INFLAMMATION

Helicobacter bilis triggers persistent immune reactivity to antigens derived from the commensal bacteria in gnotobiotic C3H/HeN mice

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Background: Infection with *Helicobacter* species has been associated with the development of mucosal inflammation and inflammatory bowel disease (IBD) in several mouse models. However, consensus regarding the role of *Helicobacter* as a model organism to study microbial-induced IBD is confounded by the presence of a complex colonic microbiota.

Aim: To investigate the kinetics and inflammatory effects of immune system activation to commensal bacteria following *H bilis* colonisation in gnotobiotic mice.

Methods: C3H/HeN mice harbouring an altered Schaedler flora (ASF) were selectively colonised with *H bilis* and host responses were investigated over a 10-week period. Control mice were colonised only with the defined flora (DF). Tissues were analysed for gross/histopathological lesions, and bacterial antigen-specific antibody and T-cell responses.

Results: Gnotobiotic mice colonised with *H bilis* developed mild macroscopic and microscopic lesions of typhlocolitis beginning 3 weeks postinfection. ASF-specific IgG responses were demonstrable within 3 weeks, persisted throughout the 10-week study, and presented as a mixed IgG1:IgG2a profile. Lymphocytes recovered from the mesenteric lymph node of *H bilis*-colonised mice produced increased levels of interferon γ , tumour necrosis factor α (TNF α), interleukin 6 (IL6) and IL12 in response to stimulation with commensal- or *H bilis*-specific bacterial lysates. In contrast, DF mice not colonised with *H bilis* did not develop immune responses to their resident flora and remained disease free.

Conclusions: Colonisation of gnotobiotic C3H/HeN mice with *H bilis* perturbs the host's response to its resident flora and induces progressive immune reactivity to commensal bacteria that contributes to the development of immune-mediated intestinal inflammation.

Altered composition and functional activities of the luminal microflora have been linked to the pathogenesis of chronic enterocolitis, such as inflammatory bowel disease (IBD). A number of animal models of IBD provide compelling evidence that the bacterial flora significantly contributes to the inflammatory process. For example, both interleukin (IL)-deficient IL2^{-/-} and IL10^{-/-} mice spontaneously develop IBD when housed under conventional conditions, but there is no evidence of colitis when animals are maintained under germ-free conditions.³ The adoptive transfer of CD45RB^{hi} T cells mediates less severe wasting disease in severe combined immunodeficiency mice harbouring a specific pathogen-free (SPF) intestinal flora.⁴ Furthermore, T lymphocyte-mediated intestinal inflammation can be transferred with effector T cells primed against enteric bacteria.^{4–5} It seems that a constant bacterial stimulus is needed for the induction and perpetuation of inflammation⁶ and that resident bacteria have different capacities to induce mucosal inflammation in a susceptible host.^{7–9} These accumulated observations incriminate intestinal bacteria in the initiation and perpetuation of chronic intestinal inflammation, although the critical bacterial species or antigen(s) remain undetermined. It is likely that select bacterial components from the resident microflora and antigen-specific immune responses that they induce are crucial for the development of colitis.

Infection with *Helicobacter* species has been associated with the development of mucosal inflammation and IBD in several mouse models.^{10–15} Although some studies indicate that *Helicobacter* spp do not induce IBD in germ-free IL10^{-/-} mice¹⁶

or worsen IBD in IL10^{-/-} mice that have been reconstituted with SPF flora,³ others have shown that *Helicobacter* spp can be an important factor contributing to murine colitis in an SPF environment.^{17–18} It is clear that determining the pathogenic potential of *Helicobacter* spp or their ability to interact with resident flora and induce inflammation is confounded by the presence of a complex colonic microbiota. We have previously shown that *H bilis* induces host responses and typhlocolitis at a single time point in gnotobiotic C3H/HeN mice.¹⁹ However, the temporal relationship between immune reactivity and the development of intestinal inflammation in this unique model has not been examined. The aim of the present study was to investigate the kinetics of the immune response and onset of mucosal inflammation subsequent to colonisation of gnotobiotic mice with *H bilis*. The results demonstrated that the introduction of a bacterial provocateur into the colonic flora of gnotobiotic mice induced antigen-specific antibody and T-cell responses to members of the resident flora, suggesting a mechanism by which dysregulated immune responses to the enteric flora may arise in patients susceptible to IBD.

Abbreviations: ASF, altered Schaedler flora; DF, defined flora; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IBD, inflammatory bowel disease; IFN γ , interferon γ ; IL, interleukin; MLN, mesenteric lymph node; NI, non-infected; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline Tween 20; PI, postinfection; SPF, specific pathogen-free; SPL, spleen

Figure 1 Caecal pathological scores over time from non-infected (NI) and *H. bilis*-colonised defined flora C3H/HeN mice. Plots show the range (grey boxes), means (solid lines inside boxes) and median values (dashed lines inside boxes) derived from triplicate experiments. As there were no differences over time in the responses of the control mice, scores depicted for NI mice represent compiled observations made from appropriately matched gnotobiotic control mice across all time points. (A) Macroscopic caecal lesions (range 0–3). (B) Histological scores obtained using a blinded evaluation of tissue sections as described in the Materials and Methods section. Maximum total histological score (determined by the summation of the individual parameters) was 20. PI, postinfection. *A significantly different ($p < 0.05$) value in comparison with NI mice.

MATERIALS AND METHODS

Animals

C3H/HeN mice (6–8 weeks old) populated with a defined flora (DF); eg, altered Schaedler flora (ASF) comprised of eight bacterial species) were obtained from Taconic Farms (Albany, New York, USA). Members of the ASF include ASF356, *Clostridium* cluster XIV; ASF360, *Lactobacillus acidophilus*; ASF361, *Lactobacillus murinus*; ASF457, *Mucispirillum schaedleri*; ASF492, *Eubacterium plexicaudatum*; ASF500, low-G+C content-positive bacteria; ASF502, *Clostridium* cluster XIV; and ASF519,

Bacteroides distasonis. All mice were certified as free of *Helicobacter* spp by the vendor and re-tested on-site prior to study enrolment. Cohorts of mice were bred and maintained within the murine gnotobiotic facility at the College of Veterinary Medicine, Iowa State University, IOWA, USA. Animals were housed in Trexler plastic isolators and fed an irradiated diet (eg, Harlan-Teklad) and autoclaved water. All animal-related procedures were approved by the animal care and use committee at Iowa State University.

Experimental design

Gnotobiotic C3H/HeN mice were assigned to one of two study groups: (1) control mice receiving sham inoculations or (2) infected mice selectively colonised with *H. bilis*. Representative experimental and control mice (6–12 mice per time point) were euthanised by CO₂ asphyxiation on days 21, 42 or 70 after *H. bilis* colonisation. Samples were processed for evaluation of gross lesions, bacteriology, histopathology, antigen-specific serum IgG concentrations, levels of cytokines secreted from antigen-stimulated cells and PCR analysis for confirmation of bacterial colonisation.

Infection with *H. bilis*

An *H. bilis* isolate (ATCC strain 51630) was kindly provided by Dr Nancy Lynch (College of Medicine, University of Iowa, Iowa, USA). Organisms were streaked onto Columbia agar plates supplemented with 5% horse serum, grown under microaerophilic conditions (80% N₂, 10% H₂ and 10% CO₂) and maintained at 37°C. Bacteria were collected from 3–5 plates and suspended in tryptic soy broth on the day of inoculation. Before inoculation, organisms were collected under sterile conditions and examined for their purity, morphology and motility by dark-phase microscopy. Samples were confirmed to be urease positive. Organisms were then suspended in broth to an approximate concentration of 10⁸–10⁹ CFU/ml. Gnotobiotic mice were orally infected with a bacterial inoculum (0.3 ml) of *H. bilis* administered every other day for three doses. Confirmation of *H. bilis* colonisation was made by PCR evaluation of faeces or caecal contents.

H. bilis and ASF-specific PCR

Faecal samples or caecal contents were analysed using PCR for *Helicobacter* spp and *H. bilis*, as described previously.^{20, 21} For generic *Helicobacter* PCR, 5 µl of quantitated faecal DNA was used as a template, whereas for *H. bilis* PCR, 10 µl of quantitated faecal DNA was used as a template for all samples. At the termination of each

Figure 2 Histological changes in the caecal mucosa of defined flora (DF) C3H/HeN:Tac mice after colonisation with *H. bilis*. All images are at 40×. (A) Control—no infection. (B) Caecal tissue from a mouse infected with *H. bilis* depicting moderate typhlocolitis characterised by moderate crypt hyperplasia with elongation of the mucosal glands, infiltration of the lamina propria with moderate numbers of lymphocytes, plasma cells and occasional neutrophils, and marked submucosal oedema. (C) Caecal tissue from a mouse infected with *H. bilis* showing severe typhlocolitis as evidenced by marked infiltration of the lamina propria and submucosa by large numbers of neutrophils with fewer lymphocytes and plasma cells. The mucosal surface is hyperplastic with elongation of the mucosal glands. Here, enterocytes along the outer aspects of the glands are multifocally ulcerated. The inset depicts the accumulation of neutrophils within the submucosa.

Figure 3 Measurement of serum immunoglobulin (Ig) G1 and IgG2a responses to altered Schaedler flora antigens for samples collected from non-infected (NI) and *H. bilis*-colonised defined flora mice as described in fig 1. The magnitude of the antibody response was determined by ELISA as described in the Materials and Methods section. The data are presented as pooled group means (SEM) derived from triplicate experiments. Separate experiments contained 5–8 mice per treatment group per time point. * $p < 0.05$ versus NI mice at each time point. † $p < 0.05$ versus IgG1 response measured in *H. bilis*-colonised mice at 3 and 10 weeks postinfection (PI).

experiment, confirmation that all eight members of the ASF continued to colonise the mice was performed by PCR analysis of faecal DNA. PCR protocols were developed and optimised to detect the 16S rRNA sequence of each ASF bacterium using specific primer pairs with minor modification.^{19 22}

Preparation of *H. bilis* and ASF antigens

H. bilis was cultivated on Columbia agar plates supplemented with bovine blood and horse serum and the bacteria were harvested from the plates using sterile phosphate-buffered saline (PBS). The cells were washed in sterile PBS using centrifugation. The cell pellet was frozen, lyophilised and then stored at -20°C until use. Each member of the ASF flora was grown anaerobically at 37°C in Schaedler broth supplemented with 5% calf serum or 5% sheep blood. Cultivation of the extremely oxygen-sensitive ASF bacteria (eg, ASF356, ASF492, ASF500, ASF502) was performed in an anaerobic chamber using pre-reduced medium. As above, the cells were harvested from broth by centrifugation, washed in PBS, lyophilised, and stored at -20°C before use.

Bacterial antigens (eg, *H. bilis* and all eight ASF strains) used for enzyme-linked immunosorbent assay (ELISA) or for stimulation of lymphocyte cultures were prepared from the lyophilised cells. Cells were weighed and resuspended in PBS to a final concentration of 2 mg/ml (dry weight to volume). The cell suspension was then placed on ice and sonicated for 5 min to prepare whole-cell sonicates. Sonication was performed using 30 s pulses to prevent the suspension from overheating (ie, denaturing of the proteins). Cell disruption was monitored microscopically. Protein content was determined by bicinchoninic acid analysis according to the manufacturer's instructions (Pierce Laboratories, New Haven, Connecticut, USA). For the

preparation of the antigen used in these studies, the whole-cell sonicates were sterilised by ultraviolet irradiation and sterility was confirmed bacteriologically (eg, aerobic and anaerobic blood agar cultures).

Macroscopic caecal lesions

Gross caecal lesions were scored using previously published criteria.²³ Macroscopic caecal lesions were scored 0–3 as follows: 0, no gross lesions; 1, excess caecal mucus with no evidence of atrophy; 2, excess intraluminal mucus with atrophy localised to the caecal apex; and 3, generalised caecal atrophy with increased intraluminal mucus.

Histopathological analysis

Samples of caecum and proximal colon were placed in 10% neutral buffered formalin, routinely processed, sectioned and stained with H&E. Sections of the caecum and proximal colon were scored by a pathologist (JH) who was blinded to the treatment group.¹⁹ Mucosal inflammation was scored 0–20 based on the severity of mucosal epithelial damage, architectural/glandular alterations and the magnitude/character of lamina propria cellular infiltrate.

Serum IgG

Blood samples were obtained by cardiac puncture at the same time that tissues were collected for analysis. Antigen-specific serum IgG1 and IgG2a responses were determined by ELISA as described previously.²⁴ In brief, 96-well plates were coated overnight with ASF or *H. bilis* bacterial lysates (10 $\mu\text{g}/\text{ml}$ in carbonate buffer, pH 6.0). Plates were washed three times with phosphate-buffered saline Tween 20 (PBST) and non-specific binding sites were blocked with 2% gelatin/fetal bovine serum (FBS) in PBST for 2 h at room temperature. Plates were washed three times with PBST; diluted serum (1:200 in PBST with 2% FBS) was added to each well and incubated at 4°C overnight, followed by four more washes. Alkaline phosphatase-conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotech, Birmingham, Alabama, USA; 1:1000 dilution) was added and incubated for 2 h at room temperature. Wells were developed using *p*-nitrophenyl phosphate at room temperature. Colour changes were measured using an ELISA plate reader at 405 nm.

Cytokine measurements

Mesenteric lymph nodes or spleens were removed and single cell suspensions were prepared by homogenisation. Cells were washed and resuspended in tissue culture medium (RPMI 1640) supplemented with 5% FBS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$

Figure 4 Measurement of serum IgG1 and IgG2a antibody responses to *H. bilis* antigen collected at various time points following colonisation of defined flora mice with *H. bilis* as described in fig 1. The antibody response was determined by ELISA as described in the Materials and Methods section. The data are presented as pooled group means (SEM) of the mean derived from triplicate experiments. Separate experiments contained 5–8 mice per treatment group per time point. NI, non-infected; PI, postinfection. * $p < 0.05$ for IgG1 at 6 weeks PI vs 3 weeks PI and 10 weeks PI. † $p < 0.05$ for IgG2a at 6 weeks PI vs 3 weeks PI and 10 weeks PI.

ml gentamicin and 2 mmol L-glutamine. Lymphocytes were incubated at 2.5×10^6 cells/ml and stimulated with or without 50 µg/ml whole-cell lysates of the ASF bacteria or *H. bilis* for 72 h. Cell cultures were treated with 0.5 µg/ml concanavalin A to evaluate the overall nature of the T-cell response. Cells were cultured in triplicate; cell-free supernatants were harvested after 72 h of culture and then analysed for the concentration of TNF α , interferon γ (IFN γ), IL6, IL4, IL10 and IL12 using a multiplexed flow cytometric assay (The FlowMetric System, Luminex, Austin, Texas, USA).

Statistical methods

Results were first analysed for data quality using summary statistics (means, medians, SD and histograms). Although antibody responses to each of the eight ASF bacteria were measured for all mice individually, there was variability from mouse to mouse in the magnitude of the antibody response to a given bacterial lysate. For this reason, the mean of the composite

antibody responses (eg, pooled average of the antibody response of individual mice to each of the eight separate ASF antigens) across three independent experiments was used for statistical comparisons. Furthermore, data analyses indicated that responses of the non-infected (NI), control mice were similar at all time points; therefore, these data were combined and are singly referred to as NI control in the figures. The initial group comparisons for gross/histopathological scores and serum antibody responses were made using the robust Kruskal–Wallis non-parametric analysis of variance. If comparisons were statistically significant, then pairwise means were tested using the Tukey–Kramer type I error-protected t test.

For analysis of cytokine concentrations, the study design had four factors: organ (two levels), cytokine (five levels), ASF bacteria (eight levels) and time (three and four levels). Technical difficulties (eg, sample limitations) precluded analysis of IL12 concentrations from both mesenteric lymph node and splenic cultures in mice colonised for 10 weeks with *H. bilis*.

Mesenteric lymph node culture									Splenic culture								
Reactive ASF bacteria*									Reactive ASF bacteria*								
356	360	361	457	492	500	502	519		356	360	361	457	492	500	502	519	
								INF- γ									
								NI							x		
x							x	3w-PI									
								6w-PI									x
	x			x	x	x	x	10w-PI		x			x	x	x	x	
								TNF- α									
								NI							x		
								3w-PI	x		x	x	x	x	x	x	
		x	x	x			x	6w-PI			x	x			x	x	
								10w-PI		x			x	x	x	x	
								IL-6									
								NI					x	x	x	x	
				x			x	3w-PI					x			x	
			x	x			x	6w-PI		x		x	x	x	x	x	
	x			x	x	x	x	10w-PI		x			x	x	x	x	
								IL-10									
								NI							x	x	
								3w-PI								x	
								6w-PI			x	x	x		x	x	
								10w-PI					x		x	x	
								IL-12									
								NI							x	x	
								3w-PI	x		x	x	x	x	x	x	
		x	x	x		x	x	6w-PI	x		x	x	x	x	x	x	
NP	NP	NP	NP	NP	NP	NP	NP	10w-PI	NP	NP	NP	NP	NP	NP	NP	NP	NP

Figure 5 Stem-leaf diagram depicting the induction of cytokine responses following stimulation of mesenteric lymph node (MLN) or spleen (SPL) cells with bacterial antigens. Tissues were recovered from non-infected control (NI) or *H. bilis*-colonised (3, 6 or 10 weeks postinfection (PI)) gnotobiotic mice. Culture supernatants were collected after 72 h in vitro stimulation with individual bacterial lysates prepared from the eight members of the altered Schaedler flora (ASF) and assayed for cytokine production as described in the Materials and Methods section. ASF bacteria that induced a ≥ 10 -fold increase in the median cytokine concentration (determined as the ratio of antigen-specific cytokine concentration to background (no stimulation) concentration) are denoted by an X in the appropriate column. ASF356, *Clostridium* cluster XIV; ASF360, *Lactobacillus acidophilus*; ASF361, *Lactobacillus murinus*; ASF457, *Mucispirillum schaedleri*; ASF492, *Eubacterium plexicaudatum*; ASF500, low-G+C-content bacteria; ASF502, *Clostridium* cluster XIV; ASF519, *Bacteroides distasonis*. IL, interleukin; INF γ , interferon γ ; NI, non-infected; NP, not performed; TNF α , tumour necrosis factor α ; w-PI, weeks postinfection.

Figure 6 Induction of cytokines from mesenteric lymph node (MLN) and spleen (SPL) cells after stimulation with *H. bilis* lysate. Tissues were recovered from non-infected (NI) control and *H. bilis*-colonised (3–10 weeks postinfection (PI)) defined flora mice as described in fig 1. The data are presented as the median fold increase in cytokine concentration (determined as the ratio of *H. bilis*-specific cytokine concentration to background (no stimulation) concentration) subsequent to stimulation.

Thus, IL12 temporal responses were evaluated over three time periods (controls, and 3 and 6 weeks postinfection (PI)), whereas the temporal responses of the remaining cytokines were analysed over all four time periods. Comparisons among levels of factors (eg, spleen vs lymph node) were performed by testing the fold increase difference in proportions of median responses (eg, determined as the ratio of antigen-specific cytokine concentration to background (no stimulation) concentration) that were at least 10 times the background level. The normal approximation to the binomial distribution was assessed as valid and was used as the inferential procedure.²⁵ These analyses were planned comparisons (rather than post hoc), so that it was not necessary to adjust the *p* values to control for type I error inflation.²⁶ A *p* value <0.05 was considered statistically significant for all inferences.

RESULTS

Colonisation with *H. bilis* and ASF bacteria

Mice inoculated with *H. bilis* were found to be colonised by faecal PCR 12–14 days PI and remained so for the duration of the study. DF control mice remained *Helicobacter* free as determined by faecal PCR. To demonstrate that *H. bilis* did not displace any of the ASF, species-specific PCR products for all eight ASF bacteria were reliably obtained at all time points by amplification of DNA extracted from caecal contents using the ASF-specific primers (data not shown).

Gross and histopathological lesions

Mild typhlocolitis was grossly observed in mice 3 weeks after colonisation with *H. bilis* as evidenced by significantly (*p*<0.05) increased caecal scores relative to control mice (fig 1A). By 6 weeks after colonisation, macroscopic changes were characteristically mild to moderate and were accompanied by regional mesenteric lymphadenopathy. Similarly, *H. bilis*-colonised mice developed mild-to-moderate histopathological inflammation, which peaked 3 weeks after bacterial inoculation. Histological scores obtained by blinded microscopic examination were significantly (*p*<0.05) increased at 3, 6 and 10 weeks PI compared with DF controls (fig 1B). Histological inflammation was characterised as lamina propria infiltration with predominantly mononuclear cells, crypt hyperplasia, lymphoid hyperplasia and submucosal oedema (fig 2A–C). Mucosal infiltration with a mixed cellular infiltrate (eg, neutrophils; see inset, fig 2C) was a salient feature in caecal tissues derived from 6- and 10-week *H. bilis*-colonised mice. Mean (SD) histological scores 2 weeks after *H. bilis* colonisation were not significantly different from DF

controls (4.0 (0.3) and 3.7 (0.1), respectively). No gross or histological lesions were detected in caecal tissues of control mice. These results indicated that *H. bilis*-colonised mice developed gross and histological signs of typhlocolitis beginning at 3 weeks after inoculation and that mucosal inflammation was sustained over 10 weeks.

Antigen-specific IgG responses in serum

Previously, we had shown that *H. bilis*-colonised mice develop IgG responses to their commensal flora at 6 weeks PI.¹⁹ To address the kinetics of the induction of host immune responses to members of the normal flora, serum antibody responses to each of the eight ASF as well as *H. bilis* were evaluated by ELISA at three time points after colonisation with *H. bilis*. Serum IgG1 and IgG2a directed against lysates of ASF bacteria significantly (*p*<0.05) increased in 3, 6 and 10 week *H. bilis*-colonised mice relative to DF controls (fig 3). Additionally, the serum IgG1 response was significantly (*p*<0.05) greater in 6-week colonised mice versus IgG1 levels observed in 3- and 10-week colonised mice. Although *H. bilis* colonisation induced significant serum antibodies in all eight ASF, the serum IgG responses to ASF457, ASF500, ASF502 and ASF519 were significantly (*p*<0.05) greater in *H. bilis*-colonised mice compared with the IgG responses to the other four members of the ASF (data not shown). Both IgG1 and IgG2a levels to *H. bilis* were significantly (*p*<0.05) increased in 6-week colonised mice compared with responses observed at 3 and 10 weeks after colonisation (fig 4). These results indicated that *H. bilis*-colonised mice developed antigen-specific immune responses to their resident microflora. The IgG responses developed rapidly, persisted throughout the 10-week study, showed selective reactivity over time and demonstrated a mixed IgG1:IgG2a phenotype.

Antigen-specific cytokine responses from lymphocyte cultures

To define the antigen-specific T-cell response, in vitro cytokine production after stimulation of unfractionated mesenteric lymph node (MLN) or splenic (SPL) lymphocyte populations with ASF-specific or *H. bilis* bacterial lysates was measured. There were appreciable differences in cytokine secretion between control (non-colonised) and *H. bilis*-colonised mice at 3, 6 and 10 weeks PI. Using 10-fold increases in cytokine production as a minimal discriminator, statistical analyses indicated that antigens derived from the resident ASF bacteria induced in vitro cytokine production by either MLN or SPL cells at one time point or another (fig 5). The only exception was the increased induction of IL10 by MLN cells stimulated with ASF antigens. A comparison of local versus systemic responses (eg, MLN vs SPL) across all time points from *H. bilis*-colonised mice showed that ASF-stimulated SPL lymphocytes secreted significantly (*p*<0.05) more IFN γ , TNF α , IL6, IL10 and IL12 compared with supernatants of MLN lymphocytes stimulated with ASF-specific antigens. Further evaluation of SPL responses showed that antigens from ASF360, ASF500, ASF502 and ASF519 induced significantly (*p*<0.05) greater cytokine responses (eg, pooled responses for all five cytokines at all time points) compared with the other four ASF bacterial antigens. Local (ie, MLN) cytokine production in response to ASF bacterial stimulation increased more slowly than the corresponding splenic responses, was predominantly T helper 1 (Th1) biased (owing to an absence of IL4 and IL10), and increased over time after *H. bilis* colonisation while remaining low to undetectable in gnotobiotic control mice.

Lymphocyte stimulation with *H. bilis* lysate induced Th1-biased cytokine responses, which were more immediate and of greater magnitude (ie, fold increase in cytokine secretion) over time in comparison with ASF-stimulated cytokine responses

(fig 6). Together, these results indicated that antigen-specific T-cell responses are induced and sustained after *H. bilis* colonisation in gnotobiotic C3H mice. Furthermore, cytokine secretion in response to ASF or *H. bilis* antigenic stimulation shows different kinetics of onset and suggests that these responses contribute to the development of, or sensitivity to, colitis.

DISCUSSION

The presence of a complex microbiota makes it difficult to determine which components of the bacterial flora selectively contribute to the development of intestinal inflammation. Reconstitution studies in different rodent models suggest that a subset of luminal bacteria, particularly anaerobes, have variable ability to induce experimental colitis.^{7–29} Similarly, infection with *Helicobacter* species has been implicated as a cause for spontaneous colitis (IBD) in various mouse strains raised in conventional facilities.^{13–18, 30–34} Collectively, these results indicate that multiple bacterial species (including *Helicobacter*) contribute to chronic mucosal inflammation and the establishment of IBD in a susceptible host.

We previously demonstrated that *H. bilis* could induce host immune responses to the commensal microflora and cause colitis in gnotobiotic C3H/HeN mice harbouring an ASF.¹⁹ However, these earlier data did not evaluate whether immune activation and mucosal injury vary over time. In this study, we specifically addressed this question by assessing the temporal alterations in host responses and severity of colitis subsequent to *H. bilis* colonisation. Our results indicate that *H. bilis* colonisation induced rapid and sustained immune reactivity to the resident flora, that host responses preceded the onset of gross and histological inflammation, and that the host immune response to members of the commensal ASF bacteria were manifested differentially over time with respect to the development of typhlocolitic lesions. Using caecal contents, preliminary results from quantitative real-time PCR (qRT-PCR) analyses indicate that *H. bilis* colonisation did not affect the relative concentration of the individual ASF members over time (data not shown, manuscript in preparation). These results suggest that the induction of antigen-specific response to members of the ASF was not associated with an increase in caecal bacterial load.

Gross and histopathological caecal lesions were observed in most *H. bilis*-colonised mice, with peak inflammatory scores observed at 3 and 6 weeks PI. Consistent with previous observations, microscopic changes in *H. bilis*-colonised mice were characterised by increased mucosal cellular infiltration, crypt hyperplasia and lymphoid hyperplasia.^{14–18, 24} Although mild histological lesions occurred early in these gnotobiotic C3H/HeN mice and were sustained for the 10 weeks of this study, others have shown that *H. bilis* induces more severe lesions that have an accelerated or delayed onset of expression in multiple-drug resistance-deficient *la*^{−/−24} and *IL10*^{−/−18} mice, respectively. Of note, *TCRα*^{−/−} mice also develop colitis of mild severity when colonised with *H. bilis* for 17 or 29 weeks.¹⁸ The mechanisms responsible for variation in the pathological observations among these published studies compared with our results remain unclear. However, differences in host genetic background, variability in the duration of *H. bilis* infection and differences in the number, composition and/or location of the resident microflora may have influenced the development of intestinal inflammation in *H. bilis*-colonised mice.^{3–18, 24}

Our results indicate that antigen-specific immune response to individual ASF members developed subsequent to colonisation with *H. bilis*, because control gnotobiotic mice did not show immune reactivity to their commensal bacteria nor did they develop microscopic colitis over the 10 weeks of this study. *H. bilis*-colonised mice produced increased IgG1 and IgG2a when either ASF or *H. bilis* bacterial lysates were used as the target

antigens. Furthermore, these antibody responses in infected mice increased significantly ($p < 0.05$) above control levels by 12 days PI (data not shown), and this observation is consistent with that of Kim *et al.*²⁹ in that immune responses preceded the development of histopathological changes. In contrast, serum samples collected from gnotobiotic mice at day 17 after treatment with a single round of dextran sodium sulphate (1.5% or 2% in water) did not induce antibody responses to their resident (ASF) flora (data not shown). This is in contrast to the observed increase in the antibody responses within 12 days after *H. bilis* colonisation as noted above, and indicates that a mild inflammatory insult alone is not sufficient to induce immune responses to the resident ASF. The antibody responses to various bacterial species have been reported only to a limited extent in experimental colitis. Brandwein *et al.*³⁵ showed selective serum antibody reactivity to commensal (eg, *Enterococcus* and *Enterobacteriaceae*) bacterial antigens in spontaneously colitic C3H/HeJBir mice, whereas high concentrations of IgG directed against luminal commensal bacteria have been observed in *IL10*^{−/−} mice housed within an SPF environment.³ Also, Mizoguchi *et al.*³⁶ reported higher levels of IgG1 and IgG2a in the sera of diseased *TCRα*^{−/−} mice compared with *TCRα*^{+/−} or *TCRα*^{−/−} mice without disease. Recent observations that *IL10*^{−/−} mice monoassociated with either *Enterococcus faecalis* or *Escherichia coli*, but not with *Pseudomonas fluorescens*, develop colitis indicate that luminal bacteria have selective ability to induce disease in a susceptible host.²⁹ Similarly, our findings show that IgG production to commensal bacteria precedes the development of histological lesions in *H. bilis*-colonised mice and potentially suggests a causal role for the resident microflora in the pathogenesis of intestinal inflammation.

The patterns of cytokine secretion observed in our study were similar to that described in human IBD^{37–38} and in other models of *Helicobacter*-induced disease.^{18–24, 39} In comparison with control mice, unfractionated MLN and SPL cells isolated from *H. bilis*-colonised mice secreted higher levels of IFN γ , TNF α , IL6 and IL12 but showed only modest secretion of IL10 when stimulated with ASF bacterial lysates. Since responses after concanavalin A stimulation were equivalent between MLN and SPL cells from control and *H. bilis*-infected mice, these observations suggested that the observed host immune responses to the ASF were antigen-specific and not a result of generalised hyper-responsiveness or hypersensitivity induced after *H. bilis* colonisation. Additionally, these data indicated that in vitro production of proinflammatory cytokines was greater in the SPL for some responses and in the MLN for others, and ASF-stimulated SPL cells produced significantly ($p < 0.05$) greater amounts of cytokines in comparison with identically stimulated MLN cells from *H. bilis*-infected mice. These results, and the inability to detect IFN γ , TNF α , IL6 or IL12 in cultures of MLN and SPL cells from NI C3H/HeN mice, indicate differential ability of individual members of the enteric flora to induce antigen-specific proinflammatory cytokine responses in DF mice colonised with *H. bilis*. Histological evidence of inflammation was not detected until 3 weeks after *H. bilis* colonisation, suggesting that ASF antigen-specific induction of cytokine secretion contributes to the initiation of colitis in this model. Of potential relevance to our results, Kim *et al.*²⁹ recently showed that *E. faecalis*-monoassociated *IL10*^{−/−} mice develop bacterial antigen-specific Th1 and 2 lymphocyte responses that are associated with the onset of colitis in these mice.

IFN γ , IL6 and IL12 levels measured in MLN and SPL cell cultures of *H. bilis*-colonised mice were much higher after stimulation with *H. bilis* lysate compared with NI control values. When compared with ASF-induced cytokine responses, those responses to *H. bilis* lysate were generally more rapid in onset and of greater magnitude, and progressively increased over the PI period than the ASF-induced cytokine responses. The

mechanisms by which *H. bilis* colonisation stimulates the development of intestinal inflammation are probably heterogeneous. We and others¹⁸ assume that *H. bilis* activates the innate immune system resulting in impaired intestinal barrier function and the induction of cytokines or toxins that may eventually produce mucosal inflammation.^{39–40, 41–44}

In conclusion, our results indicate that colonisation of DF C3H/HeN mice with *H. bilis* perturbed the host's immune response to or immune recognition of its resident flora and resulted in the induction of persistent immune reactivity to antigens derived from the commensal bacteria. Furthermore, detection of bacterial antigen-specific antibody and T-cell responses preceded gross and histological evidence of colitis, suggesting that host adaptive immune responses to the resident enteric flora participate in the immune-mediated intestinal inflammation. The use of a gnotobiotic murine model that develops colitis after selective microbial colonisation offers a unique tool for investigating the role of host responses, aberrant or otherwise, to antigens derived from intestinal bacteria in the pathogenesis of mucosal inflammation.

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