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Annual two-dose tetanus toxoid vaccination induces protective humoral immunity to all age groups of rhesus macaques

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Abstract: A tetanus outbreak occurred during 2014–2015 in the rhesus macaques reared in an open enclosure in our facility. As the soil of the facility was suspected to be contaminated with *Clostridium tetani* spores, there was a risk of further tetanus occurring among the macaques. To protect them from tetanus, a tetanus toxoid vaccination was recommended; however, the vaccinated elderly animals might not be effectively protected due to insufficient humoral immune responses. Hence, we evaluated the dynamics of antibody responses among rhesus macaques of all age groups vaccinated with two-dose tetanus toxoid at a 1-year interval during a 3-year follow-up study. The vaccination developed anti-tetanus toxin-specific antibodies in animals of all age groups, the antibody levels peaked 1 year after the second vaccination, and the peak levels decreased with age. However, the levels among elderly individuals (aged ≥13 years) were still higher than the threshold level, which was supposed to protect them from tetanus development. Although the rhesus macaques in our facility had a risk of occasional exposure to the spores due to the outbreak, no incidence of tetanus has ever occurred to date. These results indicate that the vaccination protocol is effective in protecting not only younger but also older animals from tetanus.
Key words: antibody response, *Clostridium tetani*, rhesus macaques, tetanus, tetanus toxoid vaccine

Introduction

Tetanus or lockjaw is a highly lethal zoonotic disease caused by a neurotoxin, tetanospasmin, produced by the anaerobic bacterium *Clostridium tetani*. This bacterium is widely distributed in soils around the world in the form of spores that are highly resistant to heat and desiccation. The infection generally occurs by contamination of the wound with the spores or as a postpartum infection and is not transmissible from person to person [1, 2]. Considering the difficulty in treating patients who develop the clinical symptoms of tetanus and their high mortality [3], vaccination against tetanus toxins (tetanus toxoid vaccination) is the primary method of prophylaxis for people living in areas with possible soil contamination. An antitoxin antibody level exceeding 0.01 international units (IU)/ml in vaccinated individuals is generally considered to be protective [4].

The incidence of tetanus has been reported in not only humans but also numerous nonhuman primates (NHPs) such as rhesus macaques [5–8], Japanese macaques [9], bonnet macaques [10], toque macaques [10], baboons [11], and squirrel monkeys [12]. NHPs infected with *C. tetani* generally develop symptoms similar to those seen in humans and can be treated in the same manner as in human patients [13]. As described earlier in the case of humans, antibody responses to tetanus toxin are scarcely observed even in monkeys that survived after a tetanus

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Supplementary Movie: refer to J-STAGE: https://www.jstage.jst.go.jp/browse/expanim

infection, and hence the diagnosis of tetanus is based on clinical symptoms [7, 8, 12]. In contrast, the tetanus toxoid vaccination successfully protects NHPs from the onset of tetanus. An anti-toxin antibody level exceeding 0.01 IU/ml in vaccinated NHPs is considered to be protective against tetanus, which is consistent with results in humans [7, 8].

Colony-wide vaccination is recommended to prevent the occurrence of tetanus disease in NHPs reared in outdoor facilities [7, 14]. However, there is no unified protocol for tetanus vaccination for NHPs [7, 11, 13, 15]. Besides, in humans, there are conflicting reports regarding the impact of aging on the response to tetanus toxoid vaccination [16], whereas there have been no reports to date in NHPs. Therefore, as a part of our health control program in the rhesus macaques colony, we conducted the two-dose tetanus toxoid vaccination with a one-year interval and assessed the dynamics of immune response among animals of all age groups during a follow-up study period of 3 years.

Materials and Methods

Animals and husbandry

Rhesus macaques (*Macaca mulatta*) were bred and reared in an open enclosure of our Center in accordance with the Guidelines for Care and Use of Nonhuman Primates (Version 3) by the Animal Welfare and Animal Care Committee of the Center. Open enclosures (729–960 m^2) hold naturalistic groups of 40–60 NHPs. The animal's weight varied between 1kg (0-year-old) to around 15 kg (adult males). All animals are fed on 200-g pellets (AS, Oriental Yeast Co., Ltd., Tokyo, Japan) twice a day and sweet potatoes three times a week, with occasional special treats of apples, bananas, peanuts, and dried bananas. Environmental enrichment such as various feeders, wooden toys, climbing structures, and swings was provided depending on the housing condition. Twice per day during feeding time competent caretakers observe the macaques for clinical signs of illness and disease including symptoms of tetanus.

Tetanus status in NHP facility

We found one rhesus macaque reared in an open enclosure to have suspected tetanus in 2012. The animal developed the typical symptoms of tetanus, including posterior bow tonic posture, convulsions, and the extension of both hands and feet (Fig. 1; see Supplementary Movie), followed by death on the next day of disease onset. Thereafter, six more animals reared in the same facility developed the symptoms of suspected tetanus during 2014–2015 (Table 1). The diseased animals were treated with the anti-tetanus serum and tetanus toxoid vaccine, combined with symptomatic treatment such as

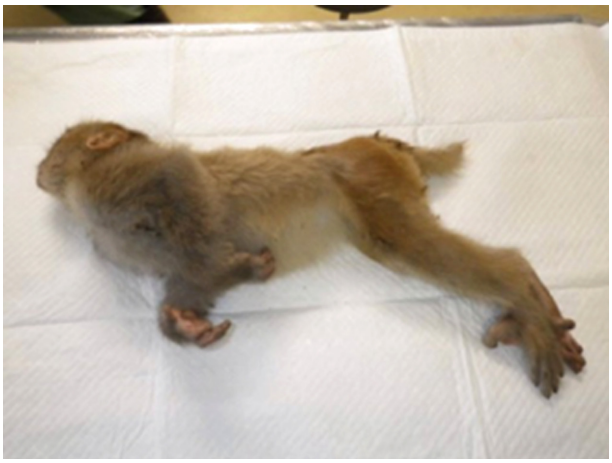

Fig. 1. A case of rhesus macaque developing a typical severe tetanus symptom. The animal was hospitalized with the symptoms of extensor rigidity of the hind legs.

Table 1. History of the rhesus macaques suspecting tetanus in a free-ranging facility during the period of 2012 to 2015

Monkey ID	Date of birth	Date of disease onset	Age of the disease onset (years)	Symptoms	Outcome
Mm1966	2011/6/23	2012/8/27	1	Posterior bow tonic posture, convulsions, extension of both hands and feet	Dead one day after the onset
Mm1825	2008/5/8	2014/2/6	5	Hospitalized due to trauma, staggering, tonic convulsions	Dead one day after the onset
Mm1830	2009/5/20	2014/3/17	5	Paralysis, whole body muscle rigidity, trembling linbs	Cured by treatment
Mm1787	2007/5/10	2014/6/7	7	Tail trauma, slow movement, slightly bent wrists, nasal wing enlargement	Dead four days after the onset
Mm1791	2007/5/21	2014/7/18	7	Hand paralysis, facial stiffness, nasal wing enlargement	Cured by treatment
Mm1679	2004/4/19	2015/8/19	11	Stiff movement, stiff mouth	Dead one day after the onset
Mm1910	2009/8/20	2015/10/29	6	Stiff movement, difficulty of eating	Cured by treatment

administration of antibiotics and wound management (Table 1). Eventually, three of the six monkeys died, but two were fortunately cured; one developed paralysis, whole body muscle rigidity, and trembling limbs, but these symptoms were relieved 1–2 weeks after treatment and were finally cured 1 month later. An asymptomatic monkey was found to be seropositive using a kit for the rapid measurement of the anti-tetanus antibody as well as the modified ELISA kit as described below, confirming the diagnosis of the case as tetanus (data not shown). We suspected that the soil of the facility was contaminated with *C. tetani* spores; hence, there was a concern about the further incidence of tetanus among the rhesus macaques.

Blood collection

During the annual health checkups blood samples (5 ml per macaque) were collected using a 5 ml syringe from the rhesus macaques conducted under anesthesia consisting of the combination of ketamine (5 mg/kg), medetomidine (0.025 mg/kg), and midazolam (0.125 mg/kg), followed by the administration of atipamezole as an antagonist of medetomidine at the end of the procedure. After collection, the blood was centrifuged at 2,000 rpm for 15min, then serum was collected and stored at −80°C until use.

Tetanus vaccination

A total of 29 rhesus macaques aged more than 1 year were administered tetanus inactivated toxoid vaccine (adsorbed tetanus toxoid, Denka Co., Ltd., Tokyo, Japan) intramuscularly under anesthesia, in the autumn of 2015 during a routine health checkup, followed by secondary vaccination 1 year later. In 2016, an additional four monkeys that became 1-year-old were also included in the cohort, followed by secondary vaccination in 2017. Age grouping and the number of males and females in each group are shown in Table 2.

Detection of antibodies against tetanus

The tetanus quick stick test (Gamma, Beaufays, Belgium) was used for the rapid detection of serum anti-tetanus toxin antibodies in the NHPs with tetanus symptoms. For the longitudinal measurement of antibodies of the vaccinated animals, we used TETANUS ELISA IgG (Vircell Microbiologists, Granada, Spain) with modifications. This kit did not cross-react with the rhesus macaque sera that were confirmed to be seropositive for the anti-tetanus toxin antibody by the rapid measurement kit as described earlier, so that the anti-monkey IgG heavy and light chain antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA) was used in place of the anti-human IgG antibody included in the kit, which resulted in reasonable and quantitative reactivity. The antibody index was calculated based on the internal control and according to the following formula:

Antibody index = (sample optical density (OD)) / (cut off serum mean OD)

An antibody index value of 0.794 corresponded to 0.1 IU/ml of antibodies in the sera, and an index of >1.0 is considered to be protective, as indicated in the description of the ELISA kit. We then evaluated the IU/ml in the sera of rhesus macaques using serial dilutions of the standard IgG reagent with known IU/ml, which enabled us to estimate the relative association between the index value and IU/ml. In particular, the results of IU/ml in this study were relative but not absolute values considering that the anti-macaque IgG was used in place of the anti-human IgG antibody included in the kit. We also tested the sera collected from some animals before vaccination and confirmed that they were negative in the ELISA kit.

Table 2. List of the rhesus macaques immunized with the two-dose tetanus toxoid vaccine

Age group	Number of monkeys	Male/Female
1 years old	7	2/5
2–3 years old	7	2/5
4–6 years old	7	1/6
7–12 years old	5	0/5
13 years or older	7	1/6
total	33	6/27

Results

The vaccination protocol resulted in seropositivity for the tetanus antibody among 65% of the rhesus macaques in 2016, 93% in 2017, and 100% in 2018, indicating that the vaccination successfully induced the tetanus toxin-specific antibody in all animals (Fig. 2). 1 out of 29 rhesus macaques was positive for the anti-tetanus toxin antibody before vaccination (Fig. 2). It was confirmed that the animal neither represented the case of cure by treatment nor had ever developed tetanus, as shown in Table 1.

The effect of age on the antibody response of the rhesus macaques after the two-dose vaccination shows the kinetics of the relative level of the anti-tetanus toxin antibodies among different age groups, with the average indicated as a thick line (Fig. 3). The induced antibodies reached their peak levels 1 year after the second vaccination, which was generally consistent among all animals. The 1-year age group developed the highest level of the antibody, which decreased in parallel with age (Fig. 3).

The average antibody index for animals in the 1-year age group was 7.45, whereas animals aged 2–3, 4–6, 7–12, and 13 years or elder individuals showed antibody indexes of 5.29, 2.94, 3.30, and 3.14, respectively (Fig. 3).

Finally, we evaluated the IU/ml of animals' sera 1 year after the second vaccination showing the highest antibody index using serial dilutions of the standard IgG reagent with known IU/ml. The antibody level was significantly lowered in the monkeys 4–6 years of age than younger groups, while further ageing did not significantly influence the antibody level (Fig.4). Importantly, 13-year-old or elder animals mostly maintained >0.1 IU/ml of the antibody, which is higher than the threshold level required for protection against tetanus toxin (Fig. 4).

Discussion

We experienced a tetanus outbreak occurred during 2014–2015 in the rhesus macaques reared in an open

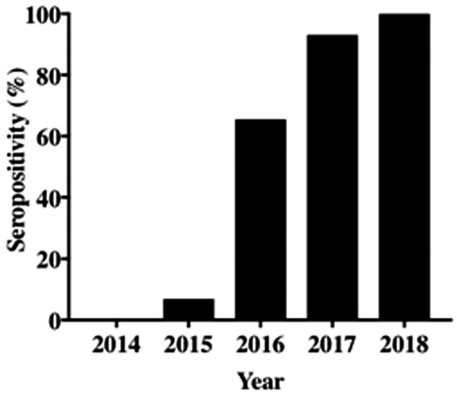

Fig. 2. An annual increase in the rate of antibody positivity among the vaccinated rhesus macaques. The x-axis shows the year in which the antibodies were measured. The y-axis indicates the percentages of rhesus macaques in the open enclosure that were positive for tetanus antibodies. In 2014, before the vaccination, one animal was positive.

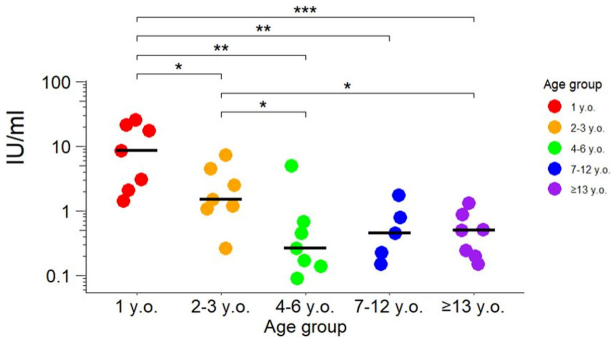

Fig. 4. Distribution of the level of serum anti-tetanus antibody among different age groups of rhesus macaques 1 year after the second vaccination. The x-axis represents the age of animals at the first vaccine administration, and the y-axis shows the IU/ml of the serum antibody. Data were analyzed in pair-wise fashion using the two tailed T test. *P*-values indicated as $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***). Non-significant correlations are not shown.

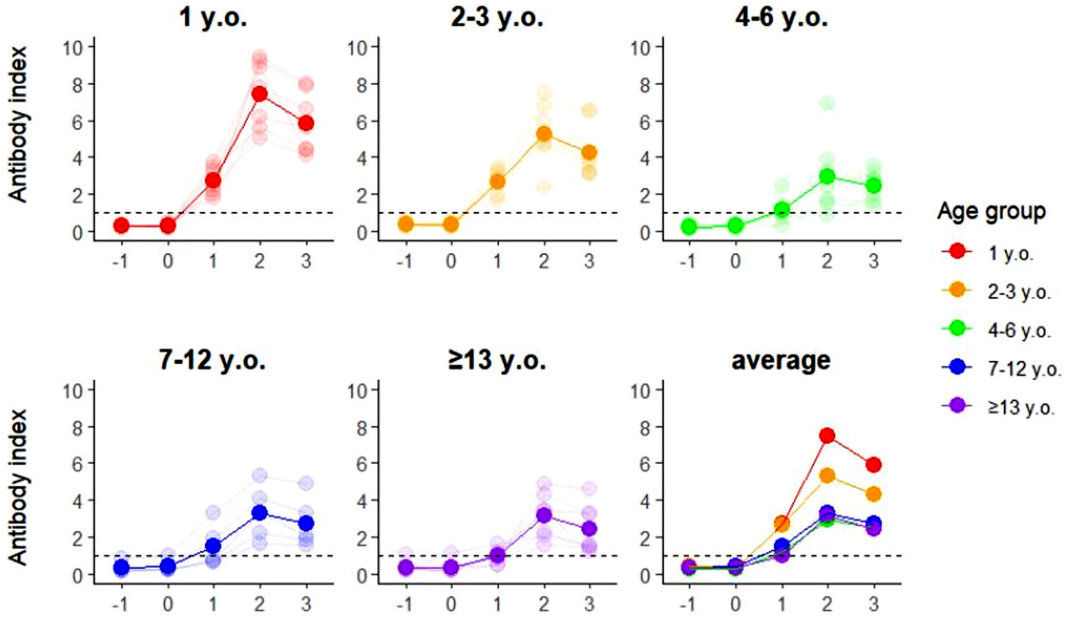

Fig. 3. Age-dependent changes in the level of serum anti-tetanus antibody among the different age groups of rhesus macaques after the two-dose tetanus toxoid vaccination. The x-axis indicates the years before and after the administration of the first dose, where 0 is the year in which the first dose was administered. The y-axis shows the antibody indexes. The graphs are shown for the animals grouped based on their age, as indicated in Table 2. Thick lines represent the average antibody indexes of each animal group.

enclosure in our facility. As the soil of the facility was suspected to be contaminated with *Clostridium tetani* spores, there was a risk of further tetanus occurring among the macaques. In this study, we demonstrated that the two-dose vaccination protocol successfully induced anti-tetanus toxin antibodies in all vaccinated rhesus macaques. We thus implement this two-dose vaccination together with a booster vaccination every 5 years in the free-ranging areas where incidence of tetanus was observed, including the area mentioned above and another one in which a few Japanese macaques previously developed tetanus symptoms (data not shown). Eventually, as of 2022, there has been no incidence of tetanus among rhesus macaques in our facility. Thus, the vaccination appears effective in protecting our macaques from tetanus.

There are conflicting reports in human cases in terms of the impact of aging on the response to tetanus toxoid vaccination [16], whereas no detailed information has ever been reported in NHPs. Our results demonstrated that the efficacy of the vaccine was dependent on the age of animals, with a decline in the peak antibodies response in animals ≥4 years old, which can be explained by a lower level of antibody response after vaccination in adulthood. It is important to note that, further ageing did not significantly influence the antibody level (Fig. 4). In fact, the animals aged 13 years or elder successfully exhibited an anti-tetanus toxin antibody level of >0.1 IU/ml, which is the threshold level required for protection against tetanus toxin (Fig. 4). Our results show that the two-dose vaccination protocol is effective in all age ranges.

Although we observed no incidence of tetanus among the rhesus macaques since 2015, it is unclear whether the protective immunity would persist for a while or decrease soon in the future. It is reasonable to postulate that older animals, at least partially, may lose their protective immunity at present or in near future. It remains to be elucidated when the protective immunity will wane below the threshold level and additional booster vaccination will be necessary. Irrespective of the present situation of the protective immunity, it is reasonable to administer the third (booster) vaccination sooner or later to reduce the risk of recurrence of the tetanus outbreak.

It is reasonable to assume that the soil at the enclosure had been contaminated by tetanus spores before we had initially introduced the NHPs in our facility, as the spores are known to be frequently prevalent in the soil. As some rhesus macaques had developed tetanus, the animals would spread the spores in the soil of the facility, which augmented the frequency of other animals being exposed to the spores when suffering the injury. Repeated cycles of these exposures probably caused the frequent occurrence of tetanus since 2012, as shown in Table 1. We must consider that the soil of the open enclosure should still be contaminated with the spores, and hence animal caretakers as well as researchers who enter the area may have a certain risk of developing tetanus. Therefore, it is strongly recommended for them to consider the tetanus toxoid vaccination, especially for people who are older than mid-twenty years of age [4].

Interestingly, 1 of 29 rhesus macaques was positive for the anti-tetanus toxin antibody before vaccination (Fig. 2). It was confirmed that the animal was not the case of cure by treatment as shown in Table 1, which suggests a history of previous asymptomatic infection with *C. tetani*, although we cannot exclude the possibility of the nonspecific reaction resembling that observed previously in humans [3].

A high rate of tetanus onset was previously reported among rhesus macaques in the Cayo Santiago colony, Puerto Rico [7]. Interestingly, no detectable anti-tetanus toxin antibody was observed among the serum samples collected from six animals that recovered from tetanus [7]. In this point of view, the rhesus macaque that recovered from tetanus was seronegative several months after the recovery, which was consistent with the report and suggests that aging after sexual maturation may not influence antibody induction by the vaccination. However, we also observed that other animals that died of tetanus became seropositive (data not shown). These results confirmed the previous information on humans that the serological examination is not useful for diagnosing tetanus [3].

The average peak of the anti-tetanus toxin antibody was observed 1 year after the second vaccination (Fig. 3). At this point, the 1-year age group exhibited the highest antibody level (Fig. 4). These results indicate that vaccination of infant individuals should be recommended for the induction of efficient protective immunity to *C. tetani* infection, which is consistent with the previous report [8]. Due to these findings in this study, we implement the two-dose tetanus toxoid vaccination as our routine protocol for the 1 and 2 years of age rhesus macaques kept in the open enclosure with booster vaccination every 5 years, and as of 2022, no tetanus cases were observed in the facility, indicating successful protection of the animals from the disease.

Conclusions

The rhesus macaques of all age groups, including the older ones, vaccinated with the two-dose tetanus toxoid at a 1-year interval successfully developed anti-tetanus

toxin-specific antibodies, whose levels were supposed to be enough for protecting them against tetanus development. In fact, no animals have eventually developed tetanus to date, irrespective of the potential risk of occasional exposure to the spores in the facility with a history of recent incidences of tetanus. We conclude that the vaccination protocol should be effective in protecting not only younger but also older animals from tetanus.

Author Contributions

Conceptualization and Supervision, H.A. and J.S.; Investigation, M.Mu., A.Ko, A.Ka, M.Mo., A.I., T.N., A.W., T.M-N., and J.S.; Writing, M.Mu., A.Ko, and H.A.

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Institutional Review Board Statement

This study was approved by the Institutional Committee for Animal Experiments without an animal experiment application because the study was associated with veterinary medicine and care.

Informed Consent Statement

Not applicable.

Data Availability Statement

For other information contact the corresponding author.

Conflicts of Interests

The authors declare no conflicts of interest.

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