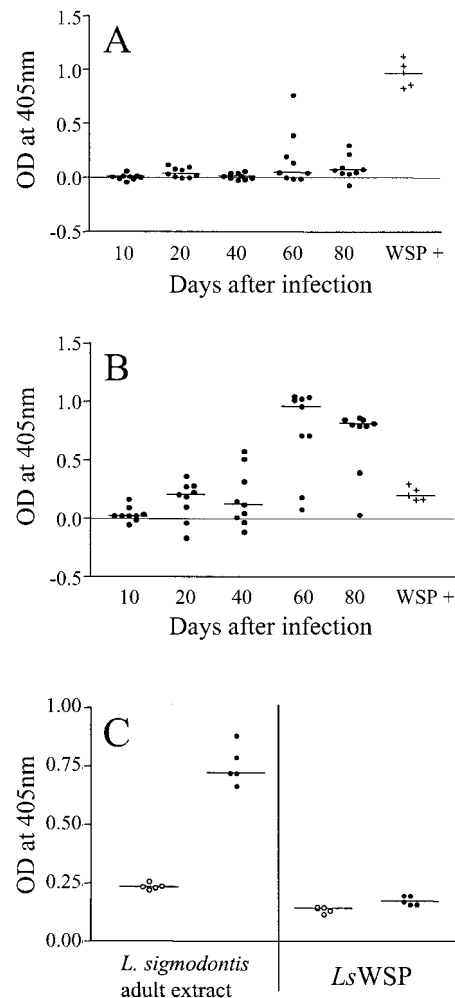


**Figure 3.** Total IgG response to *Litomosoides sigmodontis Wolbachia* surface protein *(LsWSP; A)* and adult *L. sigmodontis* extract *(B)* elicited from BALB/c mice challenged with a primary infection of *L. sigmodontis.* At all points after infection, the average response of all the naive mice was subtracted from the response of each individual infected mouse at the relevant time point. Two experiments including 5 naive mice and 5 infected mice are represented at each time point examined. Each data point represents a 1:200 dilution of serum from 1 mouse. The bars on each graph show the median for each group. Antisera to recombinant *LsWSP* (WSP+) were included in all ELISAs as a positive control for WSP. Five mice implanted with adult *L. sigmodontis* for 21 days were tested for antibody responses to adult *L. sigmodontis* extract and *LsWSP (C).* White circles, Naive mice (*n = 5*) that underwent surgery but did not receive parasites.

dominantly after parasite death. One cause of the variability may be the onset of the production of Mf, because primary infections with L. sigmodontis result in only 50% of BALB/c mice becoming microfilaremic [17]. Alternatively, variation in the number of nematodes surviving to adulthood may contribute to the variation in these experiments. We therefore decided to examine the responses to LsWSP by use of a more homogenous system, in which adult parasites are implanted directly into the

peritoneal cavity of BALB/c mice [13]. Implantation of Mf-producing adult parasites induced a very weak but statistically significant response against *Ls*WSP ($P < .01$, Mann-Whitney $U$ test), compared with that in naive mice, whereas large amounts of anti-*L. sigmodontis* adult IgG antibodies were produced ($P < .01$, Mann-Whitney $U$ test; figure 3*C*). These data indicate that Mf-producing adults alone may not be a major inducer of anti-*Ls*WSP responses.

***Mouse responses to LsWSP in different parasite life cycle stages.*** *Wolbachia* are vertically transmitted and thus are present in all stages of filarial nematodes [18]. Our studies thus far indicated that L3-stage *Wolbachia* are immunogenic (figures 2*A* and 3*A*) and are potentially the most important stage in inducing anti-WSP responses. In an attempt to further clarify the contribution of the *Wolbachia* within adult parasites and Mf to the observed response to *Ls*WSP, we injected extracts of each of these stages of *L. sigmodontis* in emulsions of CFA and measured antibody responses to *Ls*WSP.

Strikingly, the only mice that had responses to *Ls*WSP significantly greater than those of the control mice were those injected with L3 extract ($P < .05$, Mann-Whitney $U$ test; figure 4*A*). As controls for this experiment, the serum samples were

tested for antibody responses to the L3-stage larvae, adult parasites, and Mf. As expected, all mice injected with L3 extract produced antibodies to this antigen, as determined by ELISA ($P < .05$, Mann-Whitney $U$ test; figure 4*B*). All mice injected with Mf extract, except for 1, produced cross-reactive antibodies to L3 extract ($P < .05$, Mann-Whitney $U$ test; figure 4*B*). The mice injected with adult extract produced antibodies to adult extract, as well as L3 extract (both $P < .05$, Mann-Whitney $U$ test; figure 4*C*), confirming the result of our human studies that the L3 and adult stage of filarial nematodes are highly cross-reactive. Mice injected with Mf extract recognized the L3 antigen (figure 4*B*) better than the Mf antigen ($P > .05$, Mann-Whitney $U$ test; figure 4*D*). This result, although difficult to explain, was observed in 2 separate experiments. Interestingly, the single mouse injected with Mf extract that produced a recall response to Mf extract was the mouse that did not produce a cross-reactive antibody response to L3 extract. The mice injected with both larvae and adults produced cross-reactive antibodies to Mf extract (both $P < .05$, Mann-Whitney $U$ test; figure 4*D*), showing that the lack of antibodies to Mf extract in mice injected with this antigen was not due to failure of the assay. These results are not surprising, because Mf are juvenile
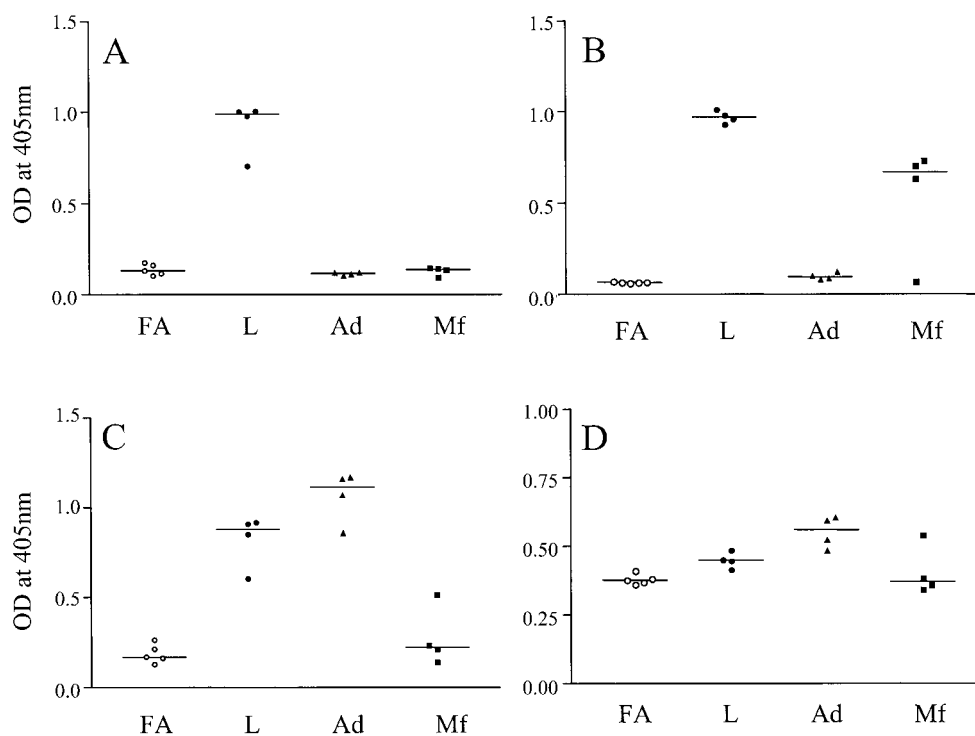


**Figure 4.** Antibody responses to *Litomosoides sigmodontis Wolbachia* surface protein *(Ls*WSP; *A),* infective larval stage (L3) extract *(B),* adult extract *(C),* and microfilarial extract *(D)* elicited from BALB/c mice injected subcutaneously with extracts of different stages of *L. sigmodontis.* For clarity, only data from serum samples taken 4 weeks after the final dose of extract (see Methods) are shown. A 1:200 dilution of serum is shown. Each data point refers to an individual mouse. The bars represent the median of each group. Ad, injected with adult extract and Freund's adjuvant emulsion ($n = 4$); FA, injected with PBS and Freund's adjuvant emulsion ($n = 5$); L, Injected with L3 extract and Freund's adjuvant emulsion ($n = 4$); Mf, injected with microfilarial extract and Freund's adjuvant emulsion ($n = 4$).

larvae that are likely to share antigenic components with the L3 stage and because the adult extract was made from mixed adults, including gravid Mf-producing females.

## DISCUSSION

The data from the present study demonstrate that *Wolbachia* are an immunogenic component of filarial nematodes and that responses are made against *Wolbachia* in both human and murine filarial infection. Anti-WSP responses have recently been shown to be produced in *Dirofilaria immitis* infection of cats [19] and *B. malayi* infection of rhesus monkeys [20], indicating that responsiveness to WSP is a feature of filarial nematode infection.

The damaging inflammatory responses that lead to lymphatic damage and elephantiasis have often been attributed to the death of adult parasites [21]. Recently, it has been hypothesized that the death of filarial nematodes is largely responsible for the release of *Wolbachia* that subsequently causes the damaging inflammatory immune responses observed in patients with elephantiasis [22]. Indeed, the death of filarial nematodes significantly increases the levels of *Wolbachia* DNA in the bloodstream of humans [23]. In support of this hypothesis, our data indicate that human immune responses to *Bm*WSP are correlated with antifilarial adult responses (figure 2*B*). In addition, the death of adult parasites in *L. sigmodontis* infection appears to induce an immune response to *Wolbachia* (figure 3*A* and 3*C*). However anti-*Bm*WSP responses in humans were also correlated with anti-L3 stages (figure 2*A*), and GLM analyses indicated that responses to *Bm*WSP were more likely to be generated by the L3 stage than by the adult stage of *B. malayi*. Indeed, further experiments with the *L. sigmodontis* murine model strongly supported this finding, because, per gram of nematode, WSP within the L3 stages induced the strongest response (figure 4*A*). Thus, the positive correlation between anti-*Bm*WSP and anti-adult responses are likely to be due to cross-reactivity between L3 and adult stages of the parasite, as shown in figure 4*C*.

We statistically tested other factors—such as age, sex, and geographic location of the subjects—that could be responsible for our finding that anti-*Bm*WSP responses are mainly generated from the L3 stage. Anti-L3 responses increase with age [24], and it is also well documented that patients with elephantiasis tend to be older [25, 26]. Therefore, it was possible that variation in age, rather than responses to larvae per se, could better explain the observed responses to *Bm*WSP. However, responses to the L3 stage of *B. malayi* were more tightly correlated with responses to *Bm*WSP than any of these variables.

The endemic healthy group was the only clinical group tested in which responses to *Bm*WSP did not positively correlate with responses to the L3 stage of *B. malayi*. This group had the same amount of within-group variation in antibody responses to the L3 stage, but less variation in anti-*Bm*WSP responses, compared with the other 2 clinical groups ($P < .05$, Bartlett's test statistic for homogeneity of variance on OD values of 8.53). Because the variation in responsiveness to L3 antigen and exposure to infective mosquito bites are comparable in all 3 clinical groups tested, low biting rate is an unlikely explanation for why there is little variation in responses to *Bm*WSP among endemic healthy subjects. Instead, it may reflect a very rapid killing of the infective larvae in this potentially immune population.

Primary *L. sigmodontis* infection in BALB/c mice indicates that a response to WSP can be induced before adult exposure (figure 3*B*), but this response (observed at day 20 after infection), although statistically significant, compared with that of naive mice, was weak. This may be because only a small number of larvae (25 at the L3 stage) were used to induce primary *L. sigmodontis* infection. When adult-stage parasites reached patency and started to die (at day 60 after infection), some mice produced stronger responses to *Ls*WSP. The data from the implantation studies with *L. sigmodontis*, as well as the injection of parasite extracts, suggest that WSP is not a major immunogen when an individual is exposed to adult or microfilarial stages in the absence of exposure to L3-stage parasites. The extract immunization studies would favor the hypothesis that the L3 stage of the parasite is intrinsically more immunogenic with regard to WSP, either because it contains the highest amount of *Wolbachia* per gram of nematode or because of a reduced level of competing immunodominant antigens.

Together, the data from human and mouse studies suggest that exposure to L3-stage parasites is required to generate a strong anti-WSP response. The requirement for the L3 stage is supported by the GLM of human antibody responses to WSP and is made considerably more convincing by murine studies demonstrating that infective larvae are intrinsically the most immunogenic when it comes to anti-WSP responses. This is not to say that the adult parasites and/or Mf do not contribute to the immune responsiveness. The death of parasites at these stages may be an important factor in creating the appropriate immunological environment for full responsiveness. This is suggested by the lower responsiveness in endemic healthy subjects, who may not harbor adult parasites and are certainly less likely to be exposed to large numbers of dying parasites, and is further supported by primary infection of mice with *L. sigmodontis*, in which robust responses to WSP were not observed until mature adults were present. Although the death of adult parasites may contribute to the development of full anti-WSP responses, our data support the hypothesis that the incoming larvae are the major inducers of the response. The significantly stronger responses to *Bm*WSP in those with chronic disease may arise because of the immune hyperactivity that is commonly observed in this clinical group [27, 28].

The present study, which used a combination of human and

mouse studies, provides evidence that the L3-stage parasite is a key player in the generation and maintenance of an anti-WSP response. If, as recent evidence suggests, exposure to WSP is an initiator of inflammatory disease [29], our data suggest that this exposure is most significant in earlier stages of infection. In the case of lymphatic filariasis, death of postinfective L3-stage parasites is likely to occur in the lymphatics and may be a more important driver of disease than the adult-stage parasite. This notion is supported by epidemiological studies suggesting that the level of exposure to infective L3-stage parasites is directly related to both the acute and chronic disease associated with lymphatic filariasis [30–32]. Increasing efforts toward designing vaccines against both elephantiasis and river blindness are under way. The present study emphasizes the importance of focusing on transmission-blocking strategies that reduce exposure to the L3 stages, not only to prevent transmission but, perhaps, as key step in reducing disease.

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