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THE IMMUNOMODULATOR ACTIVITY OF NANOSILVER'S INULIN BASED ON IMMUNOGLOBULIN G LEVEL OF MICE INDUCED BY MEASLES VACCINE

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ABSTRACT

Vaccination is an effort to increase a person's immunity against a disease actively. The process of the formation of antibodies by the body after vaccination requires complementary treatment for immunostimulators. Silver in the medical field developed as a therapeutic agent. When in the form of nanoparticles, it can reduce toxicity and act as an immune catalyst. Inulin acts as a bioreductant to synthesize silver because it is more environmentally friendly than physical and chemical methods. Nanosilver biosynthetic (AgNPs) using gembili inulin confirm as an antibacterial agent. This study aims to determine the immunomodulator activity of nanosilver based on Immunoglobulin G of Balb/c mice induced by the measles vaccine and the stability in 21 days. The research method was experimental in the laboratory. AgNPs biosynthesis process using inulin gembili as a bioreductant, then characterize, animal conditioning, treatment of an animals, ELISA test, and stability test of nanosilver. The data was analyzed statistically using one-way ANOVA. The results of the ELISA readings showed an increase in the Optical Density (OD) value of the nanosilver sample at a dose of 4 mg/kg BW was higher than the other treatments but not significantly different with the herbal stimulant. There is no effect of storage time on the stability of nanosilver during 21 days at a temperature of 4 °C on the maximum wavelenght shift. However, it is still in the nanoparticle size range. In addition, there is a change in the color of the solution from light brown to dark brown, which indicates that a nanosilver formation process has continued. It is necessary to confirm the acute toxicity test of the nanosilver in the future.

KEYWORDS: Immunomodulator, Inulin Gembili, Nanosilver, ELISA, Biosynthesis.

INTRODUCTION

Vaccination is an effort to actively increase a person's immunity against a disease. The measles vaccine has an efficacy of about 85%, but some children still do not have immunity and are susceptible to measles (Menkes, 2017). The process of antibody formation by the body after work requires complementary medication to help increase the body's immunity. Silver is one of the metals used for medical treatment for more than 100 years and has natural antibacterial

and antifungal properties. Silver has better efficacy in increasing the body's immunity through nanoparticles. According to Harso's research (2017), nanosilver works as a catalyst for the body's immune system to kill viruses, pathogens, and bacteria in the human body. The use of nanosilver in the right dose so as not to cause toxicity. According to Chakraborty et al. (2016), nanosilver affects mice's blood and lipid profiles. The toxicity test results showed that nanosilver with a dose of more than 8 mg/kg BW, namely doses 9 and 10, had a toxic effect on rats. This is supported by the research of Tiwari et al. (2011) concluded that nanosilver with a dose of more than 10 mg/kg BW causes liver toxicity. However, the nanosilver formation process in previous research was by chemical methods. The method of forming nanosilver with reductants from plant extracts has been developed because it is simpler, more practical, safer, and does not harm the environment. In this study, reductants from plant extract will be used to form nanosilver.

Gembili tubers (*Dioscorea esculenta* L.) are Indonesian plants that contain up to 14.8% inulin fiber (Yuniastuti et al., 2017). Synthesis of nanosilver can be carried out using polysaccharides as reducing agents and capping agents. Inulin is a polysaccharide group compound with probiotic properties, which can activate good bacteria in the digestive tract and help increase the body's immunity. Therefore, gembili inulin has the potential to be a reductant for nanosilver synthesis. The immune system activity test using the antibody titer method describes the specific immune system (Roitt, 1985). Immunoglobulin G (IgG) is the main component of serum immunoglobulin. The method used in measuring antibody titers to IgG is the Enzyme-Linked. Mechanism Immunosorbent Assay (ELISA). This study aims to determine the dose of nanosilver that can increase the immunoglobulin G titer of Balb/c mice induced by the measles vaccine compared to herbal immunostimulants. The stability of the nanosilver solution will also be evaluated within 21 days of the experimental animal treatment period.

MATERIALS AND METHOD

Materials

Material: Inulin gembili (Industrial Plalangan village, Gunungpati, Semarang, Indonesia), Balb c male mice No. 759/SKKH/IX/2021 (CV Dunia Kaca, Surakarta Indonesia), standard inulin (SIGMA Aladrich, USA), AgNO3 powder (Repacking Merck, Rofa Lab. Center Bandung, Indonesia), herbal stimulant (Dexa Medica, Indonesia) , prednisone (Phapros, Indonesia), distilled water (Repacking CV. Agung Jaya, Indonesia), whatman paper No. 1 (UK), dry measles vaccine (PT. Biofarma, Indonesia), special vaccine solution (PT. Biofarma, Indonesia), Crystalline NaCl (Merck KgaA, USA), NaOH (Merck KgaA, USA), ELISA kit consisting of Mouse IgG Standard, Standard Diluent, Biotinylated Antigen, Avidin-HRP Concentrate, Biotinylated Antigen Diluent, Avidin HRP Diluent, Substrate Solution A, Substrate Solution B, Stop Solution, and Wash Buffer (BT Lab, China). **Instrument**: Hotplate (Maspion, Indonesia), analytical balance (US Solid Precision, US), refrigerator (Aqua, Japan), syringe 3 mL (One Med, Indonesia), micro hematocrit (Vitrex Medical, Denmark), centrifuge (Hettich, Germany), ELISA reader (Spark, Europe), incubator (Sakura EM200T, Japan), well (BT Lab, China), vortex (Barnstead, USA), sealer (BT Lab, China), ependorff micropipette (Multipette, Germany), UV-Vis spectrophotometer (Genesys 150, USA), Particle Size Analyzer (HORIBA, USA).

Methods

Sample preparation

A total of 10 grams of inulin gembili powder was dissolved in 250 mL of distilled water at room temperature and stirred until dissolved (solution a). A total of 500 mL of distilled water was heated to a temperature of 40 °C, 0.085 grams of silver powder (solution b) was added. A total of 7.0 mL of solution a was added to 36 mL of solution b.

Mixing was carried out at 60 °C for 15 minutes. An indication of the success of the biosynthesis process is a change in color from clear to brown. The solution is left for 24 hours to optimize the biosynthesis process (Ermawati et al., 2021). The optimal absorption of the nanosilver solution was measured using a spectrophotometric device at a wavelength of 300-500 nm. The wavelength is the SPR (surface plasmon resonance) nanosilver range. The nanosilver size characterization was carried out with the PSA (Particle Size Analyzer) instrument by taking 1.0 mL of colloid, the results of the biosynthesis, then putting the cuvette to be tested on the PSA (Ermawati et al., 2021).

Stability test of nanosilver solution

The stability test of nanosilver during storage time was carried out by scanning at the maximum wavelength in the SPR range of the nanosilver using UV-VIS spectrophotometer after the nanosilver was stored for 21 days with a wavelength range of 380-780 nm. The biosynthetic nanosilver was stored in a refrigerator at 4 °C, and on days 0, 1, 7, 14, and 21, a wavelength scan was performed using a UV/Vis spectrophotometer instrument (Ermawati et al., 2021).

Imunommodulatory test

The immunomodulator dose effectiveness test was carried out by grouping the test animals into two large groups: the control group and the treatment group. The minimum number of subjects is determined based on Federer's formula, namely (t-1)(n-1) ³15, t is the number of treatments. At the same time, n is the number of repetitions in each treatment (Wahyuningrum and Probosari, 2012). Obtained n^3. This study used three mice each group. The following is a table of doses for each group.

1) AgNO3 1.0 mg/KgBW mice (Amri et al., 2020)
2) Herbal Immunostimulant 19.5 mg/KgBW mice (BPOM, 2015)
3) Inulin 5 mg/KgBW mice (Ermawati et al., 2016)
4) Prednisone 3 mg/KgBW mice (Ermawati et al., 2016)
5) Aquades 25 mg/KgBW mice (Ermawati et al., 2016)
6) Nanosilver 2 mg/KgBW
7) Nanosilver 4 mg/KgBW
8) Nanosilver 8 mg/KgBW
9) Nanosilver 12 mg/KgBW

On days 7 and 14, all mice were vaccinated. Blood samples were taken on days 0, 12, and 19. Blood samples were taken through the orbital sinus vein using a microhematocrit 2.5 mL of blood from the micro-hematocrit is accommodated in Eppendorf. The blood is then allowed to stand for 15 minutes and then centrifuged at 14,000 rpm, and then serum will be obtained to be checked for IgG ELISA reader levels at a wavelength of 450 nm.

ELISA reader

Serum analysis using an ELISA reader begins with conditioning the reagents at room temperature before use. The assay procedure was carried out by adding 50 mL of standard solution into 96 wells and 50 mL of sample into 96 wells. Added 50 mL Biotinylated Antigen. Incubated for 60 minutes at 37°C. Well-washed five times using 300 mL wash buffer. Added 50 mL Avidin-HRP. Incubated for 60 minutes at 37°C. Well-washed five times using 300 mL wash buffer. Add 50 mL of substrate solution A and 50 mL of substrate B solution into the well. Incubated for 10 minutes at 37°C. Add 50 mL of stop solution. Well plates were read on ELISA with a wavelength of 450 nm for 10 minutes (Elabscience, 2021).

Data Analysis

Theoretically, the data obtained from the test results are compared with parameters from several libraries. Statistically, this experiment determined immunoglobulin G levels using the ELISA reader method. Storage stability data analysis was carried out by data normality test, Then the One-Way Anova test. If the p-value <0.05, the results are significantly different, while if the p-value is >0.05, the results are not significantly different. If the data is significantly different, a follow-up test (Post Hoc) is performed using the Least Significant Difference (LSD) method. For data that do not meet the normality test, non-parametric tests were performed using the Wilcoxon Test.

RESULT AND DISCUSSION

The biosynthesis process was carried out at 60°C for 15 minutes. The higher temperature used, affects to the smaller size of nanosilver formed (Wisnuwardhani et al., 2019). This is in line with the research by Ermawati et al. (2021), that biosynthesis carried out at a temperature of 60 °C the reaction will be completed faster, and particle size is smaller than the biosynthetic process carried out at room temperature. The nanosilver biosynthesis process with bioreactors of gembili's inulin is due to the presence of the–OH group in the extract plant (inulin) acts as a capping agent that will reduce Ag+ to Ag0, resulting in stable nanosilver and antibacterial activity (Ermawati et al., 2021). The biosynthesis of nanosilver with gembili's inulin as a bioreductor showed that the solution turned brownish. The color change of the solution to brownish yellow indicates that a nanosilver has been formed (Kaviya et al., 2018). The maximum wavelength obtained in the nanosilver at SPR range of nanosilver is 418 nm. This is following the SPR range for the solution silver nanoparticles that are between 400–500 nm (Oktavia and Sutoyo, 2021). According to Solomon et al. (2007), the absorbance value shows a trend of the amount of nanosilver produced and the reaction process as time progresses. The higher the absorbance value, the more silver nanoparticles formed. The conclusion of the test using UV-VIS spectrophotometry. It is known that the biosynthetic solution results in an indication of size nanometers. The results of the spectrophotometric analysis in this study show a sloping absorption, which is one characteristic of silver nanoparticles with spherical morphology (Kaviya et al., 2018).

The size and distribution of the nanoparticles can be measured using a Particle Size Analyzer (PSA). Characterization of nanosilver using PSA aims to determine the size distribution and particle size uniformity. A technique used in PSA is Laser Diffraction. The principle of PSA is the dissipation of laser light by dispersed particles passing through the beam laser. The Z-Average is the average diameter of the particle distribution; the narrower the curve formed, the better the result (HORIBA, 2017). Based on nanosilver analysis has a Z-average value of 481.4 nm. The results show that the nanosilver particle size is included in the size range of nanoparticles, which are solid colloidal particles with a diameter of 1-1000 nm. The Polydisperse Index (PDI) value obtained from this analysis is 0.544. The polydisperse index describes the particle size distribution. The smaller value of polydispersity indices, the more uniform the particle size (Yuwono et al., 2015). PDI value >1 exhibits an extensive size distribution and contains particulate matter large or aggregates that can experience sedimentation, while a PI value <0.7 indicates a uniform or homogeneous particle size (Nurviana et al., 2020).

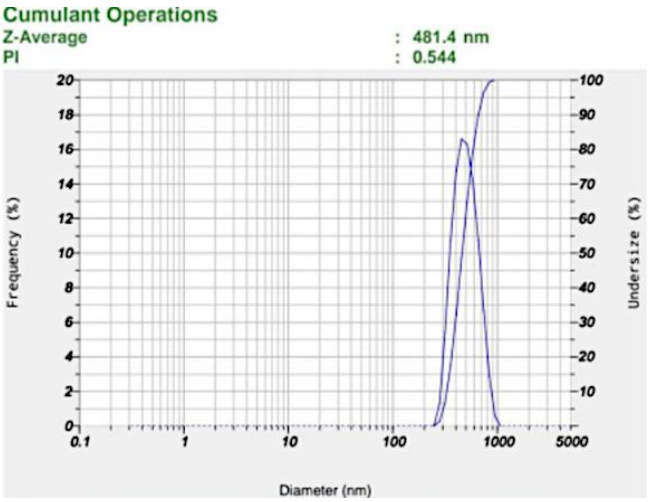

Figure 1: Results of particle size and polydisperse index of nanosilver biosynthetic.

The immunomodulator test of nanosilver aims to determine the nanosilver dose that provides an immunomodulatory effect. Vaccines act as an antigen that can stimulate an immune response humoral through the production of antibodies that provide immunity (Radji, 2009). Vaccination was carried out twice during the treatment period. It is estimated that B cell sensitization has formed, proliferated, differentiated, and developed into plasma cells that produce antibodies. That antibodies were Immunoglobulin M (IgM) and Immunoglobulin G (IgG). IgM is the first Immunoglobulin produced as an immune response to an antigen, followed by a switch to the production of IgG or other classes of antibodies (Abbas et al., 2007). Blood sampling was carried out through the orbital sinus vein section at the corner of the eye because it was easier to collect and minimized the possibility of lysis (Fatimah et al., 2018). The immunomodulator test of nanosilver was carried out by the male mice Balb-C strain aged 8-12 months with a body weight of 20-25 kg, which had got ethical clearance No. 00029/04/LPPT/VIII/2021 from the Laboratory Integrated Research and Testing Gadjah Mada University, Yogyakarta, Indonesia.

Table 1: The results of OD levels of IgG before and after vaccination.

Groups	Optical Density (OD) of Imunoglobulin G (%)		
Nanosilver 2 mg/Kg bw	0.91 ± 0.06	1.17 ± 0.29	0.79 ± 0.09*
Nanosilver 4mg/Kg bw	0.97 ± 0.05	0.89 ± 0.08	1.14 ± 0.15
Nanosilver 8 mg/Kg bw	1.09 ± 0.12	0.89 ± 0.05	0.90 ± 0.09*
Nanosilver 10 mg/Kg bw	0.92 ± 0.13	0.76 ± 0.03*	0.90 ± 0.05*
Herbal immunostimulant	1.00 ± 0.06	0.76 ± 0.04*	0.98 ± 0.17
Inulin gembili starch	0.82 ± 0.11	0.84 ± 0.33*	0.80 ± 0.06*
Silver nitrate solution	0.93 ± 0.24	0.92 ± 0.05	0.77 ± 0.14*
Prednisone	0.94 ± 0.19	0.92 ± 0.10	0.89 ± 0.06*
Water	0.89 ± 0.03	0.78 ± 0.16*	0.76 ± 0.06*

***Significant difference compared with hebal immunostimulant**

The aim of the immunomodulator test of nanosilver is to determine the nanosilver dose that provides an immunomodulatory effect. Vaccines act as an antigen that can stimulate an immune response humoral through the production of antibodies that provide immunity (Radji, 2009). Vaccination was carried out twice during the treatment period. It is estimated that B cell sensitization has formed, which will proliferate, differentiate, and develop into plasma cells that produce antibodies, namely Immunoglobulin M (IgM) and Immunoglobulin G (IgG). IgM is the first immunoglobulin produced as an immune response to an antigen followed by a switch to the production of IgG or other

classes of antibodies (Abbas et al., 2007). Blood sampling was carried out through the orbital sinus vein section at the corner of the eye because it was easier to collect and minimized the possibility of lysis (Fatimah et al., 2018).

The control group was divided into five controls by five mice (AgNO3 control, distilled water control, inulin control, herbs' immunostimulant control, and prednisone control). Distilled water as a negative control, herbal immunostimulant as a positive control, and prednisone as an immunosuppressor control. The treatment group consisted of four treatments, with five animal mice in each group (nanosilver doses of 2 mg/kg BW, 4 mg/kg BW, 8 mg/kg BW, and 12 mg/Kg BB). On days 7 and 14, all mice were vaccinated. Vaccination in mice using dry measles vaccine produced by Biopharma, Indonesia. Vaccination aims to produce immunity by introducing non-toxic antigens or attenuated viruses so that the body has immunity against certain diseases.

Blood sampling was taken on the 0, 12th, and 19th days. Blood samples were taken through the sinus vein orbital using microhematocrit. Blood of 1.5 mL was collected using hematocrit and accommodated in 2.0 mL of Eppendorf. The blood was allowed to stand for 15 minutes and then centrifuged at 14,000 rpm until serum was obtained. IgG level was analyzed using the IgG ELISA Kit with ELISA reader method at a wavelength of 450 nm. Table-1 shows the results of Immunoglobulin G Optical Density (OD) before vaccination, after the first vaccination, and after the second vaccination.

Nanosilver at a dose of 4 mg/KgBB; Nanosilver dose of 8 mg/KgBB; Nanosilver dose of 12 mg/KgBB; and distilled water showed an increase in OD values. As for on treatment of Nanosilver dose of 2 mg/KgBB; inulin; AgNO3; Prednisone, and Destilled water decreased the OD value. The increase in the OD value is directly proportional with an increase in immunoglobulin G levels, the higher the OD value, the greater the immunoglobulin G level. Vice versa, the lower the OD value, the higher the level of immunoglobulin G small (Serang et al., 2019). Distilled water as a negative control significantly differed from the Nanosilver of 4 mg/KgBB. Herbal immunostimulants as the positive control showed a significant increase in OD levels of 29.04%. Herbal immunostimulants work by boosting the immune system. However, the herbal immunostimulant as a positive control was not significantly different from the Nanosilver at a dose of 4 mg/kg BW, with an increase in OD levels of 28.43%. It means that the ability of herbal immunostimulants to increase IgG titers is equivalent to Nanosilver at a dose of 4 mg/Kg BW. The first time of serum after vaccination causes a primary immune response by the appearance of IgM so that IgM production is greater than IgG. The second serum caused a secondary immune response by increased IgG production, so IgG levels were higher than IgM. IgM is formed 4-5 days after the Percentage Shift in OD Levels exposure to antigens, while IgG is formed after five days of exposure and reaches a peak 2-3 weeks after exposure (Marliana & Widhyasih, 2018). The amount of IgG nonlinear in the treatment group was due to the not maximal IgG level formed and the factor from test animals exposed to fungal skin diseases during the research process.

The One-Way ANOVA statistical analysis stated that the significant value was <0.05, so the analysis was continued in the Post Hoc test with the LSD method. The Post Hoc Test with the LSD method aims to see a significant difference in each group. The results of the LSD Post Hoc test showed that there was a significant difference between the Nanosilver at a dose of 4 mg/KgBB group with the other controls, but not significantly different with the immunostimulant herbal control.

Table 2: The results of stability test of gembili's nanosilver based on maximum wavelength shift for 21 days.

Replication	Maximum wavelength shift (nm)				
	Day-0	Day-1	Day-7	Day-14	Day-21
1	410	410	412	418	418
2	410	410	412	418	418
3	410	412	418	418	420

The stability test of nanosilver during storage was carried out by measuring the maximum wavelength shift. The test was carried out while the nanosilver solution was stored for 21 days. Nanosilver was stored in a refrigerator at 4 °C, and on days 0, 1, 7, 14, and 21, the absorbance was scanned using a UV/Vis spectrophotometer. The maximum wavelength measurement results show that during 21 days of storage, the λ max position of the nanosilver shifted about 2 nm. Statistical analysis showed that the data were not generally distributed from the normality test, so further analysis was carried out using the Wilcoxon Test. Based on the results of the Wilcoxon Test, the opposing ranks on days 0, 1, 7, 14, and 21 were 0, namely the value of N, mean rank, and a sum of ranks. A value of 0 indicates no decrease in lambda values from day 0 to day 21. Based on the statistical analysis output results, Asymp. Sig (2-tailed) has a value of 0.001, meaning there is a difference between the maximum wavelength on days 0, 1, 7, 14, and 21. This is not because the nanosilver is unstable but because the biosynthesis process is still ongoing. According to Mulfinger et al. (2007), the absorbance value shows a trend in the amount of nanosilver produced, and the reaction process increases with increasing time. The higher the absorbance value, the more nanosilver is formed. During the stability test for 21 days, there was a change in the color of the solution from light brown to dark brown. The nano silver solution did not form aggregates during 21 days of storage (Figure 2b), meaning that the nanosilver solution was stable during storage at low temperatures.

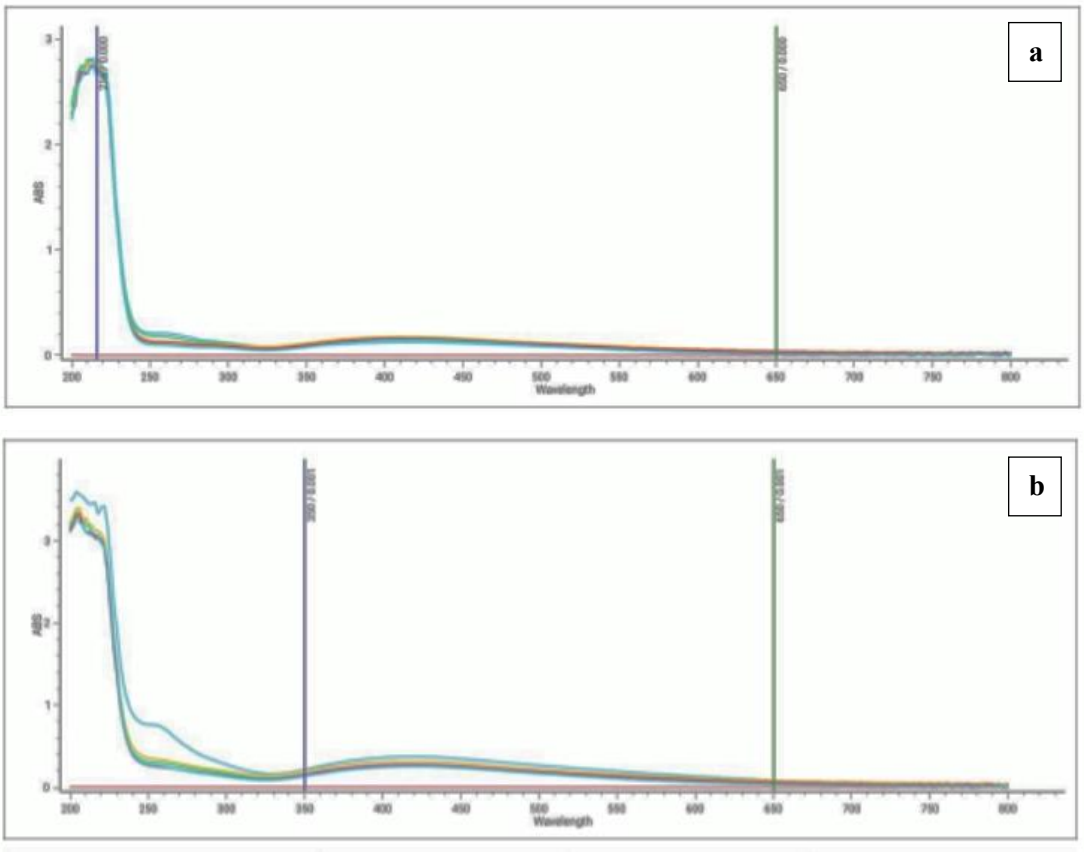

Figure 2: Results of nanosilver solution stability test based on the maximum wavelength shift.

CONCLUSION

In this study, the effective dose of nanosilver using bioreductor gembili's inulin as an immunomodulator was 4 mg/Kg BW. This dose was not significantly different from the herbal immunostimulant control. That dose can increase the mice's immunoglobulin G levels equivalent to herbal immunostimulants. The stability of the nanosilver solution is affected by the storage time. During the 21-day storage period at 4 °C, there was a shift in maximum wavelength on days 0, 1, 7, 14, and 21, but still in the nanoparticle size range. In addition, there was a change in the color of the solution from light brown to dark brown.

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