Exclusion of \textit{Kif1c} as a candidate gene for anthrax toxin susceptibility

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Different strains of mouse possess varying degrees of susceptibility to anthrax lethal toxin (LT). Previous studies have suggested a responsible locus \textit{Ltxs1} that contains 10 or more known genes, but functional relevance has been reported for two genes, \textit{Kif1c} and \textit{Nalp1b}. In this study, we attempted to determine the involvement of \textit{Kif1c} in anthrax susceptibility using \textit{Kif1c} knockout mice. We established \textit{Kif1c} knockout mice with LT-sensitive 129/Sv-derived embryonic stem cells followed by 13 backcrosses with LT-resistant C57BL/6J mice (B6) to be congenic. These knockout mice and their primary macrophages showed significantly higher sensitivity to LT than wild-type B6. However, when we replaced the remaining 129/Sv genome adjacent to the targeted \textit{Kif1c} locus with the B6 genome, this sensitivity was lost. This suggested that the sensitivity to LT in the originally established \textit{Kif1c} knockout mice was not due to the loss of the \textit{Kif1c} gene, but was because of the presence of the 129/Sv-derived genes adjacent to the disrupted \textit{Kif1c} locus. Thus, \textit{Kif1c} was excluded as a candidate anthrax susceptibility gene.

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1. Introduction

Anthrax is a major bioterrorism threat but the mechanisms of pathogenesis still remain unclear. Thus, elucidation of the mechanisms responsible for cellular resistance is of great interest. One of the major virulence factors of anthrax is lethal toxin (LT), which comprises protective antigen (PA) and lethal factor (LF). PA oligomerizes to form a pore on the cell surface and is then endocytosed along with LF, resulting in the translocation of LF into the cytosol [1,2]. Different strains of mice possess varying degrees of sensitivity to LT [3]. Positional cloning studies have suggested that two genes responsible for this sensitivity are located within the \textit{Ltxs1} region on mouse chromosome 11, but their identities have not been determined [4–6].

One candidate gene, \textit{Kif1c}, is a microtubule-dependent molecular motor protein of the kinesin superfamily [7,8]. There have been over 45 types of kinesin superfamily proteins (KIFs) identified that are involved in intracellular transport. Although \textit{Kif1c} knockout mice were viable, fertile, and showed no obvious abnormalities [9], it was suggested that \textit{Kif1c} plays a role in macrophage function [10]. Another candidate gene, \textit{Nalp1b}, is a member of the Nod-like receptor (NLR) protein family and has been suggested to be involved in caspase-dependent apoptosis signaling [11,12]. As these two genes are both closely located within the \textit{Ltxs1} locus (approximately 0.51 cm interval), they have been difficult to separate from each other on the genome [13,14]. In the present study we attempted to verify whether \textit{Kif1c} was involved in anthrax susceptibility using \textit{Kif1c} knockout mice. We established two different \textit{Kif1c} knockout models after examining the polymorphisms between mouse strains.

2. Results and discussion

2.1. Establishment of mouse genetic models for anthrax toxin susceptibility

Gene targeting for the \textit{Kif1c} gene [9] resulted in the incorporation of the LT-sensitive 129/Sv genome in the proximity of the targeted locus. This was retained after 13 backcrosses with the LT-resistant C57BL/6 mouse strain (B6). The 129/Sv-derived \textit{Nalp1b} allele was tightly linked to the disrupted \textit{Kif1c} locus, as their genetic interval is less than 0.51 cm [4]. In vitro fertilization (IVF)-mediated speed congenics was performed to segregate these two loci from each other (see Section 3.1). We detected one \textit{Kif1c} c/c, \textit{Nalp1b}B/B mouse out of 287 \textit{Kif1c} c/c mice. Accordingly, we established two different \textit{Kif1c} knockout mouse strains (Fig. 1A) with the B6 genetic background: one strain carried \textit{Nalp1b} alleles from LT-sensitive 129/Sv mice (\textit{Kif1c} c/c, \textit{Nalp1b}B/B) and the other carried those from LT-resistant B6 mice (\textit{Kif1c} c/c, \textit{Nalp1b}B/B). This allowed us to examine the role of \textit{Kif1c} in anthrax toxin susceptibility without being affected by \textit{Nalp1b}.**
2.2. Anthrax toxin susceptibility of mouse genetic models

Challenges with LT were performed in vitro and in vivo. The in vitro assay involved primary macrophages isolated from mouse bone marrow being challenged with LT for 2 h and the number of surviving cells counted (Fig. 2A). In the in vivo experiment, individual mice were intravenously injected with LT and survival rates were determined (Fig. 2B). As a result, Kif1c\(^{-/-}\) macrophages or individuals carrying 129/Sv-type Nalp1b alleles (Kif1c\(^{-/-}\) Nalp1b\(^{129/129}\)) were significantly more sensitive to LT than those carrying wild-type B6 (Kif1c\(^{+/+}\) Nalp1b\(^{B6/B6}\)) alleles. However, when the 129/Sv-type Nalp1b alleles were substituted with the B6-type alleles by IVF-mediated congenics, those with the Kif1c\(^{+/+}\) Nalp1b\(^{B6/B6}\) genotype became resistant to LT similar to the wild-type B6 (Kif1c\(^{+/+}\) Nalp1b\(^{129/129}\)) mice. Therefore, macrophages or mice carrying the B6-type Nalp1b polymorphism were resistant to LT at a similar level as wild-type B6 mice, irrespective of KIF1C protein expression, whilst those carrying 129/Sv-type Nalp1b were sensitive to LT similar to 129/Sv mice. These results were reproducible and statistically significant as indicated in Fig. 2A and B, suggesting that sensitivity to LT in Kif1c\(^{-/-}\) Nalp1b\(^{129/129}\) mice was not because of the loss of the Kif1c gene, but the presence of the loci containing the 129/Sv-type Nalp1b gene.

This result was somewhat unexpected considering that KIF1C was reported to be essential for podosome formation in macrophages [10]. However, our new mouse models clearly excluded the Kif1c gene as a candidate for susceptibility genes (Fig. 2). In this study, we swapped the LT-sensitive (129/Sv-type) Nalp1b gene for LT-resistant (B6-type) Nalp1b using meiotic recombination, which means that other genes around the Ltxs1 region would have also been involved in the swapped fragment. Therefore, we can not rule out the possibility that other genes other than Nalp1b caused the observed sensitivity in the Kif1c\(^{-/-}\) Nalp1b\(^{129/129}\) mice although no other candidate genes have been reported around this region. The present study might provide a valuable insight into the molecular pathology of anthrax toxin, which in turn could help to develop strategies for the prevention and treatment of anthrax.

3. Materials and methods

3.1. Mouse molecular genetics

Kif1c knockout mice carrying 129/Sv-type Nalp1b alleles (Kif1c\(^{-/-}\) Nalp1b\(^{129/129}\)) were established as previously described [9], followed by 13 backcrosses with B6 mice. The Nalp1b loci were altered from 129/Sv-type Nalp1b to B6-type Nalp1b by meiotic recombination through IVF [15] using fresh sperm from Kif1c\(^{-/-}\) Nalp1b\(^{B6/129}\) mice and oocytes from super-ovulated B6 females. A total of 1922 fertilized blastocysts were transferred to the uteri of pseudopregnant females to deliver 582 pups. These were genotyped by PCR using primer pairs for the neo transgene (Primer 2 in Fig. 1A) [9] and the 129/Sv-type Nalp1b gene (Primer 3) [4]. Among the 582 pups obtained, 286 were Kif1c\(^{+/+}\) Nalp1b\(^{129/129}\) and one was Kif1c\(^{+/+}\) Nalp1b\(^{B6/B6}\) which contained a crossover between the Kif1c and Nalp1b genes. Kif1c\(^{-/-}\) Nalp1b\(^{B6/B6}\) mice were generated by intercrossing Kif1c\(^{-/-}\) Nalp1b\(^{B6/B6}\) mice followed by PCR genotyping with Kif1c primers (Primer 1) [9].

3.2. Anthrax challenge of macrophages

Macrophage challenge was performed as previously described [16], except where noted below. Briefly, femur bone marrow cells were differentiated in vitro into macrophages with RPMI medium supplemented with 10 ng/mL recombinant mouse macrophage colony stimulating factor (R&D Systems, USA), 10 U/mL penicillin, and 10 μg/mL streptomycin in 10 cm Petri dishes. After 6–9 days of incubation (37 °C, 5% CO\(_2\)), 4.4 × 10\(^4\) cells were plated into each well of a 96-well dish (Nunc, USA) and incubated overnight. These cells were treated with LT (1 μg/mL PA plus 0.1 μg/mL LF; List Biological Laboratories, USA) and stained with 15 μg/mL neutral red dye (Sigma, USA) solution for 2 h. The percentage of living cells from three fields of view over two wells were scored for each genotype.

3.3. Anthrax challenge of mice

Female mice aged 8–12 weeks were intravenously injected with LT (125 μg PA plus 25 μg LF) as previously described [17]. The percent viability was calculated using the formula: 100 × (number of animals living on a given day/number of animals injected on day 0).
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