INTRODUCTION

One of the main obstacles to the acquisition of antimalarial immunity is the high degree of antigenic diversity in potential target antigens, which enables parasites to evade immune responses elicited by past exposure to variant forms of the same antigen. The merozoite surface protein 2 (MSP-2) of *Plasmodium falciparum*, a well-known target of naturally acquired clinical immunity to malaria, contains repeat arrays flanked by unique variable domains and conserved N- and C-terminal domains. This protein is encoded by highly divergent alleles grouped into dimorphic families or lineages (FC27 and 3D7). The repeat array of FC27-type alleles consists of two blocks of relatively conserved sequences: 1–4 copies of a 32-mer motif followed by 0–5 copies of a 12-mer motif. In contrast, the GSA-rich repeat motifs of 3D7-type alleles are highly variable in length (2–10 amino acids), sequence, and number of copies. Both naturally acquired and vaccine-induced antibodies to MSP-2 recognize predominantly variant families and display little cross-reactivity between dimorphic families. Significantly, immunization with a multivalent vaccine prototype containing the 3D7 variant of MSP-2 partially protected Papua New Guineans from infection with parasites carrying homologous (3D7-type), but not heterologous (FC27-type), versions of this antigen.

Symptomatic malaria in African children are usually caused by parasites expressing variant surface antigens (VSA) exported to the membrane of infected red blood cells, to which these children had not been previously exposed. Therefore, the age-related acquisition of clinical immunity to malaria would basically consist in filling the gaps in the repertoire of antibodies to VSA. Although naturally acquired antibodies to MSP-2 and other merozoite surface antigens may protect against clinical disease, there is no clear evidence that they do so in a similar variant-specific manner. In this report, we analyze naturally acquired antibody responses to five MSP-2 variants in a cohort of malaria-exposed rural Amazonians, to address three issues: 1) the variant-specificity of these antibodies, 2) the production of antibodies with new specificities after exposure to new MSP-2 variants, and 3) the association between gaps in the repertoire of antibodies to MSP-2 and disease severity.

SUBJECTS, MATERIALS, AND METHODS

Study area and population. The study protocol was reviewed and approved by the Ethical Review Board of the Institute of Biomedical Sciences of the University of São Paulo, Brazil (no. 318, 2002). Blood samples for serum separation and DNA extraction were collected during the first 15 months of follow-up (March 2004–May 2005) of an ongoing, population-based cohort study in Ramal do Granada (9°41′–9°49′S, 67°5′–67°7′W) in the eastern part of the state of Acre in northwestern Brazil. Both *P. falciparum* and *P. vivax* are transmitted year-round; the incidence of clinical *P. falciparum* malaria confirmed by microscopy was 1.39/100 person-months at risk (95% confidence interval = 1.12–1.75/100 person-months at risk) during the study. The study area, cohort recruitment strategies, and baseline data are described in detail elsewhere.

Briefly, the 114 households identified by a census performed by our field team were visited, and 466 dwellers of all age groups (98.5% of the residents in the area) were enrolled between March and April 2004. A questionnaire was given to all study participants to obtain demographic, clinical, and socioeconomic information (including the self-reported number of past malaria episodes confirmed by microscopy) and assess their cumulative exposure to malaria. Because most adults were migrants from malaria-free areas, their ages do not necessarily correlate with cumulative exposure to malaria; this was therefore estimated as the length of residence in malaria-endemic areas (either in Acre or elsewhere in Amazonia). The 399 study participants ≥ 5 years of age were considered eligible for collection of 5-mL venous blood samples; 356...
(89.2%) had their baseline serum samples tested for antibodies to MSP-2.

Clinical and laboratory surveillance of symptomatic malaria was started in March 2004. Asymptomatic malaria infections were detected during four cross-sectional surveys of the whole study population carried out in March–April 2004, September–October 2004, and February–March 2005. This combined strategy identified 168 P. falciparum infections among subjects ≧ 5 years of age; 86 (51.1%) were missed by standardized thick smear microscopy, and were diagnosed only by nested polymerase chain reaction (PCR) amplification of a species-specific segment of the 18S ribosomal RNA gene, as described by Kimura and others with the modifications by Win and others. One-third of these subjects were co-infected with P. falciparum and P. vivax. As in other studies in Amazonia, nearly all mixed-species infections were identified only by PCR because the predominant species was usually found by conventional microscopy. Plasmodium falciparum isolates from 86 infections (either asymptomatic or not) were obtained for MSP-2 gene typing. DNA templates for PCR amplification, which were isolated from 200 μL of whole blood using GFX genomic blood DNA purification kits (Amersham Pharmacia Biotech, Piscataway, NJ), were digested in 100 μL of sterile distilled water and stored at −20°C. Serum samples were collected during 69 P. falciparum infections (either asymptomatic or symptomatic) diagnosed during the follow-up in subjects ≧ 5 years of age.

**Clinical assessment.** The prevalence and intensity of the symptoms associated with P. falciparum episodes diagnosed in our cohort subjects ≧ 5 years of age during 15 months of follow-up were assessed essentially as described by Karunaweera and others. Briefly, a semiquantitative questionnaire addressing nine common symptoms (fever, chills, sweating, headache, myalgia, arthralgia, abdominal pain, nausea, and vomiting) was applied to all patients. The same physician (M.dS-N) assessed all infections to minimize inter-observer variation. According to the patient’s perception, each clinical manifestation (except for fever) was considered to be absent, mild, moderate, or severe; fever was classified as absent, mild, or severe. Numerical scores of 0, 1, 2, or 3 were assigned to symptoms reported to be absent, mild, moderate, or severe, respectively. Asymptomatic subjects were given scores of 0 for each symptom. To minimize recall bias, patients were interviewed during the acute malaria episode or up to one week after treatment. Severe malaria episodes were not diagnosed in our study population during the follow-up.

**MSP-2 gene typing.** Nested PCR amplification and digestion with restriction endonuclease Hinf I (New England Biolabs, Ipswich, MA) were used to type the MSP-2 gene of 86 P. falciparum isolates. An MSP-2 gene fragment of approximately 380–740 basepairs was amplified by a nested PCR with oligonucleotide primer pairs that target conserved domains on blocks 1 and 5. Primer sequences and amplification protocols are described in detail elsewhere. The PCR products were digested with Hinf I for 2 hours at 37°C and separated by electrophoresis on 10% polyacrylamide gels. Family-specific restriction fragments resulting from Hinf I digestion of FC27-type PCR products are two fragments of 137 basepairs and 108 basepairs, and those resulting from digestion of 3D7-type alleles are two fragments of 70 basepairs and 108 basepairs. Multiple-clone infections with parasites from both families can be identified by the presence of FC27-type and 3D7-type restriction patterns.

**MSP-2 antigens.** Two FC27-type and three 3D7-type versions of MSP-2 were expressed as recombinant proteins fused to the C-terminus of Schistosoma japonicum glutathione S-transferase (GST). The FC27-type alleles expressed comprise blocks 2, 3, and the 5’ end of block 4 (according to the classification of Snewin and others) of the MSP-2 gene of isolates S20 (nucleotides 1–418 of the sequence AF177389) and FC27 (nucleotides 201–662 of the sequence J03828). S20 has one copy of the 32-mer motif followed by two slightly different 12-mer motifs, and FC27 has two identical copies of the 32-mer motif followed by a single copy of the 12-mer motif. The three 3D7-type antigens comprise the blocks 2 and 3 of the isolates AM89 (nucleotides 1–294 of the sequence AY331635), FUP/CP (nucleotides 52–318 of the sequence M61121), and 3D7 (nucleotides 133–312 of the sequence M28891). All 3D7-type antigens differ in the repeat motifs and in the flanking unique sequences; the repeat array of AM89 comprises the motifs GA, GAGASGSA, GSGD, GASGSA, and SGSA, which are common in 3D7-type MSP-2 sequences from Brazilian isolates, and those of isolates FUP/CP and 3D7 contain 12 and 5 copies, respectively, of the motif GGSA. The origin and year of collection of the original P. falciparum isolates are as follows: S20, Brazilian Amazonia (1987); FC27, Papua New Guinea (1979); AM89, Brazilian Amazonia (1989); FUP/CP, Uganda (1966); and 3D7, collected in The Netherlands but is of probable west African origin (1979).

**Enzyme-linked immunosorbent assay for total IgG antibodies to MSP-2.** Assays with all recombinant antigens were performed as described. Briefly, 96-well microplates (Nunc MaxiSorp, Roskilde, Denmark) were coated with 0.1 μg/well of recombinant antigens or GST alone, and test samples were assayed in duplicate (50 μL/well, 1:100 dilution). After incubation for 1 hour at 37°C, antibody binding to solid-phase antigens was detected with peroxidase-conjugated goat immunoglobulin anti-human IgG (1:2,000 dilution) (Sigma, St. Louis, MO). After use of o-phenylenediamine and hydrogen peroxide at acid pH as substrate, absorbance values were measured at 492 nm. Corrected absorbance values were obtained by subtracting absorbance readings obtained for each serum sample from the control antigen (GST alone). Reactivity indices (RIs) were calculated as the ratio between the corrected absorbance values of each test sample and a cut-off value for each antigen, corresponding to the average corrected absorbance for samples from 40 malaria-naive blood donors plus 3 SD. The cut-off absorbance values ranged between 0.108 and 0.152 for different antigens. Positive samples had RIs > 1.

**Data analysis.** Proportions were compared with chi-square tests or chi-square tests for linear trend, and pairwise correlations were evaluated with Pearson’s and Spearman’s correlation models. Symptom scores were compared in different groups of subjects with the nonparametric Mann-Whitney U test. We used Somer’s d statistic to test for a dose-response relationship between ordinal variables (namely, symptom scores, levels of parasitemia, age, and time of residence in Amazonia); these last two variables were stratified into quartiles for this purpose. Analyses were made with SPSS version 13.0 (SPSS Inc., Chicago, IL). Statistical significance was defined at the 5% level.
RESULTS

Antibody recognition of MSP-2 variants at the cohort baseline. Table 1 shows the proportions of study subjects ≥ 5 years of age who had IgG antibodies detected to each MSP-2 variant at the cohort baseline in March–April 2004. FC27-type antigens were recognized by a significantly larger proportion of study subjects (22.2–28.1%) than were 3D7-type antigens (6.2–7.3%) (P < 0.00001 in all between-family pairwise comparisons, by chi-square test). The difference in the overall proportion of responders to S20 (28.1%) and FC27 (22.2%) did not reach statistical significance (χ² = 2.99, P = 0.084); 90 subjects (25.3%) recognized only one FC27-type variant (either S20 or FC27) and 16 subjects (4.5%) recognized both FC27-type variants. No subject recognized all 3D7-type variants; 46 (12.9%) recognized one of them (either AM89, FUP/CP, or 3D7) and 12 (3.4%) recognized two of them.

A current P. falciparum infection was detected by conventional microscopy or nested PCR in 32 subjects studied at the baseline (prevalence = 9.0%); 14 had P. falciparum-P. vivax co-infections and 18 had only P. falciparum infections. S20 was the only antigen to be recognized by a significantly larger proportion of P. falciparum-infected subjects compared with non-infected ones (χ² = 7.21, P = 0.007). Parasites from 86 P. falciparum infections diagnosed in cohort subjects at the baseline and over the next 15 months of follow-up had their MSP-2 gene typed; 46 (53.5%) carried FC27-type alleles, 32 (37.2%) carried 3D7-type alleles, and 8 (9.3%) carried both FC27- and 3D7-type alleles. Subjects with mixed-clone infections (as determined by MSP-2 gene typing) were slightly older (mean ± SD age = 29.7 ± 19.0 years) and reported longer time of residence in Amazonia (mean ± SD = 19.0 ± 11.2 years) compared with those with single-clone infections (mean ± SD age = 26.8 ± 18.5 years) and a mean ± SD time of residence in Amazonia = 15.6 ± 12.0 years, but these differences did not reach statistical significance (by Mann-Whitney U test).

To investigate whether age or cumulative exposure to malaria correlated to the levels of antibodies to MSP-2, we selected 324 subjects who were free of P. falciparum infection at baseline and calculated regression coefficients with both parametric (Pearson’s r) and nonparametric (Spearman’s ρ) models. Weak but significant positive correlations were found between age and levels of IgG antibodies to S20 (r = 0.127, ρ = 0.139), FC27 (r = 0.113), and FUP/CP (ρ = 0.117). The time of residence in Amazonia correlated significantly with the levels of IgG antibodies to S20 (r = 0.300, ρ = 0.237), FC27 (r = 0.276, ρ = 0.144), AM89 (r = 0.113), and FUP/CP (ρ = 0.127). Therefore, age and cumulative exposure to malaria was a better predictor of levels of antibodies to FC27 than those of antibodies to 3D7. This is probably due to 1) the predominance of FC27-type alleles in the local parasite populations and 2) the higher diversity in 3D7-type alleles, which may prevent the adequate boosting of pre-existing variant-specific immune responses upon re-exposure to parasites of the same family.

We next investigated the pairwise correlations between levels of antibodies to each MSP-2 variant (Table 2). Levels of antibodies to S20 and FC27, as well as those to FUP/CP and 3D7 (3D7-type variants that share the same GGSA repeat motif), were strongly and positively correlated, and correlations between levels of antibodies to structurally distinct 3D7-type alleles (AM89 versus FUP/CP, AM89 versus 3D7) were weak. Correlations between levels of antibodies to MSP-2 variants belonging to different dimorphic families were also usually low (Table 2). These findings provide further support to the notion that malaria-exposed Amazonians recognize MSP-2 in a family-specific manner and, within the 3D7 family, in a variant-specific manner.13

Antibodies elicited by exposure to new MSP-2 variants. The specificities of MSP-2 antibodies detected during acute infections often mismatch the MSP-2 gene type found in infecting parasites.9,11,13,28–30 These findings have been ascribed to the fact that repeated infections with different antigenic variants might boost existing antibody responses instead of generating antibodies with new specificities, a phenomenon known as clonal imprinting or original antigenic sin.13 To test this hypothesis, we compared the specificities of antibodies to MSP-2 in sequential serum samples collected from 35 participants in the cohort study.

The levels and specificities of variant-specific antibodies remained remarkably stable over time, despite the documented exposure to new MSP-2 variants during the follow-up (Figure 1). Subjects who reported no previous malaria failed to seroconvert against the MSP-2 variant (either FC27, 3D7, or both) to which they were exposed (Figure 1A). Exposure to a new MSP-2 variant elicited varying antibody response patterns among subjects who reported one or more past slide-confirmed malaria episodes (Figure 1B and C). Overall, of 16 subjects without pre-existing 3D7 antibodies (but reporting past malaria episodes), only six subjects (#374, #326, and #439 [Figure 1B] and #400, #425, and #339 [Figure 1C]) seroconverted when exposed to 3D7-type parasites. The finding of two seroconversions to 3D7-type antigens in subjects with pre-existing FC27-specific antibodies (#439 [Figure 1B] and #425 [Figure 1C]) argues against the hypothesis of clonal imprinting, although the numbers are too small for meaningful statistical comparisons. Of five subjects without pre-existing FC27 antibodies (but reporting past malaria episodes), three (#411 [Figure 1B] and #419 and #372 [Figure 2] had pre-existing FC27-specific antibodies. These findings provide further support to the notion that malaria-exposed Amazonians recognize MSP-2 in a family-specific manner and, within the 3D7 family, in a variant-specific manner.13

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td>Number (%) of subjects from rural Amazonia with IgG antibodies to five MSP-2 variants, as detected by ELISA at the cohort baseline*</td>
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<tr>
<td><strong>Infection status</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Plasmodium falciparum infection</td>
</tr>
<tr>
<td>No P. falciparum infection</td>
</tr>
<tr>
<td>Total</td>
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* MSP-2 = merozoite surface protein-2; ELISA = enzyme-linked immunosorbent assay.
We also tested for trend in the prevalence and severity of symptoms (Figure 2B, C, and D). More than two-thirds (36 of 53) of subpatent *P. falciparum* infections detected only by PCR were asymptomatic; the proportion of symptomatic infections increased linearly with increasing parasitemias ($x^2$ for trend = 44.99, degrees of freedom = 1, $P < 0.0001$). Only 20.8%, 26.4%, and 26.4% of the subjects with subpatent parasitemias reported fever, headache, and myalgia, respectively (Figure 2B). Subjects with low parasitemias detectable by conventional microscopy (10–300 parasites/µL of blood) (Figure 2C), especially those with relatively high parasitemias (> 300 parasites/µL of blood) (Figure 2D), reported more severe symptoms than those with parasites that were detected only by PCR (Figure 2B) ($P$ value range = 0.007–<0.0001, by Somer’s $d$ test). Interestingly, levels of parasitemia did not correlate with patient’s age ($d = 0.40$, $P = 0.662$), time of residence in Amazonia ($d = -0.009$, $P = 0.924$) or levels of antibodies to *MSP-2* variants ($P$ value range = 0.158–0.631, by Spearman’s rank correlation test). These data suggest that the severity of symptoms in uncomplicated *P. falciparum* infections in rural Amazonia correlates positively with the parasitemia present at the time of diagnosis, but does not necessarily reflect different degrees of clinical immunity acquired after years of exposure to malaria.

**Variant-specific antibodies to *MSP-2* and clinical disease.** We next examined the possible clinical consequences, in terms of symptom severity, of mismatches between variant-specificity of antibodies and *MSP-2* type of infecting parasites. We compared the prevalence and severity of symptoms of *P. falciparum* reported by 23 subjects with IgG antibodies to at least one variant of the *MSP-2* type(s) detected in infecting parasites (age range = 11–65 years, mean = 37.9 years) with a range of 5–53 years, mean = 19.5 years of time of residence in Amazonia) (Figure 3A) and 16 subjects with no antibodies to any of the antigens representing the *MSP-2* type(s) found in infecting parasites (age range = 10–57 years, mean = 26.2 years) with a range of <1–32 years, mean = 13.0 years of residence in Amazonia) (Figure 3B). Subpatent infections detected only by PCR occurred in 26.1% and 43.7% of the subjects in each group, respectively ($x^2 = 0.65$, $P = 0.42$). The severity of all symptoms was identical in both groups of patients ($P$ value range = 0.192–0.890, by Mann–Whitney U test). These results suggest that, in contrast with available data for antibodies to VSA, the absence of naturally acquired antibodies to antigens representing a particular *MSP-2* type is not a strong predictor of the clinical outcome of infections with parasites carrying that variant.

**DISCUSSION**

We report contrasting patterns of antibody recognition of the highly divergent dimorphic families of *MSP-2*, *FC27*, and *3D7*, in rural Amazonians exposed to low levels of *P. falciparum* transmission. The immunodominant repeats of *FC27*-type variants are structurally conserved in Brazil and, with few exceptions, comprise one copy of the 32-mer motif and two or three copies of 12-mer motifs. The *FC27*-type antigens are recognized by 21–26% of non-infected Amazonians (Table 1) and by 34–70% of those with current *P. falciparum* infections (Table 1).

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**Table 2**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>S20</th>
<th>FC27</th>
<th>AMB9</th>
<th>3D7</th>
<th>FUP/CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>S20</td>
<td>–</td>
<td>0.884†</td>
<td>0.215†</td>
<td>0.353†</td>
<td>0.356†</td>
</tr>
<tr>
<td>FC27</td>
<td>0.840†</td>
<td>–</td>
<td>0.222†</td>
<td>0.325†</td>
<td>0.357†</td>
</tr>
<tr>
<td>AMB9</td>
<td>0.338†</td>
<td>0.386†</td>
<td>–</td>
<td>0.044</td>
<td>0.137†</td>
</tr>
<tr>
<td>3D7</td>
<td>0.356†</td>
<td>0.364†</td>
<td>0.142†</td>
<td>–</td>
<td>0.608†</td>
</tr>
<tr>
<td>FUP/CP</td>
<td>0.430†</td>
<td>0.422†</td>
<td>0.228†</td>
<td>0.664†</td>
<td>–</td>
</tr>
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</table>

* $P < 0.05$

† For definitions of abbreviations, see Table 1.

†–$P < 0.05$.
Cross-reactivity exists between antibodies to the FC27-type antigens S20 and FC27, which differ in the number and sequence of 32-mer and 12-mer motifs. Levels of antibodies to FC27-type antigens among subjects with several past malaria episodes tend to be stable over time, even in the absence of frequent re-exposure to variants of this dimorphic family (Figure 1B and C), which suggest that long-term memory responses have been induced.

In contrast, repeat arrays in 3D7-type alleles are substantially more polymorphic both in Brazil and worldwide. Of 40 3D7-type MSP-2 alleles from parasites from Brazil so far sequenced, 29 (72.5%) had GASGSA repeats (as those...
found in the AM89 allele), 9 (22.5%) had GGSGSA repeats, and only 2 (5.0%) had the GGSA repeats found in the 3D7 and FUP/CP alleles. There is much less cross-reactivity of antibodies to 3D7-type antigens detected in malaria-exposed Amazonians (Table 2). The 3D7-type antigens are recognized by only 6–8% of noninfected Amazonians (Table 1) and by 3–46% of those with current *P. falciparum* infection (Table 1). The low proportion of responders to 3D7-type antigens in our population may be a consequence of several factors: 1) few subjects seroconvert when exposed to 3D7-type variants (i.e., these antigens may be poorly immunogenic in natural infections), 2) those who seroconvert usually have short-lived antibodies, possibly because short repeats within 3D7-type variants may stimulate T cell-independent antibody responses, with no memory B cells, and 3) some subjects may recognize divergent variants other than those represented in our panel of recombinant antigens, and thus have false-negative results. Because of the poor antibody recognition of 3D7-type recombinant antigens, we found several examples of infections with 3D7-type parasites in subjects who have only antibodies to FC27 (Figure 1B and C). Such mismatches have been hypothesized to result from clonal imprinting, with clear implications for the development of subunit malaria vaccines that include variable regions of MSP-2. To test this hypothesis, antibody responses must be measured in cohorts of subjects exposed to parasites of known MSP-2 gene type. We present the first direct test of the clonal imprinting hypothesis in an area of hypoendemic malaria transmission.

According to the clonal imprinting hypothesis, when subjects who have been primarily exposed to FC27-type variants are subsequently infected with 3D7-type parasites, cross-reactive antibodies are preferentially boosted, resulting in responses with fixed specificity to FC27-type epitopes. This would explain why five of seven individuals currently infected with 3D7-type parasites fail to seroconvert to homologous antigens but maintain heterologous variant-specific antibodies (Figure 1B and C). However, because 3D7-type alleles are found in nearly one-third of local parasites, we would expect to see a few examples of FC27-infected subjects with antibodies of fixed specificity to one or more 3D7 variants, resulting from primary exposure to this family, but this has not been observed (Figure 1B and C). In addition, we observed two examples of seroconversion to 3D7-antigens in the presence of pre-existing FC27-specific antibodies (subjects #439 [Figure 1B] and #425 [Figure 1C]), which argues against the notion that the selective non-responsiveness to 3D7-type variants might be a fixed phenotype. Alternatively, we suggest that the poor recognition of 3D7-type antigens results from the extensive diversity and/or the poor immunogenicity of this family of variants, coupled with the impaired development of memory B cells caused by repeat arrays. No conclusion drawn from this and other previous studies addressing the clonal imprinting hypothesis is definitive because the numbers of individuals and sequential serum samples so far examined remain limited. Further data from our ongoing cohort study in rural Amazonia will hopefully provide new insights into this issue of major practical relevance for vaccine development.
to VSA,\textsuperscript{16} it seems unlikely that MSP-2 is a target of variant-specific antibodies of major functional importance. Although asymptomatic malaria infections in native Amazonians has been ascribed to naturally acquired immunity,\textsuperscript{35} we found that the parasite burden at the time of diagnosis, rather than correlates of cumulative malaria exposure (age and time of residence in Amazonia) or acquired immunity (presence of variant-specific antibodies to MSP-2), is a major predictor of the clinical expression of uncomplicated infections with \textit{P. falciparum}. We are currently investigating in our cohort subjects whether low-grade parasitemias detectable only by PCR, if untreated, will eventually result in fully symptomatic disease after a given clinical threshold is reached.

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