Lyme disease in transgenic mice expressing the *Borrelia burgdorferi* flagellin epitope implicated in human neuroborreliosis

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Abstract

Because of an association of human neuroborreliosis with the development of an antibody response against an antigen in neural tissue that cross-reacts with an epitope on the flagellin protein of *Borrelia burgdorferi*, C3H transgenic mice were created that expressed the flagellin epitope (amino acids 213–224) as a fusion protein with myelin basic protein. The transgenic mice expressed the flagellin epitope selectively in myelinated regions of the nervous system. Both transgenic and non-transgenic mice developed an antibody response to the flagellin epitope during *B. burgdorferi* infection and both developed arthritis and carditis. However, no lesions were found in the central nervous system of either type of mouse for up to 8 weeks after infection. The data indicate that expression of the flagellin 213–24 epitope in mice does not result in neurologic disease, suggesting that *B. burgdorferi* flagellin antibodies may not be directly implicated in neuroborreliosis.

Keywords: Lyme disease; *Borrelia burgdorferi*; Flagellin; Nervous system; Spirochete

1. Introduction

Neurologic manifestations of Lyme disease include meningitis, facial nerve palsy, cranial neuritis, motor or sensory radiculoneuritis, or subtle encephalitis [1]. Disease may be due, in part, to presence of the causative spirochete, *Borrelia burgdorferi*, within the nervous system, the virulence of particular organisms, and the host immune response to the pathogen [2–5]. Antibodies to the *B. burgdorferi* flagellin, elicited during the course of infection, have been shown to bind to human axons and therefore may be implicated in the pathogenesis of neurologic disease [1,2,6]. The specificity of these cross-reactive antibodies has been mapped to a 12-amino acid (aa) epitope (aa 213–24) in the central region of the *B. burgdorferi* flagellin [7]. Moreover, the antigen to which these antibodies bind in human tissue has been identified as an endogenously expressed 60-kDa heat shock protein [8,9]. To further explore the hypothesis that these cross-reactive flagellin antibodies are implicated in the pathogenesis of neuroborreliosis, we generated transgenic mice that expressed the flagellin epitope (F213–24) as a fusion protein with myelin basic protein (MBP). We postulated that flagellin...
antibodies elicited during the course of *B. burgdorferi* infection would bind the F213–24 epitope in the transgenic mice, thereby initiating an inflammatory response and implicating these antibodies in disease pathogenesis.

The murine model was used for our studies because mice develop disseminated *B. burgdorferi* infection with ensuing arthritis, carditis and vasculitis [10]. One feature not seen in the mouse model is neurologic disease, despite extensive examination at different stages of infection up to 1 year [10,11]. Spirochetes can be cultured from the central nervous system early in the course of infection but are apparently cleared from brain [12]. The lack of neurologic disease in a model which consistently expresses joint and cardiovascular disease suggests that humans may have unique features that predispose them to neuroborreliosis. The cross-reactive nervous tissue-flagellin antigens may be the basis of this difference.

2. Materials and methods

2.1. Generation of F213–24-transgenic mice

Transgenic mice were created that expressed the flagellin epitope (F213–24) that binds with *B. burgdorferi* antibodies that have been implicated in the pathogenesis of neuroborreliosis. Oligonucleotides corresponding to nucleotides 637–672 of the gene encoding the *B. burgdorferi* flagellin (Plus: 5′ ATGGCTGAGGTTCAACAGGAGGAC-TCAACAGCCAGCA 3′, minus: 5′ CCATTGCT-GCTGTGAGCT CCTCCCTGTGAACCC-TCAG 3′) were flanked by overhanging *Esp3I* restriction enzyme digestion sites to facilitate subcloning. Plus- and minus-strand oligonucleotides (1 mM) were mixed together in water, heated to 90°C for 5 min and cooled to room temperature overnight.

The annealed oligonucleotides were cloned into pMP 302 to create a plasmid construct that would synthesize a fusion protein in which the flagellin epitope is linked to the carboxyl terminus of the 14-kDa MBP (Fig. 1E). Expression of the MBP F213–24 fusion protein was controlled by the MBP promoter [13]. Splicing and polyadenylation signals from the rabbit β-globin gene and simian virus 40 early genes flank the MBP gene to facilitate expression of the transgene. An *Esp3I* site, located 12 nucleotides before the end of the MBP gene, was used as the insertion site for the sequence corresponding to the F213–24 epitope. The mixture was electroporated into *Escherichia coli* strain DH5α, and positive colonies identified on Luria broth medium with ampicillin.

To prepare the DNA for microinjection into mouse embryos, the recombinant plasmids were purified by double-band sorting the plasmids over a CsCl gradient by equilibrium centrifugation. The plasmid DNA was linearized by digestion with *HindIII* and purified. The micro-injected C3H embryos were implanted into pseudo-pregnant C3H females and newborn mice were analyzed for the gene encoding the MBP F213–24 fusion protein using the polymerase chain reaction (PCR). Primers for PCR corresponded to a region of the F213–24 insert 5′ GGAGCTCAACAGCCAGCA 3′ and a sequence of the pMP 302 plasmid 5′ AGCCACTCAGCAGCTGTGTC 3′ 508 nucleotides downstream from the F213–24 insert.

Expression of the MBP F213–24 fusion protein in the transgenic pups was assessed by RNA-PCR. Total RNA was isolated from including the spleen and brain of transgenic mice and non-transgenic littermates by acid guanidinium thiocyanate-phenol-chloroform extraction. Ten mg of pooled RNA were treated with RNase-free DNase (Promega, Madison, WI) for 3 h at 37°C, and cDNA was synthesized by reverse transcription reaction with Moloney murine leukemia virus reverse transcriptase ((MMLV-RT) Stratagene, La Jolla CA) and 3′ primers for F213–24. Subsequently, MMLV-RT was inactivated by heating for 5 min. at 95°C, then 5′ primers for F213–24, and then PCR was carried out under following conditions; 94°C for 1 min, 45°C for 1 min, 72°C for 2 min, and 30 cycles.

2.2. *B. burgdorferi*

A clonal population of *B. burgdorferi* strain N40 with proven infectivity and pathogenicity in C3H mice was used throughout the study [10]. *B. burgdorferi* were cultivated in modified Barbour-Stoenner-Kelly medium (BSK) II and the concentration of *B. burgdorferi* was adjusted to 10⁶ organisms per
ml. Mice were inoculated with an inoculum of 0.1 ml (10⁷ spirochetes) by intradermal injection.

2.3. Mice

Three-week-old female C3H/HeNCr mice were obtained from the Frederick Cancer Research Center in Frederick, MD, USA. Mice were housed in filter frame cages and euthanized with CO₂.

2.4. Immunoblots

*B. burgdorferi* lysates, murine tissue, or epitopes of flagellin expressed as glutathione transferase fusion proteins were resolved by SDS-PAGE [7]. The fusion proteins expressed flagellin fragment F (aa 197–241), the central region that reacts with *B. burgdorferi* antibodies, and F5 (flagellin aa 212–25), the epitope that includes the region that binds with cross-reactive antibodies [7]. Proteins transferred to nitrocellulose were probed with murine sera or monoclonal antibody (1:100 dilution) in phosphate buffered saline (PBS) with 5% bovine serum albumin (BSA) for 1 h. The strips were washed with PBS and incubated with horseradish peroxidase-labeled goat anti-mouse Ig (Southern Biotechnology Associates Inc., Birmingham, AL) and developed with an enhanced chemiluminescence (ECL) detection kit (Amersham, Arlington Heights, IL, USA).

2.5. Infection of mice with *B. burgdorferi*

Transgenic mice were infected with *B. burgdorferi* to determine whether expression of the MBP F213–24 fusion protein predisposed the animals to the development of neurologic disease. Groups of 5 transgenic and non-transgenic mice were inoculated with *B. burgdorferi*. Mice were killed at 2 and 8 weeks after inoculation. In the course of murine Lyme borreliosis, 2 weeks represents the time point when arthritis and carditis are most severe, and 8 weeks represents a point at which the disease has resolved [11]. Blood, spleen, urinary bladders and ear punch were cultured for 2 weeks in BSK II medium for the presence of spirochetes. Sections of the heart, knees, tibiotarsi, spinal column (cervical, thoracic and lumbar regions, including spinal cord, spinal nerve roots, ganglia and peripheral nerves) and 3 regions of brain were formalin fixed and embedded in paraffin. Histosections were blindly examined for evidence of arthritis, carditis or inflammation of the nervous system. An animal was considered to have arthritis when at least one joint showed evidence of inflammation, and carditis when inflammation was present in and around the aortic root, heart base or epicardium [11].

3. Results

F213–24-transgenic mice were created that expressed the MBP F213–24 fusion protein, under control of the MBP promoter, in the nervous system. Selective expression of the transgene in the nervous system was determined by RNA-PCR immunoblot and immunohistochemistry. F213–24 specific mRNA was only detected in the brain of transgenic mice (Fig. 1A). In addition, the MBP 213–24 fusion protein was also detectable, by immunoblots, in the brain of transgenic mice probed with the F213–24 flagellin epitope specific monoclonal antibody H9724 (Fig. 1B), and also by immunohistochemistry of transgenic mouse brain (Fig. 1C).

To determine whether the presence of the MBP F213–24 fusion protein in the brain predisposed mice to the development of inflammatory or other changes in the central nervous system, we infected animals with 10⁷ *B. burgdorferi* N40 [10] and examined the mice at 2 and 8 weeks. Two weeks represents the time point when arthritis and carditis are most severe, and 8 weeks represents a point at which the disease has resolved [11]. Both transgenic mice and non-transgenic littermates were readily infected, and *B. burgdorferi* could be cultured from tissues of the animals (Table 1). Sections of the heart, knees, tibiotarsi, spinal column (cervical, thoracic and lumbar regions, including spinal cord, spinal nerve roots, ganglia and peripheral nerves) and 3 regions of brain were formalin-fixed and embedded in paraffin. Histosections were blindly examined for evidence of arthritis, carditis or inflammation of the nervous system. Transgenic and non-transgenic mice developed arthritis and carditis that was most severe at 2 weeks, and disease was resolving in both groups at 8 weeks. Neither F213–24-transgenic nor their non-transgenic littermates developed any microscopically evident
changes in the spinal cord or brain at either the 2 or 8 week time point.

The immune response to \textit{B. burgdorferi} was also similar in F213--24-transgenic mice and non-transgenic littermates. Following infection, both groups of mice developed an antibody response to \textit{B. burgdorferi}, and antibodies to flagellin, P39 and OspC were readily detectable at 2 weeks, at a serum dilution of 1:2,000. Moreover, antibodies elicited during the course of infection in the F213-24-transgenic mice bound to the central region flagellin, including a small region that includes the F213-24 epitope (Fig. 1D, lane 1), similar to the response in control animals. For these studies, the flagellin glutathione transferase fusion proteins expressing flagellin fragment F (aa 197--241), the central region that reacts with \textit{B. burgdorferi} antibodies, and F5 (flagellin aa 212--25), the epitope that includes the region that binds with cross-reactive antibodies [7], were probed with murine sera (1:100 dilution) in phosphate buffered saline (PBS) and developed with an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL).

4. Discussion and conclusions

The experiments using the F213-24-transgenic mice explore the postulate that antibodies to flagellin, generated during \textit{B. burgdorferi} infection, and that cross-react with human axons, contribute to the pathogenesis of neuroborreliosis. Studies implicated the flagellin antibodies in the pathogenesis of the neurologic manifestations of Lyme disease, and the specificity of the flagellin antibodies that bind human axons has been mapped to the central region of flagellin [6--9,14]. Although the C3H mouse model of Lyme borreliosis partially mimics human infection, the animals do not develop neurologic disease. Therefore transgenic mice were developed that expressed the flagellin epitope F213--24 directly in the nervous system to further explore the hypothesis of cross-reactive antibodies initiating neurologic disease. It was postulated that the presence of the F213--24 epitope in the nervous system would result in specific antibody binding, and initiate an inflammatory response. The data show, however, that the presence of this epitope within the nervous system was not sufficient to result in the genesis of inflammation.

The pathogenesis of neuroborreliosis is clearly multifactorial, and due in part to the virulence of the infecting spirochete, as well as the host response. Moreover, the human clinical manifestations of neuroborreliosis are protean, and the pathogenesis of distinct syndromes is likely to be different. There are several reasons why we may not have observed inflammatory changes in the nervous system in the F213--24-transgenic mice, and the studies do not, therefore, fully exclude the possibility that these cross-reactive antibodies are involved in the pathogenesis of disease. The chimeric protein may not
have been expressed in the same location as the human cross-reactive antigen. However, the MBP 231–24 fusion protein was controlled by the MBP promoter, and therefore expressed in tissue where MBP is synthesized. Moreover, the antigen that binds with the flagellin antibodies may be an intracellular 60-kDa heat shock protein [8,9]. As the 14.5 kDa MBP is positioned beneath the plasma membrane it is likely that the MBP 231–24 fusion protein was also intracellular. Therefore *B. burgdorferi* specific antibodies may not have access to the relevant antigen unless spirochetes present in the nervous tissue cause cell destruction. Furthermore, the antibodies elicited during infection in mice may not have initiated the inflammatory response. Both transgenic and non-transgenic mice; however, developed similar humoral responses, including antibodies against flagellin and the region of flagellin that contains the cross-reactive epitope.

In addition, the manifestations of neuroborreliosis may not have been evident at the selected time points. Neurologic disease in humans occurs in both early- and late-stage disease and we therefore chose time points that represent the acute and chronic stage of murine infection. It is possible that central nervous system inflammation in human neuroborreliosis is initiated by a combination of spirochete and host factors, and that cross-reactive anti-*B. burgdorferi* antibodies may take part in the pathogenesis by perpetuating the inflammation. In as much as murine disease resembles the human illness, these time points are likely to reflect the range of murine infection.

Although many possibilities can account for the lack of extension of the human correlation to the transgenic model, the data still suggest that the appearance of flagellin antibodies that cross-react with human axons may not be directly implicated in the pathogenesis of disease. The human antigen that binds to the flagellin antibodies is a 60-kDa heat shock protein [8,9]. As this protein is ubiquitous, it is unlikely that antibodies directed toward this antigen would cause disease manifestations directed toward a single organ system. Much work needs to be done to elucidate the factors, both with respect to the infecting spirochete, and host response to the pathogen in the pathogenesis of neuroborreliosis, and further evidence is needed to support the contention that antibodies elicited during infection are directly implicated in the pathogenesis of neuroborreliosis.

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