Juvenile Rhesus Monkeys Have Lower Type 2 Cytokine Responses than Adults after Primary Infection with Schistosoma mansoni

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Adults and children have differences in their susceptibility to schistosomiasis. The relative influences of age-dependent innate resistance and acquired immunity in the differences between susceptibility to schistosomiasis are difficult to assess in humans. Therefore, we exposed juvenile and adult female rhesus monkeys to primary infection with Schistosoma mansoni. In contrast to the adult animals, the juvenile rhesus monkeys had low levels of interleukin (IL)–4 and IL-5 production by peripheral blood mononuclear cells after schistosome infection, as well as lower levels of parasite-antigen–specific antibody (IgG, IgM, and IgA) responses, and produced limited antigen-specific or total IgE. Juvenile animals had statistically nonsignificant increased worm burdens and tissue or fecal egg counts, compared with that of adults, whereas circulating schistosome antigens were significantly higher in infected juvenile monkeys. These results suggest that juvenile rhesus monkeys have reduced type 2 cytokine responses after primary schistosome infections and perhaps are more susceptible to parasite infection.

Schistosomiasis affects ≥250 million people worldwide. The disease is caused by infection with trematode parasites of the Schistosoma species. Extensive epidemiology studies on human populations in endemic countries have shown that adults generally have lower intensities of schistosome infection than do children. The reduced levels of Schistosoma mansoni infections in adults are particularly pronounced in reinfection studies, in which the population is chemotherapeutically cured and the levels and rate of acquisition of new infections (“reinfection”) is examined after drug treatment. Adult resistance and child susceptibility to reinfection after chemotherapy have been described for all 3 Schistosoma species that most commonly infect man. For all 3 parasite species, the immunological correlates of this age-dependent resistance are associated with type 2 responses: eosinophilia with S. haematobium [1]; antiparasite IgE responses for S. haematobium [2], S. japonicum [3], and S. mansoni [4, 5]; and peripheral blood mononuclear cell (PBMC) production of interleukin (IL)–4 and IL-5 for S. haematobium [6] and S. mansoni [7].

Observation of the effects of host age, per se, on the development of innate or acquired protective responses in human schistosomiasis is usually confounded by differences between adults and children in regards to wa-
ter contact (exposure to infection) and by the unknown influence of previous infections. A recent study [8] in a Ugandan fishing community showed that lower reinfection in adults after treatment, compared with that in children, could not be fully explained by lower exposure to infection. This clearly demonstrated that adults do have partial innate and/or acquired resistance to reinfection with S. mansoni after treatment. Two separate studies on human populations that were newly exposed to S. mansoni suggested that adults were less susceptible than children to infection within their first few years of exposure; therefore, adult resistance to infection has a component that is either innate or acquired rapidly [9, 10]. However, it also has been observed that peak intensity of infection occurs slightly earlier in children living in high transmission areas versus children living in areas of lower transmission, which has been taken by some to demonstrate that the development of resistance to infection is dependent on a longer accumulated “experience” of infection [11, 12]. Whatever the prerequisite conditions for, or dynamics of, the development of adult resistance to schistosomiasis, the relative contributions of innate and required mechanisms are difficult to identify and quantify in human studies.

We have reported elsewhere [13] that the peak of reinfection is coincident with puberty (∼12–14 years of age), which suggests that, after puberty, a mechanism may mediate the development of resistance to infection in adults. The corollary of this is that “juveniles” (i.e., those aged <12 years) may be more susceptible to schistosome infection than adults and/or are unable to develop the acquired immunological responses that provide protection in adults. In this study, we have used rhesus monkeys (Macaca mulatta), a widely used primate model for studies on primary infection with schistosomes [14], to investigate the nature of age-related differences in susceptibility to primary infection with S. mansoni.

MATERIALS AND METHODS

Animals and parasitology. Ten rhesus monkeys were used in this study, were matched for sex (all females), and were housed in the Biomedical Primate Research Center (Rijswijk, The Netherlands). The 5 adult monkeys were aged 13–19 years (mean age ± SD, 16.1 ± 3.1 years) and weighed 5.5 ± 1.1 kg. Five juvenile monkeys were selected by age to be at the prepubertal to midpubertal stages of development (range, 1–3 years; mean ± SD, 2.4 ± 0.6 years) [15] and weighed 3.0 ± 0.9 kg. Before the start of the study, a veterinarian examined the animals. Levels of serum dehydroepiandrosterone (DHEA) were measured to ensure the correct categorization of animals as juvenile or adult. DHEA levels in all adults were <0.8 μM/L (below detection of assay), whereas the mean ± SD level in juveniles was 1.24 ± 0.31 μM/L. These levels of DHEA support the juvenile and adult status of the animals [16, 17].

A Puerto Rican strain of S. mansoni, maintained at the Department of Parasitology (University of Leiden, The Netherlands) was used for infections. Animals were percutaneously exposed to 1000 S. mansoni cercariae on the shaved abdomen. After 30 min, the cercarial suspension was recovered, and the number of nonpenetrating cercariae counted. Blood samples were obtained from animals on the day of infection and at weeks 5, 6, and 7 during infection, as well as week 8 after infection when animals were killed. During the last 3 days of the experiment, fecal samples were collected, and egg counts were performed by use of a filtration method [18]. Animals were perfused to recover worms from the portal vein [19]. The liver, colon, and ileum were removed and weighed. Multiple samples were dissected from different parts of each organ, and ~10%, by weight, of each organ was digested in 4% KOH for tissue egg counts [18]. Egg counts are expressed as eggs per gram (epg) of feces or tissue. Tissue samples also were obtained for histological and pharmacological analysis (J. Bogers, W. Jacobs, T. Moreels, J. De Man, P.G.F., D.W.D, J.A.M.L., A. Thomas, P. Pelckmans, E. Van Marck, unpublished observations). Levels of circulating anodic antigen (CAA) in the serum of monkeys, for quantification of worm burdens, were measured as described elsewhere [20].

Antibody responses. Antigen-specific antibody (IgG, IgM, IgA, and IgE) responses to schistosome adult worm antigens (AWAs) or soluble egg antigens (SEAs) were measured by use of ELISA, as described elsewhere [5]. SEAs and AWAs were prepared as described elsewhere [21]. Serum was added in serial dilutions from 1:50 to 1:6400 for IgG, IgM, and IgA; for IgE, serum was added from 1:25 to 1:3200. Total serum IgE was measured in a capture ELISA developed using commercial antibodies (BD PharMingen) and IgE standard (Dade Behring). Serum IgE values are expressed as international units per milliliter.

Cellular immunology and cytokine assays. Pre- and postinfection peripheral blood was taken by venipuncture (maximum 1% volume of the bodyweight [range, 30–50 mL] was removed per bleed). Serum was isolated and used for antibody analysis, as described above. Heparinized blood was used for isolation of PBMCs, using Ficoll-Hypaque density gradient centrifugation. Cells were cryopreserved and stored at −150°C until used.

Pre- and postinfection PBMCs from individual monkeys were simultaneously thawed and cultured in duplicate, using 4 × 10^6 cells/well. Cells were stimulated with medium alone, concanavalin A (Con A; 5 μg/mL), SEAs (5 μg/mL), or AWAs (10 μg/mL). After 72 h, culture supernatants were collected and stored frozen at −20°C. Interferon (IFN)–γ, IL-12, IL-4, and
IL-5 were detected in supernatants from all animals (before and after infection) by use of ELISA for monkey cytokines (U-CyTech; University of Utrecht, The Netherlands). Cytokine ELISAs (U-CyTech BV Diagnostics) were performed according to the manufacturer’s instructions. Levels of cytokines (pg/mL) were interpolated from standard curves. Data are expressed as mean ± SD cytokine production from each group of 5 animals.

**Statistical analysis.** Where normality and homoscedasticity of the data could be assumed, statistical differences between groups was determined by use of the Student’s t test. For a number of variables, Bartlett’s test demonstrated a difference in variance between groups. These were analyzed using Welch’s t test. All data are presented as mean ± SD, except total IgE, of which data were presented as mean ± SE.

**RESULTS**

**Cytokine responses before and after infection.** Before and after infection, PBMCs were stimulated in vitro with mitogen (Con A) or parasite antigens, SEAs or AWAs, and the production of type 1 (IFN-γ and IL-12) and type 2 (IL-4 and IL-5) cytokines were analyzed by ELISA. Cells from “naive” (before infection) juvenile and adult animals had no differences in their cytokine production after mitogen stimulation, and parasite antigens elicited limited cytokine release (figure 1). Con A stimulation of PBMCs from infected adult monkeys demonstrated that infection caused a marked increase in type 2 cytokine responses, which was characterized by elevated IL-4 and IL-5 production, with a reduction in IFN-γ and IL-12 levels, com-
pared with preinfection responses. Postinfection cells from adult monkeys responded to parasite antigens, which induced IL-4 and IL-5 production and limited IFN-γ (figure 1). This generalized elevation in type 2 cytokine production and reduction in type 1 responses in primary infected adult rhesus monkeys is similar to what was found in schistosome-infected humans and mice [22, 23]. In contrast, cells from infected juvenile monkeys had no marked change in mitogen-stimulated cytokine responses, compared with uninfected cellular responses (figure 1). Antigen-specific stimulation of PBMCs from infected juvenile animals did, however, elicit low levels of IFN-γ, IL-4, and IL-5 production (figure 1). Statistical comparison of the cytokine responses in mitogen-stimulated PBMCs from infected animals showed that the juveniles had significantly ($P<.05-.01$) reduced IL-4 and IL-5 and greater IFN-γ and IL-12 production, compared with the adult monkeys. Data on cytokine production by PBMCs demonstrated that juvenile monkeys failed to develop the switch to a type 2 cytokine phenotype after schistosome infection, which had occurred in adult animals.

**Antibody responses.** Pre- and postinfection serum samples were used to determine parasite-antigen–specific antibody responses. Serum samples from naive juvenile and adult animals had comparable levels of background antibody responses to schistosome antigens (figure 2). Postinfection serum samples demonstrated that, although both groups of animals developed antibody responses to AWAs and SEAs, the adult animals developed relatively greater levels of IgG, IgM, and IgA than did juvenile animals (figure 2). Similar to the other antibody isotypes studied, naive juvenile and adult monkeys had limited IgE antibody responses to schistosome antigens (figure

**Figure 2.** Antibody responses to parasite antigens before and after primary *Schistosoma mansoni* infection of juvenile and adult rhesus monkeys. Serum IgG, IgM, and IgA responses against adult worm antigens (AWAs) and soluble egg antigens (SEAs) were determined by use of ELISA. Data are group mean OD ± SD, with 5 monkeys/group.

**Figure 3.** Parasite antigen-specific and total IgE before and after primary *Schistosoma mansoni* infection of juvenile and adult rhesus monkeys. A, Antigen-specific IgE was determined by use of ELISA, and data are group mean OD ± SD. B, Total IgE was quantified by use of capture ELISA, and data are mean IgE ± SE.
after primary schistosome infection, adult animals developed elevated IgE titers to AWAs and SEAs. In contrast, infection of juvenile animals caused only a small increase in levels of IgE antibodies to AWAs and SEAs (figure 3A). Although both groups of monkeys had comparably low levels of circulating total IgE before infection, the adult animals had a 5-fold elevation in IgE after infection (figure 3B). In contrast, primary schistosome infection did not cause an increase in the levels of total IgE in juvenile monkeys (figure 3B). The antibody data correlates with the cytokine data, and, together, they indicate that juvenile animals had markedly reduced type 2 responses, including IgE production, after primary schistosome infection.

**Infection and parasitology.** After application of 1000 cercariae to the shaved abdominal skins of juvenile and adult animals, there was no statistical difference between groups in the percentage of cercariae that failed to penetrate the skin (mean ± SD of cercariae nonpenetrating: 3.1% ± 1.4% for juvenile animals and 2.6% ± 1.6% of adult animals; \( P < .146 \), Student’s \( t \) test). Eight weeks after infection, animals were perfused, and the number of worms recovered was counted. Juvenile monkeys had 48% more worms (male and females) than adult monkeys (figure 4A). The increase in worms recovered from juvenile monkeys was not statistically different from the worm burden in adult animals (\( P < .146 \), Welch’s \( t \) test). Similarly, the mean ± SD numbers of male (228.4 ± 55.6) and female (401.6 ± 17.2) worms recovered from juvenile monkeys were also not significantly different from adults (male worms: 141.4 ± 81.5, female worms: 283.4 ± 177.1; \( P < .89 \) and \( P < .212 \), respectively). To further monitor the development of the infection in juvenile and adult animals, we measured serum levels of CAA before (week 0) and at weeks 5, 6, 7, and 8 after infection. Levels of CAA in the serum of schistosome-infected humans and experimental animals correlated well with other estimates of the number of worms present [24, 25]. Throughout the course of infections, juvenile monkeys had greater levels of CAA than did adult monkeys (figure 4B). Levels of CAA in juvenile monkeys were statistically elevated, compared with levels in adults at weeks 6, 7, and 8 after infection (figure 4B; \( P < .05 \)). For both groups of animals, CAA levels continued to increase until the end of the experiment (week 8 of infection), which suggests that there was no death or reduced fitness of worms in either group during the course of the experiment.

Although juvenile monkeys generally excreted more eggs or had more eggs present in each organ compared with adults, the differences between both groups was not statistically significant (table 1). Collectively, the worm data (perfused worm recovery and CAA levels) and egg data (eggs excreted and tissue egg counts) support the premise that juvenile rhesus monkeys are more susceptible to a primary infection with *S. mansoni* than are adult monkeys.

**DISCUSSION**

In the present study, we have shown that, in contrast to adults, juvenile (prepubescent) rhesus monkeys failed to develop type 2 cytokine responses after primary *S. mansoni* infection. Primary schistosome infection of adult monkeys evoked the characteristic type 2 cytokine responses normally associated with schistosomiasis. Thus, infection of adults generated increased IL-4 and IL-5 and reduced IL-12 and IFN-\( \gamma \) production by
PBMCs. Antiparasite humoral immune responses, in particular antigen-specific IgE, also increased in adults after infection. In contrast, PBMCs from schistosome-infected juvenile monkeys had low levels of type 2 cytokine production. Juveniles showed markedly reduced parasite-antigen–specific antibody responses and significantly limited overall IgE production. These data suggest that juvenile rhesus monkeys have reduced antischistosome immune response after primary schistosome infection. It has been hypothesized that the increase susceptibility to re-infection in young children is caused by an inability to induce appropriate—that is, protective—immune responses [7]. In human schistosomiasis haematobium infections, it has been shown that, after drug treatment, children have different immunological responses than do adults [26]. Our data suggest that juvenile monkeys have diminished antiparasite immune responses after primary schistosome infection. We cannot yet determine whether this defect is schistosome specific or a generalized impairment in immune function.

Although the parasitological data suggest that juvenile monkeys are more susceptible to primary infection with S. mansoni than are adult monkeys, the difference between the 2 groups was not statistically significant. The lack of statistical differences between groups in all parameters, except CAA levels, was undoubtedly due to the relatively small (n = 5) group size, but, interestingly, the marked variability in adult animals also was a factor. However, a caveat with the interpretation of CAA levels is the potential contribution of different blood volumes between adult versus juvenile animals. Nonetheless, in the study reported here the greater susceptibility of juveniles to primary schistosome infection was consistently indicated by a number of criteria: perfused worm burden, CAA levels during infection, fecal eggs excreted, and numbers of parasite eggs trapped in various organs.

A number of physiological nonimmunological responses could account for the differences in susceptibility between juvenile and adult, including age-associated changes in skin thickness, body fat, size of the animal, and circulating putative or unknown schistosomicidal factors. In mice, the most widely used experimental animal model in schistosomiasis research, age-associated hormones, such as testosterone and DHEA, have been implicated as putative schistosomicidal compounds [27, 28]. More recently, testosterone has been found to be directly toxic to larval schistosomes in vitro [29]. However, it should be noted that rhesus monkey adults actually have lower levels of DHEA than do prepubescent animals [16, 17]. The use of mice to address puberty-associated differences in susceptibility to schistosomiasis infection is confounded, because the full development of schistosomes in the mammalian host takes longer than sexual maturation of mice. However, earlier studies have shown that juvenile mice, as well as hamsters, are more susceptible than adult animals to schistosome infection [30]. These previous studies focused on the role of age-associated differences in skin thickness as an innate barrier to infection [31]. In the present study, we did not observe any differences between adult and juveniles in the number of nonpenetrating cercariae. However, other innate differences in susceptibility cannot be excluded as causes, and more research is required to determine the role of physiological differences between juveniles and adults with respect to susceptibility to schistosome infection. It is appropriate to highlight that experimental animals, including the monkeys used in this study, are bred from animals not infected with schistosomiasis. In marked contrast, in schistosomiasis endemic populations, women can be infected while pregnant; thus, in utero exposure to schistosome antigens can occur, with prenatal modulated immune responses having been demonstrated to persist into childhood [32]. Thus, in utero sensitization to schistosomes could be a significant factor in the subsequent susceptibility of children to schistosome infection.

There is an ongoing debate as to whether the increased resistance of adult humans to schistosome infection is due to the age of the host, per se, or to previous experience of infection(s) [33, 34]. Human field studies in a Ugandan fishing community have shown the age intensity of infection profile cannot be fully explained by the levels of exposure [8], although the effects of duration of infection have been questioned in studies of a new epidemic in Senegal [9] and Kenyan migrants [10]. The results in this study show that changes associated with the age of the host are important factors in the generation of antiparasite immune responses and, perhaps, also in the susceptibility to primary schistosome infection.

### Table 1. Fecal and organ egg counts in juvenile and adult rhesus monkeys after primary Schistosoma mansoni infection.

<table>
<thead>
<tr>
<th></th>
<th>Fecal eggs, epg</th>
<th>Organ eggs, epg</th>
<th>Liver</th>
<th>Intestine</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rhesus monkey</strong></td>
<td></td>
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</tr>
<tr>
<td>Juvenile</td>
<td>72.6 ± 54.3</td>
<td>66.4 ± 69.2</td>
<td>123.8 ± 121.3</td>
<td>1.9 ± 1.2</td>
<td>1.1 ± 1.1</td>
</tr>
<tr>
<td>Adult</td>
<td>43.4 ± 62.0</td>
<td>41.8 ± 61.6</td>
<td>24.2 ± 23.9</td>
<td>1.3 ± 1.1</td>
<td>0.1 ± 0.1</td>
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**NOTE.** Data are mean ± SD eggs per gram (epg) from groups of 5 animals per group. There was no statistically significant difference between groups in all parameters.
Acknowledgements
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References