

EVALUATION OF IMMUNE RESPONSE ABILITY OF CIMAVAX-EGF® VACCINE ON ICR MOUSE

Nguyen Thi Ly*, Nguyen Kim Bach, Be Thị Tham,
Nguyen Thi Hong Dinh, Do Linh Trang

** National Institute for Control of Vaccine and Biologicals*

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Abstract: The study evaluated the immune response of CIMAvax-EGF® vaccine on Mlac: ICR mouse line has compared with the control serum samples on NMRI mouse by ELISA. The study results showed that with the designed immunogenic doses (0.1; 0.15; 0.2; 0.25 & 0.3 ml of CIMAvax-EGF® vaccine/mouse) after 14 days, using 03 serum dilutions include: 1/100, 1/1000 & 1/10000 for ELISA test gave similar results to the NMRI mouse serum standard sample provided by the manufacturer. The optimal immunogenic dose in Mlac: ICR mouse line is 0.2ml of CIMAvax-EGF® vaccine/mouse.

From the above research results, it was possible to use Mlac: ICR mouse line of National Institute for Control of Vaccines and Biologicals (NICVB) at NICVB's laboratory for potency test of CIMAvax-EGF® vaccine produced by Cuban.

Keywords: *CIMAvax-EGF®, ICR mouse, ELISA.*

1. Introduction

According to the report in 2016, lung cancer took about 12% of total cancer diseases in the world, with around 1.83 million patients and the lethal rate reached to 28% of total cancer cases. The number of victims died because of lung cancer was equal to the sum of lethal cases of Colorectal, Breast, and Prostate cancer [1].

In Vietnam, lung cancer also took the first place of total 10 common cancer in both gender. According to the report, until 2013, the patient of lung cancer increased 4 times, up to 20.000 cases annually, in which included 17.000 deaths.

Currently, there are some therapies for lung cancer such as surgery, radiotherapy, chemotherapy, targeted therapy, however they

have high cost, cause pain and side effects for 5 or 7 survival years (50%) or 6 survival months (10%) for late diagnostic patients [2]. The preventive solution like health education, risk factors isolation are not effective. Therefore, the presence of the first Cuban vaccine against non-small-cell lung carcinoma, CIMAvax-EGF®, is a huge step for lung cancer prevention and treatment [3,4,5,6,7]. To ensure safety and efficiency of vaccine, quality control before distribution is a necessary. Currently, for immune response ability test or protective efficiency of this vaccine, Cuba has used NMRI mouse line. However, National Institute for Control of Vaccines and Biologicals (NICVB, Vietnam) has not had this mouse line. To ensure safety and protective efficiency of vaccine, quality control before distribution is a need. Currently, potency test of this vaccine is carried out on NMRI mice in Cuba. However, National Institute for Control of Vaccines

* Corresponding author
E-mail address: ly.nicvb@gmail.com
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and Biologicals (NICVB) has not had NMRI mouse line and the cost for this mouse line is expensive for circulatory test. Therefore, to supply the proactive needs of quality control of this vaccine, a study of available mouse line in NICVB is necessary and the research team carried out a project with the title:

“Evaluation of immune response ability of CIMAvax-EGF® on ICR mouse”

Objectives:

1/ Evaluate immune response of ICR mouse after CIMAvax-EGF® injection with different doses/ volumes.

2/ Determine dose/ volume of optimal injection for ICR mouse.

2. Subject and research method

2.1 Research subject, duration, and location

- Research subject: Immune response after CIMAvax-EGF® injection on Mlac:ICR (ICR) mouse, female, weight 18-20gram: 100 mice.

- Research location: Quality control of Viral vaccine Department, NICVB;

- Research duration: 09/2019-10/2019.

2.2 Research method: Descriptive, retrospective and prospective methods.

2.3. Materials

2.3.1. Materials and chemicals

Sample: CIMAvax-EGF® (15 vials) (lot 12 1711-0411S/E); Control sample: Positive control (*antisera rEGF of mice which were injected standard vaccine*) – Serum samples of NMRI mice extracted from Cuba's lab (Manufacturer); Negative control (*serum of NMRI mouse without injection*).

BSA(Sigma); p-nitrophenylphosphate (pNPP); Anti-total mouse Ig conjugated

with alkaline phosphatase antiserum; rhEGF (in-house) or commercial product; Na₂CO₃ (Sigma); NaHCO₃ (Sigma); Thiomersal (Sigma); KCl (Sigma); HCl (Sigma); Na₂HPO₄ (Sigma); MgCl₂ (Sigma); Diethanolamine (Merck); Tween 20 (Sigma); NaOH (Sigma); FBS (Gibco).

2.3.2 Equipment and consumable supplies

Anaesthetic bottle CO₂ (Vietnam); Anaesthetic tool (Vietnam); Fixed tool for blood collection (Vietnam); sterilized syringe 3ml (Vinahankook); blood collection syringe (USA, code: X86829); eppendorf tube 2ml (Eppendorf, code: 30120094); incubator (Sanyo, code IC14 – VR), fridge (Panasonic, code CI13-VR); vortex (IKA, code: VT03-VR); ELISA system (HUMAN, code: ES08-VR); Clean cabinet (TelStar, code: LH-20); tip, pipette for experiment.

2.4. Research design

Vaccine is injected on ICR mouse with 5 different dilution. After 14 days, the blood is collected and serum is extracted and used for ELISA for immune response ability on ICR mouse. If OD of samples of each dilution ≥ OD cut-off value, the result is considered as positive and able to immune response.

Positive control are serum of NMRI mice injected a lot of CIMAvax-EGF® that Cuban scientists brought to Vietnam and one serum sample of NMRI mouse injected CIMAvax-EGF® in Vietnam; negative control is serum of both ICR & NMRI mice without injection.

2.5. Experimental procedure [7,8]

a. Vaccine reconstitution

Vaccine is reconstituted with Montanide ISA 51 VG vial (ratio 1:1).

b. Mouse injection

- Serial dilution for injection on ICR mouse: 0.1 ml/mouse; 0.15 ml/ mouse; 0.2 ml/ mouse; 0.25 ml/ mouse; 0.3ml/ mouse follow in Figure 1.

- Serial dilution for injection on NMRI mouse: 0.2ml/ mouse (positive control).

- Injection: intramuscular injection.

- Number of mouse: 5 mice/dilution.

- After injection, mice are taken care within 14 days.

- 5 mice without injection are taken as negative control.



Figure 1. CIMAvax-EGF® vaccinated on mice

c. Blood collection

After 14 days, blood is collected, extracted serum and stored at -20°C.

d. Determine Anti-rhEGF by ELISA

The procedure for determining anti-CIMAvax-EGF® antibodies using the Elisa method is as shown in table 1.

* Plate preparation: Add 50mL/well coating reagent rhEGF 10mg/mL, incubate plate at 37°C in 1 hour or at 2-8°C overnight. Wash the plate, add 100mL/well blocking reagent. Incubate plate at 37°C in 1 hour.

* Sample dilution: Each serum of injected ICR mouse is diluted with blocking media to 3 serial dilutions: 1/100, 1/1000 và 1/10000. Negative and positive control is diluted to 1/100 (C⁺).

* Adding sample: Add 50 µL/well of diluted samples, each dilution is replicated twice. Add at least 5 wells for negative control and 2 wells for positive control. Incubate at 37°C in 1 hour.

Table 1. Procedure for determining anti-CIMAvax-EGF® antibodies using the Elisa method

Plate														
	Dil4	1/10000		1/1000		1/100		1/10000		1/1000		1/100		3Dil
	Mouse	1	2	3	4	5	6	7	8	9	10	11	12	Mouse6
A	1	Sample						Serum from NMRI mice after 0.2 ml/ mouse injection in CuBa						1
B	2													2
C	3													3
D	4													4
E	5													5
F	C ₁ ⁻													C ⁺
G	C ₂ ⁻													Blank
H	C ₃ ⁻													

Note: Sample is serum collected from ICR mice injected vaccine in NICVB, Vietnam at 0.1; 0.15; 0.2; 0.25 and 0.3 ml/mouse; or serum from NMRI mice injected vaccine in NICVB, VN at 0,2 ml/mouse.

C₁⁻ : Negative control serum from NMRI mouse of CuBa; C₂⁻ : Negative control serum

from NMRI mouse of Vietnam; C₃⁻ : Negative control serum from ICR mouse of NICVB, VN.

* Adding conjugate: Wash the plate, add 50 µL/well diluted conjugated solution (Anti-total mouse Ig conjugated with alkaline phosphatase antiserum) for all wells. Incubate at 37°C in 1 hour.

* Adding pNPP and read the result: Wash the plate, add 50 μ L/well diluted pNPP with concentration at 1mg/mL for all wells. Incubate at room temperature for 30 minutes, protect from light. Add 50 μ L/well NaOH 3M for stopping the reaction. Read the plate at wavelength of 405 nm within 15 minutes.

* Result analysis:

- Calculate the result:

+ Calculate mean, standard derivation, and coefficient of variation CV of OD value in each sample dilution.

+ Cut-off value is equal to double average OD value of negative control.

+ The sample is positive when OD

value of sample is greater than OD cut-off.
- Criteria:

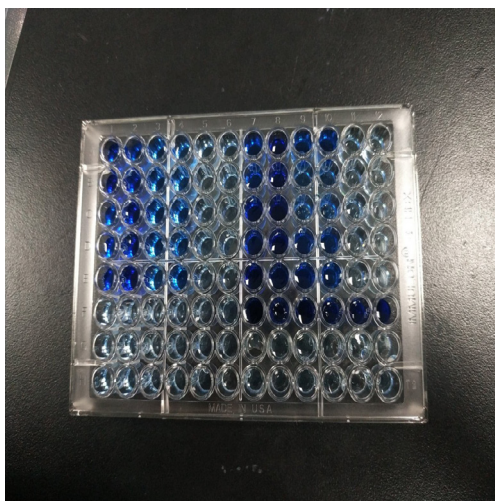
+ The experiment is valid when CV of OD of negative control is equal or smaller than 10%; average OD value of positive control/ average OD value of negative control > 10.

+ Vaccine is passed when the number of vaccine injected mice at dilution > 1/100 gives positive result $\geq 80\%$.

3. Result of study

3.1. Elisa test process and results

The Elisa test procedure evaluates the corresponding immune response to different concentrations of CIMAvax-EGF® and gives the results shown in Figure 2 (A&B).



2-A: Plate of Elisa test

Report

Date: 12/05/2017 Time: 13:22:21

Humalinder H5

Well	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Program	002	002	002	002	002	002	002	002	002	002	002	002
Sample	002	002	002	002	002	002	002	002	002	002	002	002
Abx	0.189	0.116	0.371	0.299	1.325	1.321	0.053	0.053	0.053	0.054	0.053	0.053
CTA	0.189	0.116	0.371	0.299	1.325	1.321	0.053	0.053	0.053	0.054	0.053	0.053

Operator: Print date: 12/05/2017

2-B Report of Elisa test

Figure 2 (A-B). Plate illustration and report of Elisa Procedure

Note: Each Plate corresponds to each concentration (ml) of CIMAvax-EGF® immunization in Mlac: ICR mice and NMRI mice

Plate 1 (P1): Dose of 0.1 ml/ICR mice

Plate 2 (P2): Dose of 0.15 ml/ICR mice

Plate 3 (P3): Dose of 0.2 ml/ICR mice

Plate 4 (P4): Dose of 0.25 ml/ICR mice

Plate 5 (P5): Dose of 0.3 ml/ICR mice

Plate 6 (P6): Dose of 0.2 ml/NMRI mice

3.2. OD of Elisa test results

The OD of Elisa test results corresponding to each dilution of immunogenic CIMAvax-

EGF® (Plate) and positive, negative controls and blank well are presented in Table 2.

Table 2: Result OD Elisa test of negative, positive control and blank

Criteria	Standards	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6
OD	$\leq O D_{tb}$	0.107	0.110	0.099	0.100	0.096	0.102
Blank	Negative	Pass	Pass	Pass	Pass	Pass	Pass
OD _{C+}	≥ 1.5	1.766	1.752	1.786	1.837	1.828	1.719
		Pass	Pass	Pass	Pass	Pass	Pass
OD _{C1}	$\leq 1/10 OD_{C+}$	0.119	0.122	0.119	0.118	0.121	0.120
		Pass	Pass	Pass	Đạt	Pass	Pass
OD _{C2}		0.117	0.112	0.117	0.105	0.104	0.122
		Pass	Pass	Pass	Pass	Pass	Pass
OD _{C3}		0.108	0.116	0.113	0.110	0.113	0.102
		Pass	Pass	Pass	Pass	Pass	Pass
SD _{C1}	/	0.010	0.008	0.012	0.008	0.002	0.007
SD _{C2}	/	0.004	0.009	0.009	0.009	0.007	0.001
SD _{C3}	/	0.009	0.009	0.006	0.005	0.009	0.107
%CV _{C1}	$\leq 10\%$	8.290	6.528	9.650	6.393	1.540	5.902
		Pass	Pass	Pass	Pass	Pass	Pass
%CV _{C2}		3.730	8.210	7.508	8.303	7.131	8.428
		Pass	Pass	Pass	Pass	Đạt	Pass
%CV _{C3}		7.944	7.617	5.523	4.802	7.800	7.326
		Pass	Pass	Pass	Pass	Pass	Pass

Results in Table 2 show that all six immunogenic dilutions of CIMAvax-EGF® (plates) meet the requirements according to the manufacturer's standards and were done paralleled with NMRI mouse serum as positive control.

3.3. Results of immune response results

table on Mlac mice: ICR corresponding to CIMAvax-EGF® concentrations

Results of immune response results table on Mlac mice: ICR corresponding to CIMAvax-EGF® concentration (plates) including (0.1; 0.15; and 0.2ml/mice) in Table 3.

Table 3. Elisa results correspond to CIMAvax-EGF® immunogenic concentrations (0.1; 0.15; and 0.2ml/mice) on Mlac:ICR mice

Sample	Dose 0.1 ml/ ICR mice			Dose 0.15 ml/ ICR mice			Dose 0.2 ml/ ICR mice		
Cut-off	OD _{TB} (-) x2= 0.238			OD _{TB} (-) x2= 0.244			OD _{TB} (-) x2= 0.244		
Dilution	1/10000	1/1000	1/100	1/10000	1/1000	1/100	1/10000	1/1000	1/100
Mice	X _{TB} OD	X _{TB} OD	X _{TB} OD	X _{TB} OD	X _{TB} OD	X _{TB} OD	X _{TB} OD	X _{TB} OD	X _{TB} OD

1	0.124 (-)	0.402 (+)	1.355 (+)	0.114 (-)	0.291 (+)	1.021 (+)	0.208 (-)	0.711 (+)	1.539 (+)
2	0.124 (-)	0.229 (-)	0.751 (+)	0.127 (-)	0.412 (+)	1.551 (+)	0.114 (-)	0.288 (+)	1.063 (+)
3	0.125 (-)	0.172 (-)	0.683 (+)	0.114 (-)	0.609 (+)	1.914 (+)	0.153 (-)	0.361 (+)	0.978 (+)
4	0.118 (-)	0.331 (+)	1.031 (+)	0.128 (-)	0.623 (+)	1.718 (+)	0.123 (-)	0.295 (+)	0.361 (+)
5	0.131 (-)	0.332 (+)	1.515 (+)	0.225 (-)	0.439 (+)	1.239 (+)	0.180 (-)	0.512 (+)	1.481 (+)
Immune response	0/5	5/5	5/5	0/5	5/5	5/5	0/5	5/5	5/5
% Positive	0%	100%	100%	0%	100%	100%	0%	100%	100%

Results of immune response results table on Mlac mice: ICR corresponding to CIMAvax-EGF® concentration (plates) including (0.25 ml and 0.3 ml/mice) in Table 4.

Table 4. Elisa results correspond to CIMAvax-EGF® immunogenic concentrations (0.25 ml; and 0.3 ml/mice) on Mlac:ICR mice

Sample	Dose 0.25 ml/ ICR mice			Dose 0.3 ml/ ICR mice		
Cut-off	Cut-off = OD _{TB} (-) x2= 0.236			Cut-off = OD _{TB} (-) x2= 0.242		
Dilution	1/10000	1/1000	1/100	1/10000	1/1000	1/100
Mice	X _{TB} OD	X _{TB} OD	X _{TB} OD	X _{TB} OD	X _{TB} OD	X _{TB} OD
1	0.215 (-)	0.756 (+)	1.656 (+)	0.260 (+)	1.221 (+)	2.676 (+)
2	0.119 (-)	0.310 (+)	1.147 (+)	0.109 (-)	0.259 (+)	1.171 (+)
3	0.114 (-)	0.371 (+)	1.053 (+)	0.149 (-)	0.404 (+)	1.693 (+)
4	0.109 (-)	0.294 (+)	0.666 (+)	0.125 (-)	0.276 (+)	1.228 (+)
5	0.173 (-)	0.531 (+)	1.582 (+)	0.133 (-)	0.367 (+)	1.473 (+)
Immune response	0/5	5/5	5/5	1/5	5/5	5/5
% Positive	0%	100%	100%	20%	100%	100%
P	P<0.05					

Results in Table 4 show that the immune response of NMRI mice made at the manufacturer and in Vietnam is equivalent, with no difference.

Table 5. Elisa results compared to CIMAvax-EGF® immunogenic concentrations 0.2 ml/ mice on NMRI mice, Manufacture with NMRI mice, Vietnam

Sample	Dose 0.2 ml/ NMRI mice manufacture			Dose 0.2 ml/ NMRI mice, Vietnam		
Cut-off	Cut-off = $OD_{TB}(-) \times 2 = 0.240$			Cut-off = $OD_{TB}(-) \times 2 = 0.238$		
Dilution	1/10000	1/1000	1/100	1/10000	1/1000	1/100
Mouse	$X_{TB} OD$	$X_{TB} OD$	$X_{TB} OD$	$X_{TB} OD$	$X_{TB} OD$	$X_{TB} OD$
1	0.108 (-)	0.261 (+)	0.959 (+)	0.134 (-)	0.295 (+)	1.000 (+)
2	0.109 (-)	0.338 (+)	1.078 (+)	0.144 (-)	0.409 (+)	1.529 (+)
3	0.085 (-)	0.511 (+)	0.927 (+)	0.104 (-)	0.587 (+)	1.819 (+)
4	0.126 (-)	0.425 (+)	0.911 (+)	0.142 (-)	0.604 (+)	1.621 (+)
5	0.107 (-)	0.357 (+)	1.061 (+)	0.152 (-)	0.439 (+)	1.169 (+)
Immune response	0/5	5/5	5/5	0/5	5/5	5/5
% Positive	0%	100%	100%	0%	100%	100%

From the results in Tables 3,4 and 5, it shows that the immune response in Mlac:ICR mice of CIMAvax-EGF® with doses (0.1, 0.15, 0.2, 0.25, 0.3 ml/mice) all showed immunogenic results. pandemic. However, at the dose of CIMAvax-EGF® 0.2ml/mice, both Mlac:ICR and NMRI have the same and best immunogenicity results when performed at different laboratories (at the Manufacturer, Cuba; at Vabiotech and NICVB).

4. Discussion

From Table 2, OD value of positive control between plates were equivalent and passed the given criteria: OD value between negative control C1, C2, C3 in one plate or between plates were all passed the criteria of ELISA, %CV < 10%. Therefore, ELISA experiment was valid.

From Table 3, 4 and 5 OD values had linearity based on serial dilutions ($1/100 > 1/1000 >$

$1/10000$) and concentration of immune antigen. It was determined that Mlac:ICR mouse had ability for immune response with Cimavax in all 5 diluted doses, at the highest dilution 0.3 ml/ mouse (the concentration of antigen is $15 \mu\text{g/ml}$), OD value was higher than the lowest dilution 0.1 ml/mouse (the concentration of antigen is about $5 \mu\text{g/ml}$). At the dose 0.1 ml/mouse: the mice had immune response at 1/100 dilution but for other dilution $\leq 1/1000$, they were not enough response as criteria ($\geq 80\%$). From dose 0.15 ml to 0.3 ml, mice had immune response in both dilution 1/100 and 1/1000. To determine the immune response on Mlac:ICR mice, we followed the recommendation of manufacturer which was 0.2 ml/mouse to qualify the vaccine because it was a stable and optimal diluted injection with high immune response on NMRI mice until expired vaccine period. This dose was more economized than dose of 0.25 ml/mouse and 0.3ml/mouse

without any difference of result. The dose 0.1 ml/mouse and 0.15 ml/mouse had a passed result in the dilution at 1/100 but it was evaluated when vaccine was stored at stable condition with long shelf-life, and it is not considered for on-going-expired vaccine test or clinical study [5; 6].

Therefore, dose and dilution are two other factors that affect the ability of immune response on mouse. If unsuitable dose or dilution was selected, the result may be out of the criteria (if it is too low, there will be no immune response and if it is too high, there will be no more immune response than at 0.2 ml/mice). This result is also equivalent with result of manufacturer. Besides, we used NMRI mice line to compare the equivalence between NICVB and Manufacture, Cuba and if there is no immune response on Mlac:ICR mice, NMRI mice will be used for circulatory test in NICVB. Therefore, this study tested on Mlac:ICR mice line of NICVB, Vietnam to use the available animal resource, reduce duration, expense of research instead of testing on NMRI mice as manufacturer.

5. Conclusion

On base results of study shown that Mlac:ICR mice line of NICVB, Vietnam had immune response with CIMAvax-EGF®, the optimal dose of CIMAvax-EGF® for immunovaccination on ICR mouse is 0.2 ml/mice.

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