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NOTE

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Incidental induction of secondary bowel disorders in guinea pigs during a batch safety test of veterinary vaccines

Naoyuki AIHARA[1,2], Mariko OCHIAI[1], Noriyuki HORIUCHI[1,3]*, Atsushi YAMAMOTO[1], Kasumi SUDO[1], Yoko ISHIKAWA[1] and Koji OISHI[1]

[1]National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, 1-15-1 Tokura, Kokubunji, Tokyo 185-8511, Japan

[2]Laboratory of Veterinary Pathology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan

[3]Research Center for Global Agromedicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

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ABSTRACT. Veterinary vaccines are subjected to a safety testing using laboratory animals via intraperitoneal injection per batch. From April 2010 to March 2011, 7 guinea pigs in 4 batch tests exhibited unrecoverable weight loss and/or were found dead. Six guinea pigs had developed intussusception, whereas another one had developed an intestinal obstruction consequent to adhesion. A histopathology revealed that these lesions were associated with inflammatory foci. Other animals than the 7 guinea pig also developed similar inflammatory foci but did not develop bowel disorders. In the retesting of these batches, animals did not exhibited clinical signs, though inflammatory foci were detected. The clinical signs, detected in the primary test, might be due to bowel disorders secondary to an inflammatory response, rather than toxicity.

KEY WORDS: aluminum-based adjuvant, guinea pig, intestinal obstruction, intussusception, laboratory animal batch safety test

During vaccine production, several tests are applied to the final product to meet regulations requiring quality analysis on a batch-to-batch basis [4]. Some tests continue to use animals, although the widely accepted concept of the 3R approach—namely that animal experimentation must be reduced, refined, and replaced—has led to the replacement of most vaccine quality tests with *in vitro* assays. Existing vaccine authorities have made consistent efforts to follow the 3R approach [1, 5–7]. However, many countries continue to subject final veterinary vaccine products to laboratory animal-based safety testing on a batch-to-batch basis.

In Japan, final products must be subjected to laboratory animal-based batch safety tests, according to the "Minimum Requirements for Veterinary Biological Products", which is mandated by the Law on Securing Quality, Efficacy and Safety of Products including Pharmaceuticals and Medical Devices [10]. Here, one of two tests, the abnormal toxicity test (ATT) or toxicity limit test (TLT; also known as the general safety test, innocuity test, or test for freedom from abnormal toxicity), is applied to the final product as appropriate (see Table 1 for an outline) to identify unexpected toxicities. Briefly, the ATT is applied to veterinary vaccines lacking any hazardous components, whereas the TLT is applied to vaccines containing a component considered virulent in mice and/or guinea pigs or those that are otherwise not suitable for the ATT. Using these tests, final products that cause abnormal weight loss and/or death in an animal model at a certain dose are disqualified. In this study, we describe the disqualification of certain batches after guinea pigs developed intussusception or intestinal obstruction associated with the injected vaccines during batch safety tests. However, further investigation revealed these cases to involve secondary bowel disorders caused by inflammatory responses to the injected vaccines, rather than the toxicity of a contaminant within the tested vaccines.

We tested a total of 186 final product batches of veterinary vaccines. Of these, 118 were subjected to ATT using 244 guinea pigs and 1,170 mice, and 68 were subjected to TLT using 50 guinea pigs and 640 mice. Female Hartley strain specific pathogen-free guinea pigs (average body weight: 350 g) were purchased from Tokyo Laboratory Animal Science Co., Ltd. (Tokyo, Japan). Female specific pathogen-free ddY mice (age: 5 weeks) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All animals were weighed and checked for clinical symptoms during the test period and were euthanized and subjected to necropsy and histopathology at the end of the test. Animals that died during the test period were also subjected to necropsy and histopathology. All procedures applied to the animals were approved by the Committee on the Ethics of Animal Experiments of National

*Correspondence to: Horiuchi, N.: horiuchi@obihiro.ac.jp

Table 1. Laboratory animal-based batch safety test methods applied to veterinary immunological products in Japan

Test	Animals	Method	Criterion
Abnormal toxicity test	2 guinea pigs and 10 mice	Guinea pig: 2 animals, 5 m*l* intraperitoneal injection, 7 days of observation Mice: 10 animals, 0.5 m*l* intraperitoneal injection, 7–10 days of observation	The product passes the test if no abnormal health signs are observed.
Toxicity limit test	5 guinea pigs or 10 mice	Guinea pig: 5 animals, 5 m*l* intraperitoneal injection, 7 days of observation Mice: 10 animals, 0.5 m*l* intraperitoneal injection, 7 days of observation	The product passes the test if the animals survive without abnormal signs during the observation and the body weight returns to the initial level within a specified time.

Veterinary Assay Laboratory (Permit Number: 22-031), and all applicable international, national, and institutional guidelines for animal care were followed.

Most batches of final products passed batch safety tests. On the other hand, a few batches failed the tests due to weight loss and/or succumbed to death of experimental animals (Table 2). All the vaccines, which failed in the tests, contained aluminum-based adjuvants. Thirty two guinea pigs and 30 mice were subjected to the tests of failed batches and injected with the same samples in each test. Although most of the subjected animals did not develop clinical signs, 7 guinea pigs developed clinical signs and/or were found dead; among them, the 2 guinea pigs with progressive weight loss were subjected to euthanasia via overanesthesia with isoflurane (Mylan Inc., Canonsburug, PA, U.S.A.). Necropsy revealed that all animals in the experiments harbored diffuse small white nodules (diameters: 1–2 mm) on the serous surfaces of abdominal organs. The 6 guinea pigs with the abnormal symptoms had developed intussusception and another one had developed an intestinal obstruction associated with intestinal adhesion. The intussusceptions occurred at the terminal ileum in 4 cases (Fig. 1a) or at the ileocecal region in 2 cases. The other animals, which had not showed clinical signs in the experiment, had not developed intussusception or intestinal obstruction despite the white nodules on serous surface.

Histopathologically, the white nodules on the serous surface were granulomatous inflammatory foci that consisted of centrally located eosinophilic amorphous deposit, which were surrounded by macrophages, lymphocytes, and fibrin deposits. These granulomatous foci did not invade the muscular layer of intestine. The similar granulomatous inflammatory foci were detected within the sites of intussusception (Fig. 1b) and the focus of intestinal adhesion (Fig. 1c) in the symptomatic 7 guinea pigs. The eosinophilic amorphous deposits, which were found within the granulomatous foci, yielded partly positive response to aluminum staining via the hematoxylin-lake method (Fig. 1d) [11].

The failed batches were subjected to the batch safety test again. No animals developed clinical signs in the tests. Necropsy revealed similar white nodules on the serous surface, which were granulomatous foci with eosinophilic deposit on histopathological evaluation. On the other hand, intussusception or intestinal adhesion was not detected.

To summarize our findings, seven guinea pigs exhibited bowel disorders, and all failed vaccines contained an aluminum-based adjuvant—the most commonly used adjuvant in human—and veterinary vaccines to stimulate an immune response [2]. The adjuvant-induced inflammatory nodules were detected in all the guinea pigs and mice injected with the failed vaccines. However, these nodules did not directly cause clinical symptoms; only inflammatory foci with associated bowel disorder led to clinical symptoms such as intestinal disorders that caused weight loss and/or death in the guinea pigs. Accordingly, we attribute the failures of these batches to the induction of inflammatory foci that led to secondary bowel disorder (e.g., intussusception or intestinal obstruction), rather than unexpected contaminant-related toxicity. The results of the retest, wherein none of the injected animals developed clinical signs despite harboring inflammatory nodules within the abdomen, also support our conclusion. The secondary bowel disorder was observed in the guinea pigs but not in the mice, and might be attributed to the variation in anatomical features between the two species; however the detailed mechanisms were not detected.

Although the exact mechanism leading to intussusception has not been elucidated, the secondary development of intussusception consequent to a pathological alteration is widely accepted [8]. Briefly, some types of bowel wall or luminal lesions may alter the peristaltic activity and initiate intussusception, after which the intestine begins to undergo invagination. Carcinomas, polyps, Meckel's diverticulum, colonic diverticulum, strictures, and benign neoplasms have all been identified as triggers for this process [9]. In this study, however, both intussusception and intestinal obstruction likely developed in response to the adjuvant-induced inflammatory nodules.

As noted, other animals injected with the failed vaccines also developed inflammatory nodules without a subsequent secondary bowel disorder. Additionally, no secondary bowel disorders were recorded in animals injected with vaccines that lacked aluminum adjuvant, despite the development of inflammatory foci (unpublished data). Although a previous report investigated the toxicity of aluminum adjuvant [3], no reports have described an associated bowel disorder. Accordingly, we remain unable to explain why only 7 guinea pigs injected with aluminum-containing adjuvant vaccines developed secondary bowel diseases in this study.

To our knowledge, this is the first report to describe intussusception and intestinal obstruction in guinea pigs following intraperitoneal vaccine administration. Although the exact mechanisms of the secondary bowel disorder associated with these injected vaccines have not been elucidated, vaccine inspectors should pay attention to disorders secondary to inflammatory response when encountering unfavorable batch safety test results in laboratory animals.

Table 2. Abnormal findings in guinea pigs

Tested vaccines	Type of vaccine	Type of test	Adjuvant	Clinical sign	End point	Macroscopic lesion
A	Monovalent bacterial inactivated vaccine	ATT	Aluminum hydroxide gel	Unrecoverable weight loss (7.6% decrease from 0 dpi to 7 dpi)	Euthanized due to progressive weight loss	Intestinal obstruction associated with adhesion
B	Monovalent bacterial inactivated vaccine	ATT	Aluminum hydroxide gel	-	Found dead at 2 dpi	Intussusception (terminal ileum)
C	Multivalent viral inactivated vaccine	ATT	Aluminum phosphate gel	-	Found dead at 1 dpi	Intussusception (terminal ileum)
D	Multivalent viral inactivated vaccine	TLT	Aluminum hydroxide gel Saponin	-	Found dead at 1 dpi	Intussusception (terminal ileum)
D	Multivalent viral inactivated vaccine	TLT	Aluminum hydroxide gel Saponin	Unrecoverable weight loss (0.9% decrease from 0 dpi to 7 dpi)	Found dead at 7 dpi	Intussusception (ileocecum)
D	Multivalent viral inactivated vaccine	TLT	Aluminum hydroxide gel Saponin	-	Found dead at 1 dpi	Intussusception (terminal ileum)
D	Multivalent viral inactivated vaccine	TLT	Aluminum hydroxide gel Saponin	Unrecoverable weight loss (16.3% decrease from 0 dpi to 7 dpi)	Euthanized due to progressive weight loss	Intussusception (ileocecum)

ATT: abnormal toxicity test, TLT: toxicity limit test, dpi: day post injection.

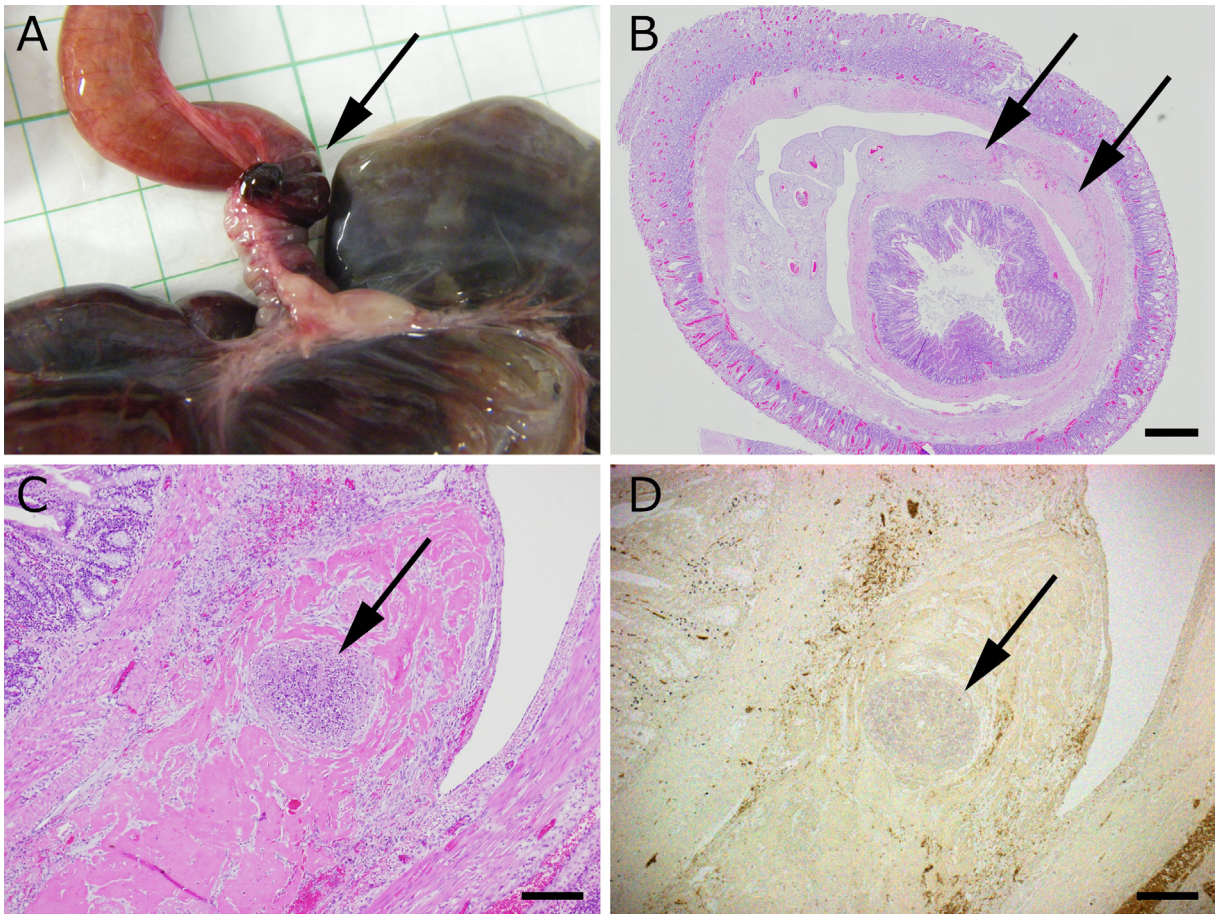

Fig. 1. (A) Gross appearance of the intussusception. An intussusception (arrow) occurred at the terminal ileum. (B) Photomicrograph of the intestine at the site of intussusception: centrally located penetrating intestine and laterally located intussuscepted intestine. The intussuscepted intestine was inverted due to intussusception. Inflammatory nodules (arrows) were identified between the serous surfaces of the intestines. HE. Bar, 1 mm. (C) Photomicrograph of the intestinal adhesion: fibrin exudate, infiltration of inflammatory cells, and nodular lesion (arrow) similar to the inflammatory focus detected at the intussusception were noted. HE. Bar, 200 μm. (D) Photomicrograph of the inflammatory nodule stained with aluminum stain (hematoxylin-lake method). Positively stained substance was detected in the nodules. Bar, 200 μm.

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