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Preparation of Different Formulations of Experimental Herpes Simplex Viral Vaccine Type -2 Using Aluminum and Calcium Phosphate gel as Adjuvants and Related Stability and Immunogenicity

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Abstract- Herpes simplex virus type 2 (HSV-2) is a one of the most prevalent worldwide sexually transmitted infection. HSV-2 causes recurrent genital ulcers, neonatal herpes, and also, increases the risk for HIV acquisition. Many HSV-2 vaccines have been tested in humans, but were not consistently effective. In the present work we tried to produce a model vaccine for HSV-2 formulated in different formulae as an attempt for development of an effective prophylactic and therapeutic HSV-2 vaccine. Beta propiolactone (BPL) as virus inactivant was compared with the conventional formalin. Also, calcium phosphate nano-particles (CPN) as a vaccine adjuvant was compared with current used aluminum phosphate gel (alum) micro-particles. Stability of adjuvanted and non adjuvanted vaccine models was monitored at different thermal conditions. Subcutaneous route was conducted in balb-c mice to determine the immune response of vaccine formulations. Data revealed that Al-BPL-HSV-2 vaccine showed the highest and longer durative immune response compared with other vaccine candidates. Different formulations of the test vaccine showed the same pattern of stability at different thermal conditions (4°C and 37°C and 45°C). Also, Al-BPL-HSV-2 vaccine formulation could efficiently develop antibody level that could neutralize the challenge dose of HSV-2 and no viral infection could be detected in the vaginal tissues using direct immune-fluorescent assay.

Index Terms- Herpes simplex virus-2; Calcium phosphate nanoparticles; Alum; Adjuvant; Beta-propiolactone; Formalin; Stability.

I. Introduction

HSV-2 is a sexually transmitted virus that is the major cause of genital herpes, a highly prevalent infection among people worldwide [1]. HSV-2 infects epithelial cells in the skin and mucosal surfaces during primary infection, then travels via retrograde transport along nerve axons to the dorsal root ganglia (DRG), where latency is established [2].While epithelial cells are destroyed during lytic HSV-2 replication, neuronal cells are not destroyed and provide a reservoir for latent virus. Reactivation can be caused by numerous factors, including stress, hormonal changes and immunosuppression [3].

Transmission of HSV-2 from mother to infant during birth is the most serious complication of genital herpes, and women who acquire HSV-2 during pregnancy are at the highest risk of transmitting the infection [4]. Neonatal herpes can result in long-term neurologic, blindness or mortality [5]. Furthermore, a recent study concluded that HSV-2 infection to be associated with a three fold increase in susceptibility to human immunodeficiency virus (HIV-1) in both men and women from the general population [6]. Although the great efforts to develop a safe and effective vaccine, up to date no proven effective vaccine is available [7].

Vaccine effectiveness depends on its ability to provide enough protection in order to avoid infection, minimize post-infection complications, reduce the viral shedding or block its transmission [8]. Most of the ongoing vaccine research designed for induction of increased systemic humoral and cellular immunity [9]. The immune response to the vaccine was based on a combination of many key factors such as the dose numbers, volume, route of administration, the antigen contents, and adjuvant used [10-11].

Inactivation with formaldehyde is commonly used for the production of commercial human and animal viral vaccines such as those against polio, hepatitis A, and influenza viruses. However, little is known about the molecular mechanisms underlying virus inactivation by formaldehyde [11]. Also, BPL is widely used for the inactivation of infectious agents in many vaccine preparations as well as in disinfection, plasma sterilization and tissue transplants as it reacts with nucleophilic reagents (including nucleic acids and proteins) leading to alkylated and/or acylated products. BPL is very reactive with different chemical moieties of various biological molecules including proteins (mostly methionine, cysteine and histidine) and nucleic acids (mainly adenosine, cytidine and guanosine moieties of the vRNA . BPL seems to be more effective in inactivating enveloped viruses than non-enveloped viruses [12-13].

An effective vaccine usually requires an adjuvant to enhance the immune response. The adjuvant induced immunity differs according to the adjuvant types, the ratio between the adjuvant and vaccine, the route of administration, the charge and particle size of the adjuvants. Calcium phosphate and aluminum compounds have been approved for human use as adjuvants in several European countries [14].

Alum is the most commonly used adjuvant. In the United States, alum compounds are the most extensively used adjuvants in licensed vaccines for humans [15]. Although they effectively enhance immune responses, there are several disadvantages associated with their use [16]. They include the severity of local tissue irritation, the longer duration of the inflammatory reaction at the injection site, strong Th_2 (T-helper cells) responses, minimal induction of cell-mediated immunity, and a propensity to elicit undesirable immunoglobulin E (IgE) responses [17]. Despite of its widespread use in human vaccines, the mechanism of action for aluminum adjuvant is not completely well understood. The "depot effect" is a commonly suggested mechanism for adjuvancy. Adsorptive capacity and strength of adsorption between antigen and alum adjuvant has been shown to correlate with adjuvancy [17]. Replacement of aluminum salts with calcium phosphate has long been described [18].

CPN is another mineral adjuvant has been used and approved as a vaccine adjuvant in several European countries [19]. CPN is an ideal biomaterial because it is a natural constituent of the human body and is generally regarded to be safe, biocompatible and biodegradable. CPN as an alternative adjuvant to aluminum compounds induced a higher IgG2a level and a lower IgE response [20]. CPN may be an effective substitute in vaccines that require cell-mediated or cytotoxic T-cell response [21].

The present work aimed to prepare HSV-2 cell culture derived experimental vaccine inactivated with different inactivants namely formalin and beta-propiolactone, evaluation of CPN as an alternative adjuvant compared with current used alum, and thermal stability and related immune response.

II. Materials and Methods

Determination of virus infectivity

HSV-2 was kindly provided by Dr. Aly Fahmy, Head of R&D sector. The Holding Company for Biological products, Vaccines and Drugs (***VACSERA***), Giza, Egypt. The virus seed stock was prepared according to [22], where growth medium was decanted from the African green monkey kidney (VERO) precultured cell flasks kindly provided by the cell culture department, virology sector. The cell line was maintained according to [23-24]; the monolayer was washed with sterile PBS (phosphate buffered saline), pH 7.2. HSV-2 used as 0.1 MOI / ml (multiplicity of infection) was dispensed to each 175 cm^2 TC (tissue culture) flask. Bottles were shacked at 15 min interval for assurance of well virus distribution, 100 ml of maintenance media were dispensed to each flask. Vero cells with growth media were distributed. Once cells have been seeded, the cells were allowed to grow overnight. In the next day, growth medium were removed from cells. 10-fold dilutions of HSV-2 of virus stock were prepared (10^{-1} through 10^{-7}). 0.1 ml of each dilution was inoculated to the precultured vero cell plate in quadrate. Un-inoculated control well was left as a negative control. Plates were incubated in CO2 incubator for 7 days with daily examination using inverted microscope to check for development of CPE (cytopathic effect). At the end of examination duration the viral infectivity titer was determined according to Reed and Muench (1938)[25].

Animals

18-20 gm female balb-C mice were used for vaccination and chemical inactivant toxicity monitoring. Experimental trials using animals started post Faculty of Pharmacy - Cairo university ethical committee approval. Balb-C mice obtained from the animal house unit, VACSERA, Egypt. The animals maintained under standard laboratory conditions (12 hrs light and dark cycles) had free access to standard meal and water supply.

Inactivation of HSV-2 using chemical inactivants

BPL and formalin (F) were purchased from (Sigma - Aldrich, USA). Vaccine pool was liquated in 2 aliquots, first aliquot of HSV-2 was inactivated using BPL prepared as 0.0035 M [23-24]. BPL was stepwise dispensed to virus aliquot with continuous stirring. Second aliquot was inactivated using 0.2% F [21] as it was diluted in HBSS (Hank's buffered salt solution) pH 7.2 as 37 - 40 % final concentration and added to the virus suspension to obtain at final concentration 0.2 %.

Determination of Inactivation kinetics of HSV-2

Inactivation kinetics relative to time post treatment with F and BPL was determined [26-27] where 1 ml of chemically treated virus aliquots was collected at time interval (every 1 hr for F and 15 minutes for BPL). Virus samples were 10 fold serially diluted 10^1-10^8 in 199-E (medium -199 Earle's salts). Prepared dilutions were dispensed onto precultured vero cells in 96 well plates (TPP-Swiss). Infected cultured plates were incubated in CO_2 incubator (Jouan –France) and daily examined for detection of CPE. 50 % end point induced, CPE was determined [28].

In case of formalin treatment, when residual live virus could not be detected residual formalin was neutralized using 1/16 of 35% Na-bisulphite as 0.0289% final concentration [29].

Determination of chemical inactivants toxicity

Intraperitoneal route was conducted using balb-c mice to determine the toxicity of formalin and BPL as currently used inactivants according to the OECD-423 guidelines [21,25]. Balb-C mice were allocated into groups (10 /cage). General symptoms of toxicity and mortality were observed for 24 hrs through 7 days post chemical administration.

Intravaginal HSV-2 challenge model

After 3 weeks of final boost, immunized female balb-c mice were challenged intravaginally using 100 TCID50 (tissue culture infective dose) HSV-2. Vaginal tissues were examined after 1 week for detection of non-neutralized HSV-2 using immune-fluorescent assay (IFA) compared to non-immunized balb-c mice as negative control.

Preparation of Adjuvants

CPN adjuvant: Calcium phosphate nanoparticles was prepared by mixing buffers A and B, prepared by the rapid dissolution of 18.36 gm Na_2HPO_4-$12H_2O$; 12.5 gm NaOH; 7.5 gm $NaHCO_3$ in 325 ml of distilled water and 10.75 gm $Ca(NO3)_2$ 4H2O in 125 ml of distilled water. All buffers were sterilized by membrane filtration (0.022 Stereoscopy vacuum filtration system (Millipore –USA)). Vaccines were homogenized in a 0.07 M dibasic sodium phosphate sterile solution. The antigen suspension was mixed with solution A prior to mixing to solution

B. The precipitate of gel-like amorphous calcium phosphate formed was agitated for approximately 30 seconds. Then HSV-2 antigen were absorbed on the CPN adjuvant [30].

Aluminum phosphate (Alum) adjuvant: both of 0.63 M $AlCl_3.6H_2O$ and 0.3 M $Na_3PO_4.12H_2O$ were prepared in 40 ml normal saline each. Prepared solutions were 0.2µm filtered. Contents were stirred continuously during the procedure at 40 to 60 rpm (recycle/minute). 0.3 M $Na_3PO_4.12H_2O$ solution was added to the mixing bottle. Then 300 ml normal saline was added. The antigen was also added followed by addition of 0.63 M $AlCl_3.6H_2O$ solution to the mixing bottle. The pH was maintained between 6.5–6.8. The final volume was adjusted with sterile normal saline. The suspension was mixed for 2 hrs at 37°C [30].

Formulation of HSV-2 vaccine

Both F and BPL inactivated HSV-2 virus were adjuvanted using CPN and alum, non adjuvanted inactivated virus was involved. Six formulae of test vaccine were prepared namely, BPL-HSV-2, CPN-BPL-HSV-2, Al-BPL-HSV-2, F-HSV-2, CPN-F-HSV-2 and Al-F-HSV-2. The adjuvants used according to the recommendation of the World Health Organization [32].

Vaccine stability

90 healthy balb-c mice took part in this study. They were randomized into six groups to receive the same lot vaccine stored in six different ways. the first group received vaccine that freshly prepared, the second group received vaccine heated at 45°C for 1 week (accelerated stability), the third group received vaccine incubated at 37°C for 1 week, the fourth group received vaccine incubated at 37°C for 2 week, the fifth group received vaccine incubated at 37°C for1 month (Real time stability) and finally the sixth group received vaccine refrigerated (4°C) for 1 year. The six participant groups did not differ with respect to mean ages or sex distribution. 0.5 ml vaccine doses were administered subcutaneously (s.c) in the according to a 0 and 24 weeks schedule separating them in first 12 weeks and second 12 weeks by injection of 0.5 ml vaccine as poster dose . Blood was drawn for measurement of anti-HSV-2 at 2,4,8,12,16,20,24 weeks. Evaluation of test vaccine stability and related immune response was performed according to [30].

Evaluation of immune response

Blood samples were collected through retro-orbital plexus of immunized mice. Sera were collected using cold centrifugation for 15 minutes at 5000 rpm (recycle per minute) and analyzed for HSV-2 antibodies using enzyme-linked immunosorbent assay (ELISA). ELISA plates (Nunc – Denmark) were coated with cell lysate antigen prepared according to [24] overnight at 4°C. Serially diluted test sera were incubated with pre coated ELISA plates at 37°C for one hour. Plates were washed using washing buffer (PBS+ 0.05% Tween-20) using automated Bioteck plate washer (Bioteck –Elx-800, USA). Anti-mouse conjugate labeled with peroxidase enzyme (Sigma immuno-chemicals) was dispersed as 100µl as 1/1000 final dilution. Plates were incubated for 1hr and unbound conjugate was washed. TMB (tetramethylbenzidine) substrate buffer (Sigma – Aldrich, USA) was added as 100µl/ well. Plates were kept in dark for 20 minutes. The reaction was stopped using 100µl of 2N hydrochloric acid HCl (Sigma –Aldrich, USA). Developed color was measured at 450 nm using ELISA reader (Tecan Sunrise – Austria), antibody level was plotted against weeks.

Determination of IFN-γ and IL-10 (interferon and interleukin 10)

IFN-γ and IL-10 concentrations detected post immunization were monitored in sera samples from immunized mice groups using ELISA (standard kits from Life science Inc., USA).

III. Statistical Analysis

Statistical analysis was performed using analysis of variance (one-way ANOVA), P value less than 0.05 was considered significant. Data collected from three repeated experiments.

IV. Results

Determination of virus infectivity

HSV-2 was of an infectivity titer in the order of 7.5 $\log_{(10)}$ / ml determined using cell culture assay.

Inactivation kinetics of HSV-2 virus

Data recorded revealed that HSV-2 virus was completely inactivated within 2 hrs (120 min) post treatment with 0.0035M BPL and 5 hrs (300 min) post treatment with 0.2 % formalin; recording a mean depletion rate of HSV-2 was 0.65 $\log_{(10)}$ / hr and 3.2 $\log_{(10)}$/ hr in case of formalin and BPL respectively [Fig.1] . Data revealed that BPL was significantly effective and faster inactivant than formalin ($P < 0.05$).

Determination of chemical inactivants toxicity

It was clear that the used concentration of 0.2% of neutralized formalin and 0.0035 M BPL were completely safe to tested mice , Mice behaved normally with no signs of illness and no mortality detected throughout 7 days post administration.

Intravaginal HSV-2 challenge model

Data revealed that the developed immune response to BPL-Al- HSV-2 vaccine could completely neutralize the infective dose of HSV-2 used in intravaginal infection compared with control non immunized balb-c mice [Fig.2.]

Evaluation of immune response to experimental vaccine candidates

Data recorded revealed that the none adjuvanted BPL and F prepared vaccine candidates were potentially immunogenic and there was a noticed elevated antibody level relatively to time till the 8th week followed by declined phase of antibody level and a 2nd elevation occurred post boostering extended till the 16th week. There was no significant difference of antibody level post immunization with BPL and F inactivated vaccines ($P>0.05$).

It was clear that freshly prepared BPL and F-HSV-2 inactivated vaccine candidates showed a non significant difference in the immune response than that stabilized at 37°C for 1 week ($P>0.05$). While for freshly prepared BPL and F-

HSV-2 inactivated vaccine candidates showed a significant immune response than those stabilized at 37°C for 2 and 4 weeks , 4°C for a year and at 45°C for a week ($P<0.05$),

Although Al-BPL-HSV-2 formulation had given a higher immune response than Al-F-HSV-2 vaccine candidates at different thermal conditions (freshly prepared, 37°C 1,2 and 4 weeks, 45°C and 4°C) but it was insignificant difference ($P > 0.05$).

Regarding the alternative adjuvant namely CPN, the immune response of both CPN-BPL-HSV-2 and CPN-F-HSV-2 were elevated antibody level relative to time till the 8th week post immunization followed by significant decreased immune response till 12th week ($P<0.05$). While post boostering there was a noticed elevation that not extended too much and declined after 16th week followed by significant decrease till 24th week ($P<0.05$) at different thermal conditions. There were a non-significant difference between the two formula as using the same adjuvant but using different viral inactivants ($P>0.05$).

Alum adjuvanted formulations as formalin or BPL inactivated at different thermal conditions had given more significant increase in immune response than those calcium phosphate adjuvanted ($P<0.05$) [Fig.3-8] .

All formulation behave the same thermal pattern as there was a non significant difference between freshly prepared formulations and those thermally treated for 1 week at 37°C ($P > 0.05$). While there was significantly elevated antibody level between freshly prepared formulations and those incubated at 37°C for 2 and 4 weeks post immunization ($P < 0.05$). Also, highest difference in antibody level was showed when compared to the same formulations stabilized for a year at 4°C and accelerated stabilized at 45°C for a week ($P<0.05$) .

Plasma, IFN-γ and IL-10 levels

Regarding the cellular immune response post vaccination with test vaccine candidates there was a noticed significant elevation of IL-10 and IFN –γ level relatively to time compared with that induced post immunization with inactivated non adjuvanted vaccines. As, Al-BPL-HSV-2 induced IL-10 and IFN-γ significantly elevated compared with Al-F-HSV-2 ($P<0.05$) [Fig. 9,10].

V. Discussion

Data reported revealed that AL-BPL-HSV-2 vaccine candidate showed a longer durative and higher immune response with a better stability either real time stability or accelerated stability (4°C , 37°C and 45°C) compared with other vaccine formulations prepared using CPN as adjuvant , and the variation of antibody level may be attributed to variation in viral load efficacy/ capacity that may be due to the nature of adjuvant ,chemical structure of alum than CPN, particle size and physiochemical structure. Our data were in the opposite to records of [16,22,25] as they used different vaccine candidates and the virus particle size and out shell charges may have a role in these variation. Also, our data were in agreement with them as they recorded the better potential of BPL as inactivant than formalin. The present data was in agreement with [34] despite their use of thermal treatment of viral inactivation at 33°C for 72 hrs, while we used F and BPL for 24 hrs at 37°C. Regarding the formulation of HSV-2 vaccine, our best formula was Al-BPL-HSV-2 and no viral infection detected post challenge representing a higher immune potentials that could neutralize the challenge dose of HSV-2. These records were partially agreed with data recorded by [37] regarding the efficacy of HSV-2 vaccine protection and immune potential. But, they disagreed with them in the formulation pattern and route of administration of vaccine, where they reported that the best protection against HSV-2 disease and shedding was obtained by intramuscular (i.m) injection of FI-HSV2/ MPL/Alhydrogel.

Also, they postulated that the route of administration used of formulated vaccine candidate (FI-HSV2/MPL/Alhydrogel) could affect the immune response, but not resulting a high level of protection, and in 2 independent experiments, i.m immunization elicited significantly higher levels of virus neutralizing antibodies compared to the s.c rout of administration. The immunological mechanism for the better protection against disease found by i.m delivery of the same FI-HSV-2 vaccine is unknown, but is likely related to differential effects of administration route on the levels, functions, or localization of vaccine induced immunity [37].

In the mean time our data was aligned with [37] despite we arranged for only one challenge trial and vaccine was subcutaneously administered as a whole viral antigen adjuvanted with both CPN and alum, while they reported that this vaccine prevented HSV-2 disease despite the mild inflammation, reduced virus shedding by 3 to 4 Logs, protected the majority of animals from detectable vaginal virus even after two challenges, and provided complete protection against HSV-2 DNA in DRG. The high level efficacy was dependent upon formulation with MPL/Alhydrogel (monophoshoryl lipid) or MPL/Adju-Phos. The long-term challenge experiment with FI-HSV2/MPL/Adju-Phos (formalin inactivated) demonstrated that responses were durable, as similar high level protection was observed at 3 and 9 weeks post-boost. Also, [34] as they evaluated the immune response post vaccination with hydragel adjuvanted FI-HSV-MPL / Alhydrogel and reported that antibody level cold reduce the virus infectivity titer by 4 $\log_{(10)}$. Also, the protection in animal models appears to be at least as high as that engendered by attenuated or replication-defective HSV-2 vaccines, strategies that have the potential for the establishment of latency or recombination with a wild-type virus. While vaccination with inactivated virus eliminates many safety concerns, a major criticism of the first-generation inactivated HSV-2 vaccines has been the poor durability of elicited responses. However, that formulation with adjuvants such as MPL/Alhydrogel challenges this dogma. These exciting results demand further consideration of inactivated HSV-2 vaccines formulated with the modern, clinically approved adjuvants. In the present study s.c route of administration was conducted and that was in agreement with [36] where they used i.m ad s.c. routes of administration and compared between their effect on immune response. And they found that the route of administration of the same tube of formulated FI-HSV2/MPL/Al hydrogel could affect the immune response in unknown way as recorded by [37].

Regarding the stability assessment of test vaccine our stabilization / preservation condition were in agreement with [34] despite use of different inactivated viral vaccine model and different formulation using CPN and Al recording that yeast-

derived recombinant DNA HB vaccine (Engerix-B) is apparently stable for 30 days at 20–25°C, for one week at 37°C, and for three days at 45°C, the corresponding half lives being calculated as nine months, 31 days and 13 days according to the manufacturer protocols. In the mean time there were no differences in immune responses between healthy persons immunized with a recombinant vaccine heated to 37°C for one week and similar persons given a control vaccine stored at 4°C , One month at 37°C and 45°C for one week ; the antibody distribution and geometric mean antibody titers were similar in the two groups. The total incidence, severity and types of symptoms were similar in persons immunized with the two vaccines, and no severe reactions were reported [38-40].

VI. Conclusion

Finally from the presented data, it could be concluded that formalin and BPL are good inactivants despite the inactivation potential of both was different since BPL showed a faster inactivation activity than formalin. Al-BPL-HSV-2 prepared vaccine showed a significant elevated immune response than CPN-BPL-HSV-2 prepared vaccine. Boostering is needed in case of using both vaccine candidates either adjuvanted or not. Vaccine immune potential maintained active post stability testing at different thermal conditions for tested durations.

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Figure legends

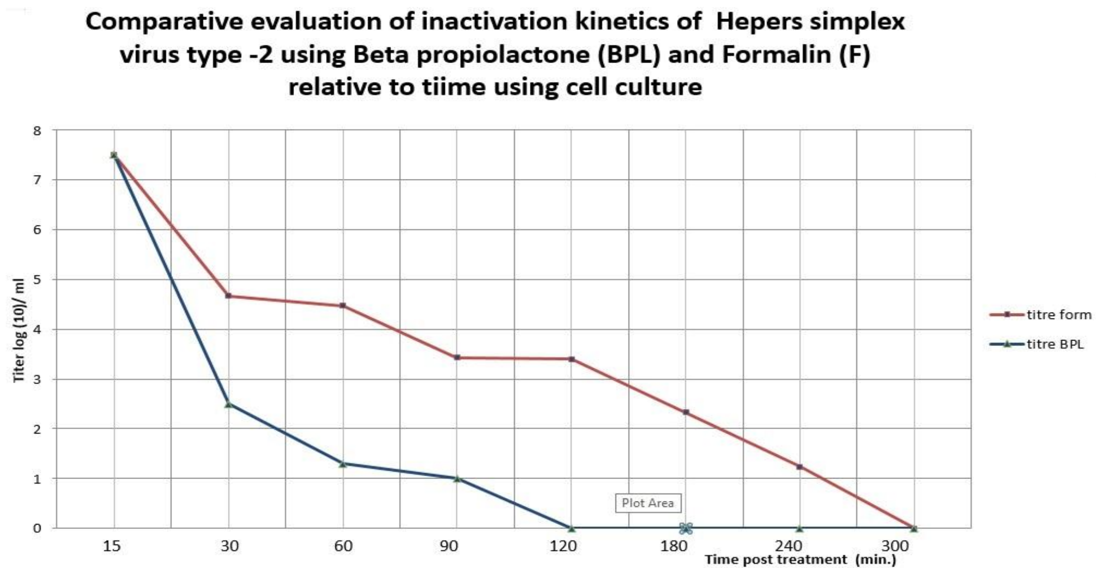

Fig.1. Inactivation kinetics of HSV-2 using F and BPL.

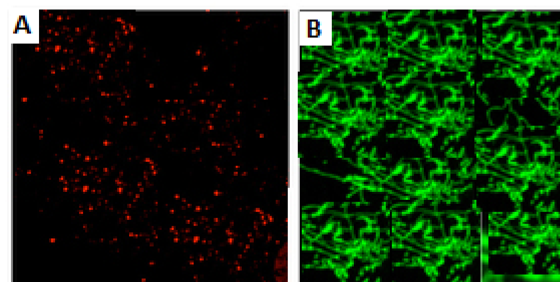

Fig.2. Detection of residual live HSV-2 virus in mice vaginal tissue post challenge

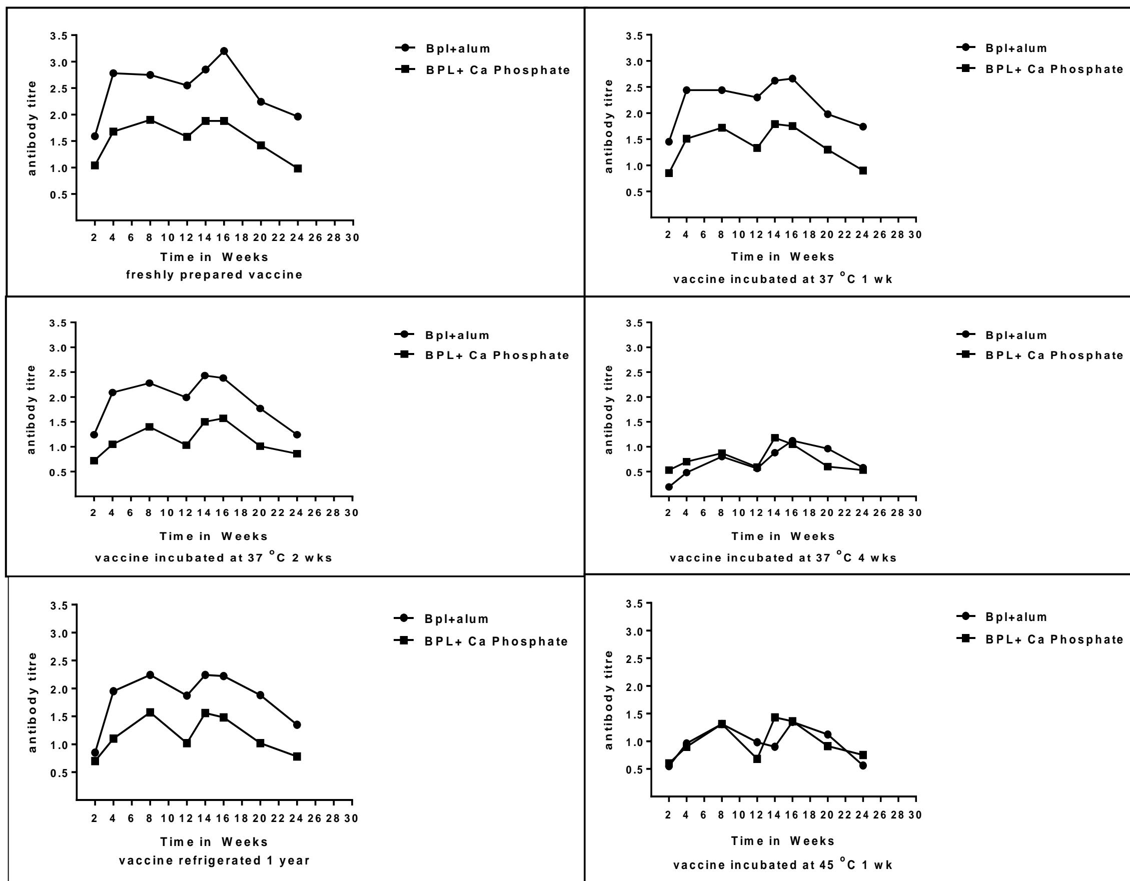

Fig.3-8. Evaluation of immune response post vaccination with Alum -BPL-HSV-2 vaccine versus Ca -BPL-HSV-2 relative to time

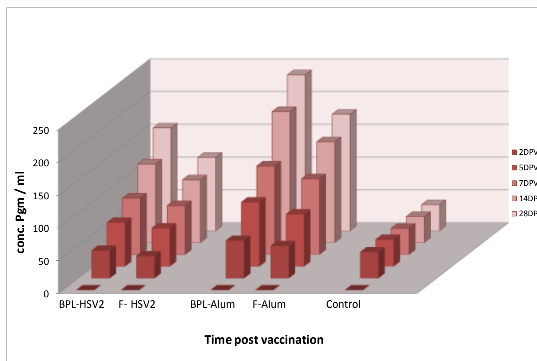

Fig.9. Evaluation of Interleukin level post vaccination with BPL and Formalin inactivated

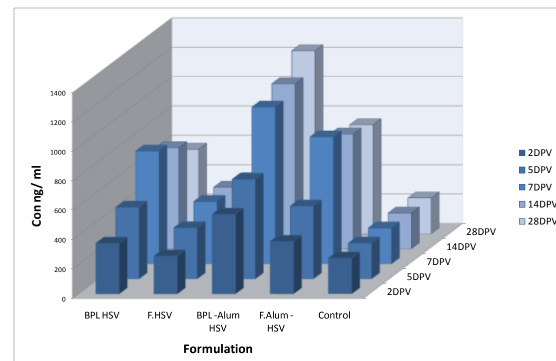

Fig.10. Evaluation of interferon level post alum adsorbed and non adsorbed vaccine candidates

N.B

Tables showing the effect of different thermal conditions related to time interval on the immune response (not included in paper).

TCID50: tissue culture infectious dose which will infect 50% if the cell monolayers challenged with the defined inoculum