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ANTIBODY TITER AFTER ANTI-IDIOTYPE RABIES VACCINATION WITH NANO-CHITOSAN ADJUVANT

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Abstract. *Background.* The rabies virus neutralizing antibodies titers is a public health problem in the world, including Indonesia. Rabies is zoonotic and causes death in humans with a case fatality rate of 100%. Anti-idiotype antibody (Ab2) from chicken immunoglobulin (IgY) can be a substitute antigen for the rabies virus. This research aims to study the potential of rabies vaccine based on anti-idiotype antibody (Ab2) with nano-chitosan as an adjuvant. *Materials and methods.* The production of Ab2 is derived from purified and characterized chicken immunoglobulins. Nano-chitosan is made from chitosan from shrimp shell waste. Vaccination tests were carried out on rats compared with commercial vaccines as a positive control and physiological solutions as a negative control. The characterization results of IgY Ab2 were IgY rabies with BM ~180 kDa, heavy chains (BM ~60 kDa), and light chains (BM ~30 kDa) of IgY. Nano-chitosan is less than 100 nm in size, can dissolve well, and does not cause side effects in experimental animals. *Results.* The vaccine formula uses a concentration of 0.5%, where the ratio of nano-chitosan and Ab2 is 1:1. The Ab2 concentration used was about 1000 units per mL of the Ab2 suspension. *Conclusion.* This study concludes that anti-idiotype antibodies dissolved in nano-chitosan adjuvant can induce the formation of antibodies with titers that are not statistically different from the antibody titers induced by commercial rabies vaccines. Nano-chitosan has a potential vaccine adjuvant candidate, safe to use, and can enhance the immunogenicity of an antigen applied subcutaneously. According to the results of this study, it is recommended that post-vaccination antibody titers be measured regularly: for example, every one week for six weeks after vaccination. This measurement determines the time of the peak of the antibody titer so that the time for revaccination can be determined.

Key words: *anti-idiotype, antibody, chicken immunoglobulins, chitosan, rabies, vaccine.*

ОЦЕНКА ТИТРА АНТИТЕЛ ПОСЛЕ АНТИИДИОТИПНОЙ ВАКЦИНАЦИИ ПРОТИВ БЕШЕНСТВА С АДЪЮВАНТОМ НАНОХИТОЗАНОМ

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Резюме. *Введение.* Бешенство — зоонозная инфекция с летальностью 100%. Антиидиотипические антитела (Ab2) из куриного иммуноглобулина (IgY) могут использоваться в качестве замещающего антигена вируса бешенства. Целью настоящего исследования было изучение потенциала вакцины против бешенства на основе антиидиотипических антител (Ab2) с нанохитозаном в качестве адъюванта, с оценкой уровня титра антител, нейтрализующих вирус бешенства. *Материалы и методы.* Ab2 получают из очищенных куриных иммуноглобулинов. Нанохитозан производится из хитозана панциря креветок. Оценка вакцинации проводилась на крысах в сравнении с коммерческими вакцинами в качестве положительного контроля и физиологическим рас-

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твором в качестве отрицательного контроля. Характеристики использованной вакцины: IgY Ab2 IgY против антигена бешенства с Mr ~180 kDa, тяжелые цепи (Mr ~60 kDa) и легкие цепи (Mr ~30 kDa) IgY. Нанохитозан имеет размер менее 100 нм, хорошо растворяется и не вызывает побочных эффектов у экспериментальных животных. *Результаты.* Вакцина используется в концентрации 0,5%, где соотношение нанохитозана и Ab2 составляет 1:1, при концентрации Ab2 около 1000 единиц на мл суспензии Ab2. *Выводы.* Настоящее исследование позволяет заключить, что антиидиотипические антитела, растворенные в адъюванте нанохитозана, могут вызывать образование антител с титрами, которые статистически не отличаются от таковых, индуцированных коммерческими вакцинами против бешенства. Нанохитозан является потенциальным кандидатом в качестве адъюванта вакцины, безопасен в использовании и может усиливать иммуногенность антигена при подкожном введении. Согласно результатам данного исследования, титры антител после вакцинации рекомендуется измерять регулярно: например, каждую неделю в течение шести недель после вакцинации, что позволяет определить время пика титра антител для установления времени ревакцинации.

Ключевые слова: *антиидиотип, антитело, куриные иммуноглобулины, хитозан, бешенство, вакцина.*

Introduction

Rabies is a public health problem in the world, including Indonesia. Rabies causes the death of more than 59 000 people or nearly one death every 9 minutes worldwide. In Indonesia, in the last five years (2015–2019) cases of bites of rabies-transmitting animals were reported to be 404 306 cases with 544 deaths. The incidence of rabies in Indonesia from 2020 to April 2023 was reported to be at an average of 82 634 bite cases per year. However, from January to July 2023, Indonesia witnessed 74 cases of human rabies, which were associated with suspected rabid animals among a total of 66 170 bite cases reported [26].

There were five provinces with the highest fatalities: North Sulawesi, West Kalimantan, South Sulawesi, North Sumatra, and East Nusa Tenggara. The last rabies outbreak in 2019 was reported in West Nusa Tenggara. In 2023, it is worth noting that out of the 34 provinces in Indonesia, only 8 regions are currently free of rabies. Eight free rabies provinces are Riau Islands, Bangka Belitung Islands, DKI Jakarta, Central Java, DI Yogyakarta, East Java, Papua and West Papua [26]. Rabies is zoonotic, meaning that the disease can be transmitted from animals to humans and causes death in humans with a case fatality rate of 100% [5]. There is no effective drug to cure rabies, but rabies can be prevented by vaccination.

Louis Pasteur developed the first rabies vaccine in 1885, which was made using the dried spinal cord of rabbits infected with rabies [10]. However, the presence of nerve tissue in such vaccines can cause post-vaccination neurological reactions such as meningoencephalitis, meningoencephalomyelitis, myelitis, and paralysis [24]. Over time, vaccine production technology has improved, and most rabies vaccines produced worldwide are now cell-culture vaccines. These include human diploid cell vaccine (HDCV), purified Vero cell vaccine (PVRV), and purified chick embryo cell vaccine (PCECV), all of which have elements of animal origin and human serum albumin [23]. As a result, systemic allergic reactions such as swelling and redness can occur [24]. To overcome this challenge, anti-idiotype antibodies (Ab2) can be utilized as replacement antigens in making vaccines that are free from viruses and animal components, which can cause side effects [27].

Paryati et al. reported that anti-idiotype antibodies derived from chicken immunoglobulins could be used as a substitute antigen for the rabies virus in the vaccination process. Furthermore, diagnostic kits have been successfully prepared using anti-idiotype antibodies as a substitute for antigens; therefore, it is hoped that serological examinations can be carried out without harmful infectious substances [17].

Rabies vaccination trials using anti-idiotypic antibodies carried out on rabbits and dogs showed a protective immune response against rabies with an antibody titer higher than 0.5 IU/mL serum. Still, this titer was much lower when compared with the immune response that occurred in the viral vaccine [16]. This more inadequate immune response is probably due to the smaller antigen size; therefore, it is necessary to increase the size, such as adding adjuvants.

Adjuvants are immunostimulants in one or more compounds commonly added to vaccines to increase the immune response. At least 30 different adjuvant formulas are being developed commercially and some seem to show promise for wider clinical use [19]. Adjuvants are frequently utilized when the body quickly neutralizes the antigen or is incapable of responding to antibody production. The use of adjuvants can increase the titer two times higher than without adjuvant [6]. Adjuvant use can also reduce the dose of antigen required in response to antibodies. In addition, adjuvants can balance the response of humoral and cell-mediated antibodies [12].

Chitosan from shrimp shell waste can be a candidate for adjuvant. Chitosan is non-toxic, easy to synthesize, safe to use, and able to induce an immune response by activating macrophages [3]. The administration of chitosan by inhalation and orally has been carried out and produces good effects. To date, there have been no reports on the use of intravenous chitosan. Biologically, chitosan can form a protective layer and can be properly absorbed by the body. Chitosan has a structure similar to glu-

cosamine in the extracellular matrix [17]. Chitosan can be made in nanoparticles, which can be used for drug and vaccine delivery through inhalation, orally, intravenously, and as a vector for nonviral gene delivery.

Chitosan has unit characteristics as a polymer, which is mucoadhesive or can adhere to mucosal surfaces [14]. This mucoadhesive characteristic is due to the ionic interaction between the quaternary ammonium group of chitosan and the negatively charged mucus. When attached to the mucosal surface of chitosan, it can temporarily open tight junctions between glycoprotein epithelial cells, namely, anionic sialic acid. This temporary opening allows a longer time for drug interaction and transport into the cell [3]. The process of inserting nanoparticles into cells is through a mechanism called pinocytosis. The mechanism starts with the attachment of chitosan nanoparticles to the cell. Chitosan administration can be degraded in vivo by lysozyme and glucosaminidase in animal cells [8].

Chitosan can modulate the maturation of dendritic cells to induce interferon interactions and stimulate the increased activity of T lymphocytes and B lymphocytes. This process occurs due to cGAS-STING cytoplasmic DNA sensors, which cause activity in dendritic cells. This process depends on the presence or absence of cytoplasmic DNA [13]. If chitosan can cause an increase in the activity of T lymphocytes and B lymphocytes, it is hoped that it can increase the rabies virus neutralizing antibodies titers. To date, only aluminum hydroxide adjuvant has been widely used in rabies vaccines. Other types of adjuvants such as immunostimulating complexes and Freud's adjuvant also have disadvantages because they are quite expensive and cause an inflammatory response in experimental animals. Nano-chitosan has the potential as an adjuvant that can increase immune response at an economical price. This research aims to study the potential of rabies vaccine based on anti-idiotype antibody (Ab2) with nano-chitosan as an adjuvant.

Materials and methods

Vaccine preparation. Before vaccination, pure anti-idiotype antibodies (Ab2) with characteristics of Ab2 ~180 kDa molecular weight, ~60 kDa heavy chains, and ~30 kDa light chains [15] and chitosan nanoparticles measuring less than 100 nm were prepared. Anti-idiotype antibodies with a concentration of 1000 mg/mL were mixed with a 0.5% concentration of nano-chitosan in a ratio of 1:1, made sufficiently.

Vaccination procedure in rats. A total of 27 rats were randomly divided into three groups, each group consisting of nine rats. Group 1, as a negative control group, was injected with 0.5 mL of physiological NaCl. Group 2 was injected with a mixture of 0.5 mL of anti-idiotype rabies antibody and 0.5 mL of nano-chitosan adjuvant. Group 3 was injected with 0.5 mL of commercial rabies vaccine (Nobivac rabies). The commercial vaccine used is an inactivated virus vaccine which is used to vaccinate dogs with a dose adjusted to the rat's body weight. All the groups of rats were injected three times on days 0, 7, and 14. Vaccination was carried out subcutaneously in areas of loose skin tissue: for example, at the nape [25].

The treatment procedure for experimental animals received ethical clearance from the Health Research Ethics Committee of the Faculty of Medicine, Padjadjaran University, with ethical approval No. 1114/UN6.C.10/PN/2017.

The aspect of using experimental animals in this research is to pay attention to the welfare of experimental animals, based on the Declaration of Helsinki by the World Medical Association in 1964, revised in 1975 in Tokyo, and the International Guiding Principles of Biomedical Research Involving Animals by the Council for the International Organization of Medical Sciences and WHO in 1985 with the basic principles of 3R (Replacement, Reduction, and Refinement).

This research was carried out by determining the minimum sample limit using the Federer formula, so that the average in 1 group consisted of 9 animals. After an acclimatization period of 7 days before treatment, the rats were placed in cages measuring 30 × 50 × 30 cm at a room temperature of ±26°C, proper lighting and ventilation, and the cages were ensured to be cleaned so that the rats could still move and carry out their natural behavior and were given food and drink bottles. The rats were given rat food according to the rat's body weight, 20–25 grams/day, and given drinking water of 10 mL/100 grBB/day ad libitum. To reduce pain during blood collection, anesthetics, and analgesics are given to experimental animals according to the Formulary of Laboratory Animals guidelines. After the research is complete, the rats will be euthanized using the carbon monoxide gas inhalation technique following the American Veterinary Medical Association (AVMA) guidelines [2, 4, 9, 11].

Antibodies titer examination. Two weeks following the last dose of vaccine, rat blood was obtained. For four weeks, it was collected once a week. A micropipette was used to draw blood from the retro-orbital plexus. Serum was separated from other blood components to be tested for rabies virus neutralizing antibodies titers. This examination was performed using the agar gel precipitation test (AGPT) method. AGPT composition consists of 0.8 g of agarose, 2.4 g of PEG, 40 mL of aquadest, and 40 mL of phosphate-buffered saline with a pH of 7.4. The ingredients were mixed and heated to a boil and allowed to stand for 5–10 minutes. Then, the AGPT medium was poured as much as 3 mL into a 6-cm-diameter petri dish until it became solid. It was next placed in a refrigerator at

8°C. After chilling, a well was made for rabies virus antigen and six well for blood serum of rats surrounding the antigen, as seen in Figure (see cover III).

The determination of antibody titer is based on the formation of precipitation in the form of a white line (positive result) between the antigen and serum with a specific dilution. A $\log_2$ serial dilution was carried out in this study starting from 2^0, 2^1, 2^2, 2^3, 2^4, and 2^5. If no precipitation line was formed on the AGPT test or a negative result, the antibody titer was considered zero or absent.

The limitation of this study is that the measurement of antibody titer is still using the semiquantitative method (AGPT). This test is less sensitive; hence, false-negative observations are possible.

Statistical analysis. The study commenced with data processing using the Shapiro–Wilk Test, which is generally employed for samples less than or equal to 50. The results of the normality test with the Shapiro-Wilk Test indicated that the data is abnormally distributed, as the obtained p-value was less than 0.05. Therefore, the Kruskal–Wallis Test, a non-parametric statistical test, was utilized. Additionally, the Mann–Whitney test was conducted to identify the group that had a more significant impact on increasing antibody titers. This test involved the comparison of serum rats antibody titers in a positive control group immunized with commercial rabies vaccine with a treatment group immunized with anti-idiotypic antibody vaccine and the addition of chitosan nano adjuvants.

Results

Vaccination in rats. Observations of the three groups of vaccinated rats showed that all the rats looked healthy. Their appetites did not change with normal behavior during the treatment period up to 6 weeks after vaccination. The rats' body weight increased normally. In group 2 vaccinated with anti-idiotype antibodies with the addition of a nano-chitosan adjuvant, there was no change in the skin tissue of rats at the subcutaneous injection site.

Antibodies titer examination. The results of antibody examination in rat serum since the second week after vaccination using the AGPT method are shown in Figure (see cover III). A positive reaction indicates the presence of antibodies against rabies, demonstrated by the presence of a white precipitation line between the antigen and serum.

In Table 1, the antibody titer is shown based on the formation of a precipitation line in AGPT. Based on Table 1, there are differences in antibody titers in each treatment group. The highest antibody titer was obtained in the group of rats vaccinated with the commercial rabies vaccine, followed by the group of rats immunized with anti-idiotype antibodies with nano-chitosan adjuvant and the negative control group.

Table 1. Rabies antibody levels based on AGPT examination

Sample (N = 9)	Positive control (log2)	Negative control (log2)	Ab2 + nano-chitosan (log2)
1	0	0	1
2	2	0	0
3	0	0	1
4	1	0	0
5	0	0	1
6	1	0	0
7	0	0	0
8	2	0	1
9	1	0	0
Mean	0.7778	0.0000	0.4444

Statistical analysis. The statistical tests carried out using the Kruskal–Wallis Test indicate that the use of anti-idiotype rabies serum antibody vaccine along with nano chitosan adjuvant had a significant effect on raising rats serum antibody titers. The calculated p-value was found to be 0.032 ($p \leq 0.05$), which indicates a statistically significant result. The relevant data regarding the Kruskal–Wallis Test results have been presented in Table 2.

Additionally, the Mann–Whitney test was used to determine the group that had the most significant impact on increasing antibody titers. The re-

Table 2. Kruskal–Wallis test for the variety of rabies virus neutralizing antibodies titers based on the AGPT method

Antibody titers	N	Mean Rank	Kruskal–Wallis H	df	Sig
Positive Control	9	17.44	6.912	2	
Negative Control	9	9.50			0.032*
Ab2+nano-chitosan	9	15.06			
Total	27				

Note. *p < 0.05: there is a significant difference between groups.

Table 3. The Mann–Whitney test of rabies virus neutralizing antibodies titers between groups

	Treatment group	N	Mean Rank	Sum of Rank	Sig.
Antibody titer	Positive Control	9	12.00	108.00	0.021*
	Negative Control	9	7.00	63.00	
Antibody titer	Positive Control	9	10.44	94.00	0.406
	Ab2+nano-chitosan	9	8,56	77.00	
Antibody titer	Ab2+nano-chitosan	9	11.50	103.50	0.0288*
	Negative Control	9	7.50	67.50	

Note. *p-value < 0.05: there were significant differences between groups.

sults of the Mann–Whitney Test have been presented in Table 3, which shows a significantly higher antibody titer ($p < 0.05$) in the commercial vaccine group than in the negative control group. The group of rats that received anti-idiotype antibody vaccine (Ab2) with the addition of nano-chitosan adjuvant showed a higher antibody titer than the control group ($p < 0.05$). Although the commercial vaccine group obtained the highest antibody titer, it was not significantly different ($p > 0.05$) from the antibody titer of the group of rats given anti-idiotype antibody vaccine (Ab2) with the addition of nano-chitosan adjuvant. The group of rats given anti-idiotype antibody vaccine (Ab2) with the addition of nano-chitosan adjuvant showed a higher antibody titer than the control group ($p < 0.05$).

Discussion

Observations in the vaccinated rats showed no physical changes, and the rats gained normal body weight. This condition indicates that there is no treatment effect on the physical health and behavior of experimental animals. The skin area where the injection was given did not show any changes, and there was no inflammation or signs of allergy, indicating that the vaccine given was safe and had no adverse effects.

In accordance with Figure on the results of the AGPT test, it shows a homologous reaction between rat antibody and rabies virus antigen, which is indicated by the presence of a precipitation line. These figures indicate that anti-idiotype (Ab2) antibodies can induce the formation of antibodies in rat that can recognize and bind homologously to the rabies virus. The AGPT test is a serological test with the appropriate antigen deposition technique for antibodies. The AGPT test is semiquantitative and less sensitive. A low antibody titer will be difficult to visually observed because it does not produce a clear line of precipitation.

Roitt and Delves stated that precipitation is a secondary reaction due to the primary interaction between specific antigens and antibodies [21]. Specific antigen-antibody interactions entail various noncovalent interactions between the antigen determinant, the antigen epitope, and the hypervariable regions of the antibody molecule [20].

The specific reaction between rats serum and anti-idiotype antibodies indicates that the rats serum has formed antibodies similar to or similar to rabies antibodies and can bind specifically with the original antigen [22]. It suggested that the anti-idiotype antibody contains an internal image that can induce a specific antibody against the original antigen. Anti-idiotype antibody vaccines can mimic the structure of the antigen so that it can be used as an immunogen.

Macrophages will phagocytose the antigen, but the process of antigen destruction in the macrophages is slowed down due to the addition of adjuvants. The antigen is gradually released to prolong exposure to antigens and the immune response and can intervene in selective immune systems: for example, T cells and B cells. Furthermore, antigens phagocytized by macrophages will be exposed to T lymphocytes via the major histocompatibility molecules complex class II [22]. The introduction of antigens by the presenter cells causes T lymphocytes to be activated, which triggers the process of active proliferation and differentiation from other T lymphocytes in lymphoid tissue [20].

Other T lymphocytes are activated and secrete protein mediators called cytokines. Cytokines act on other cells of the immune system. The cytokines secreted by T lymphocytes include IL-2, IL-3, IL-4, IL-5, IL-6, and other factors that play a role in the response of B lymphocytes4. Activated B lymphocytes then proliferate and differentiate into mature plasma cells and produce antibodies that are rapidly secreted into lymph fluid and transported into the blood circulation [18, 19].

Table 1 shows that there are differences in antibody titers between treatment groups. The highest antibody titer was obtained from a group of rats vaccinated using an anti-idiotype antibody vaccine mixed with a nano-chitosan adjuvant. Chitosan has unit characteristics as a polymer, which is mucoadhesive or can adhere to mucosal surfaces. The characteristic of this adhesive mucus is due to the ionic interaction between the quaternary ammonium group of chitosan and the negatively charged mucus. When attached to the mucosal surface, chitosan can temporarily open tight junctions between epithelial glycoprotein cells, namely, anionic sialic acid. This temporary opening allows a longer time for drug interaction and transport into the cell [1].

Chitosan can modulate dendritic cell maturation to induce interferon interactions and stimulate increased activity of T lymphocytes and B lymphocytes. This process occurs due to cGAS-STING cytoplasmic DNA sensors, which cause activity in dendritic cells. This process depends on the presence or absence of cytoplasmic DNA. Because chitosan can increase the activity of T lymphocytes and B lymphocytes, it is hoped that it can increase the rabies virus neutralizing antibodies titers [8]. The addition of nano-chitosan adjuvant can increase growth stimulation and antibody formation by B lymphocytes so that antibody levels can increase rapidly in the body.

Conclusion

This study concludes that anti-idiotype antibodies dissolved in nano-chitosan adjuvant can induce the formation of antibodies with titers that are not statistically different from the antibody titers induced by commercial rabies vaccines. Nano-chitosan has a potential vaccine adjuvant candidate, safe to use,

and can enhance the immunogenicity of an antigen applied subcutaneously.

According to the results of this study, it is recommended that post-vaccination antibody titers be measured regularly: for example, every one week for six weeks after vaccination. This measurement determines the time of the peak of the antibody titer so that the time for revaccination can be determined. To enhance the precision of antibody titer measurements, it is advisable to employ more sensitive assays, such as the enzyme-linked immunosorbent assay (ELISA).

The use of nano chitosan as an adjuvant in the concept of rabies vaccination based on anti-idiotype antibodies is worth considering because it can increase antibody titers and is safe for humans. Nevertheless, additional research is necessary to fully understand the scope of its efficacy and safety.

Additional information

Availability of data and materials. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest. The authors declare that they have no competing nor financial interests.

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Illustration for the article “Antibody titer after anti-idiotype rabies vaccination with nano-chitosan adjuvant” (authors: Paryati S.P.Y., Ramadhanti S., Hasan K.) (pp. 788–794)

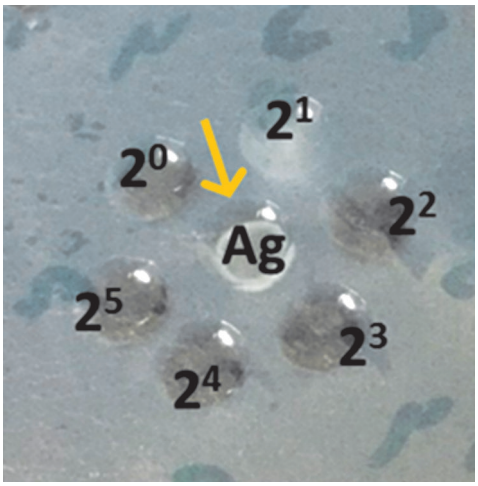

Figure. Positive reactions are indicated by the presence of a white precipitation line between the antigen (Ag, rabies virus) and antibody (serum at dilution 21) (arrow)