Persistent *Yersinia enterocolitica* infection in three rat strains

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Yersinia enterocolitica may persist for prolonged periods of time in humans sometimes resulting in the development of reactive arthritis. To elucidate factors predisposing for persistence we developed animal models. In Lewis and Fischer rats, viable bacteria could be demonstrated for prolonged time and abscesses could be found in the liver, spleen and lungs. Splenic abscesses were observed for more than 20 weeks. *Yersinia enterocolitica* persisted in Lewis and Fischer rats, but only Lewis rats developed reactive arthritis. In Brown Norway rats abscesses developed early during infection but in contrast to the other strains disappeared after 3 weeks. Culture of homogenized abscess-containing tissue of all three rat strains yielded *Yersiniae*. Immunofluorescence studies of the abscesses showed diffuse staining inside the abscesses only, indicating the presence of *Yersinia enterocolitica* antigen. Brown Norway rats, in contrast to Lewis and Fischer rats, developed a different serological reaction pattern against *Yersinia enterocolitica* antigens and this correlated with the disappearance of the abscesses.
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Introduction

Gastrointestinal infection with *Yersinia enterocolitica* is self-limiting in most cases because the host's inflammatory response is usually able to eliminate the bacteria. In some cases infection with *Y. enterocolitica* as well as infection with a variety of micro-organisms, such as *Salmonella* and *Chlamydia*[1,2] may lead to the development of a persistent infection with or without the ensuing development of sequellae such as reactive arthritis. It is unknown why some people develop such a chronic infection whereas others do not. Persistence of the causative organism or bacterial antigens somewhere within the body, thereby maintaining a stimulus for a prolonged immune response and inflammation, might explain the persistence of *Y. enterocolitica*-specific IgA antibodies in patients with chronic infection or *Y. enterocolitica*-associated reactive arthritis.[3,4] A prerequisite for the development of reactive arthritis seems to

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be the persistent presence of bacteria or bacterial antigens in the body.[4–7] Typically, no bacteria can be cultured from affected joints which separates reactive arthritis from septic arthritis. However, bacterial antigens (immune-complexes) have been demonstrated.[5,7] In addition, the majority of patients developing reactive arthritis as well as other spondylarthropathies have the MHC class I antigen typing of HLA-B27.[8–11]

Persistent *Y.* infection in animal models followed by the development of reactive arthritis has been described previously. Lewis and spontaneously hypertensive (SHR) rats[6,12–15] as well as several strains of mice[16] develop arthritis after infection with *Y. enterocolitica*. Here we describe and compare a new rat model that might provide useful information to study the pathogenesis of persistent *Y. enterocolitica* infections and ensuing sequellae.

Results

Characteristics of Y.enterocolitica infection in different rat strains

Lewis rats. Two weeks after inoculation with *Y. enterocolitica* multiple macroscopic abscesses were found in the spleen of all animals. In some of the animals abscesses were also found in the liver (40%) and lungs (13.3%). *Yersiniae* were recovered from all abscesses. Liver and lungs without macroscopic visible abscesses (i.e. day 7 after infection) also yielded positive cultures. Internal organs from uninfected rats as well as blood and Peyer's patches from infected rats did not contain any viable *Yersiniae*. Splenic abscesses persisted for more than 138 days and reached maximal sizes of 3.5 cm diameter. Throughout this period *Yersiniae* could be cultured from the abscesses. Abscesses in liver and lungs disappeared 5 to 6 weeks after infection and these organs became culture negative.

Microscopic examination of splenic tissue revealed abscesses encapsulated with a thick fibroblastic layer (Fig. 1A). Infiltration of granulocytes was a prominent feature

(a) (b)
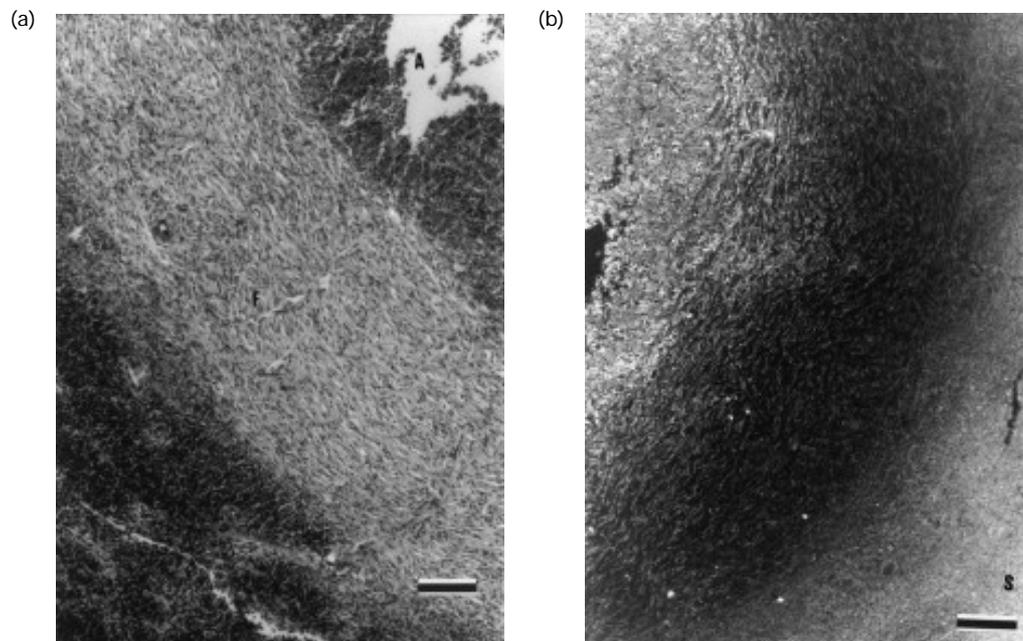
Fig. 1. Spleen of Lewis rat with large abscess 5 weeks after i.v. infection with 10^4 cfu *Yersinia enterocolitica* O:8WA (5 μm section). (a) hematoxylin-eosin stain; (b) immunofluorescence. a = abscess, f = fibroblast capsule, s = normal splenic tissue. Bar represents 25 μm.
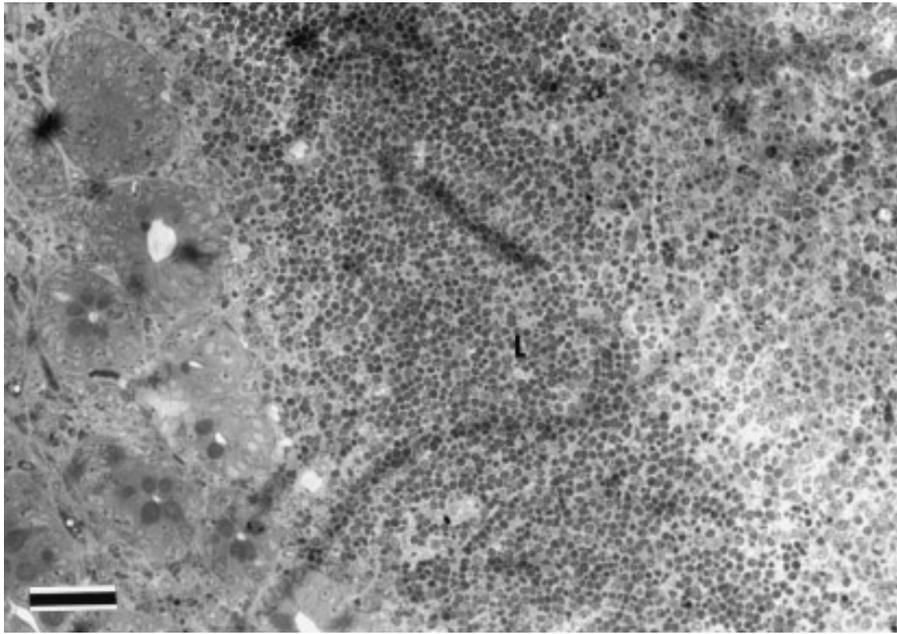
Fig. 2. Peyer's Patch of Lewis rat 2 weeks after i.v. infection with 10^4 cfu *Yersinia enterocolitica* O : 8WA. Toluidine Blue stain (1 μm section). Notice proliferation of lymphatic tissue (L). Bar represents 25 μm.

of the abscesses, however intact bacteria could not be distinguished. Immuno-histological staining of the splenic tissue revealed strong, diffuse fluorescence within the abscesses (Fig. 1B). No fluorescence was observed in the fibroblastic layer or in the unaffected splenic tissue, indicating that bacteria or bacterial antigens are located inside the abscesses. As in the spleen, immunofluorescence of liver sections was confined to abscesses (results not shown). Although no bacteria could be demonstrated within the Peyer's patches, histological examination revealed proliferation of lymphatic tissue (Fig. 2).

Serum samples taken at sequential timepoints after infection showed the development of an antibody response against five outer proteins (Yops) of *Yersinia* (Yops O, M, H, D and N) within two weeks after inoculation of bacteria (Fig. 3A). These antibody levels persisted for more than 138 days.

Three weeks after injection of bacteria 12 out of 15 Lewis rats had developed arthritis. Swelling of a single, or in two cases both hind paws could be observed. In the fifth week after infection the swelling began to subside and 2 weeks later no distinction could be made between rats with and without an episode of arthritis. Before, during and after the episode of arthritis no bacteria could be cultured from the affected joints.

Fischer rats. After inoculation of bacteria the same pathology could be observed in Fischer rats as in Lewis rats with the exception of the development of reactive arthritis which was absent in Fischer rats. Macroscopic abscesses were found in the spleen of all animals, and sometimes in liver (33.3%) and lungs (20%). Antibody reactivity in Fischer rats did not differ from that observed in Lewis rats (Fig. 3B).

Brown Norway rats. In Brown Norway rats abscesses developed in spleen (100%), liver (20.6%) and lungs (13.3%) and bacteria could be cultured from these organs. Histology showed the same characteristics as described for Lewis and Fischer rats.
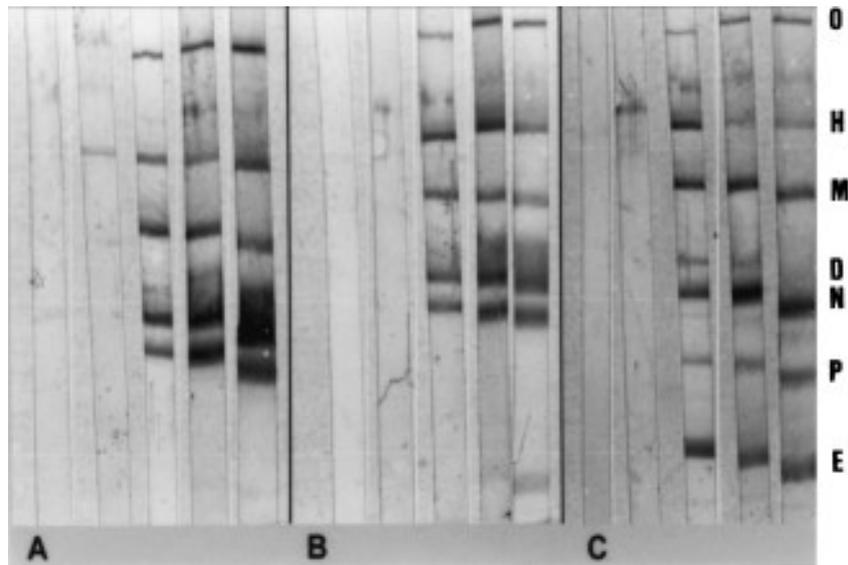
Fig. 3. Immunoblot of serum from individual rats after infection with *Yersinia enterocolitica* O:8WA. From left to right lanes indicate serum samples taken on day 0, 7, 14, 22 and 35 after infection. A = Lewis rats, B = Fischer rats, C = Brown Norway rats. Mol. weight Yops: YopO = 84 kD, YopH = 51 kD, YopM = 48 kD, YopD = 37 kD, YopN = 35 kD, YopP = 30 kD and YopE = 25 kD.

However, after 21 days abscesses decreased in size and all organs became culture negative.

All Brown Norway rats produced antibodies against Yops O, M, H, D, N and against two additional Yops, YopP and YopE (Fig. 3C), and this response became prominent at the time that abscesses began to decrease in size. During the course of the infection humoral reactivity against Yops O, M, H, D and N ultimately decreases as indicated by the fading of the immunoblot pattern against these Yops. No reactive arthritis developed in Brown Norway rats.

Discussion

Humans infected with *Y. enterocolitica* serotypes O:3, O:8 or O:9 sometimes develop a chronic infection with persistent levels of antibodies (IgA subclass) against *Yersinia enterocolitica* antigens.[4] Persistent infection with either of the three serotypes may be accompanied by the development of sequellae such as reactive arthritis.[17] Studies in rat models have also shown that infection with *Y. enterocolitica* may lead to a chronic infection with persistence of the bacteria somewhere within the affected host.[12] Additional evidence for the prolonged presence of bacteria in infected rats is presented here. After infection, all three rat strains developed splenic abscesses from which *Yersiniae* could be cultured. Furthermore, strong immuno-fluorescence was found inside the abscesses whereas unaffected tissue lacked any label. This indicates that viable bacteria are located in the abscesses rather than in normal splenic tissue. Although no bacteria could be demonstrated inside Peyer's Patches, proliferation of the lymphatic tissue indicates an inflammatory response.

Yops are strong antigens and elicit a specific IgM-, IgA- and IgG-antibody response in orally infected rabbits,[18] in humans suffering from *Yersinia* infection[19] and in rats.[6] We find persistent antibody levels directed against several Yops in infected Lewis and Fischer rats in contrast to Brown Norway rats where antibody reactivity began

to fade from the fifth week of infection and onwards. This might reflect persistence of bacteria inside the infected host since the vanishing of Yop-specific antibodies in sera from Brown Norway rats coincided with the disappearance of abscesses and bacteria. Development of a humoral response against YopP and YopE was found exclusively in Brown Norway rats and was associated with disappearance of splenic abscesses. Failure to mount an anti-YopP and/or anti-YopE response by Lewis and Fischer rats may therefore be correlated with persistence of bacteria suggesting that YopP and/or YopE may be suitable target-antigens for effective elimination of the bacteria by the immune system. Furthermore, antibody reactivity against YopE might render the bacteria susceptible to phagocytosis since YopE is supposed to be involved in resistance against phagocytosis through depolymerization of the cellular actin network.[20]

Differences in kinetics of antibody production against Yops have been shown by Gaede *et al.*[6] They found a weaker and delayed antibody response against Yops in Fischer rats compared to Lewis rats after infection with *Y. enterocolitica* O : 8WA. In their study Lewis rats developed reactive arthritis whereas Fischer rats did not. Also in our experiments Lewis rats developed reactive arthritis in contrast to Fischer rats but no evidence for a delayed antibody response was found in Fischer rats which make it unlikely that a relatively early antibody response may explain the development of reactive arthritis in Lewis rats.

As described by Hill and Yu[12] the development of reactive arthritis after infection of rats with *Y. enterocolitica* O : 8WA is closely linked to the prolonged presence of the causing organism somewhere in the affected host. In their experiments Lewis rats developed a persistent infection in the spleen followed by arthritis whereas Fischer rats cleared the bacteria from spleen and liver within 2–3 weeks and did not develop arthritis. They indicated that the persistence of bacteria in Lewis rats might be the reason for the development of reactive arthritis. In addition, experiments with SHR rats[15] suggested that the development of arthritis depended on persistence of the bacteria in the affected host. However, in our experiments Lewis as well as Fischer rats developed a persistent infection but only Lewis rats developed reactive arthritis. The reason why in our experiments Lewis rats developed reactive arthritis and Fischer rats did not still remains unclear since *Yersinia* persisted in both rat strains. As described in human reactive arthritis the genetic background (MHC type) may play a role.[9] However, SHR (Spontaneously Hypertensive Rats) and WKY rats (Wistar Kyoto, the normotensive strain of SHR) that share the same histocompatibility antigens, differ in their susceptibility to *Y. enterocolitica*-induced arthritis.[13] Furthermore, the microbial status of the host may play a role as described by Gripenberg-Lerche and Toivanen.[21,22] This indicates that the development of reactive arthritis following a persistent infection with *Y. enterocolitica* may depend on the immune-status rather than on the MHC type which may explain the difference between our results and those observed by others.[6,12]

Materials and methods

Experimental animals. Male rats weighing at least 300 g were used in all experiments. Lewis and Fischer rats were obtained from colonies maintained at the central Animal Laboratory, University Hospital Nijmegen and Brown Norway rats were obtained from TNO, Rijswijk, The Netherlands. Rats were housed in plastic cages under specific pathogen free conditions and received water and food *ad libitum*. For persistence studies five rats of each strain were used and the experiment was performed three times.

Bacteria. *Yersinia enterocolitica* serotype O : 8WA (ATCC 9610) was used in all experiments. This strain contains the virulence plasmid. Bacteria were cultured overnight in Brain Heart Infusion broth (BHI) at 26°C. The concentration of inoculated viable bacterial cells was estimated by colony counts on Columbia blood agar plates.

Infection. For all experiments animals were infected intravenously with approximately 10^4 cfu in 0.2 ml 0.9% saline.

Individual animals from each rat strain were killed 7, 14, 22, 40 and 138 days after inoculation with *Y. enterocolitica.* Spleen, liver, lungs and Peyer's patches were examined for the presence of macroscopic lesions and assayed for live bacteria. Abscess-containing tissue samples were prepared for histology.

For serological studies five rats of each strain were inoculated with *Y. enterocolitica* and serum samples were taken at serial intervals. In three independent experiments samples were taken on days 0, 7, 14, 22 and 35 after inoculation and were examined for antibody reactivity against Yops.

Antiserum. New Zealand White rabbits were obtained from colonies maintained at the Central Animal Laboratory, University Hospital Nijmegen. Rabbits were immunized with *Yersinia enterocolitica* O : 8WA (ATCC 9610) as described by Hanski *et al.*[22] Briefly, bacteria were grown overnight at 26°C in BHI broth containing 20 mM calciumchloride and 20 mM sodium-oxalate. Formaldehyde fixed bacteria (10^9 cfu in 1 ml saline) mixed with Complete Freund's Adjuvants (1 : 1) were injected intracutaneously on 10 locations on the back of the rabbits followed by booster injections with 10^5 cfu in 1 ml saline mixed with Incomplete Freund's Adjuvants (1 : 1) 2 and 4 weeks later. Eight days after the second booster the animals were bled by cardiac puncture and the serum was collected and stored at $-20°C$.

Bacterial culture. Culture of bacteria from homogenized tissue samples of spleen, liver, lungs and Peyer's patches was carried out on Columbia blood agar plates and selective CIN plates (Cefsulodin Irgasan Novobiocin, Oxoid). API 20E or 50SH was used to identify *Yersinia enterocolitica.*

Microscopy. Routine histological procedures were used for preparation of tissue samples for light microscopy. For immunofluorescence, 5 μm cryosections were allowed to dry overnight and were then incubated with rabbit-anti-*Yersinia enterocolitica* serum diluted 1 : 250 in 0.05% Tween-20 in PBS. After washing twice with PBS/Tween, the sections were incubated with swine-anti-rabbit antibodies coupled to FITC (Dako A/S, Glostrup, Denmark; dilution 1 : 50 in PBS/0.05% Tween-20). After washing twice, sections were embedded in Gurr Aquamountant (BDH Laboratory Suppliers, Poole, U. K.) and examined in a fluorescence microscope.

Serology. SDS-PAGE/Immunoblotting technique[24] was used to determine the presence of antibodies directed against plasmid encoded Yops. Briefly, *Yersinia* was grown overnight at 26°C in BHI broth containing 20 mM $MgCl_2$ and 20 mM EGTA followed by 3 h incubation at 37°C. After centrifugation released proteins from the supernatant were precipitated with $(NH_4)_2SO_4$ and separated by SDS-PAGE followed by Western blotting. Specific antibodies against Yops were detected by a subsequent incubation of blot strips with rat sera (dilution 1 : 250) followed by rabbit-anti-Ig coupled to alkaline phosphatase (1 : 500).

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