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EXAMINATIONS OF THE IMMUNOGENICITY OF THE EXPERIMENTAL BIVALENT SUBUNIT VACCINES AGAINST HERPES SIMPLEX VIRUS TYPE 1 AND 2

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*The objective of our study was to check the immunogenicity of experimental bivalent subunit vaccines against Herpes simplex viruses (HSV1 and HSV2). The subunit vaccines were prepared from glycoprotein antigens isolated from the external envelopes of HSV1 and HSV2. In the first vaccine the glycoproteins subunits were adsorbed on the adjuvant Al(OH)3. The secortd subunit vaccine was prepared from glycoprotein antigens with the adjuvant MPs-19 (a water soluble oligosaccharide) dissolved in phosphate buffered saline (PBS). The final concentration of $Al(OH)_3$ in the first vaccine was 5mg/ml, while the final concentration of MPS_{-19} in the second vaccine was 2 mg/ml. These vaccines contained 0.13 mg total protein for the HSV1 subunits and 0.16 mg for the HSV2 subunits ml. One group of 10 female Swiss albino mice (20 gr body weight) were each immunized s.c. with 0.5 ml of the HSV1/HSV2 subunit vaccine, containing the adjuvant Al(OH)3. The second group of 10 mice was immunized with the same quantity of the HSV1/HSV2 subunit vaccine with adjuvant MPS-19. A group 11 healthy, nonimmunized mice served as the control in the assay. The specific humoral immune response of the vaccinated mice was measured by a standard method of indirect immunofluorescence. The specific cellular immune response of the vaccinated animals was tested by measuring the lymphocyte proliferation response to HSV1 and HSV2 antigens with tritium methyl thymidine *128.5 Bq/mmol), in vitro. The titres of the specific IgG and IgM class antibodies, in the sera of both groups of immunized mice on day 10 after vaccination were: a) with adjuvant Al(OH)3: from 1:8 to 1:128 IgG; and 1:32 to 1:128 IgM for HSV1; from 1:32 to 1:128 IgG and IgM antibodies, for HSV2; b) with adjuvant MPS_{-19}; from 1:16 to 1:128 IgG and 1:64 to 1:128 IgM for HSV1; from 1:16 to 1:32 IgG and 1:64 to*

1:128 IgM for HSV2. The mean values for radioactivity in the medium after spontaneous proliferation of lymphocytes were 799 cpm for the first and 798 cpm for the second experimental group. The mean radioactivity of lymphocytes from the first group of mice stimulated with HSV1 and HSV2 antigens in, 10 days after immunization, was 1470 cpm (for HSV1) and 1845 cpm (for HSV2). The mean values of radioactivity in the proliferation test for HSV1 and HSV2 antigens in the second experimental group of mice on day 10 after vaccination were 1389 cpm (for HSV1) and 2416 cpm (for HSV2). Thus, the low subunit antigen concentrations in both vaccines, induced satisfactory humoral and cellular immune responses to HSV1 and HSV2 in the organisms of immunized mice.

Key words: HSV1, HSV2 glycoprotein antigens, subunit vaccines, immunization, swiss albino mice.

INTRODUCTION

Herpes simplex viruses 1 and 2 have 9 surface glycoprotein antigens (gA-gI) important for the biological characteristics of the virus and of the host cells. The glycoproteins gC and gE are responsible for the determination of the virus types; whilst the glycoproteins gB and gD represent key molecules in the process of inducing the synthesis of virus neutralizing antibodies for HSV1 and HSV2 in the host organisms, i. e. for stimulating protective humoral and cellular immune responses, in vivo (Arvin et al., 1991, Roizman and Sears 1990;, Savarese et al. 1994;, Stanberry et al. 1987. and Whitley, 1990.).

We decided to check the immunogenicity of two bivalent subunit vaccines against HSV1 and HSV2, prepared from purified glycoprotein antigens isolated from the excternal envelopes of the above mentioned viruses (with two different adjuvants) in a biological assay on mice.

MATERIAL AND METHODS

I Vaccines 1. The subunit vaccines were prepared from glycoprotein antigens isolated from the external envelopes of HSV1 and HSV 2. In the first vaccine the glycoprotein subunits were adsorbed on the adjuvant Al(OH)3, in 100 mmmol/1 PBS. second subunit vaccine was prepared from glycoprotein antigens with the adjuvant MPS-19 (a water soluble oligosaccharide) dissolved in PBS. The final concentration of Al(OH)3 in the first vaccine was 5 mg/ml, while the final concentration of MPS - 19 in the second vaccine was 2 mg/ml.

The HSV1 and HSV2 viruses used for the preparation of the subunit vaccines were individually propagated in a Vero cell line for 48-72 hours at 36^{0}C. The titres of the propagated viruses were: - $LD_{50}=10^{-3.5}$ for HSV1, and

$LD_{50}=10^{-4.3}$ for HSV2. These viruses were identified by the standard serum neutralisation - virus test (SNV) on the Vero cell line.

2. The glycoprotein antigens were isolated and purificated from the outer envelopes of the above mentioned viruses by preparative ultracentrifugation in salt gradients with Triton X-100, as described in by Milic et al. (1991) and Milic (1993).

3. The immunogens in the preparation were identified by SDS-PAGE electrophoresis in a discontinuous buffer system according to Laemmli (1970) with selective staining of the virus proteins (PAS-staining) according to Gordon (1983). Glycoprotein fractions of the isolated subunits stained pink.

The same methods were used for the biochemical characterization of the isolated subunit virus proteins (virus glycoproteins), i. e. immunogenic protein molecules isolated from the purified virions HSV1 and HSV2.

4. After purification and concentration of the virus, disruption of virions and the isolation of their subunits, the total protein concentration was determined in the samples of purified virus antigens, resuspended in 200 mmol/l PBS, (pH 7.0) according to Lowry et al. (1951).

5. The pyrogenic test on experimental rabbits was performed according to the Ph.Yug IV and Ph. Britannica Vet. 1985, Add, 1992.). The above mentioned vaccines were apyrogenic as they did not cause any rise in the body temperature of the immunized rabbits.

6. The vaccines were tested for abnormal toxicity according to Ph. Yug IV, in a biological test on 15 experimental mice, BALB/C strain, each weighing 20g. All the experimental mice survived intraperitoneal applications of the vaccines (i/p) at doses of 0.5 and 1 ml, without any disturbance to their health, which was monitored regularly for the 20 days of the experiment. There fore, it was concluded that the vaccines do noproduce any abnormal toxic effects.

7. The specific toxicity was tested on 25 female mice, Swiss albino strain, by using three times, five times and ten times bigger doses of the vaccine. All the animals survived the experiment with no changes in their state og health.

II. Biological assay on experimental mice. The immunogenicity of the bivalent subunit vaccines against HSV1 and HSV 2 was tested in a biological assay on 31, Swiss albino, female mice, each weighing 20 g. The first group of 10 mice was immunized s.c. with the HSV1/HSV2 subunit vaccine with adjuvant Al(OH)3 (0,5 ml/mouse). The second group of 10 mice was immunized s. c. with the same dose of the HSV2 subunit vaccine with adjuvant MPS-19.

A group of 11 healthy, nonimmunized mice served as the control in the assay.

The blood sera of the immunized experimental mice were examined for the presence and the titre of spcific virus-neutralizing antibodies of the IgG and IgM classes against HSV1 and HSV 2, before and on day 10 after vaccination, by the method of indirect imunofluorescence.

The specific cellular immune response of the vaccineted animals was tested by determining the lymphocyte proliferation reaction to HSV1 and HSV2 antigens using tritium methyl thymidine (128.5 Bg/mmol), in vitro, on day 10 of the assay.

III Commercial lyophilized antisera to human IgG and IgM classes, conjugated with FITC (INEP), were used for the determination of the virusneutralizing IgG and IgM antibodies to HSV1 and HSV2 in the sera of experimental mice by indirect imunofluorescence according to Mihajlović (1984).

IV The standard test of proliferation of lymphocytes of the cervical lymph node stimulated with antigens HSV1 and HSV2, was applied for testing the cellular immune response of the immunized animals according to Ford (1988). The incorporation of methyl-thymidine-3H, 128.5 Bq/mmol in cellular suspensions of lymphocytes in RPMI medium was recorded.

RESULTS

The total protein concentration in the vaccines was 0.29 mg/ml i. e. 0.13 mg (for HSV1 subunits) and 0.16 mg (for HSV2 subunits).

The antibody titres for IgG and IgM against HSV1 on day 10 after immunization with the HSV1/HSV2 subunit vaccine containing the adjuvant Al(OH)3 ranged from 1:8 to 1:128 (for IgG) and 1:32 to 1:128 (for IgM) - (Table 1). The antibody titres of IgG and Ig against HSV 2 in the sera of the same group of imunized mice, ranged from 1:32 to 1:128 for IgG and IgM - (Table 2).

Table 1. Antibody titres against HSV1 10 days after immunization with vaccine containing $Al(OH)_3$

Mouse	IgG					IgM				
	1:8	1:16	1:32	1:64	1:128	1:8	1:16	1:32	1:64	1:128
1				+						+
2					+	–	–	–	–	–
3				+						+
4		+							+	
5		+						+		
6	+							+		
7				+						+
8			+							+
9		+							+	
10			+							+

The antibody titres of IgG and IgM against HSV 1 in the blood sera of the second group of 10 mice immunized with the bivalent subunit HSV1/HSV2 vaccine, containing the adjuvant MPS-19, were found to be: 1:16 to 1:128 (for IgG) and 1:64 to 1:128 (for IgM) on day 10 of the experiment 0 (Table 3). The antibody titres of IgG and IgM against HSV2 in the sera of the same mice, on day 10 after immunization ranged from 1:16 to 1:32 (for IgG) and 1:64 to 1:128 (for IgM) - (Table 4).

Table 2. Antibody titres against HSV2 10 days after immunization with vaccine containing Al(OH)3

Mouse	IgG					IgM				
	1:8	1:16	1:32	1:64	1:128	1:8	1:16	1:32	1:64	1:128
1			+					+		
2					+					+
3			+							+
4				+						+
5				+						+
6				+					+	
7					+					+
8				+					+	
9				+					+	
10				+					+	

Table 3. Antibody titres against HSV1 10 days after immunizing mice with the vaccine containing MPS-19

Mouse	IgG					IgM				
	1:8	1:16	1:32	1:64	1:128	1:8	1:16	1:32	1:64	1:128
11				+						+
12			+						+	
13			+							+
14			+						+	
15				+						+
16					+					+
17			+						+	
18				+						+
19			+							+
20		+								+

Thus, the experimental bivalent subunit HSV1/HSV2 vaccines (both with adjuvants Al(OH)3 and MPS-19) induced strong humoral immune responses in the organisms of the immunized animals which resulted in the synthesis of virusneutralizing IgG and IgM antibodies against the viruses HSV1 and HSV2 in satisfactory titres (mainly from 1:32 and 1:128).

In the third (control) group of 11 healthy, nonimmunized mice, humoral immune responses to HSV1 and HSV2 were not detected.

The mean values for radioactivity in the medium duringspontaneous proliferation of mouse lymphocytes, were 799 cpm for the first and 798 cpm for the second experimental group - (Table 5 and 6).

Table 4. Antibody titres against HSV2 10 days after immunizing mice with the vaccine MPS-19

Mouse	IgG					IgM				
	1:8	1:16	1:32	1:64	1:128	1:8	1:16	1:32	1:64	1:128
11		+							+	
12			+						+	
13			+							+
14		+							+	
15			+							+
16			+							+
17			+						+	
18			+						+	
19		+								+
20			+							+

Table 5. Proliferation to HSV1 antigensProliferative response of lymphocytes of the cervical lymph node from mice immunized with the vaccine containing Al(OH)3 to HSV1 and HSV2 (c/min)

Mouse	Spontaneous proliferation in the medium	Proliferation to HSV1 antigens	Proliferation to HSV2 antigens
1	677	964	1507
2	969	1531	1816
3	867	1156	3130
4	754	2485	1857
5	909	1332	1493
6	619	1353	1269
x	799	1470	1845

Table 6. Proliferative response of lymphocytes of the cervical lymph node from mice immunized with the vaccine containing MPS-19 to HSV1 and HSV2 (c/min)

Mouse	Spontaneous proliferation in the medium	Proliferation to HSV1 antigens	Proliferation to HSV2 antigens
1	586	1413	2364
2	745	1173	1028
3	878	1833	5391
4	809	1115	1479
5	864	1280	1937
6	907	1522	2294
x	798	1389	2416

The mean radioactivity incorporated into lymphocytes stimulated with HSV1 and HSV2 antigens in the first group of mice, 10 mice days after immunization, was 1470 cpm (for HSV1) and 1845 cpm (for HSV2) - (Table 5). The mean values for radioactivity in the test for lymphocyte proliferation response to HSV1 and HSV2 antigens in the second experimental group of mice on day 10 after vaccination were 1389 cpm (for HSV1) and 2416 cpm (for HSV2) - (Table 6).

DISCUSSION

Other authors in this field (Hassan et al., 1996., Zarling et al.,1988) have shown that the glycoprotein antigens isolated from external envelopesof HSV1 and HSV2 in the subunit vaccines have an important role in inducing specific cellular immunological reactions in the immunized organisms (mice and humans).

The proliferation test results in our investigation confirmed the proliferative response of lymphocytes to HSV1 and HSV2 antigens in mice, 10 days after immunization with the bivalent subunit vaccine containing the adjuvant Al(OH) 3 at a concentration of 5 mg/ml.

A satisfactory proliferative response of lymphocytes to HSV1 and HSV2 antigens was also found in mice 10 days after immunization with the bivalent subunit vaccine containing the water soluble oligosaccharide (MPS-19) at 2 mg/ml.

Moreover, these HSV1/HSV2 subunit vaccines induced strong specific humoral immune responses in all vaccinated mice, i.e. the synthesis of virus-neutralizing antibodies against HSV1 and HSV2 viruses.

The results of these experiments have confirmed that the abovementioned experimental subunit vaccines against HSV1 and HSV2, with low concentrations of isolated viral subunits (0.13 mmg/ml for HSV1 and 0.16 mg/ml for HSV2), induce humoral and cellular immunological responses in the immunized organisms.

REFERENCES

1. *Arvin M. A., Prober G. C.,* 1991. Harpes Simplex Viruses, In: Bullows A.: Clinical Microbiology, 5th. ed., American Society for Microbiology, Washington, p. 822-828.

2. *Ford W. L.* 1978. The preparation and labelling of lymphocytes. Cellular Immunology vol. 2., p.23. 1-23.22.

3. *Gordon A. H.,* 1983. Electrophoresis of proteins in polyacrylamide and starch gels. *Ed. Work E. Elsevier.*

4. *Hassan Y., Brewer M. J., Alexander J. and Jennings R.,* 1996. Immune responses in mice induced by HSV-1 glycoproteins presented with ISCOMs or NISV delivery systems. *Vaccine, vol. 14, No 17/18, p.* 1581-1589.

5. *Laemmli U. K.*, 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, p. 680-685.

6. *Lowry C. H., Rosebraugh N. J., Farr A. L., Randall R. J.*, 1951. Protein measurement with the Folin-phenol reagent. *Journal of Biological Chemistry, 193*, 265-275.

7. *Mihajlović B.*, 1984. Priručnik za laboratorijsku dijagnostiku, str. 371-375.

8. *Milić S. N.*, 1993. Ispitivanje subjedinačnih vakcina protiv virusa PI3. Doktorska disertacija na Katedri za mikrobiologiju Veterinarskog Fakulteta u Beogradu.

9. *Milić S. N., Gordana Gađanski-Omerović, Ružica Ašanin, Marković S. B.*, 1991. sous-unites glucoproteiques du virus Parainfluenzae 3 bovin (P13). Bull. Acad. Vet. e France, 64, 365-373.

10. *Ph. Brit.(Vet.)* 1985., Add. 1992. Utilisation de D-(6-3H) glucosamine dans les procedese de prufication et de separation des

11. *Ph. Yug. IV.*

12. *Roizman B., Sears E. A.*, 1990. Herpes Simplex Viruses and their Republication, In Fields B. N., Knipe M. D., Virology 2nd ed., Raven Press, New York, p. 1795-1841.

13. *Savarese B., Barnam G., Krause R. P.*, 1994. Placebo controlled trial of vaccination with recombinant glycoprotein D of Herpes simplex virus type 2 for immunoterapy of genital herpes. Lancet, 343, 1460-1463.

14. *Stanberry L. R., Bernstein I. D., Burke L. R., Pachl C., Myers G. M.* 1987. Vaccination with recombinant herpes simplex glycoproteins progection against initial recurent genital herpes. J. Inf. Dis. 155, 914-923.

15. *Whitley R. J.*, 1990. Herpes Simplex Viruses in: Field B. N., Knipe M. D., Virology, 2nd. ed., Raven Press, New York, 1843-1887.

16. Zarling M. J., Morgan P. A., Brewer L., Ashley R. and Corey L., 1988. Herpes Simplex Virus (HSV)-Specific, proliferative and cytotoxic T-cell responses in humans immunized with an HSV Type 2 glycoprotein subunit vaccine. Journal of Virology, 62, 12, 4481-4485.

ISPITIVANJE IMUNOGENOSTI EKSPERIMENTALNIH SUBJEDINIČNIH VAKCINA PROTIV HERPES SIMPLEX VIRUSA TIPA 1 I 2

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SADRŽAJ

Cilj naših istraživanja je bilo ispitivanje imunogenosti eksperimentalnih bivalentnih subjediničnih vakcina protiv virusa HSV1 i HSV2. Ispitivanje imunogenosti ovih subjediničnih vakcina protiv Herpes simplex virusa, pripremljenih po metodi Milića, izvršeno je u biološkom ogledu na 31 eksperimentalnom mišu, Swiss albino, ženki, težine 20 grama. Subjedinične vakcine su pripremljene od glikoproteinskih antigena izolovanih iz spoljašnjih omotača virusa HSV1 i HSV2. U prvoj vakcini glikoproteinske subjedinice su adsorbovane na adjuvans Al(OH)3. Druga subjedinična vakcina je pripremljena od glikoproteinskih antigena sa adjuvansom MPS-19 (hidrosolubilnom oligosaharidnom supstancom) rastvorenim u PBS-u. Finalna koncentracija Al(OH)3 u vakcini je 5 mg/ml, dok je finalna koncentracija MPS-19 u drugoj vakcini 2mg/ml. Koncentracije proteina u vakcinama iznosile su 0.13 mg/ml za HSV1 i 0.16 mg/ml za HSV2. Prva grupa od

10 eksperimentalnih životinja imunizovana je (supkutano) sa 0.5 ml HSV1/HSV2 subjediničnom vakcinom, sa adjuvansom Al(OH)3. Druga grupa od 10 miševa imunizovana je sa istom dozom vakcine HSV1/HSV2 sa adjuvansom MPS-19, kao što je već opisano. Grupa od 11 zdravih, neimunizovanih miševa predstavlja kontrolnu grupu u ovom ispitivanju. Specifični humoralni imunski odgovor vakcinisanih miševa meren je standardnom metodom indirektne imunofluorescencije. Specifični celularni imunski odgovor vakcinisanih životinja određen je standardnom metodom proliferacije limfocita protiv HSV1 i HSV2 antigena pomoću tricium metil timidina (128.5 Bq/mmol), in vitro. Titri specifičnih antitela IgG i IgM klase, u serumima dve grupe od 10 eksperimentalnih miševa imunizovanih sa HSV1/HSV2 subjedinične vakcine (sa dva različita adjuvansa) desetog dana posle vakcinacije bili su: sa adjuvansom Al(OH)3: od 1:8 do 1:128 IgG; i 1:32 do 1:128 IgM za HSV1; od 1:32 do 1:28 IgG i IgM antitela, za HSV2; b) sa adjuvansom MPs-19: od 1:16 do 1:128 IgG i 1:64 do 1:128 IgM za HSV1; od 1:16 do 1:32 IgG i 1:64 do 1:128 IgM za HSV2. Srednje vrednosti radioaktivnosti uzoraka u testu spontane proliferacije limfocita kod miševa, u medijumu su bile 799 c/min za prvu i 798 c/min za drugu eksperimentalnu grupu. Rezultati testa proliferacije limfocita stimulisanih sa HSV1 i HSV2 antigenima u prvoj grupi miševa, 10 dana posle imunizacije bili su 1470 c/min (za HSV1) i 1845 c/min (za HSV2). Srednje vrednosti radioaktivnosti uzoraka u testu proliferacije limfocita na HSV1 i HSV2 antigene u drugoj eksperimentalnoj grupi miševa desetog dana od vakcinacije bile su 1389 c/min (za HSV1) i 2416 c/.min (za HSV2). Niska koncentracija subjediničnih antigena u navedenim vakcinama, indukuje zadovoljavajući humoralni i celularni imunski odgovor kod imunizovanih miševa protiv HSV1 i HSV2.