Hacettepe University, Journal of the Faculty of Pharmacy
Volume 24 / Number 2 / July 2004 / pp.81-94

Adjuvant Effects of Niosome and Water/ Oil/Water Multiple Emulsion Carrier Systems for Recombinant Hepatitis B Surface Antigen

Received : 23.11.2004
Revised : 05.01.2005
Accepted : 16.02.2005

Aydan Eratalay*, Filiz Öner*, Erkan Özcengiz, Reha Alpar*****

1.Introduction

Recombinant vaccine antigens are highly purified materials, so their safety profiles are much higher than conventional vaccines. However, they can not elicit strong immune responses in the absence of safe and effective adjuvant formulations[1,2].

In recent years improved adjuvant formulations have attracted increasing interest in the pharmaceutical area. The term adjuvant can be applied to an agent that augments specific immune responses to antigens. The adjuvant formulation should be efficacious, safe, stable and easy to use[3]. Adjuvants can act with different mechanisms. Chedid summarized the mechanism of adjuvant effects as follows[4];

- formation of a depot of antigen which is released slowly,
- presentation of antigen to immunocompetent cells,
- production of different lymphokines such as interleukins and tumour necrosis factor

There are two types of adjuvant formulations, the first type includes small molecule immunomodulators such as interleukins, lipids, saponins and the second type includes vehicles generally in the form of disperse systems. Metal ions, emulsions, lipid and surfactant vesicles, particulate systems and viral particles are systems known to have adjuvant properties. Most vaccines available on the market do not achieve ideal properties. At

* Department of Pharmaceutical Biotechnology, Hacettepe University, 06100, Ankara, Turkey
** Division of Vaccine and Sera , Refik Saydam Central Institute of Hygiene, 06100, Ankara,Turkey
*** Department of Biostatistics, Hacettepe University, 06100, Ankara, Turkey

present the only adjuvant licensed for human use in the United States is an aluminum salt (alum). Alum acts by stimulating antibody release but is not recognized as an adjuvant for inducing effective cell-mediated immune responses and it has some unwanted side effects[5]. Mineral oil-based stronger adjuvants, such as complete Freund's adjuvant (CFA), can cause serious adverse reactions including local granulomas, pain, fever and possibly malignancies, making it unacceptable for human use[6].

At present approximately 70 biotechnologically derived vaccine formulations are in the development stage and a few of them are on the market. Commercially available vaccines against hepatitis B virus infections developed by recombinant DNA technology are safe and immunogenic but must be injected repeatedly to provide protective antibody levels[7].

In this research, recombinant hepatitis B surface antigen was selected as a model antigen due to the increasing importance. In vitro-in vivo studies were performed in order to design suitable alternative adjuvant systems. Adjuvants were formulated in the form of W/O/W multiple emulsion, niosome and an aluminum based suspension. Antigen was included appropriately into the formulations and evaluated in vitro-in vivo.

2. *Materials and Methods*

2.1. Materials

Recombinant HBsAg was kindly provided by Smith Kline Beecham, Belgium. Nonionic surfactants Arlacel 1689 and Synperonic PE/F 68 were generous gifts from ICI-Surfactants-Kemsol, Turkey. Anti-HBs Elisa kit was purchased from Teco Diagnostics, USA. Other chemical substances used in the experiments were of analytical grade.

2.2. Adjuvant Formulations

- W/O/W multiple emulsion

A water/oil/water type multiple emulsion was prepared by modifying the two step emulsification procedure described by Florence et al[8]. First a primary W/O emulsion was prepared by addition of the water phase to the oil phase containing lipophylic surfactant and cholesterol. According to the preliminary studies the mixture was agitated for 50 minutes at 163 gain. The primary W/O emulsion was added into the outer water phase, containing hydrophilic emulsifier and HBsAg, at 56 gain mixing rate for

15 minutes and then at 92 gain rate for 10 minutes at room temperature. The general formula (w/w) is:

Primary W/O emulsion:

-Liquid paraffin	38.23 g
-Arlacel 1689	1.96 g
-Cholesterol	0.58 g
-NaCl	0.39 g
-Distilled water	58.8 g

W/O/W multiple emulsion:

-Primary emulsion	70 g
-Synperonic PE/F 68 (%8)	30 g

Stability of the emulsions (containing and not containing HBsAg) was evaluated by keeping them at 4°C, 25°C and 40°C temperatures. Macroscopic-microscopic appearances and particle size distribution were evaluated for 6 months period. pH and viscosity of the emulsions were measured to characterize the formulations. Particle size of the emulsions were measured with an electronic particle counter (Coulter Multisizer II, England). Viscosity of the emulsions were measured with a cone plate viscometer and S51 type cone (Brookfield Model DV-II cone plate, USA) at 25°C±3°C temperature.

- Nonionic Surfactant Vesicles

Nonionic surfactant vesicles were prepared by the hand-shaking method[9]. 155 µmol Brij surfactants 72:721 (1:1) and 0.031 g Cholesterol were dissolved in chloroform and the solvent was evaporated under low pressure in a rotating evaporate system. Dry surfactant film was hydrated with distilled water at 55°C and 200 µg HBsAg was added to niosome after cooling to room temperature.

Niosomes (containing and not containing HBsAg) were kept at 4°C, 25°C and 40°C to investigate the stability of samples by examining their microscopical appearance and particle size. pH and viscosity of the niosomes were measured to characterize the formulations.

- Control

0.5 mg/mL aluminium salt (alhydrogel) in distilled water was prepared as control adjuvant formulation.

2.3. Animal Model

Female mice (swiss albino) of 25±5 g and female guinea pigs of 210 to 250 g were used as animal models in the in vivo studies.

2.4. Immunization Protocol

Four groups of 10 mice were immunized intramuscularly (i.m.) with emulsion, niosome and alhydrogel formulations. All of the formulations contained 1µg/0.5mL and 10µg/0.5mL antigen. On the 30th day of the experiment, booster injections were performed. Cardiac blood samples were collected weekly up to the 13th week and stored at -20°C until assayed.

2.5. ELISA

Specific anti HBsAg antibody titers measured by ELISA kits were provided by Teco Diagnostics. Mice were bled nine times on a weekly basis and sera were stored at -20°C and tested by a solid-phase simultaneous immunoassay to detect antibody against HBsAg. In this technique, all the reagents were allowed to reach room temperature before use. 50µl serum and 50 µl positive control as well as negative control were dispensed into respective wells. 50 µl enzyme conjugate was added to each well on the flat bench microtiter plate. After mixing gently the microtiter plate was placed into a humidified box and incubated at 37°C for 60 min. Wells were washed with diluted wash buffer 6 times, then the plate was inverted vigorously to get all water out and the rim of wells was blotted on absorbent paper for a few seconds. 50 µl of substrate solution A was added to each well and then 50 µl of substrate solution B was added to each well. After mixing gently, the plate was incubated at room temperature for 15 min. 50 µl of stop solution was added to each well to stop the colour reaction, and optic density values of all samples were red at 450 nm.

2.6. Toxicity Tests

Toxicity studies were performed by serum creatine phosphokinase activity test and the weight gain test in mice and guinea pigs[10.11].

a. Serum Creatine Phosphokinase Activity Test

Mice were injected with w/o/w multiple emulsion, niosome and alhydrogel formulations which contained 1µg/0.5 mL HBsAg. Blood samples were collected 2 days after the injection. Enzyme levels were measured by using Hitachi/Automatic Analyzer.

b.Weight Gain Test

Five mice and two guinea pigs were injected by i.m. route with emulsion, niosome and alhydrogel formulations (0.5 mL for mice and 2.5 mL for guinea pigs). Weights of the animals were measured before the injection and 7th day of the experiment.

2.7. Haemolytic Activity

Haemolytic activity was determined by the method described by Reed and Yalkowsky[12]. 0.2 mililiters of citrated fresh human blood was added to 0.1mL samples of emulsion, niosome and alhydrogel formulations. After mixing gently, samples were incubated at 25°C for 2 min, and 5 mL saline was added to them. These mixtures were centrifuged at 2000 g for 10 minutes, pellets were washed four times with 5mL saline and supernatants were discarded. Pellets consisting of red blood cells and ghosts were dispersed with 4mL distilled water and centrifuged at 2000 g for 10 minutes. 1 mL of the supernatant was diluted with 4 mL of distilled water and the absorbances were recorded at 540 nm. The absorbance value at this wavelength is directly proportional to the haemoglobin concentration.

The 100 percent haemolysis level was defined as the absorbance of the haemoglobin in the supernatant at 540 nm after the erythrocytes were completely haemolysed in distilled water.

2.8. Delayed Type Hypersensitivity Test

On the 44th day of the vaccination the delayed type hypersensitivity test was performed in mice. In this procedure, the hair on the flanks of the animals is removed and the bare skin is injected with the formulation and hypersensitivity reaction on the skin was evaluated by a dermatologist.

2.9. Statistical evaluation

Two-way analysis of variance was used to evaluate the changes in differences between doses and IgG titers using SPSS 10.

3. Results

3.1. In vitro characteristics of the emulsions

Under the light microscope the characteristic structure of a multiple emulsion was observed (Figure 1). By visual observation, instability indicators such as coalescence or creaming were not observed in the multiple emulsion samples stored at 4°C, 25°C and 40°C.

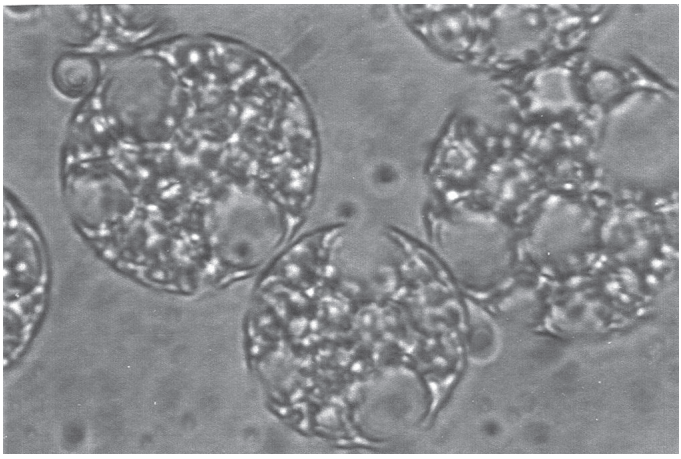

Figure 1
Photographs of the multiple emulsion droplets under the light microscope (x40)

Mean droplet size of the emulsion samples was measured as 1.7±0.24 µm. During the six months storage period the mean droplet size of the emulsion samples did not change significantly ($p<0.001$).

The pH value of the freshly prepared emulsion was 6.9 and after one month storage at 25°C temperature, pH value was slightly changed to pH 6.4.

Initial flow curve of the emulsion sample is seen in Figure 2. After storage at 25°C, viscosity of the formulation is decreased and type of the

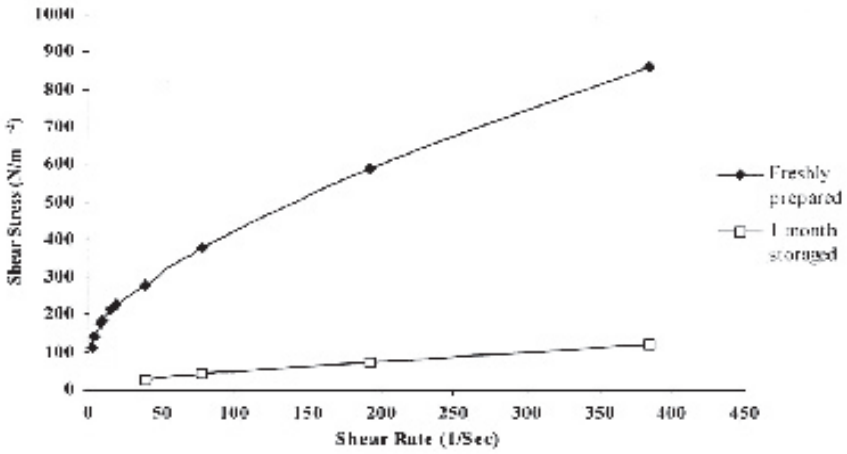

Figure 2
Flow Curve of W/O/W Emulsion

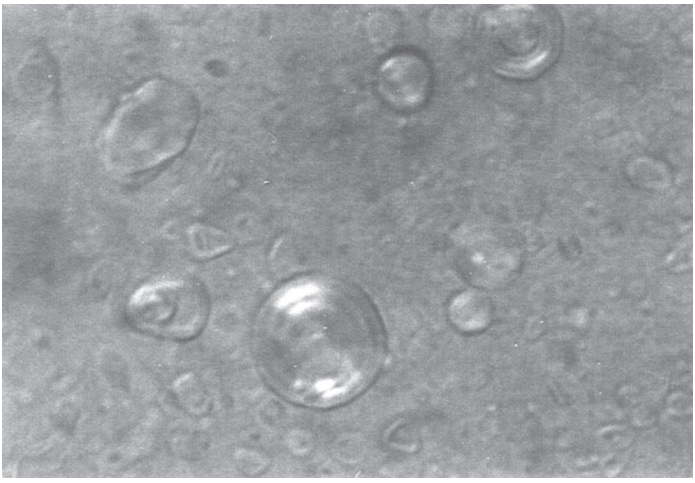

Figure 3
Photographs of the niosomes under the polarizing microscope (x100)

flow curve changed to the Bingham flow curve. Meanwhile coalescence or creaming were not observed in the samples. This change may reflect reversible flocculation of the inner phase.

3.2. In vitro characteristics of the niosomes

Vesicular structure of the niosomes was examined under polarizing microscope by observing typical polarizing crosses (Figure 3). Initial particle sizes of the niosomes were measured as 1.25 µm. During the six months period particle size of the niosomes was not changed significantly ($p<0.001$).

Initial pH value of the niosome containing liquid formulations was 6.5 and after 1 month storage at 25°C, pH was decreased to 5.6. Viscosity of niosome formulation was measured as 1.5 poise with a Ubbelohde viscometer and not changed after one month storage at 25°C.

3.3. Haemolytic activities of the formulations

Among the adjuvant formulations (W/O/W, niosome and aluminium gel), haemolytic effects of the niosome and alhydrogel formulations were higher than W/O/W emulsion formulation in an respectively increasing order. Alhydrogel is the least acceptable formulation from the aspect of the haemolytic effect. Freshly prepared formulations caused less haemolysis than aged formulations. Results were seen in Figure 4.

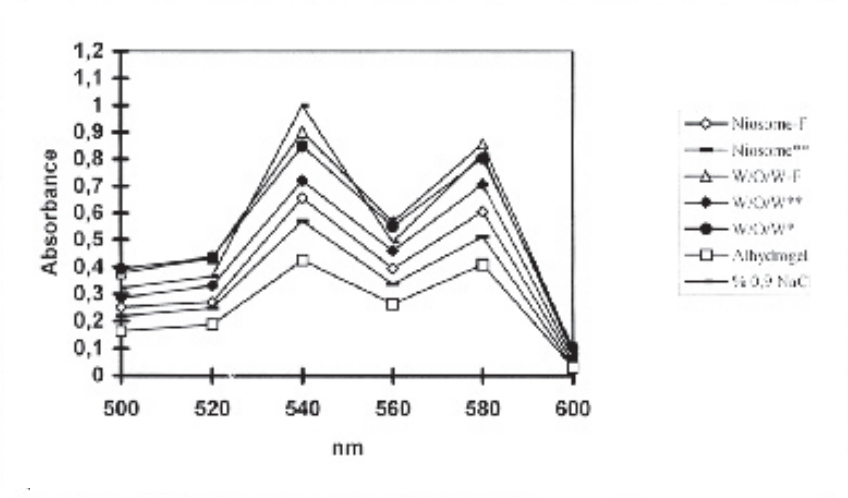

Figure 4
Haemolysis Profiles of the Formulations
F: freshly prepared **: 15 day stored *: 1 month stored

3.4. Delayed type skin hypersensitivity reaction of the formulations

Delayed type skin hypersensitivity reaction was not observed at the 44th day of the experiment with all of the formulations.

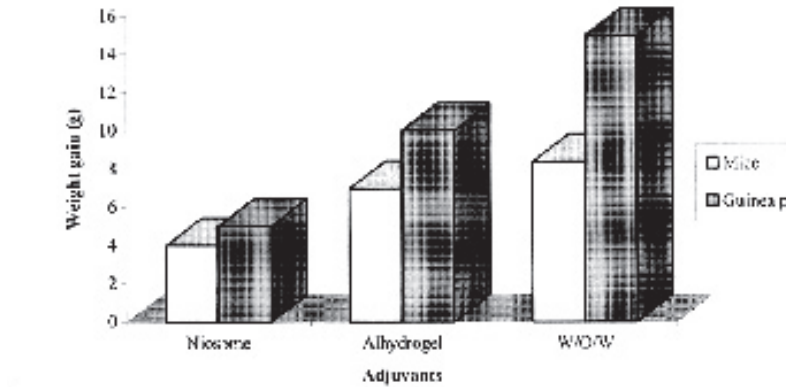

Figure 5
Weight Gain of the Animals Administered With Different Formulations

3.5. Toxicity tests

Creatine phosphokinase activities (CPA) were measured in the serum of immunized animals. 3000 IU is given as the acceptable higher limit for CPA level for a toxicity test[10]. CPA values with all formulations were measured lower than 3000 IU (Table I).

TABLE I
Creatine Phosphokinase Activities of the Injected Animals With Adjuvant Formulations

Formulations	Creatine Phosphokinase Level (IU)
W/O/W	935
Niosome	182
Alhydrogel	423

Presence of endotoxin causes weight loss in the animals[11]. In this study, tested animals didn't lose weight after 7 days of immunization (Figure 5).

3.6. Immune responses to the formulations

Results of Elisa analysis obtained with pooled sera of mice vaccinated with 1µg and 10µg of HBsAg are shown on Figure 6, 7. Mice

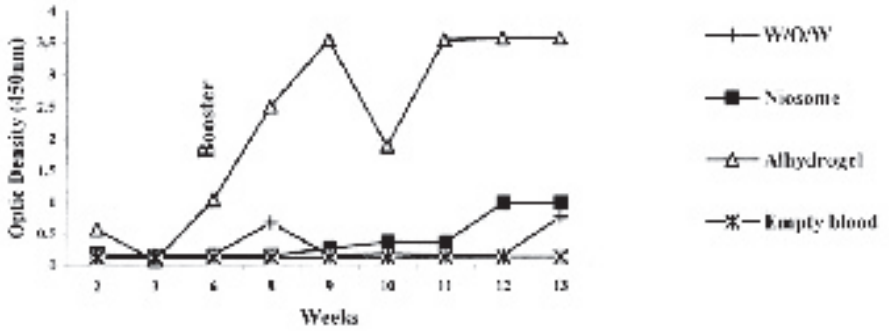

Figure 6
AntiHBs Responses to the Formulations (1µg/0.5 mL)

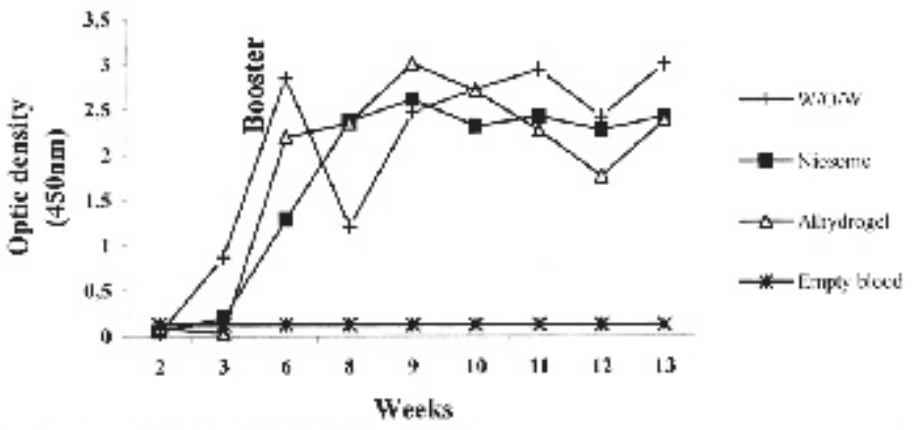

Figure 7

AntiHBs Responses to the Formulations (10µg/0.5 mL)

responded to i.m. injections of either 1µg or 10µg HBsAg containing formulations. W/O/W emulsion and noisome formulations at 1µg/0.5 mL concentrations showed lower activity than the formulations containing 10µg/0.5 mL antigen. This effect may be due to the partial inactivation of antigen by surfactants. Responses to HBsAg in the form of W/O/W emulsion was higher than the responses to other formulations with 10µg/0.5 mL antigen dose (Figure 7).

3.7. Statistical evaluation

Antibody titers from two doses of antigen formulations were compared by using ANOVA and Tukey HSD tests. Results were outlined in tables.

ANOVA results are given in Table II and III.

According to the results in Table II;

- Differences between formulations were significant (F=5.660, p=0.008)

- Differences between doses were significant (F=12.011, p=0.003)

- The interaction between formulation and dose was not significant (F=2.144, p=0.135)

According to the results in Table III; differences between the formulations and the doses, and interaction of the formulations and doses were significant (p=0.000).

TABLE II
Two-way ANOVA Results for the Differences Between the Second and First Week Antibody Titer Values

Source of variation	SS	df	MS	F	p
Formulations	1.359	3	0.453	5.660	0.008
Doses	0.962	1	0.962	12.011	0.003
Formulations-Doses	0.515	3	0.172	2.144	0.135

SS : sum of squares df : degrees of freedom p : level of significance
MS : mean squares F : F value

TABLE III
Two-way ANOVA Results for the Differences Between the Last and First Week Antibody Titer Values

Source of variation	SS	Df	MS	F	P
Formulations	15.850	3	5.283	190.603	0.000
Doses	4.056	1	4.056	146.318	0.000
Formulations-Doses	9.011	3	3.004	108.363	0.000

4. Discussion

Adjuvants are included in vaccine formulations in order to induce immune responses with faster and longer duration of immunity. In recent years, development of more protective but less toxic vaccine formulations has become possible by developing new adjuvant delivery systems.

Immunization dose, administration route, ease of use, protective efficiency and nontoxic properties are important parameters for adjuvant carriers.

Herbert W.J. used multiple emulsions as vehicles for the parenteral administration of certain vaccines. They found that this system was preferable when compared to simple emulsion systems since they were easier to inject and the resultant antibody titre was much higher[13]. Subsequently, some workers have employed multiple emulsions for the administration of vaccines and they reported advantages of multiple emulsions over other types of vaccine systems, giving rise to a good antibody response[14]. In this study we have formulated two adjuvant formulations and compared them with conventional alum adjuvant system. Homogeneity, particle size distribution and viscosity values of the formulations are observed as acceptable for the application of systems by parenteral route. Stability parameters were satisfactory for the duration of the experiments. Among the formulations, emulsion yielded the lowest

in vitro haemolysis value. High amounts of surfactant in the niosome formulation might cause higher haemolysis ratios than emulsion but may also help immunogenicity.

In this study W/O/W emulsion and niosome adjuvant formulations seemed to be good candidates as carrier systems for HBsAg[15]. Emulsion systems are seemed to be non-toxic, non-haemolytic and effective formulations.

In the recent literature nonionic surfactant vesicles seem to have significant advantages over liposomes in that they are stable in the atmosphere and do not require any special handling or storage conditions [16]. Nonionic vesicles are likely to be less toxic than vesicles produced from ionic amphyphiles[17]. In our study emulsions and niosomes displayed better antibody responses than the other formulations in every aspect except the dose related manner. Higher antibody responses were measured by i.m. injection of 10µg rHBsAg than 1µg antigen containing formulations (Fig. 6-7). These data was supported by another study reported higher immunogenicity of vaccine with 20 µg antigen load as compared to vaccine with 10 µg antigen load[18].

CPA values were observed in acceptable limits in in vivo toxicity studies in the animals given all of the formulations (Table I).

According to the results from the study multiple emulsions and niosomes may be promising adjuvant carriers for recombinant antigens.

It can be concluded from these data that protective immunity could be achieved with alternative adjuvant systems.

Acknowledgements

This work was supported by a grant from Government Planning Organisation, DPT Ankara, Turkey.

Summary

There is an increasing interest in vaccine studies in terms of inventing new adjuvants and carrier systems because of the poor immunogenicity of pure synthetic recombinant antigens and problems with aluminium based adjuvants. New alternative adjuvant systems must not cause local and systemic reactions and they have to provide long term immunization with minimum application.

In this study two new adjuvant carrier systems, niosome (nonionic surfactant vesicles) and W/O/W emulsion were compared with an

aluminium based adjuvant. Recombinant HBsAg incorporated into all adjuvant carriers as a model antigen and antibody titers were evaluated with an in vivo animal model. W/O/W emulsion formulation caused to the lowest haemolysis on the blood samples in vitro. Toxicity studies were carried out by measuring serum creatine phosphokinase activity and weight gain in mice and guinea pigs and the results were obtained in acceptable limits.

Hepatitis B antibody titers in blood serums of mice were determined by using ELISA technique. Antibody titers were obtained higher with the emulsion and niosome formulations of 10µg dose than 1µg dose.

Key words: Recombinant Hepatitis B surface antigen, adjuvant formulations, niosomes, W/O/W emulsions, alum

Özet

Rekombinant Hepatit B Yüzey Antijeni İçin Niozom ve Su/Yağ/Su Çoklu Emülsiyon Taşıyıcıların Adjuvan Etkileri

Saf sentetik rekombinant antijenlerin zayıf immünojenisitesi ve aluminyum bazlı adjuvanlarla ilgili sorunlar nedeniyle aşı çalışmalarında yeni adjuvanlar ve taşıyıcı sistemlere artan bir ilgi mevcuttur. Yeni alternatif adjuvan sistemler lokal ve sistemik yan etkilere neden olmamalı ve en az uygulama ile uzun süreli bir bağışıklık sağlamalıdırlar.

Bu çalışmada iki yeni adjuvan taşıyıcı sistem olan niozom (non iyonik yüzey aktif veziküller) ve S/Y/S emülsiyonu bir alüminyum bazlı adjuvan ile karşılaştırılmıştır. Adjuvan taşıyıcıların tümüne model antijen olarak rekombinant HBsAg eklenmiş ve antikor titreleri in vivo hayvan modeli ile değerlendirilmiştir. S/Y/S emülsiyon formülasyonu in vitro kan örneklerinde en düşük hemolize neden olmuştur. Toksisite çalışmaları fare ve kobaylarda serum kreatin fosfokinaz etkinliği ve kilo artışı ölçülerek yapılmış ve sonuçlar kabul edilen sınırlar içerisinde bulunmuştur.

Farelerin kan serumlarındaki Hepatit B antikor titreleri ELISA tekniği ile saptanmıştır. 10 µg doz içeren emülsiyon ve niozom formülasyonlarında 1 µg doz içeren formülasyonlara göre daha yüksek antikor titreleri elde edilmiştir.

Anahtar Kelimeler: Rekombinant Hepatit B yüzey antijeni, adjuvan formülasyonları, niozomlar, S/Y/S emülsiyonlar, alum.

REFERENCES

1. Stewart-Tull, D.E.S., 'Recommendations for the assessment of adjuvants (immunopotentiators)', Gregoriadis, G., Allison, A.C, Poste, G. (Eds.), Immunological Adjuvants and Vaccines, New York, Plenum Press, (1989), pp 213-226.
2. Giudice, G.D.: New carriers and adjuvants in the development of vaccines, Current Opinion in Immunology, 4, 454-459 (1992).
3. Allison, A.C., Byars, N.E.: An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and cell-mediated immunity, J. Immunol. Methods, 95, 157 (1986).
4. Chedid, L.: Adjuvants of immunity, Ann. Inst. Pasteur Immunol., 136D (3), 283-91 (1985).
5. Hem, S.L., White, J.L, 'Structure and properties of aluminium-containing adjuvants', Powell, M.F., Newman, M.J., Burdman, J.R. (Eds.), Vaccine Design, New York, Plenum Press, (1995), pp. 249-275.
6. Uchida, T., Shiosaki, K., Nakada, Y., Fukada, K., Eda, Y.: Microencapsulation of Hepatitis B core antigen for vaccine preparation, Pharm. Res. 15 (11), 1708-1713 (1998).
7. Canho, R., Grosheide, P.M., Voogd, M., Huisman, W.M., Heijtink, R.A., Schalm, S.W.: Immunogenicity of 20 µg of Recombinant DNA Hepatitis B Vaccine in Healthy Neonates: A Comparison of Three Different Vaccination Schemes, J.Med.Virol., 41, 30-34 (1993).
8. Florence, A.T., Whitehill, D.: The formulation and stability of multiple emulsions, Int. J. Pharm., 11, 277-308 (1982).
9. Bouwstra, J.A., Hofland, H.E.J., Gooris, G.S., Junginger, H.E., 'Characterization of niosomes', Bouwstra, J.A., Hofland, H.E.J., Gooris, G.S., Junginger, H.E. (Eds.), A fundamental and practical approach, Netherlands, Kluwer Academic Publishers, (1992), pp. 227-238.
10. Gray, J.E., Weaver, R.N., Moran, J., Feenstra, E.S.: The parenteral toxicity of clindamycin 2-phosphate in laboratory animals, Toxicology and Applied Pharmacology, 27, 308-321 (1974).
11. World Health Organization, Mondiale de la Santé, Manual for the Production and Control of Vaccines, (1977), pp. 70-71.
12. Reed, K.W., Yalkowsky, S.H.: Lysis of human red blood cells in the presence of various cosolvents, J. Parent. Sci. and Tech. 39 (2), 64-67 (1985).
13. Herbert, W.J. : Multiple emulsions, Lancet, 2, 771 (1965).
14. Davis, S.S.: Liquid membranes and multiple emulsions, Chemistry and Industry (1981), 684-688.
15. Eratalay, A., Öner, F., Özcengiz, E., Hıncal, A.A.: Adjuvant carriers for recombinant Hepatitis B virus surface antigen, Proc.2nd World Meeting APGI/APV, pp. 1127-1128, (1998).
16. Brewer, J.M., Alexander, J.: The adjuvant activity of non-ionic surfactant vesicles (niosomes) on the Balb/c humoral response to bovine serum albumin, Immunology, 75, 570-575 (1992).
17. Baillie, A.J., Florence, A.T., Hume, L.R., Muirhead, G.T., Rogerson, A.: The preparation and properties of niosomes-nonionic surfactant vesicles, J.Pharm.Pharmacol., 37, 863-868 (1985).
18. Chiaramonte, M., Majori, S., Ngatchu, T., Moschen, M.E., Baldo, V., Renzulli G., Two Different Dosages of Yeast Derived Recombinant Hepatitis B Vaccines: A Comparison of Immunogenicity, Vaccine, 14 (2), 135-137 (1996).