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EXAMINATION OF THE IMMUNOGENICITY OF EXPERIMENTAL POLYVALENT SUBUNIT VACCINE AGAINST INFLUENZA VIRUSES

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The immunogenicity of experimental polyvalent subunit vaccine against the influenza viruses, types A/H1N1, A1 /H3N2/ and B was studied in a biological assay on 15 experimental female rabbits,of the chin-chilla breed, weighing 700 g, and 33 experimental mice, (BALB/C females), weighing 20 g. The experimental subunit vaccine was prepared from purified glycoprotein subunits isolated from outer envelopes of the influenza viruses, types A/Singapore/6/86 H1N1, A1/Peking/353/89 H3N2 and B/Hamagota/45/90. Antigens were adsorbed on the adjuvant A1(OH)3, dissolved in 100 mmol/l of PBS. The finalconcentration of the adjuvant in the vaccine was 5 mg/ml. The haemagglutinating activity of isolated glycoprotein subunits in the vaccine was 128 HJ/0.1 ml. The samples of purified glycoprotein antigens, isolated from the above mentioned influenza viruses, with total protein concentrations of 0.20, 0.154 and 0.20 mg/ml, had significant haemagglutinating activities of 256 - 512 HJ/0.1 ml. The samples of nondisrupted and purified influenza virions (before the isolation of glycoprotein subunits), with higher total protein concentrations of 0.44. 0.30 and 0.53 mg/ml had haemagglutinating titres from 128 - 256 HJ/0.1 ml. One group of 10 experimental rabbits was immunized with 0.5 ml subunit vaccine (s/c per animal) and revaccinated with the same dose 24 days after the first vaccination. The mean geometric titres of HI antibodies (GMT) log 2/25 μl) against influenza viruses A/H1N1/, A1/H3N2/ and B in the sera of these experimental rabbits on day 7 after vaccination were 2.5; 2.0 and 2.5; on day 14: 3.5;3.0 and 3.0; on day 21: 4.0; 3.5 and 3.0; on day 28: 4.5; 4.0 and 3.5. In the blood sera of 5 control nonvaccinated rabbits, virusneutralizing HI antibodies were not detected. A group of 12 mice was immunized with 0.5 ml of the polyvalent influenza subunit vaccine (s/c per animal). The mean geometric titres of HI antibodies (GMT log 2/25 μl) against influenza viruses type A/H1/N1/, A1/H3/N2/ and B in the sera of immunized mice, on day 21 after vaccination were 4.6; 4.3 and 3.5. In the sera of 7 nonimmunized mice, virusneutralizing HI antibodies were no detected. Seven experimental mice were artificially

infected 21 days after vaccination with a suspension (1 ml) of the above mentioned types of influenza viruses adapted to mice, with the titre EID 50 = 10-8.5 (for each type) intraperitoneally (i/p) and paranasally (p/n). An 7 vaccinated and artificially infected mice survived this infection without any symptoms. A second group of7 nonvaccinated control mice was artificially infected in the same way. All the animal were taken ill with acute respiratory infection 7 days later and 3 animale died on day 10 of the experiment.

Key words: influenza viruses, glycoprotein antigens, haemagglutinating activities, polyvalent subunit vaccine, immunization, artificial infection, rabbits, mice.

INTRODUCTION

In immunoprophylaxis of infections caused by influenza viruses, classical viral vaccines are usually used, i. e. live - attenuated or inactivated vaccines, prepared from complete attenuated or inactivated viral particles.

An important disadvantage of live - attenuated vaccines is the possibility of reactivation of the vaccine viruses as under certain conditions (passage through susceptible organisms), a virus that has lost virulence could regain it. Among other defects are the appearence of pyrogenic and general or local allergic reaction in vaccinated organisms.

One defect of inactivated vaccines is the long period from the administration of a vaccine till the induction of immunity, i. e. - 14 days, during which the organisms can be infected with viruses. Other disadvantages are the weaker immunogenic effect due to the high level polyclonal reaction and the damage inflicted to immunologically important viruses during the process of inactivation.

Significant efforts to correct the above mentioned defects, have been made in this field. Namely, the possibility of the production of specific subunit vaccines from purfied glycoprotein antigens separated from the complete viral particle and the infective parts of the virus (nucleocapsid with infective RNA) and pyrogen, to make them totally harmless to immunised organisms has been investigated (Schmidt 1966; Jennings et al., 1974; Jenninngs et al; Jenninngs et al.; 1975; Brady and Furminger 1976). The published results have shown that experimental subunit vaccines have diminished immuno responsiveness compared with classical viral vaccines, as a consequence of the damage inflicted to the complex structure of immunologically important glycoprotein antigens during their purification and isolation from the complete viral particle (Brady and Furminger 1975; Brady and Furminger 1976 and Weir 1967).

The results of our investigations, carried out over many years, have confirmed that it ispossible to produce specific subunit vaccines against influenza and parainfluenza viruses from immunologically important glycoprotein subunits, isolated from virus outer envelopes in a biologically active and purified form, namely with a completely preserved antigenic structure, without the pyrogenic and infective parts of the virus. Laboratory investigations of these vaccines have

demonstrated that they possess marked immunogenic properties but are utterly harmless to the vaccinated organisms (Milić, 1993; Milić et. al., 1994; Milić et al., 1996).

This prompted the production of subunit vaccines that, in addition to isolated glycoprotein antigens of the influenza virus types A/Singapoor/6/86/H_1N_1, A_1/Peking/353/89/H_3N_2 and B/Hamagota/45/95, contain immunologically significant glycoprotein subunits of these viruses. The laboratory results obtained show that this variant of polyvalent subunit vaccines induces a specific immune response of protective character in all immunized organisms (Milić et al., 1996; Ašanin and Milić, 1995).

MATERIAL AND METHODS

I Vaccine. The experimental polyvalent influenza subunit vaccine was a sterile liquid suspension of purified haemagglutinating glycoprotein antigens (immunodominant antigenic molecules with Mr of 19. 21 and 22 kD), isolated from outer envelopes of influenza viruses, type A/ Singapoore/6/86/ H_1,N_1, A_1 /Peking/353/89/ H_3N_2 and B/Hamagota/45/90, These isolated glycoprotein subunits, with a haemagglutinating titre of 128 HJ/0.1 ml, were adsorbed on an appropriate adjuvant of $Al(OH)_3$, suspended at 100 mmol/l in phosphate buffered saline (PBS, pH 7.0), according to the method of Milić (1993), and Milić et al., (1994). The total virus protein concentration was 0.55 mg/ml, i. e. 0.27 mg per dose of 0.5 ml.The adjuvant concentration in the vaccine was 5 mg./ml.

The isolation and purification of glycoprotein antigens from theouter envelopesof the above mentioned virusses was done by preparative ultracentrifugation in linear K-Na-tartrate gradients with Triton X-100, as described by Milić et al., 1991 and Milić 1993.

The immunogen in the vaccine was identified by SDS-PAGE electrophoresis in a discontinous buffer system (Laemmli, 1970) with selective staining of virus proteins (PAS-staining method, according to Gordon, 1983) during which the glycoprotein fractions of the isolated subunits stained purple; as well as with the HI test (Mihajlović, 1984, Clarke and Casals, 1958).

The pyrogenic test on experimental rabbits, performed according to Pharmacopoeia Yugoslavica IV andPharmacopoeia Britanica (Vet.) 1985. Add 1992., showed that the vaccine was apyrogenic, as it did not cause any rise in the body temperature in the immunized animals. In the rabbits treated with a fivefold dose of the vaccine the rise of the body temperature was only 0.5^0C.

The virus strains for the production of the vaccine were individually propagated in allantochorionic cavities of 11 day-old chick embryos for 48 - 72 hours at 36^0C. The titres of the propagated viruses ranged from log= $10^{-7,5}$ to $10^{-8,3}$ $EID_{50/01\ ml}$ ($EID_{50} = 10^{-7,5}$ to $10^{-8,3}$/0.1 ml); whereas the haemagglutinating titre was 512 HJ/0.1 ml.

II Total protein concentrations in the samples of purified glycoprotein subunits were determined by the method of Lowry et al., (1951).

Hemagglutinating activities of isolated glycoprotein subunits were examined by the method of direct hemagglutination (Clarke and Casals, 1958; Mihajlović 1984).

III Biological assay on experimental animals

The immunogenicity of the polyvalent subunit vaccine was tested in a biological assay on 15 female rabbits, of the chin-chilla breed, weighing 700 g, and 33 BALB/C females mice, weighing 20 g.

1. Each of 10 experimental rabbits was immunized with 0.5 ml subunit vaccine and revaccinated with the same dose 24 days after the first vaccination. The remaining 5 nonvaccinated rabbits served as the negative control in the assay.

The virus-neutralizing HI antibody titre in the sera of immunized animals was determined using the standard haemagglutination inhibition (HI) test and "Limbro" microplates (according to Mihajlović, 1984.; Clarke and Casals, 1958.). Prior to the vaccination they were all, free from specific antibodies against the above mentioned types of influenza viruses.

During the immunization assay blood samples from the experimental animals were taken in order to examine their sera for the presence of virus-neutralizing HI antibodies against all three virus types, namely on days 7, 14, 21 and 28 after the vaccination.

2. The immunogenicity of the polyvalent subunit vaccine against all three types ofinfluenza virus was examined also in. The experimental group of 12 animals was immunized intramuscularly, in the neck area, with 0.5 ml of the subunit vaccine which contained 0.55 mg/ml of protein. Twenty one days after vaccination the animals were bled in order to examine their blood sera for the presence of virusneutralizing HI antibodies for influenza viruses using the standard HI test. The other group of 7 non immunized mice served as the control in the assay.

3. The HI test was carried out according to the standard method in Limbro microplates with 0.5% suspension of chicken erythrocytes (Mihajlović, 1984, and Clarke and Casals, 1958). The titres of virus neutralizing HI antibodies against influenza viruses, types A/Singapoore/6/86 H_1 N_1, A_1 /Peking/353/89 H_3 N_2 and B/Hamagota/45/90 in the blood sera of the examined animals were expressed in mean geometric titres (Gmt log 2/25 ml), according to Sjurin et al., (1984).

4. The artificial infection of two groups of 7 experimental mice with the virulent strains of the above mentioned types of influenza viruses, adapted to mice (challenge experiment), was done according to Ph. Brit (Vet) 1985.; Add. 1992. and Requirements for influenza vaccine (Annex 2, No 17, revised 1990.).

RESULTS

1. Total protein concentrations and haemagglutinating activities of the whole purified virions and isolated glycoprotein antigens during preparation of experimental influenza subunit vaccine.

The total protein concentrations and haemagglutinating activity of the purified virus samples resuspended in PBS and the glycoprotein subunit samples after the breakdown of thepurified viruses and the isolation of glycoprotein antigens and their outer envelopes are shown in - Tables 1 and 2.

Table 1. Protein concentrationand haemagglutinating titre in purified virion samples resuspended in 0,2 mol/IPBS

Type of influenza virus	Protein concentratiion (mg/ml)	Haemagglutinating titre (HJ/0.1 ml)
A/Singapoore/6/86 H_1N_1	0.44	256
A_1/Peking/353/89 H_3N_2	0.30	128
B/Hamagota/45/90	0.53	256

Table 2. Protein concentrationand haemagglutinating titre in purified glucoprotein subunit samples resuupendedin 0,2 mol/I PBS

Type of influenza virus	Protein concentratiion (mg/ml)	Haemagglutinating titre (HJ/0.1 ml)
A/Singapoore/6/86 H_1N_1	0.20	512
A_1/Peking/353/89 H_3N_2	0.154	256
B/Hamagota/45/90	0.20	256

As the isolated and purified glycoprotein antigens in low concentrations had marked haemagglutinating activities (256 - 512 HJ/0.1 ml), they were used for the preparation of the polyvalent influenza subunit vaccine. The haemagglutinating activityof the glycoprotein subunits in the vaccine was 128 HJ/0.1 ml.

2. The immunogenicity of the experimental subunit influenza vaccine in rabbits.

The titre of virusneutralizing HI antibodies in the sera of the immunized rabbits is presented in Table 3.

Table 3. Mean geometric titre of HI antibodies (GMT kog 2/25 μl) in immunized rabbits

Type of influenza virus	Days after vaccination				
	0	7	14	21	28
A/Singapoore/6/86 H_1N_1	0	2.5	3.5	4.0	4.5
A_1/Peking/353/89 H_3N_2	0	2.0	3.0	3.5	4.0
B/Hamagota/45/90	0	2.5	3.0	3.0	3.5
Control	0	–	–	–	0

There was no evidence of the presence of HI antibodies against influenza viruses in the blood sera of the 5 control nonvaccinated rabbits, either at the beginning or at the end of the assay.

On the basis of the results obtained it can be concluded that the extremely low glycoprotein antigen concentration in the administered dose of the vaccine stimulates the production of virusneutralizing HI antibodies against all three types of influenza viruses in the vaccinated animals at a satisfactory titre.

3. The immunogenicity of the experimental subunit influenza vaccine in mice.

The titres of HI antibodies against influenza, types A/Singapoore/6/86 H1N1, A1/Peking/353/89 H_3N_2 and B/Hamagota/45/90, in the blood sera of the immunized mice, on day 21 after vaccination are given in Table 4 and in geometric titres (GMT log 2/25 μl) in Table 5.

Table 4. HI antibody titres in mice on day 21 after vaccination

No.	Influenza virus type A/Singapoore/6/86H_1N_1	Influenza virus type A1/Peking/353/89H_3N_2	Influenza virus type B/Hamagota/45/90
1.	1 : 16	1 : 16	1 : 8
2.	1 : 16	1 : 16	1 : 16
3.	1 : 32	1 : 16	1 : 16
4.	1 : 64	1 : 64	1 : 8
5.	1 : 16	1 : 8	1 : 8
6.	1 : 32	1 : 32	1 : 8
7.	1 : 64	1 : 16	1 : 16
8.	1 : 64	1 : 8	1 : 8
9.	1 : 32	1 : 16	1 : 8
10.	1 : 16	1 : 16	1 : 16
11.	1 : 8	1 : 128	1 : 8
12.	1 : 16	1 : 16	1 : 16
Control	0	0	0

On the basis of these results, it is possible to conclude that the low concentrations of purified glycoprotein antigens used in the vaccine (0,55 mg/ml, i. e.0,27 mg per dose) induce a satisfactory immune response in vaccinated rabbits and mice.

4. Artificial infection of vaccinated and nonvaccinated mice with influenza viruses types A/H_1N_1/, A/H_3N_2/ and B - challenge experiment

Namely, 21 days after vaccination group one was artificially infected with 1 ml suspension of influenza virulent viruses of the above mentioned types, adapted to mice, with the titre $EID_{50} = 10^{8.5}$ (for each type) intraperitoneally (i/p) and paranasally (p/n). The vaccinated group of mice survived artificial infection with the three types of virus without any symptoms of the disease.

In the control group of 7 nonvaccinated and artificially infected mice (challenge experiment), all the animals were taken ill with acute respiratory infection 7 days later; 3 animals died on day 10 of the experiment. The survived artificially infected experimental mice had marked respiratory syndrome, accom-

panied with general deterioration of their health (inappetence, tremour and emaciation).

Table 5. Geometric titres of HI antibodies 21 days after vaccination of mice (GMT log 2/25 μl)

No.	Influenza virus type A/Singapoore/6/86H_1N_1	Influenza virus type A1/Peking/353/89H_3N_2	Influenza virus type B/Hamagota/45/90
1.	4	4	3
2.	4	4	4
3.	5	4	3
4.	6	6	3
5.	4	3	4
6.	5	5	4
7.	6	4	4
8.	6	3	3
9.	5	4	3
10.	4	4	4
11.	3	7	3
12.	4	4	4
Mean geometric titre	4,6	4,3	3,5

On the basis of the obtained results it can be concluded that the polyvalent subunit vaccine provides protection of the vaccinated animals from infections with virulent strains of influenza viruses.

DISCUSSION

Isolation of immunologically important glycoprotein antigens from external envelopes of influenza viruses in purified and biologically active form, with preserved antigenic structure, represents a prerequisite for the preparation of quality immunogens i. e. specific subunit vaccines as described in papers by many authors (Brady and Furminger, 1976, Lamb et al., 1985, Rothbard, 1986, Skehel and Wiley, 1987, Tang Xi Lin et al., 1988, Milić et al., 1995, Mannino, 1995 etc.).

Our results showed that the samples of isolated influenza glycoprotein subunits in low concentrations of 0.20.0.154 and 0.20 mg/ml had marked haemagglutinating activities (256 - 512 HJ/0.1 ml); whereas in the samples of purified whole virions of influenza viruses, with higher total protein concentrations of 0.40, 0.30 and 0.53 mg/ml, had haemagglutinating titres from 128 to 256 HJ/0.1 ml. The significant haemagglutination activities of glycoprotein antigens, isolated from outer envelopes of influenza viruses, made possible to use these glycoproteins for preparation a experimental influenza subunit vaccine.

The results of the biological assay on experimental rabbits and mice has confirmed that our experimental subunit vaccine against influenza viruses, types $A/H_1,H_1/$, $A_1/H_3N_2/$ and B, has marked immunogenic properties and elicits specific humoral immune response in the organisms of all immunized animals. The low concentration of viral proteins in the vaccine (of 0.27 mg/ml per one dose), with haemagglutinating activity of 128 HJ/0.1 ml, synthesis of virus neutralizing antibodies with a satisfactory titre induced in all vaccinated animals.

The results of other studies in this field (Webster and Laver, 1966, Jennings et al., 1975, Brady and Furminger, 1976) have shown that experimental influenza subunit vaccines with higher concentrations of viral proteins than in our vaccine have a weaker immunogenic effect.

Moreover, the biological assay on BALB/C mice demonstrated that our subunit vaccine protects immunized animals from artificial infection with influenza viruses of types A/H_1 $N_1/$, A_1 $/H_3N_2/$ and B.

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ISPITIVANJE IMUNOGENOSTI EKSPERIMENTALNE POLIVALENTNE SUBJEDINIČNE VAKCINE PROTIV INFLUENCE

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SADRŽAJ

Imunogenost eksperimentalne polivalentne subjedinične vakcine protiv virusa influence, tipa A/H_1N_1/, A_1/H_3N_2/ i B ispitivana je u biološkom ogledu na 15 eksperimentalnih kunića, čin-čila rase, ženskog pola, teških 700 g i 33 eksperimentalna miša, BALB/C ženki, teških 20 g. Eksperimentalna subjedinična vakcina je pripremljena od prečišćenih glikoproteinskih subjedinica izolovanih iz spoljašnjih omotača virusa influence, tipova A/Singapoore/6/86/H_1N_1/ A_1/Peking/353/89/H_3N_2/ i B/Hamagota/45/90. Antigeni su adsorbovani na adjuvans $A_1(OH)_3$, rastvoren u 100 mmol/lPBS-u. Finalna koncentracija adjuvansa u vakcini iznosila je 5 mg/ml. Hemaglutinaciona aktivnost izolovanih glikoproteinskih subjedinica u vakcini bila je 128 HJ/0,1 ml. Uzorci prečišćenih

glikoproteinskih antigena izolovanih iz predhodno pomenutih virusa influence, sa ukupnim koncentracijama proteina od 0,20; 0,154 i 0,20 mg/ml, imali su izražene hemaglutinacione aktivnosti, od 256 - 512 HJ/0,1 ml.Uzorci nerazgrađenih i prečišćenih viriona influence virusa (pre izolacije glikoproteinskih subjedinica), sa višimukupnim koncentracijama proteina od 0,44, 0,30 i 0,53 mg/ml imali su hemaglutinacioni titar od 128 - 256 HJ/0,1 ml. Jedna grupa od 10 eksperimentalnih kunića imunizovana je sa po 0,5 ml subjedinične vakcine (s/c) po životinji i revakcinisana sa istim dozama posle 24 dana od prve vakcinacije. Srednji geometrijski titri HI antitela(GMT log 2/25 ml) protiv virusa influence A/H_1N_1/, A_1/H_3N_2/ i B u serumimaovih eksperimentalnih kunića, iznosili su 7-og dana od vakcinacije 2,5; 2,0i 2,5; 14-og dana 3,5;3,0 i 3,0; 21-og dana 4,0; 3,5 i 3,0; i 28-og dana 4,5; 4,0 i 3,5. U krvnim serumima 5 kontrolnih nevakcionisanih kunića nisu otkrivena virusneutralizujuća HI antitela. Grupa od 12 miševa imunizovana je sa 0,5 ml polivalentne subjedinične influenca vakcine (s/c po životinji). Srednji geometrijski titri antitela (GMT log 2/25 μl) protiv virusa influence tipa A/H_1N_1/, A_1/H_3N_2/ i B u serumima imunizovanih miševa 21-og dana od vakcinacije, bili su 4,6;4,3 i 3,5. U serumima 7 neimunizovanih miševa nisu otkrivena virusneutralizujuća HI antitela. Dve grupe od 7 eksperimentalnih miševa veštački su inficirane 21-og dana od vakcinacije sa po 1 ml suspenzije pomenutih tipova influenca virusa, adaptiranih na miševe, titra $EID_{50} = 10^{-8,5}$, intraperitonealno (i/p) i pernazalno (p/n) po životinji. Prva grupa od 7 vakcinisanih i veštački inficiranih miševa preživela je ovu infekciju bez ikakvih simptoma. Druga grupa od 7 nevakcinisanih kontrolnih miševa, veštački je inficirana na opisani način. Sve životinje su obolele od akutne respiratorne infekcije posle sedam dana, dok su tri životinje uginule desetog dana od infekcije.