



Serological and histological evaluation of species-specific immunocontraceptive vaccine antigens based on zona pellucida 3 in the small Indian mongoose (*Herpestes auropunctatus*)

Naotoshi KUNINAGA¹⁾, Makoto ASANO^{1,2)*}, Ryota MATSUYAMA³⁾, Takahiro MINEMOTO⁴⁾, Takayuki MORI⁴⁾ and Masatsugu SUZUKI^{1,2)}

¹⁾The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan

²⁾Joint Department of Veterinary Medicine, Faculty of Applied Biological Sciences Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan

³⁾Graduate School of Medicine, Hokkaido University, Kita 15 Jo Nishi 7 Chome, Kita-ku, Sapporo, Hokkaido 060-0808, Japan

⁴⁾Graduated from Department of Veterinary Medicine, Faculty of Applied Biological Sciences Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan

ABSTRACT. The small Indian mongoose (*Herpestes auropunctatus*) was introduced to Japanese islands and has impacted on the island's biodiversity. Population control has been attempted through capturing but its efficiency has rapidly declined. Therefore, new additional control methods are required. Our focus has been on the immunocontraceptive vaccines, which act in an especially species-specific manner. The amino-acid sequence of the mongoose ovum zona pellucida protein 3 (ZP3) was decoded and two types of synthetic peptides (A and B) were produced. In this study, these peptides were administered to mongooses (each n=3) and the sera were collected to verify immunogenicity using ELISA and IHC. Treated mongoose sera showed an increasing of antibody titer according to immunizations and the antigen-antibody reactions against the endogenous mongoose ZP. In addition, IHC revealed that immune sera absorbed with each peptide showed a marked reduction in reactivity, which indicated the specificity of induced antibodies. These reactions were marked in peptide A treated mongoose sera, and the antibody titer of one of them lasted for at least 21 weeks. These results indicated that peptide A was a potential antigen, inducing autoantibody generation. Moreover, immunized rabbit antibodies recognized mongoose ZP species-specifically. However, the induction of robust immune memory was not observed. Also, the actual sterility effects of peptides remain unknown, it should be verified as a next step. In any case, this study verified synthetic peptides we developed are useful as the antigen candidates for immunocontraception of mongooses.

KEY WORDS: fertility control, immunocontraception, invasive alien species, mongoose, zona pellucida

J. Vet. Med. Sci.

81(2): 328–337, 2019

doi: 10.1292/jvms.17-0605

Received: 13 November 2017

Accepted: 28 November 2018

Published online in J-STAGE:

27 December 2018

The small Indian mongoose (*Herpestes auropunctatus*) is a carnivorous animal mainly native to the region expanding from West to Southeast Asia [35, 46]. The mongoose is listed among the “100 of the World’s Worst Invasive Alien Species” published by the invasive species specialist group of the IUCN [18]. This is the result of introducing the small Indian mongoose intentionally to 76 islands throughout the world, which has threatened the native ecosystems [3, 18, 33]. In Japan, ten-odd individuals of the small Indian mongoose were introduced to Okinawa Island in Japan in 1910 and brought in Amami-oshima Island in 1979, with the aim of biologically controlling Habu snakes and house rats [15, 45, 49, 51].

Okinawa Island and Amami-oshima Island are both located in the sub-tropical region, which has endemic ecosystems and uniquely evolved fauna. The lack of remarkable natural enemies and the comfortable climate easily allowed the mongoose population to establish its habitat and spread, which resulted in a large impact on the island’s biodiversity. Specifically, preying on the Amami rabbit (*Pentalagus furnessi*), Ryukyu long-furred rat (*Diplothrix legata*), Ryukyu robin (*Erithacus komadori*), Ryukyu

*Correspondence to: Asano, M.: asanojr@gifu-u.ac.jp

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tree lizard (*Japalura polygonata*), and other rare species, and the competition with species that prey on the same food resources such as Okinawa rail (*Gallirallus okinawae*) largely impacted the islands [36, 38, 49, 51]. Not only impacts on ecosystems but also social impacts such as risk of leptospirosis transmission and damage to poultry by mongooses were reported [14, 16, 30, 52].

The Ministry of the Environment Government of Japan (MOE) has conducted control of mongoose on both islands in cooperation with the local governments, starting from the year 2000 [34]. Further, An Invasive Alien Species Act was carried out in 2005, and small Indian mongoose was legally designated as an invasive alien species in Japan [32]. The MOE organized a group of experts in mongoose capturing called “Mongoose Busters” on each island and have succeeded in reducing the mongoose population drastically [34]. However, achieving a low mongoose population density has also lowered the capture efficiency. Therefore, the complete eradication of the mongoose population is not yet to be achieved. The other factors suppressing the capture efficiency are the increase in by-catch of native species and the appearance of trap-shy individuals, who are highly cautious of traps.

Therefore, new methods for mongoose population control are required. We focused on the immunocontraceptive methods, which induce immunological sterility by treatment with contraceptive vaccines. Immunocontraception in wildlife was initially established in 1980s to control wild horses in the U.S.A. [23, 28], and has since been applied to various mammals (e.g., cat, African elephant, and some ungulates), due to a growing concern for animal welfare [9, 24, 26]. Immunocontraception is a technique for suppressing reproduction of the target species, in which antibodies induced by the contraceptive vaccines inhibit the activity of reproduction-related factors as autoantigens. In these studies, nonspecies-specific agents such as porcine zona pellucida (PZP) or GnRH preparations, some of which have already been made into products (e.g., SpayVac™, GonaCon™), have been used as direct injectable contraceptive vaccines [27, 29, 31]. On the other hand, species-specific agents have also been developed with remote injection to control the free-ranging population [8, 19]. Since the present study targeted on free-ranging invasive animals, it was not realistic to treat mongooses with any direct injectable agents under visual recognition or capture. Furthermore, the effects on non-target species had to be minimized. Therefore, the overall objective of this study was to develop mongoose-specific immunocontraceptive agents.

We focused on zona pellucida 3 glycoprotein (ZP3) as vaccine antigens, which has high species-specificity and plays an important role in the primary binding of sperms and oocytes [2, 4]. Manufacturing of vaccine candidates was necessary to clarify whether these agents would induce antibody production in mongoose and whether, the induced antibody, would recognize the mongoose endogenous zona pellucida (ZP) as biological antigens. Additionally, we tried to identify the species-specific effects of these peptides.

MATERIALS AND METHODS

Synthesis of antigen peptides

We started by decoding the full-length sequence of mongoose ZP3. Briefly, RNA was extracted from mongoose ovary, sampled from individuals culled in MOE control program. PCR products were obtained with reverse transcriptase reaction method and RACE method; RACE-PCR products were then amplified by TA cloning. After the sequence reaction, base sequence analysis was performed by the Division of Genomics Research of Life Science Research Center of Gifu University. Thereafter, amino-acid sequences were predicted from the decoded mongoose ZP3 gene sequence and homology of the sperm-oocyte binding site was compared with known ZP3 sequences from cat (*Felis silvestris catus*), dog (*Canis lupus familiaris*), stoat (*Mustela ermine*), mouse (*Mus musculus*), and humans (*Homo sapiens*).

Through the above steps, two types of peptides, namely peptide A (CGLPGHSRRLSHPERPRRK) and peptide B (CHPERPRRKLA PRSRNRH), which have a homologous amino-acid sequence to a part of the mongoose ZP3 sperm-oocyte binding site (both 19 AA residues) were designed. These peptides were synthesized and conjugated with keyhole limpet hemocyanin (KLH) on the cysteine residue in order to promote antigenicity (Sigma-Aldrich Japan K.K.; Tokyo, Japan). Each peptide was assumed to include two epitope regions. One of them has common amino-acid sequence between peptide A and B.

Animals

Nine female small Indian mongooses were captured in Nago city, Okinawa using box traps in the MOE mongoose control project [34]. They were all determined as mature by their head/body length (265–290 mm) and body weight (275–487 g) [37]. The animals were acclimatized to captivity for at least 2 weeks, and were housed in individual cages (L 230 × H 270 × W 600 mm) in the outdoor rearing facility at the Yambaru wildlife conservation center of MOE. All animals were fed dog food and had free access to water at all times. The mongooses were randomly assigned to three groups: group A, which received peptide A (n=3; A-1~3), group B, which received peptide B (n=3; B-1~3), and control group C treated with phosphate-buffer saline (PBS; n=3; C-1~3). No animals showed noticeable body weight loss or behavioral abnormality during the experiment.

Immunization and blood sampling

Starting from February 2014, the animals were treated with peptides (150 µg/150 µl) emulsified with the same volume of Freund's incomplete adjuvant (TiterMax® Gold, TiterMax U.S.A. Inc., Norcross, GA, U.S.A.) or 150 µl of PBS with the same volume of adjuvant as control under anesthesia. Immunization was performed four times at an interval of 2 weeks in several dorsal subcutaneous sites. Two of the animals (A-1 and B-2) were used for the confirmation of the antibody's durability. Subsequently, after a decrease in the antibody titer (83 weeks after the first immunization), these two animals were treated with a single immunization to evaluate the immune memory. The mongooses were anesthetized with intramuscular injection of ketamine

hydrochloride (0.5 mg/head; Ketalar, Daiichi Sankyo, Tokyo, Japan) and medetomidine hydrochloride (0.07 mg/head; Domitor, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) using a special box cage. Subsequently, the animals were treated with atipamezole hydrochloride (0.35 mg/head; Antisedan, Nippon Zenyaku Kogyo Co., Ltd.) to promote recovery from sedation. Blood (1 ml) was collected via cardiocentesis at the time of immunization (except the second immunization) and a week after the fourth immunization. Blood samples from A-1 and B-2 were collected continuously at an interval of approximately 15 weeks and at the time of reimmunization. Blood samples were kept overnight at 4°C, and were subsequently centrifuged for 10 min at 10,000 rpm, and the serum was separated. All serum samples were subdivided and stored at -80°C until further analysis. All animals, except A-1 and B-2 were euthanized 1 week after the last immunization via exsanguination under anesthesia, and the ovaries were collected for pathological analysis. Euthanasia was performed in accordance with the guideline of The American Veterinary Medical Association (AVMA; 2007).

Enzyme-linked immunosorbent assay (ELISA)

The amount of serum antibodies against synthetic peptides was measured by using indirect ELISA. Briefly, 96-well microplates (#3590, Corning Inc., Corning, NY, U.S.A.) were coated with 100 µl of peptide A or B in carbonate-bicarbonate buffer (C3041, Sigma-Aldrich Co., St. Louis, MO, U.S.A.) and incubated for 1 hr at room temperature. After washing three times with 200–300 µl/well of PBS containing 0.05% Tween®20 (Sigma-Aldrich; PBS-T), nonspecificity was blocked with 3% skim milk in PBS (200 µl/well) overnight at 4°C. After washing three times with PBS-T, mongoose serum in PBS (1:100; 100 µl/well) was added as the primary antibody and incubated for 1 hr at room temperature. After washing three times with PBS-T (300–400 µl/well), horseradish peroxidase (HRP)-labeled goat anti-Ferret IgG antibody (1:2,500; 1 mg/ml; Bethyl laboratories Inc., Montgomery, AL, U.S.A.) in PBS (100 µl/well) was added and incubated for 1 hr at room temperature; cross-reactivity to mongoose IgG has been previously reported [17]. After washing three times with PBS-T (200–300 µl/well), tetramethyl benzidine (100 µl/well; TMB Substrate Regent Set, BD, Franklin Lakes, NJ, U.S.A.) was added to induce a chromogenic reaction for 20 min, and the reaction was subsequently stopped by 1 M of H₂SO₄. The absorbance was measured at dual wavelengths, 450/655 nm using a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, U.S.A.).

All sera were tested in triplicate on a plate to verify the technical validity of the experiment, and the average optical density (OD) and standard deviation (SD) of sera were calculated. An average OD + 3 SD of all control sera was set as the cut-off value.

IHC-labeled streptavidin-biotin (LSAB) method

Immunohistochemical analysis was performed on sections (4 µm) from fixed and paraffin-embedded non-immunized mongoose ovaries obtained from three mature female mongooses, other than from immunized individuals, captured under the same MOE control program, to verify the recognition of endogenous ZP by induced mongoose antibodies. Mongooses were then euthanized under anesthesia by exsanguination and ovaries were collected and fixed in 4% neutral buffered formalin.

According to the general methods, sections were deparaffinized and rehydrated using xylene and alcohol gradient. At this stage, one section was used for hematoxylin and eosin staining (HE) to observe the tissue structure. For IHC, antigen retrieval was performed in other sections by incubating them in citrate buffer solution (pH 5.4) in a warm bath (90°C) for 40 min. After cooling down for 20 min at room temperature, sections were washed three times with PBS (5 min), and the endogenous peroxidase activity was inhibited with 3% H₂O₂ for 15 min at room temperature. After washing three times with PBS (5 min), the slides were treated with 3% bovine serum albumin (BSA; A3590, Sigma-Aldrich Co.) in PBS (100 µl/section) and incubated overnight at 4°C to block nonspecific reactions. Subsequently, the sections were incubated with primary antibody, i.e., either serum from the treated mongooses, diluted at 1:50 with 3% BSA in PBS (100 µl/section) for 1 hr at room temperature. After washing three times with PBS (5 min), the sections were incubated with HRP-labeled protein A (1:1,000, 100 µl/section; ab7456, Abcam, Cambridge, U.K.) for 1 hr at room temperature. After washing in PBS, sections were incubated with streptavidin (1:200, 100 µl/section; Extravidin®, Sigma-Aldrich Co.) in PBS for 1 hr at room temperature to amplify the avidin-biotin reaction. Subsequently, the sections were washed in PBS, incubated in 3,3'-diaminobenzidine (DAB; Dako, Santa Clara, CA, U.S.A.) for 1 min to induce a chromogenic reaction, and counterstained with hematoxylin. All slides were sealed through a dehydrating and permeating operation.

To evaluate the specificity of the antibody, an absorption test was performed, in which absorbed sera with immunized peptide (e.g., A-1 serum-peptide A) were used as the primary antibody. Each serum and the same volume of peptide were co-incubated in PBS (1:50 diluent) for 1 hr at room temperature.

Additionally, to estimate the species-specificity of antibodies induced by peptides, IHC using immunized rabbit sera and ovary sections of various animals was performed. Briefly, the same peptides (peptide A or B) were administered to rabbits (each n=2; a-1, 2 and b-1, 2) 5 times every two weeks and sera were collected continuously. Immunized rabbit sera were used as the primary antibody (1:100 with 3% BSA in PBS, 100 µl/section). Ovary sections from Mongoose, Japanese marten (*Martes melampus melampus*), cat (*Felis silvestris catus*), dog (*Canis lupus familiaris*), Japanese wild boar (*Sus scrofa*), and rabbit (*Oryctolagus cuniculus*) were used (fixed with 4% neutral buffered formalin, 4 µm). Ovary samples were collected from spayed cats and dogs, captured wild animals based on permission, and experimental animals. Every captured or experimental animal were euthanized in accordance with the guideline of AVMA appropriately and ovaries from them were treated as ones from mongooses. Other experimental conditions were the same as described above.

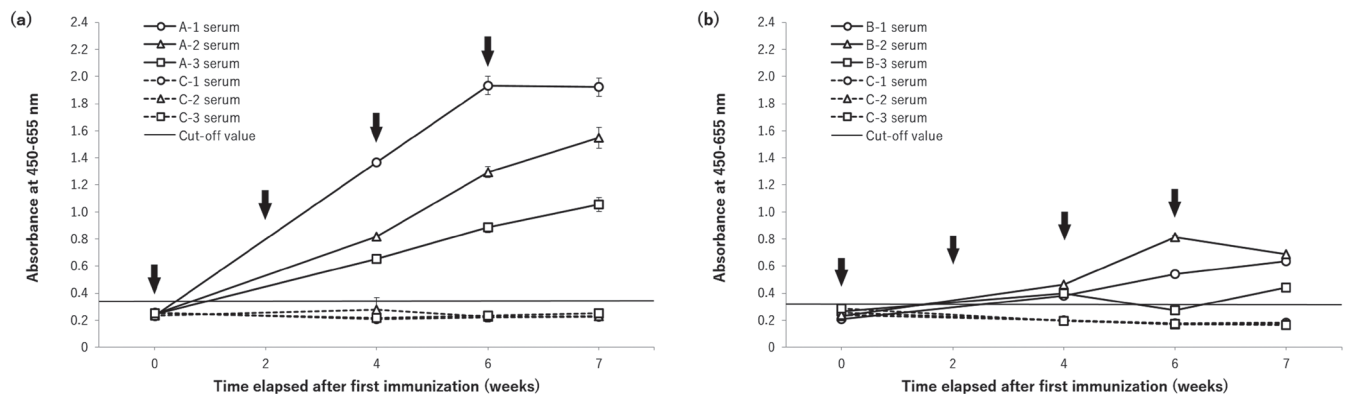


Fig. 1. Antibody titers (absorbance at 450–655 nm; mean \pm SD) in mongoose sera following immunization with peptide A (a) and peptide B (b). Horizontal lines mean the cut-off value calculated from each control on same microplate: cut-off value=mean antibody titer of control + 3 SD. Arrows indicate the times of immunization.

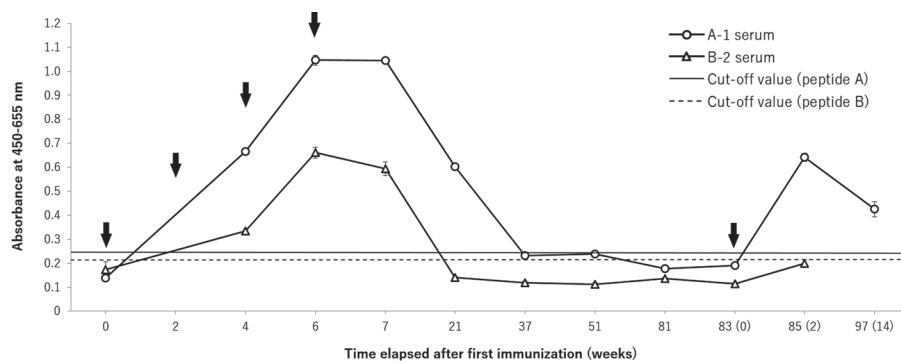


Fig. 2. Durability of antibody titers (absorbance at 450–655 nm; mean \pm SD) in mongoose following immunization with peptide A (A-1) and peptide B (B-2) and degree of immune memory against reimmunization with each peptides. The cut-off value was calculated from control on each peptide A (horizontal solid line) and peptide B (horizontal broken line): cut-off value=mean antibody titer of each control + 3 SD. Arrows indicate the times of immunization.

RESULTS

Comparisons of ZP3 homology between mongoose and the other animals

Decoded base sequence of mongoose ZP3 reached 1,278 bp and it is assumed to be translated into a 426 AA sequence. The homologies between the mongoose ZP3 sperm-oocyte binding site and the ones from cat, dog, stoat, mouse and human were 47.8, 60.9, 60.9, 17.4, and 34.8%, respectively.

Antigenicity of the synthetic peptides

Both groups of animals treated with synthetic peptides showed a higher antibody titer than the pre-immunized serum or control animals (Fig. 1). In particular, animals in group A showed a significantly higher antibody titer, notwithstanding some individual variation, which increased mostly according to immunization (Fig. 1a). On the other hand, the antibody titer in group B was generally positive, but lower than that in group A; animal B-3 displayed a temporal decrease in the titer, which was less than the cut-off value (Fig. 1b).

Longevity of the antibody titer and intensity of the immune memory

Blood samples were continuously collected over time from two animals followed-up for their antibody titer (A-1 and B-2), which exhibited the higher titer in each group. The antibodies' longevity and the reaction against single reimmunization are shown in Fig. 2. The antibody titer of both animals gradually declined after the last immunization and became lower than each cut-off value, and stabilized subsequently at a low value. The antibody titer of the A-1 serum lasted for at least 21 weeks from the first immunization; whereas, that of the B-2 mongoose lasted for at least 7 weeks.

Regarding the immune memory, the degree of the increase in the antibody titer against single reimmunization after the antibody became stably low was evaluated. The inclination of the A-1 antibody titer to increase by reimmunization corresponded to two

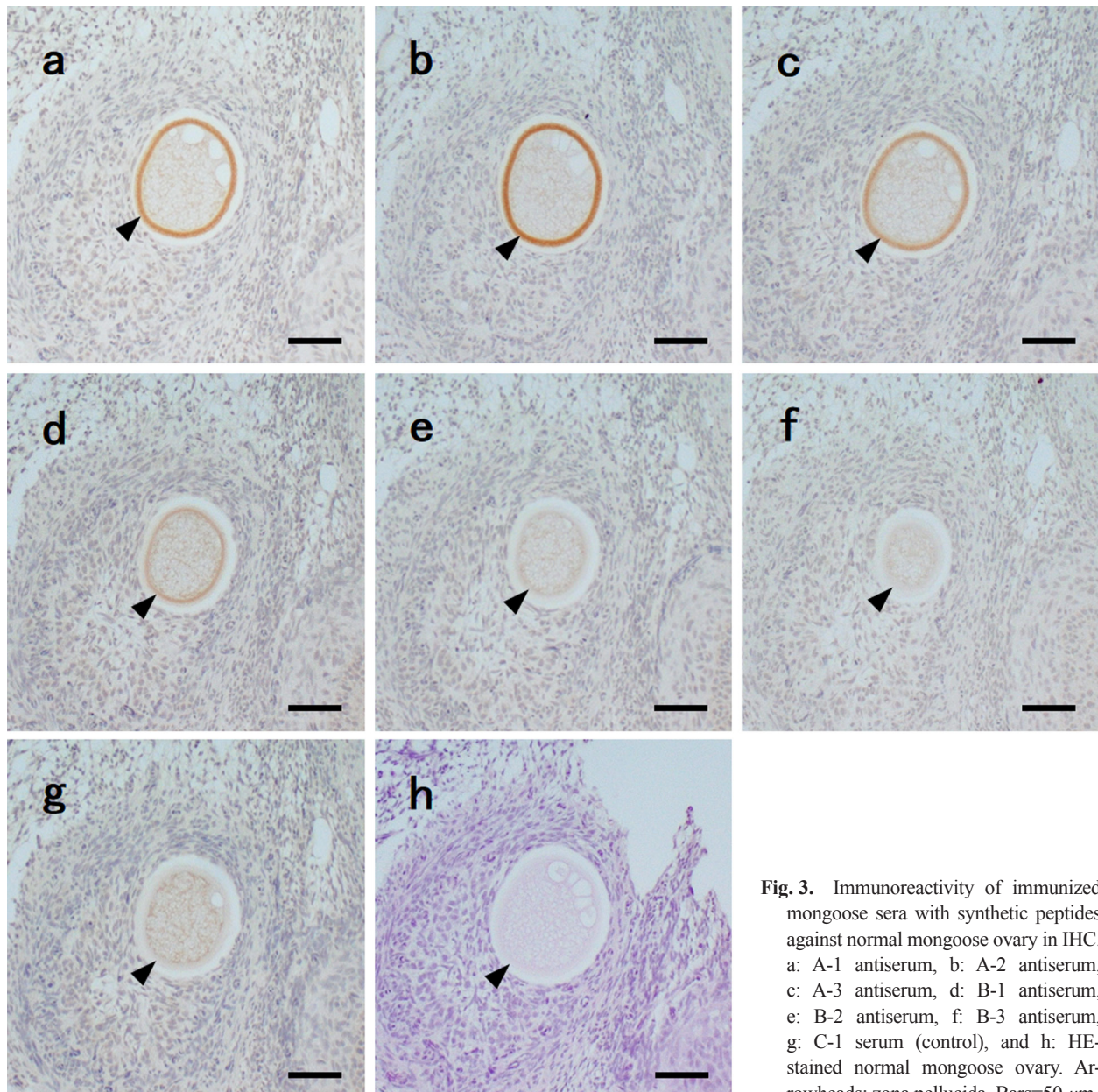


Fig. 3. Immunoreactivity of immunized mongoose sera with synthetic peptides against normal mongoose ovary in IHC. a: A-1 antiserum, b: A-2 antiserum, c: A-3 antiserum, d: B-1 antiserum, e: B-2 antiserum, f: B-3 antiserum, g: C-1 serum (control), and h: HE-stained normal mongoose ovary. Arrowheads: zona pellucida. Bars=50 μ m.

times that of the former four times of immunization. The B-2 antibody titer only slightly increased against the reimmunization, but remained under the cut-off value.

Histological findings of immunized mongoose ovaries

No infiltration of inflammatory cells nor defections were observed in HE-stained ovary tissues in the experimental animals compared with control ovaries (data not shown).

Antigen recognition by the antibodies

Each sera containing antibodies induced by ZP3 peptides were tested as the primary antibody by IHC. Based on the results, ZP-specific positive reactions were observed in the serum of both experimental groups, in comparison with serum from the control animals (Fig. 3). Marked positive reactions were observed in the A-2 serum (Fig. 3b), while the A-1 and A-3 sera showed a moderate positive reaction (Fig. 3a, 3c). The sera of animals immunized with peptide B showed a negative to mild positive reaction (Fig. 3d–f). The positive reactions were observed at every stage of follicles containing ZP, namely the secondary, antral, and atretic follicles (Fig. 4). Because of the nature of this experimental condition, especially in ovary blood vessels, diffused nonspecific reactions were observed; however, these were minimal to ignorable compared with the positive reactions.

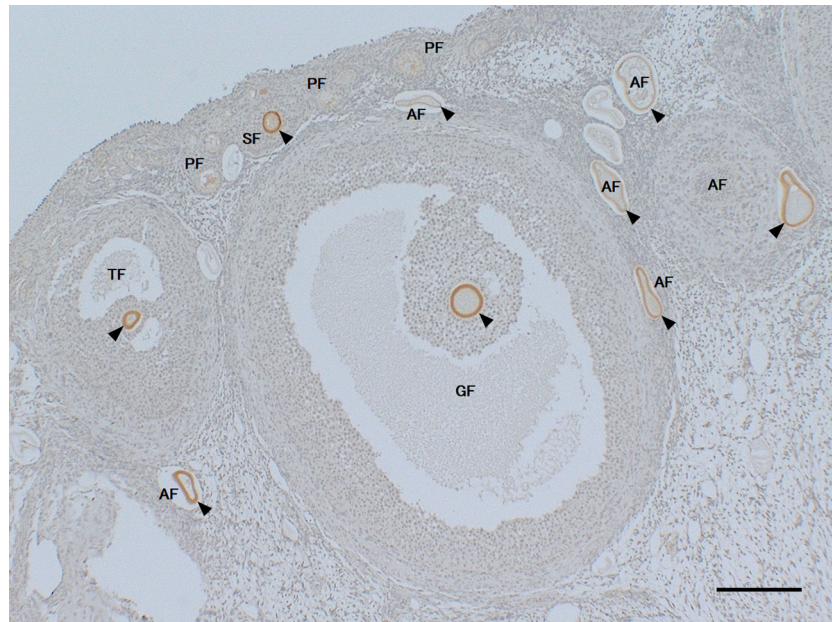


Fig. 4. Immunoreactivity of the A-2 mongoose antiserum against zona pellucida of various stages of follicles. PF: primary follicle, SF: secondary follicle, TF: tertiary follicle, GF: Graafian follicle, and AF: atretic follicle. Arrowheads: zona pellucida. Bar=100 μ m.

Specificity of antigen-antibody reaction

To confirm the specificity of the antigen-antibody reaction, sera absorbed with the peptide were tested using IHC. Based on the results, the positive reactions disappeared or decreased in the absorbed sera (Fig. 5). The A-2 serum showed the strongest reaction in IHC, and, when co-incubated with peptide A, lost the specific positive reactions with ZP, but not the nonspecific reactions (Fig. 5a, 5b). In the group B, the positive reactions did not disappear completely, but became weaker in the absorbed serum than in the non-absorbed serum (Fig. 5c, 5d).

Species-specificity of induced rabbit antibodies

Antigen-antibody reactions were observed in each serum sample derived from rabbits immunized with peptide A or B, and the reactions were specific to mongoose ovary (Fig. 6). In the mongoose ovary section, rabbit antibodies bound to the ZP. The reaction did not depend on the stage of follicles (Fig. 6a). The positive reaction was remarkable in rabbit sera immunized with peptide A than in those immunized with peptide B (results not shown).

DISCUSSION

According to the ELISA results, the immune response to the synthetic peptides A and B was confirmed in mongooses. Since peptides without KLH conjugation were used as a coated antigen, the induced antibodies were considered as the specific reactions against each peptide rather than the carrier protein. The capacity of peptide A to induce antibodies seemed to be superior to that of peptide B, even though these two synthetic peptides were suspected to include a common epitope sequence. However, there is a possibility that another epitope region in peptide A may be relatively excellent in the antigen presentation capacity of the immune system of mongoose.

The longevity of the antibody titers was at least 21 and 7 weeks in A-1 and B-2, respectively. Moreover, peptide A exhibited a longer antigenicity than peptide B. Abe (1995) reported that the life span of small Indian mongoose is 2 years or less in Amami-oshima Island [1], while the female estrus season spans mainly from March to August and generally occurs at 1 year of age [37]. Considering these reproductive biological characterizations, a single immunization by an 5-month lasting vaccine in spring would be applicable to keep the antibody titer for a most of breeding season. However, it must be noted that these results occurred after multiple direct immunizations of an antigen peptide amplified antigenicity and not suggested longevity of fertility suppressive effects furthermore in the oral vaccination. Prolongations of the lifespan and/or the breeding season were also reported in some species treated with contraceptive vaccines [22, 41, 50]; hence, the effect of increased longevity and breeding season on the duration of the contraceptive effect would have to be considered.

We did not observe any significant results in the immune memory test to be able to judge whether the immune memory had been induced. It is worth noting that the induction of immune memory is nonessential but helpful to solid fertility control. The immune memory may be improved by variations of the vaccination route or by additional modification of the antigen agents [13].

Although not shown, no inflammation or other histological disorders were observed in the ovary tissues of all treated animals.

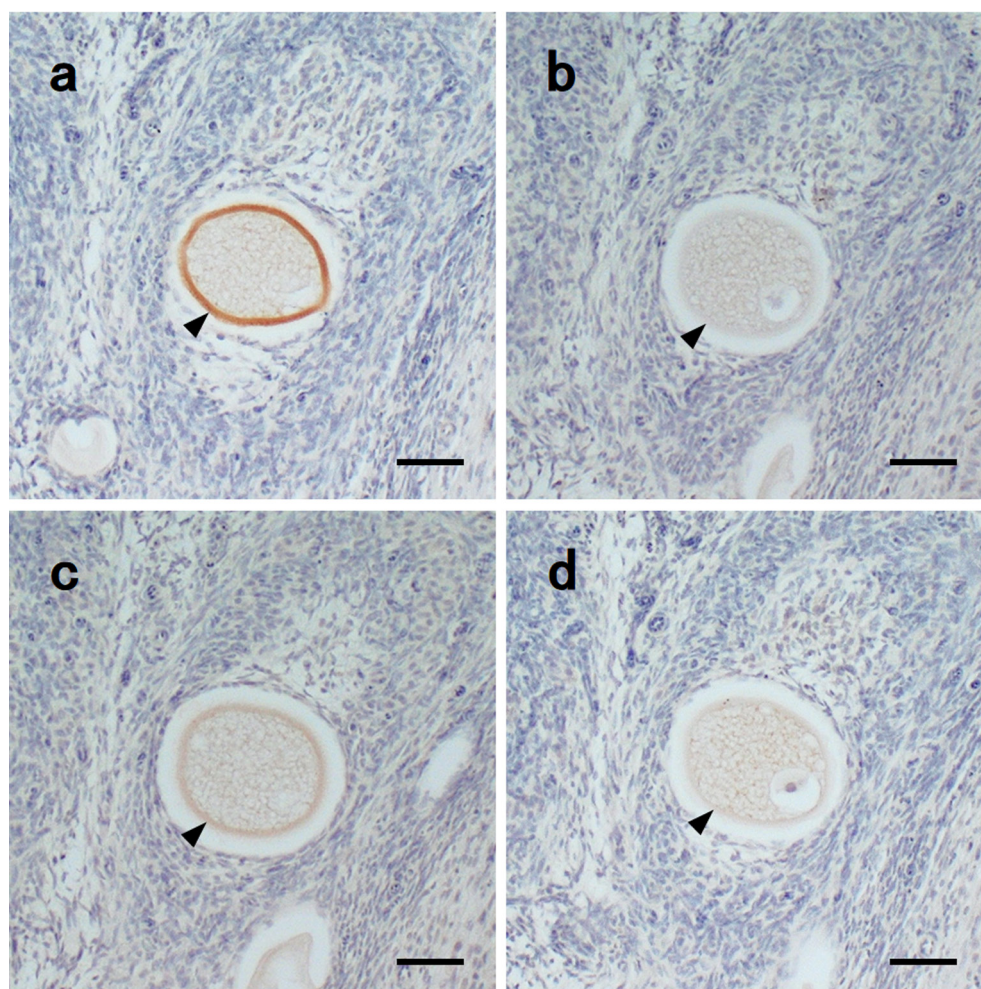


Fig. 5. Immunoreactivity of immunized mongoose sera with synthetic peptides and absorbed sera with each peptide against normal mongoose ovary in IHC. a: A-2 antiserum, b: A-2 antiserum absorbed with peptide A, c: B-1 antiserum, and d: B-1 antiserum absorbed with peptide B. Arrowheads: zona pellucida. Bars=50 μ m.

The peptides evaluated in this study did not seem to affect the ovary or oocytes histologically, although previous studies have indicated the presence or absence of ovaritis [7, 12, 20]. The correlation between ovaritis and contraceptive effect is still unknown, while absence of disorders is important from the perspective of animal welfare [21, 39, 41]. Although considered invasive alien species, the pain attendant on treatment should be minimized [40].

It is well established that ZP consists of three to four types of glycoproteins containing a ZP module (ZP 1–4); it is synthesized by oocytes from the secondary follicle period and is post-translationally modified with a sugar chain [2, 4]. Furthermore, ZP3 is a principal glycoprotein that is implicated in the primary binding of sperms and has a high species-specificity [2, 4]. Antibodies induced by treatment with synthesized ZP3 peptides bound to mongoose ZP, and recognized ZP at every stage of follicles forming ZP. Moreover, these antigen-antibody responses were specific based on the experiment using antibodies absorbed with antigen peptides. These results indicated that the exogenous antigens stimulated a specific *in vivo* response to endogenous ZP proteins in the mongooses, and that the ZP3 epitope regions targeted by the antibodies are expressed from an early ZP stage, which is unsusceptible to its maturation. Ideally, control sera in the absorption test should be co-incubated with unrelated peptide to immunization. However, all sera were diluted with a blocking solution that contained more miscellaneous proteins. Thus, the possibility that the peptide solution impeded the antigen-antibody responses nonspecifically would be rejected.

Table 1 summarized the results of the ELISA and IHC assays. There was a general correlation between the treated groups in the strength of the reaction in both assays, within group variation; for example, the A-1 serum showed the highest antibody titer in ELISA and the second strongest response in IHC. These discrepancies may be due to the differences of the target antigen or the other conditions between ELISA and IHC. The combination of tissues and primary antibodies originating from the same tissue species, i.e., mongoose-mongoose, should be avoided in IHC. The secondary antibodies also bind to endogenous immunoglobulin, which results in nonspecific reactions [11]. However, immune sera had high specificity to mongoose ZP enough. In addition, by appropriately adjusting the dilution concentration and incubation condition of the sera and secondary antibodies, the nonspecific

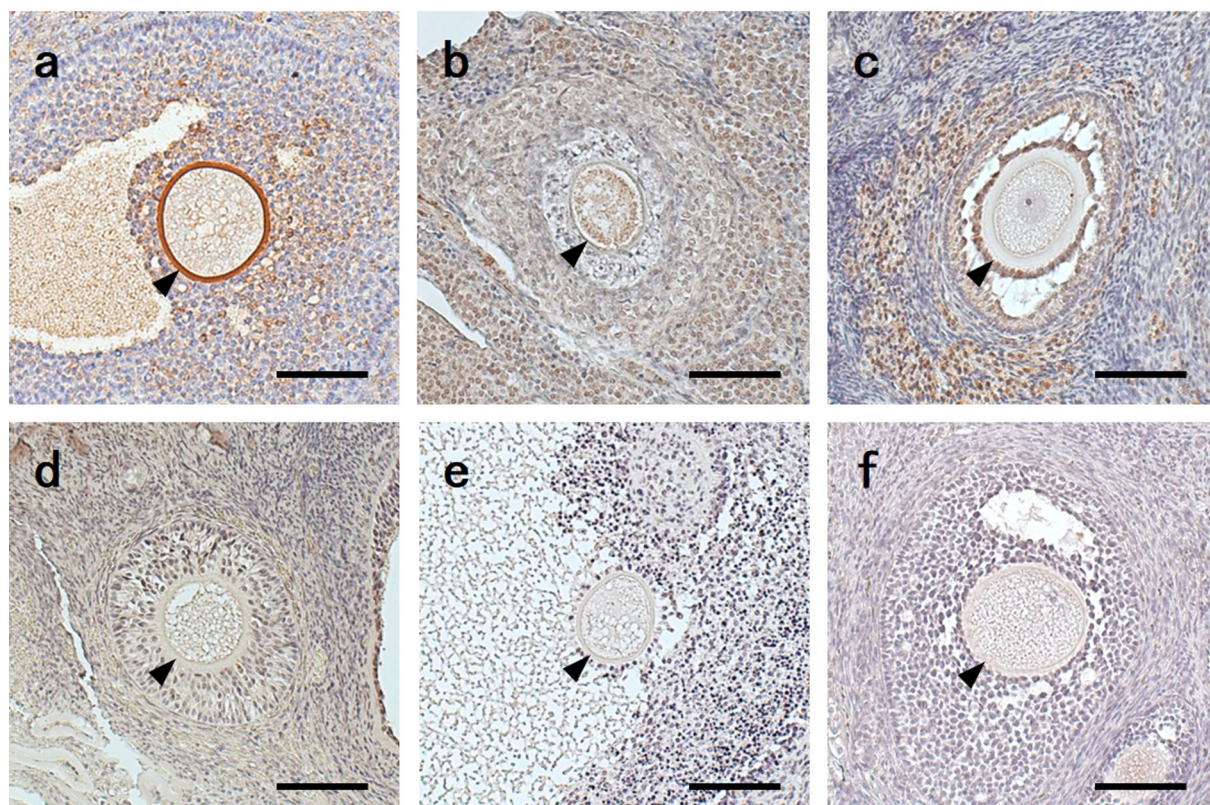


Fig. 6. Immunoreactivity of immunized rabbit sera (a-2) with synthetic peptides A against ovary of various animal species in IHC. a: small Indian mongoose, b: Japanese marten, c: cat, d: dog, e: Japanese wild boar, and f: rabbit. Arrowheads: zona pellucida. Bars=50 μ m.

Table 1. Summarized the results of ELISA and histological analysis

Group name (treatment)	Individual No.	Antibody titer (absorbance at 450–655 nm)		Reactivity in IHC	Pathological findings
		Pre-immuned	Immuned (4 times)		
Group A (peptide A)	A-1	0.231 \pm 0.017	1.922 \pm 0.067	++	ND
	A-2	0.242 \pm 0.021	1.548 \pm 0.079	+++	–
	A-3	0.244 \pm 0.011	1.056 \pm 0.052	++	–
Group B (peptide B)	B-1	0.208 \pm 0.004	0.637 \pm 0.012	+	–
	B-2	0.229 \pm 0.013	0.689 \pm 0.001	\pm	ND
	B-3	0.262 \pm 0.026	0.442 \pm 0.009	–	–
Group C (control)	C-1	0.257 \pm 0.004 (peptide A)/0.254 \pm 0.019 (peptide B)		–	–
	C-2	0.234 \pm 0.010 (peptide A)/0.239 \pm 0.005 (peptide B)		–	–
	C-3	0.253 \pm 0.014 (peptide A)/0.288 \pm 0.029 (peptide B)		–	–

+++; marked, ++; moderate, +; mild, \pm : mild to negative, –; negative, ND: no data.

reactions were minimized and the specific reactions were potentiated.

The synthetic peptides used in this study, especially peptide A, seemed useful as vaccine candidates. As a next step, we have to evaluate the actual fertility suppressive effect of the peptides. Only after a successful inhibition of pregnancy may we discuss whether the quantity of the antibody titer is effective or not. Although it has not been explained completely, the actual inhibitory pathway for fertilization by immunocontraception is assumed to be the immune response against the oocytes in follicles and/or ovulated [20]. Nevertheless, there is likely to be a correlation between the antibody titer and the fertilization suppressive effect. However, a previous immunocontraceptive study in mice has reported no correlation between them [12].

Immunized rabbit sera with the same synthetic peptides showed mongoose-specific responses in IHC using ovary tissues of various animals, including rabbit. This result may mean a certain species-specificity of synthetic peptides. However, actual specificity of contraceptive effect should be mentioned after it has been proved that these synthetic peptides do not affect any animals other than mongoose. On that account, it has to be confirmed by the vaccination of synthetic peptides to various species; especially sympatric animals with mongoose in Japan. Rare species that are difficult to use for experiments are included within

them. So, there is a necessity for obtaining a suitable permission or using some experimental animals as a substitute for them.

Remarkable differences in the immune response between animals were not observed in this small-scale study. However, the individual differences in the immune response possibility have a negative impact on the fertility control in the mongoose population. Selection pressure will be applied on the mongooses' acquired resistance to the fertility suppressive effects of contraceptive vaccines, which can lead the dominant character of the mongoose population to vaccine resistance [42]. To avoid such "adverse effects of immunocontraceptive vaccines", we have to identify the frequency of appearance of individuals potentially resistant to the fertility suppression and estimate the population suppressive effects of contraception under various patterns [42, 44].

To deliver an immunocontraceptive vaccine to free-roaming wildlife animals, which are particularly difficult to access, remote methods for vaccination (vaccine delivery systems) are necessary [43]. Some oral vaccine delivery agents have been already devised for immunocontraceptive vaccines (e.g., variety of virus vectors, bacterial ghosts, and liposomes) [5, 47, 48]. Particularly in Japan, the diffusion of recombinant biological vectors into the field is prohibited legally. Therefore, no proliferative delivery systems such as bacterial ghosts and liposomes have the potential to be applied to control the mongoose population [6, 10, 25, 48]. These should be selected after investigating further, the compatibility with vaccines and the cost effectiveness.

ACKNOWLEDGMENTS. We are grateful to Katsushi Nakata and the other staffs of Yambaru Wildlife Conservation Center (Ufugi Nature Museum) for cooperation in this study. This work was supported by JSPS KAKENHI Grant Number 26430203, the Global Environment Research Fund (4-1401, Leader: K. Goka) of the Ministry of the Environment, Japan, 2014 and a grants-in-aid of The Inui Memorial Trust for Research on Animal Science, Japan.

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