Persistent infection of rhesus monkeys with ‘Helicobacter macacae’ and its isolation from an animal with intestinal adenocarcinoma

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INTRODUCTION

Inflammatory and neoplastic conditions across several species have been attributed to enterohelobacters. Helicobacter hepaticus is associated with chronic active hepatitis, hepatic adenoma and adenocarcinoma in susceptible mice (Fox et al., 1994, 1996). In a number of strains of immune-compromised mice and stocks and strains of defined flora and gnotobiotic mice, this organism can cause typhlocolitis, enterocolitis and lower bowel cancer (Rogers & Fox, 2004). A novel Helicobacter species has been isolated from the inflamed colon of cotton-top tamarins, which are predisposed to developing inflammatory bowel disease (IBD) and colon cancer (Saunders et al., 1999). In macaques, Helicobacter cinaedi has been isolated from the colon, liver and mesenteric lymph node of a rhesus monkey with chronic colitis and hepatitis (Fox et al., 2001a). Both H. cinaedi and Helicobacter fennelliae have been used experimentally to induce diarrhoea and bacteraemia in infant pigtail macaques (Flores et al., 1990). In 2001, our laboratory isolated two novel Helicobacter species from colonic tissue of monkeys in a colony in which chronic idiopathic colitis and diarrhoea were enzootic (Fox et al., 2001b). The two organisms were designated Helicobacter sp. rhesus type 1 and 2, the former having been isolated from two animals with mild colitis but without diarrhoea and the latter from monkeys with diarrhoea and moderate to severe colitis. Slender, spiral, argyrophilic bacteria consistent with Helicobacter species were also observed in samples of colonic mucosa. Evaluation of faecal cultures with confirmation of Helicobacter species by a genus-specific PCR assay in a second colony of rhesus monkeys in which diarrhoea was not enzootic yielded Helicobacter species in 20/35 animals (57%). Of these 20 enteric helicobacters, 13 were presumptively identified as ‘Helicobacter macacae’ by RFLP analysis;
three of these strains were confirmed to be ‘H. macacae’ by 16S rRNA gene sequencing (Fox et al., 2007). Isolates of rhesus type 1 from both colonies were used for full characterization of the organism; it was subsequently named ‘Helicobacter macacae’ (Fox et al., 2007). The organism has also been isolated from the ileum and liver of a baboon (Garcia et al., 2006) and its DNA has been identified in a stool sample from a child with gastroenteritis (Haggerty et al., 2005). A decade after the original survey, a rhesus monkey from the second colony from which ‘H. macacae’ had been isolated was diagnosed with intestinal adenocarcinoma. Culture of the caecum adjacent to the neoplasm yielded an organism identical to that isolated previously. In this study, we have described the potential relationship between this organism and intestinal cancer, and report the positive findings of ‘H. macacae’ colonization in a second survey for enteric helicobacters in rhesus monkeys remaining from the original cohort.

METHODS

Animals. A 23-year-old, intact male, singly housed rhesus monkey (Macaca mulatta, animal no. 93-11) showed clinical signs of progressive inappetence, decreased faecal production and weight loss over several weeks. Pertinent physical examination findings included poor body condition and moderate thoracolumbar kyphosis. The results of a complete blood count and serum chemistry analysis included mature neutrophilia, anaemia, hypoproteininaemia and hypoglycaemia. A radiographic contrast examination was consistent with partial intestinal obstruction. The animal was euthanized and a complete necropsy was performed. Tissues were fixed in 10% neutral-buffered formalin, processed routinely and stained with haematoxylin and eosin. The liver, ileocaecal junction, caecum and colon were also stained with Warthin–Starry stain for argyrophilic bacteria. Representative samples of various gastrointestinal tissues (stomach, jejunum, ileum, ileocaecal junction, caecum, colon and liver) were collected in freeze medium (1–2 ml 20% glycerol in Brucella broth) and stored at −70 °C for subsequent culture.

Ten additional rhesus monkeys (M. mulatta, eight males and two females) remained from the original cohort of 35 animals tested in 1999. They were singly housed in stainless steel cages (31 × 29 × 64 inches) in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility. Animals were fed ad libitum with commercial primate chow (Lab Diet 5038; PMI Nutrition International) and daily fruits, vegetables and treats. All animals were used in cognitive neuroscience research and, in accordance with Institutional Animal Care and Use Committee-approved protocols, had periodic but limited restriction of access to water. Animals that were off-study had water available ad libitum. The vivarium had 10–15 complete air changes h−1, a 12 h light:dark cycle, and temperature and humidity levels of 72–78 °F and 30–70% respectively.

Microbiology. Faecal samples collected from the cage pans of nine monkeys were placed onto the following media: Skirrow’s medium, Campy CVA agar, blood agar and Glaxo. In a tenth animal (94-9) and in the animal with adenocarcinoma, evaluation for helicobacter was achieved using tissue samples from the ileum, colon and caecum obtained at necropsy. Microaerobic culture of faeces or tissue was performed as described previously (Fox et al., 2001b). Cultures were restreaked periodically onto fresh medium after the initial inoculation. Plates with optimum growth were used for DNA extraction; colonies were also Gram stained, evaluated for morphology and collected in freeze medium for storage at −70 °C. Biochemical analysis was performed as described previously (Fox et al., 2001b).

DNA extraction. The High Pure PCR Template Preparation kit (Roche Molecular Biochemicals) was used to extract DNA from bacterial pellets as described previously (Fox et al., 2007).

PCR amplification. DNA was amplified by Helicobacter genus-specific primers (C97 and COSE) and a 1200 bp product was separated using gel electrophoresis on a 1% agarose gel, as described previously (Fox et al., 2007).

RFLP. RFLP analysis of Helicobacter species DNA was performed using Alul and HhaI digestion. The restriction patterns of the digested products were compared after separation in a VisiGel 6% separation matrix as described previously (Fox et al., 2007).

16S rRNA gene sequencing. Purified DNA from a PCR of the caecal isolate of the animal with adenocarcinoma was sequenced with an ABI Prism cycle sequencing kit (BigDye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS; Perkin Elmer) as described previously (Fox et al., 2007).

RESULTS AND DISCUSSION

Necropsy and histopathology

Post-mortem examination of animal 93-11 revealed the presence of moderate small intestinal and caecal distension proximal to a focal annular stricture at the ileocaecal junction (Fig. 1a, arrow). On dissection, the ileocaecal junction exhibited a focally extensive (2–3 cm diameter) firm, raised, nodular thickening of the intestinal wall with intraluminal protrusion and cavitating constriction of the central core of the mass giving a ‘napkin ring’-like appearance (Fig. 1b). The mass was associated with visible haemorrhage and mucosal ulceration. Other pertinent gross findings included prominent, multiple, variably sized (0.5–2.5 cm diameter), tan to yellow, slightly nodular to flat or depressed, discrete to vaguely demarcated foci involving all liver lobes both on the capsular surfaces and on cut sections of the parenchyma (Fig. 1c).

Histologically, the primary intestinal mass was diagnosed as tubular adenocarcinoma on the basis of the predominant morphological pattern involving the terminal ileum and extension into the ileocaecal valve and adjacent proximal caecum (Fig. 1d–f). The neoplastic tubulo-acinar glandular structures and occasional solid nests/cords or clusters of neoplastic cells were highly invasive with transmural involvement and effacement (Fig. 1e, f). The neoplasm was moderately well differentiated and cytologically exhibited a mixed cellular pattern ranging from glands/acini lined by tall, deeply basophilic columnar cells with a large prominent nucleus to tubulo-acinar structures and loose aggregate clusters composed of cells with a pale staining to clear, small, finely vacuolated cytoplasm and a small, hyperchromatic, eccentric nucleus (Fig. 1f, g). Within and along the margins of the mass, there was...
variable moderate chronic to active lymphoplasmacytic and neutrophilic inflammation, multifocal mucosal erosion/ulceration, necrosis, haemorrhage, amyloidosis and moderate reactive fibrosis (scirrhous response). In multiple examined sections, a variable intermingling of foveolar and glandular hyperplasia to low-grade dysplasia/adenomatous glands transitioning to invasive carcinoma was present within and along the margins of the mass. Warthin–Starry staining of the ileoecal junction, caecum and colon from both neoplastic and non-neoplastic regions demonstrated mats of argyrophilic, small, pleomorphic (spiral to filamentous bacteria (8–15 μm) adjacent normal mucosa, moderate numbers of long, crypt lumens (Fig. 1h). In some areas of the tumour and the surface epithelium and occasionally within glandular/occasionally short, rod-like) bacteria closely adherent to mats of argyrophilic bacteria: one with a short spiral morphology and the other with a long, spiral to filamentous form within glandular crypt lumens (Fig. 1h). In some areas of the tumour and the surface epithelium and occasionally within glandular/occasionally short, rod-like) bacteria closely adherent to mats of argyrophilic bacteria: one with a short spiral morphology and the other with a long, spiral to filamentous form within glandular crypt lumens (Fig. 1h).

Microbiology

The results of microbiological samples of faeces or caecum from the 10 rhesus monkeys are shown in Table 1. The organisms were Gram-negative, slender and slightly curved. ‘Helicobacter macacae’ was isolated from the faeces of all four animals from which it had been isolated in 1999 and from tissue culture of the caecum of both animals evaluated at necropsy. ‘Helicobacter macacae’ was also isolated from the faeces of one animal that had been negative. Two distinct novel helicobacters were cultured from the faeces of the remaining four monkeys; two of these had been negative in 1999, one had a mixed culture of Campylobacter and Helicobacter, and the other had another novel Helicobacter that had not been characterized further. Both novel species were identical to organisms isolated previously from different animals by our laboratory (07-1672 – novel species 1, and 99-10781 – novel species 2).

PCR

All tissues obtained from the animal with adenocarcinoma were positive for Helicobacter species by PCR using genus-specific primers.

RFLP

The gel electrophoretogram of the AluI and HhaI digest of the 1200 bp fragment PCR product from archival and current isolates of ‘H. macacae’ is shown in Fig. 2. Four different RFLP patterns were observed in the nine strains isolated from faeces and the two from caecal samples of rhesus monkeys in 2009. Two belong to ‘H. macacae’ patterns B and D. These patterns shared over 99% sequence identity in their 16S rRNA gene; mutations at two AluI cutting sites contributed to the different RFLP patterns. The remaining two patterns were identical to those of isolates cultured previously from the colony and designated novel species 1 and 2.

16S rRNA gene sequencing

The ‘H. macacae’ strain isolated from the caecum of the animal with adenocarcinoma had a 16S rRNA gene sequence identical to that of the ‘H. macacae’ strain sequenced previously a decade earlier (Fig. 3). The relationship of this isolate with archival isolates is shown in Fig. 4.

This report has documented the persistent colonization of an enterohelian helicobacter in rhesus monkeys and is the first isolation and demonstration of argyrophilic bacteria with helicobacter morphology in intimate association with intestinal adenocarcinoma in this species. It should be noted that, in a manner similar to existing information in the literature, we detected two major morphological forms of argyrophilic bacteria: one with a short spiral morphology and the other with a long, spiral to filamentous form in the ileoecaecocolic junction of the animal diagnosed with intestinal carcinoma. These organisms have been classified historically as either spirochaetes (short spiral) or flagellates (long spiral). The identity of the so-called ‘flagellates’ is uncertain, as attempts to culture and isolate them have been largely unsuccessful. The short spiral bacteria in the intestine of macaques are now increasingly recognized as members of the species Helicobacter. Consequently, the role of these unidentified flagellates or long spiral forms in the causation of intestinal inflammation and progression to cancer is unknown (Zeller & Takeuchi, 1982).

The identification of ‘H. macacae’ by PCR in the liver of our index case was significant. Its presence was thought to be a result of bacterial translocation from the gut by one of two mechanisms: bacterial transport in association with metastasis of neoplastic colon cancer cells (either...
intracellularly or by superficial adherence) or independently of neoplastic cells either by the lymphatic route (through circulating lymph via lymph nodes) and/or by the haematogenous route (through the enterohepatic portal or systemic circulation) (Berg, 1995). Bacterial overgrowth and the associated loss of mucosal integrity in the context of inflammation are two key factors in bacterial translocation from the intestine to other tissues (Berg, 1995). As is common in colon cancers of humans and also observed in this report, intestinal cancers of rhesus monkeys are frequently associated with hepatic and lymph node metastasis. It is believed that this metastasis is aided by angiogenesis and lymphangiogenesis within the neoplasm followed by spread through circulating blood (systemic or portal) or lymph (via mesenteric nodes), respectively (Gout & Huot, 2008).
Table 1. Helicobacters recovered from rhesus monkeys in 1999 and 2009

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>1999</th>
<th>2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>93-11†</td>
<td>‘H. macacae’, pattern B</td>
<td>‘H. macacae’, pattern B</td>
</tr>
<tr>
<td>94-9†</td>
<td>‘H. macacae’, pattern B</td>
<td>‘H. macacae’, pattern B</td>
</tr>
<tr>
<td>90-10</td>
<td>‘H. macacae’, pattern B</td>
<td>‘H. macacae’, pattern B</td>
</tr>
<tr>
<td>94-10</td>
<td>Negative</td>
<td>‘H. macacae’, pattern B</td>
</tr>
<tr>
<td>98-6</td>
<td>‘H. macacae’, pattern D</td>
<td>‘H. macacae’, pattern D</td>
</tr>
<tr>
<td>97-7</td>
<td>‘H. macacae’, pattern D</td>
<td>‘H. macacae’, pattern D</td>
</tr>
<tr>
<td>95-9</td>
<td>Negative</td>
<td>Novel 1</td>
</tr>
<tr>
<td>98-7</td>
<td>Mixed Helicobacter/Campylobacter species</td>
<td>Novel 1</td>
</tr>
<tr>
<td>99-1</td>
<td>Novel Helicobacter species</td>
<td>Novel 1</td>
</tr>
<tr>
<td>92-8</td>
<td>Negative</td>
<td>Novel 2</td>
</tr>
</tbody>
</table>

*Monkey with adenocarcinoma.
†Monkeys from which ‘H. macacae’ was isolated using tissue. All other isolates are from faeces.
those animals newly identified as colonized with helicobacter is unknown.

The association of chronic enterocolitis with cancer is recognized in humans with IBD. Patients with both ulcerative colitis and Crohn’s disease are at higher risk for the development of cancer (Rogers & Fox, 2004). Among patients with ulcerative colitis, the incidence of adenocarcinoma is 15–20% after 30 years of disease; for those with Crohn’s disease, the 30-year incidence is 8.3% (Boivin et al., 2003; Feagins et al., 2009). In mouse models of IBD, intestinal bacteria are required for inducing inflammation that culminates in neoplasia. In interleukin (IL)-10 and IL-2 deficient mice maintained under ‘conventional’ conditions, colitis progresses to adenocarcinoma. These lesions do not occur in mice in the germ-free state. In pathogen-free states in which only commensals have colonized the mice, the number and size of lesions diminish (Boivin et al., 2003; Rogers & Fox, 2004).

Maggio-Price et al. (2009) demonstrated that infection with Helicobacter species was required to ‘trigger’ colitis and colorectal cancer in SmaD3−/−/Rag2 mice. Using Rag2-deficient mice, Erdman et al. (2009) showed that infection with H. hepaticus led to colitis, which progressed to cancer under the influence of tumour necrosis factor (TNF)-α and a Gr-1+ neutrophil-associated increase in inducible nitric oxide synthase expression. In these and similar studies, bacterial infection, its recognition by elements of the host immune system and subsequent downstream events in genetically susceptible mice led to chronic inflammation, hyperplasia, dysplasia and ultimately to adenocarcinoma.

Among the potential mechanisms by which inflammation may induce cancer are disruption of cellular homeostasis through cellular injury, alteration in cytoskeleton and cell proliferation kinetics, initiation of the innate and adaptive immune response, predisposition of tissue to genomic instability, and alteration of tumour suppressor and

Fig. 2. Gel electrophoretogram of AluI and HhaI digests of the 16S rRNA gene 1200 bp PCR fragment from current and archival rhesus monkey isolates. I, Helicobacter sp. monkey novel 1; II, ‘H. macacae’ pattern D; III, ‘H. macacae’ pattern B; IV, Helicobacter sp. monkey novel 2; A, 2009 Helicobacter isolates digested by AluI; a, 1999 Helicobacter isolates digested by AluI; H, 2009 Helicobacter isolates digested by HhaI; h, 1999 Helicobacter isolates digested by HhaI; M, 100 bp ladder.

Fig. 3. Partial 16S rRNA gene alignment of ‘H. macacae’ patterns B and D. Identical sequences are boxed; shaded areas are cutting sites for AluI restriction enzyme. A deletion mutation at cutting site 843 and a C→T substitution mutation at site 960 contribute to the generation of these two patterns.
oncogene expression (Feagins et al., 2009; Hussain & Harris, 2007). Mediators of these mechanisms include transcription factors, free radicals, cytokines, growth factors, prostaglandins and matrix metalloproteinases (Hussain & Harris, 2007). Among the enterohelical helicobacters, *H. hepaticus* is the organism that has most definitively been shown to induce a progression of inflammation to neoplasia, either through chronic active hepatitis to hepatocellular carcinoma (Fox et al., 1996; Ge et al., 2008; Ihrig et al., 1999; Rogers & Fox, 2004) or IBD to colonic cancer (Erdman et al., 2003; Poutahidis et al., 2007) in genetically susceptible mice. In rodent models of colitis and cancer, host immune modulation by enteric bacteria, with IL-10, TNF-α and IL-6 as pivotal cytokines, has been shown to influence neoplastic transformation of epithelium in microbially induced chronic inflammation (Erdman et al., 2003; Poutahidis et al., 2007). Using purified colonic epithelia from *H. hepaticus*-infected 129/SvEvRag2/2 mice, Poutahidis et al. (2007) also demonstrated dysregulation of the transforming growth factor (TGF)-β signalling pathway, disruption of which is a feature of colorectal carcinoma in humans. The importance of TGF-β has also been demonstrated in *Helicobacter* species-infected SmaD3−/− and SmaD3/Rag2 double-null mice (Maggio-Price et al., 2006, 2009).

A more thorough molecular characterization of known virulence determinants in *H. macacae* and a better understanding of its effect on the host immune response, as well as confirmatory data from *in situ* hybridization studies, will help determine a causative rather than associative role for *H. macacae* in intestinal adenocarcinoma. Studies of enteric flora in macaques in disease and health should also contribute to our understanding of *H. macacae*-associated intestinal disease. A thorough evaluation of the macaque microbiome has recently been initiated by McKenna et al. (2008). In their study, DNA was isolated from stool, intestinal and colonic contents of rhesus monkeys, conserved regions of the 16S rRNA gene were amplified by PCR and the products were finally subjected to parallel pyrosequencing with subsequent operational taxonomic unit clustering and analysis. With this approach, the authors demonstrated diminished diversity of bacterial communities and a significant association of the bacterial family Campylobacteraceae with histopathological colitis. *Helicobacter* species were also detected in colonic mucosa. Recently, Gradel et al. (2009) demonstrated an increased hazard ratio for IBD in individuals who had experienced *Campylobacter* or *Salmonella* species-associated gastroenteritis. This risk, although highest in the first year after diagnosis, persisted throughout the entire follow-up period of up to 15 years. These findings support earlier studies demonstrating an increased odds ratio for IBD after bouts of infectious gastroenteritis in military personnel (Porter et al., 2008) and enhanced risk for Crohn’s disease after *Salmonella*- and *Campylobacter*-associated enteritis (García Rodríguez et al., 2006). A study by Zhang et al. (2009) recently demonstrated an increased presence of *Campylobacter concisus* and anti-*C. concisus* antibodies in children with Crohn’s disease. *C. concisus* was one of four non-*Campylobacter jejuni* campylobacters that contributed to a PCR positivity rate of 82% in isolates from Crohn’s disease patients compared with 23% for controls. Taken together, these studies support the hypothesis that induction of enteritis with specific pathogens precipitates a cascade of events that may result in chronic inflammation and neoplasia in susceptible individuals.

Fig. 4. Dendrogram demonstrating the similarity of sequences generated from the isolate collected from the animal with adenocarcinoma (MIT 09-0022, shown in bold) and other archival isolates. The bar shows 3% sequence differences as measured by the sum of the horizontal distances connecting taxa.
Intestinal adenocarcinoma is the most frequently diagnosed tumour of older macaques. In three separate surveys of the features of intestinal adenocarcinoma in macaques, all documented a high prevalence of intestinal tumours in aged animals (>20 years) and identified the ileocaecal junction as the most common site of neoplasia (Rodriguez et al., 2002; Uno et al., 1998; Valverde et al., 2000). Interestingly, this site is also a noted area of intestinal pathology in mouse models of helicobacter-induced IBD and lower bowel cancer (Cahill et al., 1997; Erdman et al., 2003, 2009; Maggio-Price et al., 2006). Common clinical features of weight loss, scant faecal production, anorexia, anaemia, hypoalbuminaemia and hypoproteinaemia were also described. Significantly, the animals in those reports had no known exposure to ionizing radiation or carcinogens. In one study, use of single-strand conformational polymorphism analysis to identify K-ras mutations, observed in approximately 40% of colon adenocarcinomas of humans, failed to identify evidence of mutations in exon 1 or 2 in 10 macaques with intestinal adenocarcinoma (Valverde et al., 2000). In the current study, the persistence of ‘H. macacae’ in an animal with intestinal adenocarcinoma and accompanying colitis, and the demonstration of argyrophilic organisms at the site of the lesion, as well as the many examples of helicobacter-associated tumorigenesis in other species, raise the intriguing possibility of a causative or contributory role for ‘H. macacae’. The combined strategies of microbial community analysis in specific disease states, virulence factor characterization of specific micro-organisms, evaluation of tumour tissue for known cancer-associated genes and development of probes for in situ demonstration of infectious agents promise to provide important insights into enteric infection, inflammation and the role of bacteria in intestinal cancer.

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