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Age-Dependent Changes in Susceptibility of Suckling Mice to Individual Strains of *Helicobacter pylori*

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To model establishment of *Helicobacter pylori* infection in infants, suckling mice were inoculated with mixtures of strains that preferentially colonize different gastric regions and coexist in vivo. Characterization of *H. pylori* recovered 2 weeks later showed that susceptibility begins earlier for some strains than for others and that the onset of susceptibility varies among mouse lines.

Human infection with the genetically diverse gastric pathogen *Helicobacter pylori* usually begins in early childhood (4, 9, 18, 21). Urea breath test studies of infants in developing countries, where overall rates of infection are high, pointed to unexpected complexity in the establishment of infection: many infants exhibited one or more episodes of strong urea breath test positivity (implying *H. pylori* infection), then negativity (implying clearance), and again positivity (13, 26). One explanation for this pattern invokes early exposure to *H. pylori* strains that may be better suited to older family members; subsequent persistent infection reflects developmental changes in the host that increase *H. pylori* susceptibility. Such ideas cannot be tested adequately in people because ethical considerations preclude endoscopy of infants solely to collect *H. pylori* strains and monitor host responses. In this study, we used newborn mice to model human pediatric infection.

Mice were from established lines (The Jackson Laboratory) and were bred and maintained in the Washington University Animal Quarters with water and food supplied ad libitum under Animal Studies Committee-approved protocols. Strains of four *H. pylori* lineages were used: SS1 (containing *cag* PAI *vacAs2*) (15), X47-2AL (Δ*cag vacAs1*) (8), AM1 (Δ*cag vacAs2*), and AL10103 (Δ*cag vacAs2*) (6). They were grown on standard brain heart infusion blood agar with antibiotics as needed (1). SS1 and X47-2AL can coexist in adult mice, predominating in the antrum and corpus, respectively (1). Strains AL10103 and AM1 can similarly coexist (present results; M. Zhang and D. E. Berg, unpublished data). To easily distinguish SS1 and X47-2AL, we constructed derivatives resistant to clarithromycin (23S rRNA gene allele A2143G) and tetracycline (16S rRNA gene allele AGA965-967TTC) (5, 27) (strains called SS1cR, and X47tR, respectively). A kanamycin-resistant derivative of AM1 was constructed with an *frxA*::*aphA* allele (12) (AM1kR); AL10103 is Kan^s. Resistant strains attained densities similar to those of their drug-sensitive parents in adult C57BL/6J mice (scored 2 weeks after inoculation). To reduce accumulation of attenuated variants, cultures used for infection were derived from pools of *H. pylori* recovered from infected mice.

Suckling mice were inoculated with 100-μl suspensions containing 4×10^8 CFU of a single strain or 2×10^8 CFU of each of two strains grown in parallel by oral gavage with a 26-gauge plastic catheter syringe (Abbott Labs). Their snouts were cleaned with alcohol wipes to avoid infant-to-mother transmission. Mice were sacrificed by CO_2 asphyxiation 14 to 19 days after inoculation and at least 3 days after weaning (day 21) and cut open immediately afterward. The relative abundance of each input strain in the antrum and corpus was estimated separately by (i) quantitative culture of gastric homogenates on agar with or without tetracycline, clarithromycin, or kanamycin, as appropriate, and (ii) by testing 20 single colonies per antrum and corpus (40 per mouse) for drug susceptibility. The statistical significance of differences was estimated with Fisher's exact probability test.

Preliminary tests were carried out with mice from crosses of 129P3J females and BALB/cJ or C3HeJ males. They were inoculated with suspensions of freshly grown overnight plate cultures of SS1cR and X47tR (1:1 mixture). Of 24 pups inoculated at 2 weeks of age, 14 carried only SS1cR, 1 carried only X47tR, and 8 remained uninfected; only 1 of 24 mice carried both strains. In contrast, each of six mice inoculated 1 week later carried both strains. Inferences that a strain was present or absent were based on finding thousands of colonies versus no colonies, respectively, in smears of 100 μl (10%) of the gastric homogenate on agar medium.

Subsequent tests were carried out with *H. pylori* cultures that had been grown overnight (to early exponential phase) on brain heart infusion agar, suspended in tryptic soy broth with 20% glycerol, and held at −80°C before thawing for inoculation. Control experiments showed that strains SS1cR and X47tR prepared in this way each retained ~100% viability (quantitative culture) and were fully competent for adult mouse infection. The results after mixed inoculation with frozen cultures (below) and freshly grown cultures were similar.

Three inbred mouse lines were used. With C57BL/6J mice (wild type and IL-12β knockout), 24 of 41 animals inoculated between 5 and 11 days of age became infected with SS1cR

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TABLE 1. Fraction of mice inoculated with mixtures of strains SS1cR and X47tR that became infected with one or both strains

Age[b] (days)	C57BL/6J WT[a]			C57BL/6J IL-12β			FVB/NJ			BALB/cJ		
	SS1cR[c]	Both	Not infected	SS1cR	Both	Not infected	SS1cR	Both	Not infected	SS1cR	Both	Not infected
5	7/14[d]	2/14	5/14				6/10	4/10	0/10			
7	4/7	1/7	2/7	3/7	4/7	0/7	6/11	5/11	0/11	2/11	0/11	9/11
11	5/7	2/7	0/7	5/6	1/6	0/6	5/9	4/9	0/9	3/12	0/12	9/12
14	0/6	6/6	0/6	0/6	6/6	0/6	6/10	4/10	0/10	1/15	13/15	1/15
17	0/5	5/5	0/5	0/9	9/9	0/9	0/7	7/7	0/7	0/12	12/12	0/12
21				0/6	6/6	0/6	0/9	9/9	0/9	0/5	5/5	0/5

[a] WT, wild type; IL-12β, null mutation in gene for β (p40) subunit of cytokine IL-12.
[b] Age at inoculation.
[c] SS1cR indicates that only SS1cR was recovered (identified by clarithromycin resistance), both indicates that SS1cR and X47tR (identified by tetracycline resistance) were both recovered, and not infected indicates that neither *H. pylori* strain was found. X47tR was not found without SS1cR in any of these coinoculated mice.
[d] Groups in which differences were significant statistically ($P < 0.05$; Fisher's exact probability test) include SS1cR only versus X47tR only in C57BL/6J mice inoculated at 5, 7, or 11 days; in FVB/NJ mice inoculated at 5, 7, 11, or 14 days; and in BALB/cJ mice at 7 and 11 days of age; SS1cR only or both strains in C57BL/6J or FVB/NJ mice versus BALB/cJ mice at 7 days of age; SS1cR only in C57BL/6J versus BALB/cJ mice at 11 days of age.

alone and another 10 became infected with SS1cR and X47tR together, but none became infected with X47tR alone (Table 1; $P < 0.02$ at each time point). In contrast, each of 32 older mice (inoculated between 14 and 21 days of age) became infected with both strains, as was also seen with fully grown adults (1). No significant differences in the susceptibility of male versus female, nor of wild-type versus IL-12β knockout, mice were detected (Table 1). In further tests, 5 of 13 C57BL/6J mice inoculated with strain X47tR alone at 7 or 11 days of age became infected and the other 8 remained uninfected (in contrast to the 12 of 14 mice inoculated with the SS1cR-X47tR mixture, most of which retained only SS1cR [Table 1; $P < 0.05$]). These outcomes indicated that the later onset of susceptibility to X47tR was intrinsic to the strain itself and not due to competition from SS1cR.

With FVB/NJ mice, all 40 animals inoculated with the 1:1 mixture at 5 to 14 days of age became infected: 23 with SS1cR alone and the other 17 with both SS1cR and X47tR. Each of 16 mice inoculated later (17 or 21 days of age) became infected with both strains. Again, none of these 56 mice became infected with strain X47tR alone (Table 1).

With BALB/cJ mice, 5 of 23 animals inoculated at 7 or 11 days of age became infected with SS1cR only and the other 18 remained uninfected, but high susceptibility appeared soon thereafter: 13 of 15 mice inoculated at 14 days, and all 12 mice inoculated at 17 days, became infected with both strains (Table 1).

In animals with mixed infections (inferred on the basis of growth from gastric homogenate on both clarithromycin- and tetracycline-containing media), tests of single colonies (20 per gastric region) indicated on average that 61 to 80% of the *H. pylori* bacteria in the antrum and 11 to 24% of those in the corpus were SS1cR; all others were X47tR. This distribution is similar to that seen in adult mice (1).

Two other compatible mouse-colonizing strains, AM1kR (Kan^r) and AL10103 (Kan^s), were used to test the generality of the SS1cR and X47tR data above. Table 2 shows that 6 of 22 C57BL/6J IL-12β mice inoculated with these two strains at 7 to 14 days of age became infected with AM1kR only, that none became infected with AL10103 only ($P = 0.02$), and that all 10 mice inoculated at 17 days of age became infected with both strains. In equivalent tests, each of 15 adult mice of the same breed (8 to 10 weeks old when inoculated) also became infected with both strains. However, the average antrum and corpus yields of AM1 were 25 and 68%, respectively, from adult mice, but 57 and 93% from the infant mice with mixed infections. Thus, AM1 seems particularly well suited (and AL10103 seems ill suited) for very young mice.

Collectively, our results indicate that traits that change rapidly after birth can affect susceptibility to *H. pylori*, which begins earlier for some strains than others. The time of development of susceptibility varied among mouse lines but seemed unaffected by cytokine IL-12β deficiency (Table 1), a lesion that had made adult mice more permissive for infection with certain enfeebled strains (10). We propose that the development of susceptibility studied here involves a critical establishment phase of infection, whereas IL-12β may be more important in a later persistence phase. Studies of the effects of inactivating strain SS1's vacuolating cytotoxin (*vacA*) gene on mouse colonization (25) and host transcript profiles (28) also distinguish between establishment and maintenance phases of infection.

The strains for which susceptibility developed first preferentially occupy different gastric regions: SS1 in the antrum but AM1 in the corpus (1; present results). This outcome argues against a model of susceptibility resulting from developmental changes that occur first in one gastric region and then in another. A second model invokes traits that change during infancy, such as: (i) gastric acidity (16, 20), (ii) composition of

TABLE 2. Fraction of mice[a] inoculated with mixtures of strains AM1kR and AL10103 that became infected with one or both strains

Age (days)	No. of mice/total		
	AM1kR[b]	Both	Not infected
7	1/6[c]	0/6	5/6
11	2/8	1/8	5/8
14	3/8	5/8	0/8
17	0/10	10/10	0/10

[a] C57BL/6J IL12β knockout mice were used. The protocol and presentation of data are as in Table 1 (see Table 1 footnotes).
[b] AM1kR indicates that only AM1kR was recovered (identified by kanamycin resistance), both indicates that AM1kR and AL10103 were both recovered, and not infected indicates that neither *H. pylori* strain was found. AL10103 was not found without AM1kR in any of these coinoculated mice.
[c] The difference in yields of AM1kR only versus AL10103 only at 7, 11, and 14 days was statistically significant ($P < 0.05$, Fisher's exact probability test).

mucin that *H. pylori* must penetrate (3), (iii) types of epithelial surface proteins or glycans to which *H. pylori* may adhere (2, 11, 17), and (iv) responsiveness to immune and inflammatory stimuli (7). A third model focuses on mother's milk and its antibacterial factors, including iron-sequestering lactoferrins, antimicrobial peptides, fatty acids, glycoconjugates that interfere with adherence, and cytokines and other regulators of general mucosal and immune system development (19). The composition of mother's milk changes with time between birth and weaning and varies with genotype (14). A fourth model focuses on other gastrointestinal flora (22), which change with age after birth and may either inhibit or promote *H. pylori* growth (23, 24). Recent experiments have shown that a strain that colonizes mice first enjoys a competitive advantage relative to superinfecting strains that is not seen during simultaneous inoculation (Zhang and Berg, unpublished). We suggest that human infants also change in susceptibility to particular *H. pylori* strains with age and that patterns of exposure early in life may profoundly affect strains carried decades later and thereby the pace and directions of *H. pylori* genome evolution.

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