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EXAMINATION OF HUMORAL AND CELLULAR IMMUNE RESPONSES AFTER IMMUNIZATION WITH SUBUNIT VACCINE AGAINST HERPES SIMPLEX VIRUS 1 AND 2

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*The objective of our study was to examine the humoral and cellular immune response in mice after immunization with bivalent subunit vaccine against Herpes simplex viruses (HSV 1 and HSV2). These examinations were carried out in biological assay on 27 female Swiss albino mice (20 g bm). The subunit vaccine was prepared from purified glycoprotein antigens (subunits) isolated from the external envelopes of HSV 1 and HSV 2 and adsorbed on the adjuvant $Al(OH)_3$ at a final concentration of 5 mg/ml. This vaccine contained 0.14 mg/ml total protein for the HSV 1 subunits and 0.16 mg/ml for HSV2 subunits. Two groups of 9 and 7 experimental mice were immunized with the HSV1/HSV2 subunit vaccine (0.5 ml s/c). A group of 11 healthy non-immunized mice served as the control. The specific humoral immune response of the vaccinated mice was examined 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 tritiated methyl thymidine (*128.5 Bq/mmol), in vitro. The titers of the specific IgG and IgM class antibodies, in the blood sera of the first group of 9 immunized mice, on day 10 after vaccination were: from 1:8 to 1:64 IgG and 1:32 to 1:128 IgM for HSV1 and 1:32 to 1:128 IgG and IgM antibodies for HSV2. The equivalent titers for the second group of 7 immunized mice, on day 24 after vaccination were: from 1:64 to 1:128 IgG and 1:16 to 1:64 IgM for HSV1; from 1:32 to 1:128 IgG and IgM for HSV2. The mean values for radioactivity in the proliferation test of lymphocytes for HSV1 and HSV2 antigens were 1471 cpm and 1839 cpm respectively on day 10 after vaccination, The mean value for radioactivity in the medium after spontaneous proliferation of lymphocytes for this group of mice was 893 cpm. The mean radioactivity of lymphocytes from the second group of mice, stimulated with HSV1 antigens was 4158 cpm on day 24 after vaccination, with a*

spontaneous proliferation value of 364 cpm. Thus, the low subunit antigen concentrations in the bivalent subunit vaccine, induced satisfactory humoral and cellular immune responses to HSV1 and HSV2 in the vaccinated mice.

Key words: glycoprotein antigens, HSV1, HSV2, humoral and cellular immune response, immunization, subunit vaccine, Swiss albino mice.

INTRODUCTION

The immunogenic properties of the surface glycoprotein antigens of the Herpes Simplex viruses type 1 and 2 have been examined by many authors (Stanberry *et al.*, 1987, Zarling *et al.*, 1988, Roizman and Sears, 1990, Hassan *et al.*, 1996).

There are ten known antigenically distinct glycoproteins in the outer envelopes of Herpes simplex viruses: gB, gC, gD, gE, gG, gI, gH, gK, gL and gM (Little *et al*, 1981, Hutchinson *et al*, 1992, and Ghiassi *et al.*, 1996). The glycoprotein antigens gB, gD and gI of HSV1 and HSV2 represent the key molecules for stimulating protective humoral and cellular immune responses, in vivo (Paoletti *et al.*, 1984, Cremer *et al.*, 1985, Savarese *et al.*, 1994, and Ghiasi *et al.*, 1996).

We decided to examine the humoral and cellular immune response of mice vaccinated with a bivalent subunit HSV1/HSV2 vaccine, prepared from purified glycoprotein antigens isolated from the outer viral envelopes.

MATERIAL AND METHODS

Vaccine: The bivalent subunit vaccine was prepared from purified glycoprotein antigens (subunits) isolated from the external envelopes of HSV1 and HSV2. The glycoprotein antigens were adsorbed on adjuvant $Al(OH)_3$, dissolved in 100 mmol/l phosphate buffered saline (PBS, pH 7.0), according to the method of Milić *et al.*, (1991) and Milić (1993). The final concentration of adjuvant in the vaccine was 5 mg/ml. The total virus protein concentration in the vaccine was 0.30 mg/ml (0.14 mg/ml for HSV1 subunits and 0.16 mg/ml for HSV2 subunits), i.e. 0.15 mg per dose of 0.5 ml.

The HSV1 and HSV2 viruses used for the preparation of the subunit vaccine were individually inoculated and cultivated in Vero cell lines for 48-72 hours at 36°C. The titres of the propagated viruses were: - $LD_{50}=10^{-3.5}$ for HSV1, and $LD_{50}=10^{-4.2}$ for HSV2. The viruses were identified by the standard serum neutralisation - virus test (SNV) in Vero cell lines according to Mihajlović (1984).

Glycoprotein subunits from outer envelopes of the above mentioned viruses were isolated and purified by preparative ultracentrifugation in linear salt gradients with Triton X-100, as described by Milić (1993) and Milić *et al.* (1994).

The virus antigens in the preparations 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), during which the glycoprotein fractions of the isolated subunits stained pink.

The aforementioned methods were used for biochemical characterization of the virus glycoproteins isolated from the purified virions of HSV1 and HSV2.

Total protein concentrations in the samples of HSV1 and HSV2 purified subunits were determined by the method of Lowry et al. (1951).

Immunization of mice: The immunogenicity of the bivalent HSV1/HSV2 subunit vaccine was tested in a biological assay on 27 Swiss albino, female mice, each weighing 20 g. Two groups of 9 and 7 experimental mice were immunized s.c with the same doses of the subunit HSV1/HSV2 vaccine (0.5 ml/mouse). A group of 11 healthy, nonvaccinated mice served as the control in the assay.

Serum antibody determinations: The blood sera of the vaccinated mice were examined for the presence and titre of virus-neutralizing antibodies of the IgG and IgM classes against HSV1 and HSV2, before and on day 10 after vaccination (for the first group of 9 mice) or on day 24 (for the second group of 7 mice), by the standard method of indirect immunofluorescence according to Mihajlović (1984) and Sjurin *et al.* (1984).

Sera from the control group of 11 nonvaccinated mice were tested for the presence of antibodies against HSV1 and HSV2 antigens, before and at the end of the immunization assay.

Commercial lyophilized antisera to human IgG and IgM classes, conjugated with FITC (INEP) were used to detect the virus-neutralizing IgG and IgM antibodies to HSV1 and HSV2 in the sera of experimental animals by indirect immunofluorescence.

Lymphoproliferation assays: The specific cellular immune response of the immunized mice was tested by determining of the lymphocyte proliferation reaction to HSV1 and HSV2 antigens using tritiated methyl-thymidine (128.5 Bq/mmol), in vitro. The proliferative responses of lymphocytes from the first experimental group of mice to HSV1 and HSV2 were examined on day 10 after vaccination. The lymphoproliferative responses in the second experimental group of mice to HSV1 antigens were measured on day 24 after immunization with bivalent subunit HSV1/HSV2 vaccine.

The standard test of proliferation of lymphocytes of the cervical lymph node stimulated with HSV1 and HSV2 antigens, was applied for the testing the cellular immune response of the vaccinated experimental mice according to Ford (1978). The incorporation of methyl-thymidine - ^{3}H, (128.5 Bq/mmol) in cellular suspensions of lymphocytes in RPMI 1640 medium with 10 g/dl inactivated fetal calf serum (FCS), was recorded.

The lymphoproliferative responses of the immunized mice were expressed as counts per minute (c/min).

RESULTS

The titres of IgG and IgM antibodies against HSV1 in the sera of the first group of mice vaccinated with HSV1/HSV2 subunit vaccine, ranged from 1:8 to 1:64 (for IgG) and 1:32 to 1:128 (for IgM) - (Table 1).

Table 1. Antibody titres against HSV1 10 days after immunization with subunit vaccine

	IgG					IgM				
mouse	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			+						+	

The antibody titres of IgG and IgM against HSV2 in the sera of this group of immunized mice, ranged from 1:32 to 1:128 for IgG and IgM - (Table 2).

The titres of IgG and IgM antibodies against HSV1 in the blood sera of the second group of mice immunized with HSV1/HSV2 vaccine ranged from 1:64 to 1:128 (for IgG) and from 1:16 to 1:64 (for IgM) on day 24 after vaccination, The

Table 2. Antibody titres against HSV2 10 days after immunizing mice with the vaccine containing $Al(OH)_3$

	IgG					IgM				
mouse	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			+					+		

antibody titres of IgG and IgM against HSV2 in the sera of the same mice on day 24 after vaccination were found to be: from 1:64 to 1:128 (for IgG and IgM) - (Table 3).

Table 3. Antibody titres against HSV1 24 days after immunizing mice with subunit vaccine

	IgG					IgM				
mouse	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					+				+	

The bivalent HSV1/HSV2 vaccine induced strong humoral immune responses in the organisms of immunized animals on day 10 and day 24 after vaccination. Significant levels of virus neutralizing IgG and IgM antibodies against HSV1 and HSV2 were detected in the sera of all immunized animals.

Humoral responses to HSV1 and HSV2 were not detected in the third group of eleven healthy, nonvaccinated mice, which served as the control in the assay.

Table 4. Antibody titres against HSV2 24 days after immunizing mice with subunit vaccine

	IgG					IgM				
mouse	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				+				+		

The mean values for radioactivity in the medium after spontaneous proliferation of mouse lymphocytes were 893 cpm for the first and 364 cpm for the second experimental group - (Table 5 and 6).

Table 5. Proliferative response of lymphocytes of the cervical lymph node from mice to HSV1 and HSV2 10 days after immunization (c/min)

mouse	Spontaneous proliferation in the medium	Proliferation to HSV1 antigens	Proliferation to HSV2 antigens
1	673	770	1505
2	1247	1536	1499
3	1188	1533	1820
4	621	1351	1270
5	868	1160	3080
6	765	2477	1860
x	893	1471	1839

Table 6. Proliferative response of lymphocytes of the cervical lymph node from mice to HSV1 24 days after immunization (c/min)

mouse	Spontaneous proliferation in the medium	Proliferation to HSV1 antigens
1	298	4392
2	467	3702
3	450	5499
4	357	2621
5	351	5110
6	261	3626
x	364	4158

The mean values of radioactivity in the lymphoproliferation assay for HSV1 and HSV2 antigens in the first group of mice were 1471 cpm (for HSV1) and 1839 cpm (for HSV2) on day 10 after immunization - (Table 5).

The mean radioactivity incorporated into lymphocytes stimulated with HSV1 antigens in the second group of mice was 4158 cpm on day 24 after vaccination - (Table 6).

DISCUSSION

Many authors have examined the immunogenicity of some surface HSV1 and HSV2 glycoprotein antigens in biological assays on experimental animals (Lasky *et al.*, 1984, Cremmer *et al.*, 1985, Eisenberg *et al.*, 1985, Cantin *et al.*, 1987, and Ghiasi *et al.*, 1992). The results of these examinations showed that the individual recombinant herpes simplex virus glycoproteins (gD; gB; gl or gH) had weak immunogenic properties; whilst vaccination with a mixture of seven recombinantly expressed HSV glycoproteins (gB, gC, gD, gG, gE, gH and gl) induced a stronger immune response in the vaccinated mice (Ghiasi *et al.*, 1996).

The immunogenic properties of glycoprotein antigens isolated from the outer envelopes of the Herpes simplex viruses were confirmed in biological assays on the experimental animals (Ghiasi *et al.*, 1994, Ghiasi *et al.*, 1995 and Milić *et al.*, 1999).

Hassan *et al.* (1996) incorporated HSV1 antigens into immunostimulating complexes (ISCOMs) and non-ionic surfactant vesicle (NISV) delivery systems in order to improve their immunogenicity for the preparation subunit vaccines.

Investigations of the immunogenicity of isolated HSV1 and HSV2 glycoprotein subunits have shown that the size and shape of these specific glycoprotein antigen molecules have an essential role in the induction of specific humoral and cellular immune reactions in immunized organisms.

In order to improve the immunogenic properties of the purified glycoprotein subunits isolated from the peplos of HSV1 and HSV2, we adsorbed them on an adjuvant ($Al(OH)_3$) dissolved at 100 mmol/l in PBS. This method was used in the preparation of our bivalent subunit vaccines.

The results of the biological assay on experimental mice have shown that our subunit HSV1/HSV2 vaccine has marked immunogenic properties and elicits a strong humoral immune response in the organisms of all vaccinated animals, i.e. the synthesis of virus-neutralizing antibodies against HSV1 and HSV2 viruses.

Our proliferation test results confirmed the proliferative responses to HSV antigens in mice 10 and 24 days after vaccination.

On the basis of the results obtained it can be concluded that the low concentration of viral proteins in the vaccine (0.30 mg/ml, i.e. 0.15 mg per dose) induced satisfactory humoral and cellular immune responses to HSV1 and HSV2 in vaccinated mice.

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ISPITIVANJA HUMORALNOG I ĆELIJSKOG IMUNSKOG ODGOVORA POSLE IMUNIZACIJE SA SUBJEDINIČNOM VAKCINOM PROTIV HERPES SIMPLEX VIRUSA TIPA 1 I 2

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SADRŽAJ

Cilj naših istraživanja je bilo ispitivanje humoralnog i ćelijskog imunskog odgovora posle imunizacije miševa eksperimentalnom bivalentnom subjediničnom vakcinom protiv Herpes simplex virusa tipa 1 i 2 (HSV1 i HSV2). Ova ispitivanja su izvršena u biološkom ogledu na 27 ženki Swiss albino miševa, težine 20 g. Subjedinična vakcina je pripremljena od prečišćenih glikoproteinskih

antigena (subjedinica) izolovanih iz spoljašnjih omotača HSV1 i HSV2 i adsorbovanih na adjuvans $Al(OH)_3$. Finalna koncentracija $Al(OH)_3$ u vakcini iznosila je 5 mg/ml. Ukupne koncentracije antigena u vakcini iznosile su 0.14 mg/ml za HSV1 i 0.16 mg/ml za HSV2. Dve grupe od 9 i 7 eksperimentalnih miševa imunizovane su supkutano sa istim dozama od 0.5 ml HSV1/HSV2 subjedinične vakcine. dok je grupa od 11 zdravih, neimunizovanih miševa predstavljala kontrolnu grupu. Specifični humoralni imunski odgovor vakcinisanih miševa ispitivan je standardnom metodom indirektne imunofluorescencije. Specifični celularni imunski odgovor vakcinisanih životinja ispitivan je *in vitro* standardnom metodom proliferacije limfocita na HSV1 i HSV2 antigene pomoću tricijum metil timidina (128.5 Bq/mmol). Specifična antitela Ig G i Ig M klase ustanovljena su u krvnom serumu prve grupe miševa, desetog dana posle vakcinacije i njihov titar se kretao u opsegu od 1:8 do 1:64 (Ig G) i od 1:32 do 1:128 (Ig M) za HSV1 i od 1:32 do 1:128 (Ig G i Ig M) za HSV2. Titar specifičnih antitela u serumu miševa druge grupe, 24-tog dana posle vakcinacije, kretao se u opsegu od 1:64 do 1:128 (IgG) i 1:16 do 1:64 (Ig M) za HSV1 i od 1:32 do 1:128 (IgG i IgM) za HSV2. Srednje vrednosti testa proliferacije limfocita stimulisanih sa HSV1 i HSV2 antigenima kod prve grupe miševa, 10 dana posle imunizacije, iznosile su 1471 c/min (za HSV1) i 1839 c/min za HSV2. Srednja vrednost radioaktivnosti uzoraka u testu spontane proliferacije limfocita kod ovih miševa u medijumu, bila je 893 c/min. Srednja vrednost radioaktivnosti limfocita stimulisanih sa HSV1 antigenima u drugoj grupi miševa, 24-tog dana od vakcinacije iznosila je 4158 c/min, dok je vrednost spontane proliferacije limfocita ovih miševa bila 364 c/min. Niske koncentracije subjediničnih antigena u vakcini indukovale su zadovoljavajući humoralni i ćelijski imunski odgovor kod vakcinisanih miševa protiv HSV1 i HSV2.