

Humoral immune response of Swiss mice after immunization with E-selenium adjuvated and non-adjuvated locally prepared Vero-cell rabies vaccine

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ABSTRACT

The immune response pattern of Swiss mice immunized intramuscularly using both E-selenium adjuvated and non-adjuvated locally prepared Vero-cell rabies vaccine was able to elicit antibody levels more than the WHO-recommended level of less than 0.5 IU/dose. Two different immunization regimes were applied, 5 single and 3 double doses. The antibodies developed against rabies vaccine could be detected 14 days after immunization using ELISA and indirect immunofluorescence. The highest antibody level was detected 10 to 12 wks after immunization using the 5-single-dose adjuvated vaccine, recording antibody levels of the order of 1/1024. The highest antibody levels using 3 double doses of adjuvated vaccine, or 5 single doses of non-adjuvated vaccine, recorded an antibody titre of the order of 1/512, maintained during the 8th to 10th wks post immunization. The lowest recorded IF antibody level was using 3 double doses of non-adjuvated vaccine, with an antibody titre of the order of 1/128 during the 8th week post immunization. The antibody levels developed in the sera of mice immunized with rabies vaccine, either adjuvated or non-adjuvated, whatever the regimen, was able to induce protection in mice against 100 LD₅₀ of the challenge virus standard for at least 6 months.

KEYWORDS: rabies vaccine, vitamin E, antibody, ELISA, immunofluorescent assay, Challenge Virus Standard.

INTRODUCTION

Rabies is probably one of the oldest recorded infectious diseases to mankind. The development of the first rabies vaccine by Louis Pasteur by rights should have led to the elimination, or at least reduction of rabies incidence. However, this goal has not been achieved yet because rabies is maintained in many animal reservoirs, including both domestic and wild animals (Poltkin 1996). Many attempts have been made to produce cheap and safe rabies vaccine in different tissues, and the use of human-origin substrate cell line has been successful (Meslin & Kaplan 1996). Other cell-culture vaccine candidates are inactivated, concentrated and purified vaccines; these vaccines evoked much better immune response in animals and humans than that induced by duck embryo vaccine (DEV) and suckling or adult brain tissue vaccines. The antibody levels are higher and appear earlier after immunization with cell-culture vaccine than those induced post DEV (Wiktor 1973; El-Karamany 1987; El-Hariry 1998).

Selenium is an essential trace element; toxicity is seen only at very high levels. Selenium is an essential component of glutathione peroxidase, a seleno-enzyme that catalyzes the reduction of lipid hydroperoxides to the corresponding alcohols or the conversion of H₂O₂ to water. Consequently, selenium with glutathione peroxidase indirectly provides a line of defence against free radicals before they propagate in a chain reaction, damaging membranes and cell components. Selenium and vitamin E act synergistically in defence mechanisms against residual oxygen metabolites (ROM) and for protecting neutrophils and surrounding tissues (Boxer 1986). Vitamin E and selenium act coherently to reduce the body's requirements for each other and reinforce each other in their action against lipid peroxides. Vitamin E, as an antioxidant, appears to be the first line

of defence against peroxidation of fatty acids contained in cellular and subcellular membranes. Vitamin E acts as a breaker of free radical chain reactions because of its ability to transfer hydrogen to peroxy free radicals of peroxidized fatty acids

The role of trace elements in maintenance of immune-system functioning is increasingly important under increased conditions of natural-killer (NK) activity, cytotoxic T-cell activity, interleukin-2 (IL-2) receptors expression-cell proliferation and lymphokine-activated killer-cell activity. The interaction of trace elements and immunity is a complex process, because it is frequently associated to other nutritional deficiencies. α -tocopherol (vitamin E) and selenium as antioxidants work in concert although each element has different functions and pathway in the immune system. Their deficiency causes dysfunction of cellular immunity without affecting B-cell function, impairs the immune system's ability to fight infections, and reduces infectious morbidity (Pollock *et al.* 1994; Girodon *et al.* 1999; Kodama 1996). Vitamin E was used successfully as an adjuvant for vaccinating some species by enhancing the immune response in poultry, sheep, and mice as compared with the response obtained with the use of conventional adjuvants without abscess formation (Afzal *et al.* 1984; Franchini 1991).

The present work aims to evaluate the immune reactivity of Swiss mice to locally prepared Vero-cell rabies vaccine adjuvated to combined vitamin E and selenium as an immune-stimulating agent, as assessed by antibody levels. An evaluation of the efficacy of the proposed vaccination regimen as compared to that recommended by the World Health Organisation (WHO) vaccine committee is attempted.

MATERIALS AND METHODS

Vero-cell rabies vaccine (Aventis Pasteur) and E-selenium containing 50 mg kg⁻¹ - 300 IU/ml were kindly supplied from the National Control Authority laboratories, Biological Product Research Unit. Antirabies immunoglobulin-G was locally prepared and evaluated in Rabies Vaccine Research Unit (VACSERA, Egypt) by immunization of 20 Swiss mice with a mean body weight of 14-16 grams using rabies vaccine: 5 doses were administered on the 0, 3rd, 7th, 14th, 28th days. Immunized mice were challenged using 50 LD₅₀. Mice were bled 15 days later through the retro-orbit plexus using a heparinized capillary tube. Blood was left at room temperature to coagulate, then refrigerated at +4°C for 18 hrs. Sera were collected after cold centrifugation (Jouan GR 412-France). Anti-mouse peroxidase conjugate (Sigma-USA) was used as 1/1000 final concentration.

Potency of antirabies immunoglobulin was determined according to Hasse *et al.* (1985) as follows. The reference and test sera were inactivated for 30 minutes at 56°C. Several two-fold serial dilutions of reference and anti-mouse immunoglobulin under test, starting with 1/500, were prepared, so that the final dilution should show no evidence of neutralization. A half ml of each dilution was then transferred to a test tube. Virus dilutions were 0.5 ml, corresponding to 100-500 LD₅₀. This resulted in two-fold dilution of both virus and test sera. It is essential to include a reference serum, titrated in the same time as the unknown test sample. Test sera dilutions-virus mixtures were incubated at 37°C for 1-1.5 hrs (WHO 1996; Fitzgerald & Rastogi 1985). The virus-sera mixtures were removed from the incubator and placed in an ice-water bath. Mice groups of a mean weight of 14-16 grams were inoculated intracerebrally with a dose of 0.03 ml of each dilution, using at least 10 mice per dilution. This was done for both the test sera and control. Each group of mice was placed in a labelled cage and kept under observation for 14 days: the number of mice dying after the first five days was recorded. The LD₅₀ of test sera was calculated and subtracted from that of the reference serum and the result was then multiplied by the value of the reference IU content.

Antibody content was evaluated using direct ELISA (El-Karamany 1987; WHO 1996; Arai *et al.* 2002): purified rabies vaccine was used in an effectively protective dose (ED₅₀) 5 IU/ml (20-40 µg protein) to coat polystyrene 96-well plates (Costar-USA) in a 0.5 mol/L, pH 9.6, carbonate–bicarbonate buffer. Plates were kept at room temperature for 18 hrs. Immune sera samples were dispensed as 1/20 starting dilution in the 1st and 2nd plate row as 100 µl/well. Sera samples were two-fold diluted in a dilution buffer (phosphate buffer saline pH 7.2 ± 0.2 + 1% bovine serum albumin: Sigma-USA). Plates were incubated for 1.5 hrs at 37°C, then washed three times at 5-minute intervals using a washing buffer. Reference antibody against rabies vaccine was prepared in mice using 25 IU/ml. Anti-mouse labelled with peroxidase conjugate (Sigma-USA) was used as 1/1000 final dilution for 1 hr at 37°C and the plates washed as before. OPD (O-phenylenediamine 2 HCl) substrate (Abbott-USA) was added as 100 µl/well and the plates kept in the dark until the development of colour. A stop solution (2N sulphuric acid) was dispensed into the whole wells of plates. The optical density representing antigen and antibody reactivity was measured using the Organon Teknika system (USA) at a wavelength of 450 nm. The effective protective dose (ED₅₀) was evaluated from the equation:

$$\frac{(\text{OD of Test serum}) \times (\text{Conc. Stand. serum})}{\text{OD of Ref. serum}}$$

Antirabies immunoglobulin titre was determined by immunofluorescent assay (Hostnik & Grom 1997). CVS-infected BHK₂₁-cultured cells were dissociated from the surface of cell culture flask using a proteolytic enzyme (trypsin, GIBCO-UK). Dispensed cells were centrifuged for 10 min at 3500 rpm using a cooling centrifuge with a fixed angle rotor (Jouan GR412 –France). The cell pellet was re-suspended in phosphate buffer saline, and the cell count adjusted to 10000 cell/ml. Infected cells were dispensed on a Teflon- coated immunofluorescent glass slide at 25 µl/well. The prepared slides were kept in cold acetone for 1-2 hrs at -20 °C. Slides were loaded using two-fold diluted test sera. Positive and negative sera were included; slides were incubated in humidified chambers at 37 °C for 30 min, washed and dispensed to 0.01 M phosphate buffer saline solution at pH 7.6.

Anti-mouse immunofluorescent conjugate used as 1/100 (Sigma-USA) was dispensed into the reciprocal wells and kept at 37 °C for 30 min. Slides were washed in phosphate buffer saline three times at 5-min intervals. Slides were dipped in DD water for few seconds, dried and examined using an immunofluorescent microscope (Nikon–Japan). The final dilution of test sera showing a positive reaction was determined.

Results are represented as mean ± standard error (S.E.), and analysed via 2-tailed statistical tests using SPSS version 8. Group differences were considered statistically significant at the level of P<0.01 (Zar 1984).

RESULTS

Anti-rabies immunoglobulins developed in sera of laboratory animals immunized with locally prepared rabies vaccine on days 0, 3, 7, 14 and 28 using the single-dose regime, and on days 0, 14 and 28 using the double-dose regime. Vitamin E conjugated to selenium salts was chosen as an immunostimulant to evaluate the immune reactivity to E-selenium adjuvated rabies vaccine compared to non-adjuvated vaccine. Using the five single-dose regime, a significantly higher antibody potency level resulted from using E-selenium adjuvated rather than non-adjuvated vaccine (Figure 1). Similarly using the three double-dose regime, the antibody potency developed in sera of mice immunized with E-selenium adjuvated vaccine was higher than that detected in sera of mice group immunized using non-adjuvated vaccine (Figure 2). Comparing the two regimes using E-selenium adjuvated rabies vaccine, the five single doses developed a noticeably superior antibody potency than

the three double doses (Figure 3). The five single-dose regime of non-adjuvated vaccine showed lower antibody potency than that induced by a non-adjuvated three double-dose regime (Figure 4). There was a higher antibody potency titre elicited by the three double doses of non-adjuvated vaccine than the five single doses of adjuvated vaccine (Figure 5). Finally, the five single doses of non-adjuvated vaccine gave a lower antibody titre than that elicited by the adjuvated three double doses (Figure 6).

The immunofluorescent antibody titre was highest on the 10th to the 12th week, recording 1/1024 using five single doses of adjuvated rabies vaccine, and 1/512 with both the three double doses of adjuvated vaccine and the five single doses of non-adjuvated vaccine on the 8th to the 10th week post immunization. The non-adjuvated three double-dose regime showed the least immunofluorescent antibody titre on the 8th week post immunization, with a value of 1/128 (Table 1).

Table 1: Evaluation of antibody titre using indirect immunofluorescent assay post immunization with E-selenium adjuvated and non-adjuvated rabies vaccine

WPI*	2 WPI	4 WPI	6 WPI	8 WPI	10 WPI	12 WPI
Vac.Schedule						
Adj. -Rabies 5 - doses	1/64	1/256	1/256	1/512	1/1024	1/1024
Non-Adj. Rabies 5 - doses	1/32	1/64	1/128	1/512	1/512	1/256
Adj.-Rabies 3 - doses	1/32	1/64	1/256	1/512	1/512	1/256
Non-Adj.-Rabies 3 - doses	1/32	1/64	1/64	1/128	1/64	1/64

WPI*: week post immunization

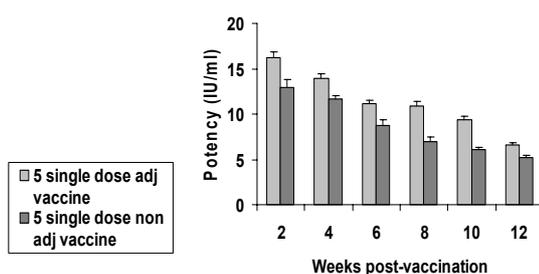


Fig.1: Evaluation of sera sample potency post-vaccination with 5 single dose regimen of E-selenium adjuvated and non-adjuvated vaccine

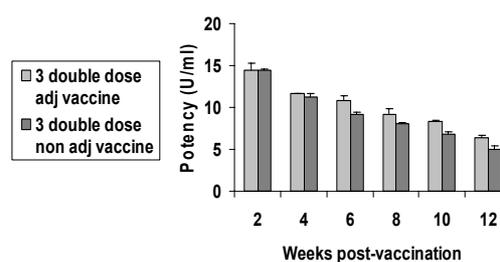


Fig.2: Evaluation of sera sample potency post-vaccination with 3 double doses of E-selenium adjuvated and non-adjuvated vaccine

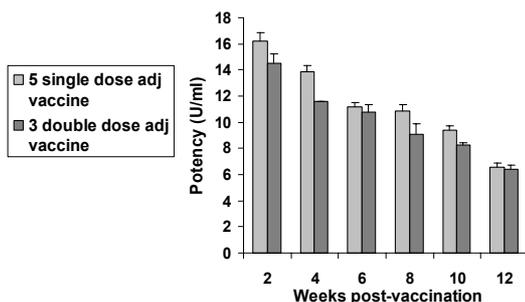
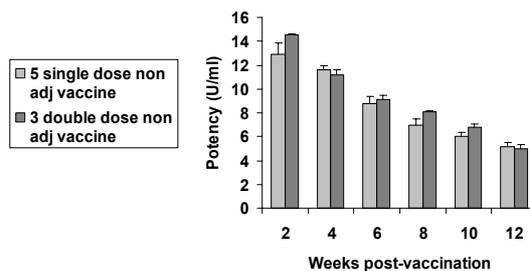
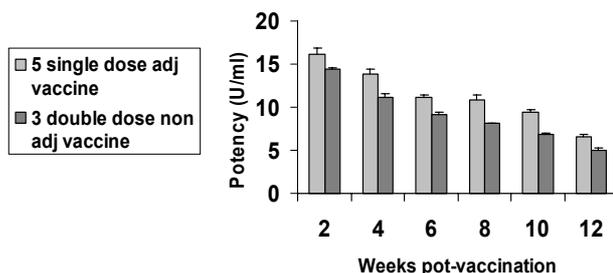


Fig. 3: Evaluation of sera samples potency post-vaccination with 5 single dose of E-selenium adjuvated vaccine and 3 double dose adjuvated vaccine using ELISA



Evaluation of sera sample potency post-vaccination with 5 single dose and 3 double dose of non -adjuvated vaccines using ELISA



Evaluation of sera sample potency post-vaccination with 5 single dose of E-selenium adjuvated vaccine and 3 double dose of non-adjuvated vaccine using ELISA

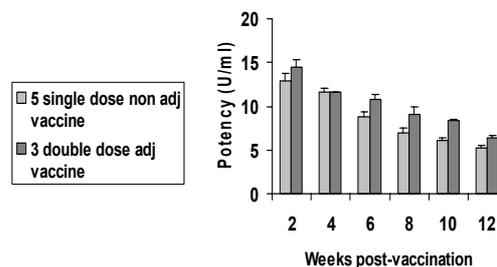


Fig.6: Evaluation of sera potency post-vaccination with 5 single dose of E-selenium of non -adjuvated vaccine and 3 double dose adjuvated regimens using ELISA

DISCUSSION

There is a growing body of research concerning the roles of trace elements (zinc, copper, selenium, and others) in immunity and the mechanisms that underlie such roles. α -tocopherol and selenium have interactive effects on the lymphocyte response to antigens, suggesting that micronutrient status is important when interpreting results of in vitro lymphocyte function (Pollock *et al.* 1994)

Selenium and vitamin E either administered individually or combined have an effect on the immune response of both cellular and humoral immunity (Girodon *et al.* 1999; Panousis *et al.* 2001). The immune-potentiating activity of antioxidants is well known: a higher antibody level against Newcastle virus vaccine (NVV) was detected 10-21 days post immunization with E-selenium adjuvated vaccine than that induced by non-adjuvated vaccine; also, chicks which received Se- (1 mg/Kg) and vitamin-E- (300 IU/ml) adjuvated vaccine showed a significantly higher immune response to NVV than those immunized with non-adjuvated vaccine. The effect of E-selenium on body weight was obvious when chicks were fed a diet containing 0.5 mg/Kg selenium and 300 IU/ml vitamin E. A significantly higher antibody titre, evaluated by the haemagglutination inhibition test and ELISA, 10 days post immunization was attributed to 0.06 mg/kg selenium and 150 IU/ml vitamin E. These data suggest that a higher growth rate concomitant with immune potentiation may be achieved by combined supplementation of Se and vitamin E (Kodama 1996). Our work agrees with this conclusion, since the antibody level post immunization of mice using the E-selenium adjuvated rabies vaccine was higher than that induced with the non-adjuvated vaccine.

Although competitive ELISA was developed as an alternative, reliable and highly correlated substitute for the mice virus neutralization test (Sugiyama *et al.* 1997), direct ELISA can be used to evaluate the neutralizing antibody developed in human sera post immunization using purified vero cell rabies vaccine, and the determined antibody levels were correlated to those evaluated using mice neutralization assay (El-Karamany 1987). The immune response to rabies vaccine, evaluated by ELISA, revealed that the antibody titre in the sera of laboratory animals of different groups was higher than the protective level (>0.5 IU/ml) right until the end of the test period (6 months). Although the antibody levels detected by the indirect immunofluorescent assay were not completely correlated to the antibody levels assayed by the rapid focus fluorescent inhibition test, they were able to prevent the development of an encephalitis post challenge of immunized mice by 100 LD₅₀ of the challenge virus standard six months post vaccination (with the exception of the antibody levels developed by non-adjuvated vaccine used as three double doses, which resulted in only partial protection).

On the other hand, neither the immune response to rabies vaccination nor the lymphocyte blastogenic response to nonspecific mitogens is affected by selenium, and the most common adjuvant (alum) showed no effect on the immune response to rabies vaccine, since the antibody level detected in sera of vaccines immunized with alum-adjuvated vaccine was less than those developed after non-adjuvated rabies vaccine (Ellis *et al.* 1997). At the same time (Berlin *et al.* 1983; Burgoyne *et al.* 1985; Umehara *et al.* 2002) there was clear synergetic activity of aluminum-hydroxide-adjuvated rabies vaccine, either when alone or combined with Avidine, and the two vaccines could provoke the immune response with a titre persistently above 2.264 log₁₀ SN₅₀ throughout the study. The mean antibody titre on the 30th day post vaccination was numerically higher for the AL(OH)₃ adjuvated vaccine, but the antibody level developed post immunization with Avidine-containing vaccine was numerically higher on day 90. Although no significant elevation was reported following re-vaccination at day 90, the antibody level induced by the Avidine-containing vaccine was higher and persisted higher until the end of the study (the difference was significant on days 180, 360 and 390 (Umehara *et al.* 2002)

Concerning different immunization regimes and related immune responses, the present results showed that the classical immunization regime of five doses of adjuvated vaccine induced a long-lasting and higher antibody level than a modified regime of three double doses. These results support those of others (Zhonghua *et al.* 1999; Madhusudana *et al.* 2001): there was an excellent immune response with more than the protective titre (> 0.5 IU/ml) on all days tested up to the end of the 3-yr study period. More significantly, protective titres were detected in all subjects by day 7 and only minimal side reactions were observed. This multi-site regime with or without passive immunization prevents the development of rabies encephalitis in people bitten by confirmed rabid dogs. The route of administration and dose number significantly influences antibody level production and protection from challenge independently. Extended challenge studies have shown that intramuscular vaccination of mice results in the highest antibody levels and a protection level equivalent to intraperitoneal vaccination; subcutaneous vaccination even if given multiply, results in a poor serum neutralizing antibody titre, and is far less protective than other routes. Further studies suggest that it is possible to improve vaccine potency in mice by a single intramuscular dose and a delayed time of challenge (Wunderli *et al.* 2003).

In the same way, immune response to Fermi-type vaccine and cell culture vaccine immune potentiation has been evaluated using the mice model and human vaccines, revealing a protective antibody titre. Mice immunized with the Fermi-type rabies vaccine produced in Ethiopia, and challenged intracranially with rabies virus at a concentration of 64 MICLD₅₀ 90 days post initial vaccination, show levels of neutralizing antibodies ranging

from 4.6 to 25 IU/ml; boosting showed a non-significantly increased immune response (Ayele *et al.* 2001). All immunized mice could withstand intracranial challenge, and virus neutralizing antibodies in the sera of human vaccines vaccinated with prescribed Fermi vaccine had more than 0.5 IU/ml detected by day 14, but nothing was detectable on day 1 (Ayele *et al.* 2001).

In conclusion, this study suggests that E-selenium as an immunostimulant can enhance the immune response of mice. A single-dose regime is preferable to a double dose. The investigated vaccines (either adjuvated or non-adjuvated) were potentially immunogenic and could induce the least protective anti-body titre (>0.5 IU/ml). Pre-clinical evaluation of E-selenium as an immunostimulant is recommended.

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