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ANTIBODY RESPONSE TO RUBELLA VIRUS PROTEINS IN DIFFERENT PHYSICAL FORMS

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The immunogenicity of different antigens, containing rubella virus hemagglutinating (HA) membrane protein, was studied using live virus, β-propiolactone-inactivated virus, detergent and lipid-free octamers and virosomes. Whole virus particles, live or inactivated, induced hemagglutination inhibition (HAI) antibodies in rabbits after one subcutaneous injection of 0.16 μg of HA protein. Hemagglutinin rosettes or virosomes failed to induce antibodies even at a dose of 120 μg. Apparently, the extraction of viral membrane hemagglutinin, for the preparation of a rubella subunit vaccine, led to destruction of the antigenicity responsible for the induction of hemagglutination inhibiting antibodies. These results are discussed in the light of earlier studies on the preparation of a rubella subunit vaccine.

Viral subunit vaccines rubella virosomes humoral antibodies

INTRODUCTION

The search for a safer and more effective vaccine to control congenital rubella virus syndrome has led us to study the antibody reponse to membrane spike proteins of rubella virus in different physical forms. Previous studies on the immunogenicity of isolated rubella virus membrane proteins [3,19,21] showed that hemagglutination inhibiting (HAI) antibody responses resulted only when viral membrane proteins were given repeatedly in massive doses with adjuvant, either aluminum phosphate or complete Freund's adjuvant.

Subunit vaccines have been prepared against several viruses [1,2,4,8–12,22]. Among these, Semliki Forest virus spike proteins have been biochemically well characterized and their immunogenicity studied in three different physical forms: detergent-solubilized monomers, detergent and lipid-free octamers (rosettes) and virosomes [2,11]. In order to investigate the structural requirements for the antigenicity responsible for the induction of humoral immunity against rubella, we have studied the immunogenicity of rubella virus proteins in different physical forms.

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MATERIALS AND METHODS

Virus production and concentration

Rubella virus, strain M-33 (ATCC VR-315) was grown in Vero cells (ATCC CCL 81; African green monkey kidney) which had been produced in a multiple-tube tissue culture propagator (Bellco Glass Co., Vineland, NJ) [12]. Viral suspensions with hemagglutinating activity (HAA) were concentrated using hollow fiber ultrafiltration [16]. Virus titers were measured by $TCID_{50}$ assay on RK 13 cells.

Preparation of antigens

Concentrated live virus was inactivated by treatment with β-propiolactone 1 : 2000 for 2 h at 37°C. Detergent-free rosettes were prepared from hemagglutinin after Sepharose 2B chromatography of purified virus [13]. Briefly, virus was disrupted by treatment with 1% Triton X-100 for 20 min. Triton X-100 was then exchanged for the dialysable detergent β-D-octylglucoside [7] by centrifugation through a 10–40% sucrose gradient containing 30 mM octylglucoside (SW40 rotor, 4 h at 40,000 r.p.m.). Rosettes were further purified and the detergent removed by chromatography on a Sepharose 6B column [14]. Virosomes were prepared from preformed liposomes made of lecithin and dicetyl-phosphate (3.5 : 1) by the method of detergent dialysis [15]. Separation of virosomes from unadsorbed hemagglutinin and free liposomes was achieved by rate zonal centrifugation through a 10–50% linear sucrose gradient containing 0.5 M NaCl (SW40 rotor, 2h, 40,000 r.p.m.). Non-infectious hemagglutinating particles were purified from the culture supernatants and separated from infectious virus by Sepharose 2B chromatography as they eluted in a second broad peak of hemagglutinating activity. Tween 80/ether rubella virus extract was prepared as described earlier [17].

Viral protein content determination

The concentration (μg HA) of each preparation was determined by a micro-single radial immunodiffusion assay [18] adapted for the quantitation of rubella virus hemagglutinin in different physical forms (virus, rosettes or virosomes). Briefly, 3 mm circular wells were cut in 1% agarose containing specific anti-rubella virus antiserum. Serial twofold dilutions of test sample and chromatography-purified reference antigen were treated with Triton X-100 for 30 min at 20°C before being added to the immunoplates. The plates were then incubated at 20°C for 24 h. After incubation the plates were revealed with 0.3% Coomassie R 250 and dried. The diamenter of reference and sample zones were measured and the concentration determined in μg of hemagglutinin (μg HA).

Antibody assay

A rubella virus HAI assay was used to measure rubella virus-specific antibodies according to the standard procedure [20]. Also, enzyme-linked immunosorbent assay (ELISA) modified for analysis of rabbit sera was used for the detection and quantitation of virus-specific antibodies [6].

Vaccination of rabbits

New Zealand albino rabbits (10–11 weeks old) were used. The antigens were administered in 0.5 ml of NTE buffer (0.15 M NaCl, 0.05 M Tris-NaCl, 0.001 M EDTA, pH 7.4). Doses of 0.016–1600 μg of HA protein were given subcutaneously in one injection. Sera were assayed every second week.

RESULTS

Table 1 shows the results obtained when live rubella virus, β-propiolactone-inactivated virus and purified hemagglutinin rosettes were injected. With live and inactivated virus, a strong HAI response results from the injection of 0.16 μg HA. HA rosettes did not induce HAI antibodies even when 1600 μg of HA protein were injected, even through antibodies were detected by ELISA in response to 16–160 μg HA protein.

In order to evaluate the adjuvant potential of liposomes and to determine whether larger polymers of hemagglutinin would be more immunogenic, we prepared HA-containing liposomes (virosomes). Results obtained are shown in Table 2. Doses of up to 120 μg HA protein in virosomes were still unable to elicit HAI antibodies. A low level antibody was observed by ELISA with virosomes made from performed liposomes. Virosomes made from dried lipid films were less efficient.

The different characteristics of the antigens used are presented in Table 3. Live and inactivated virus consisted of complete virions with their three structural proteins. Rosettes were spike proteins in octameric form and were composed of the surface glycoproteins VP_2 and VP_3. Virosomes were large polymers of spike proteins composed of VP_2 and VP_3 adsorbed onto lipid membranes and having a virus-like membrane structure. Inactivated virus, rosettes and virosomes were all non-infectious by $TCID_{50}$ assay on RK 13 cells.

In complementary experiments with the small non-infectious viral particles, injection in rabbits of a single dose of 100 μg HA induced HAI titers of 1 : 16 and ELISA titers of 1 : 280. Examination by electron microscopy of this material showed mostly viral membrane fragments with some hemagglutinin rosette structure. The injection of 100 μg HA of a Tween 80/ether rubella virus extract [17] did not produce a detectable level of HAI antibodies, though the ELISA titer was 1 : 320. The injection of two doses of 100 μg HA of the same material in Freund's complete adjuvant induced a HAI titer of 1 : 512. Electron microscopic examination of this material did not reveal appreciable amounts of rubella hemagglutinin rosettes: the structures observed were amorphous material of variable size, similar to those shown by Väänänen and Vaheri [21].

TABLE 1

Immunological evaluation of different polymers of rubella hemagglutinin[a]

	Doses ($TCID_{50}$)	Protein concentration (μg HA)	2 Weeks		4 Weeks		6 Weeks		8 Weeks		10 Weeks		12 Weeks	
			HAI	ELISA	HAI	ELISA	HAI	ELISA	HAI	ELISA	HAI	ELISA	HAI	ELISA
Live virus	100,000	16	16	320	16	160	8	160	16	80	16	80	32	80
	10,000	1.6	–	160	–	80	8	80	16	40	16	80	32	80
	1,000	0.16	–	40	–	40	32	40	64	40	32	40	64	40
	100	0.016	–	80	–	–	–	–	–	–	–	–	–	–
Inactivated virus		160	–	320	–	160	16	160	16	80	32	80	64	80
β-propiolactone		16	–	160	–	80	16	160	16	40	16	160	32	–
1/2000		1.6	–	160	–	40	–	80	16	80	32	40	32	40
		0.16	–	80	–	–	–	–	16	40	32	40	16	40
Hemagglutinin rosettes		1600	–	640	–	80	–	80	–	80	–	40	–	40
		160	–	320	–	80	–	80	–	80	–	80	–	40
		16	–	160	–	40	–	40	–	–	–	–	–	–
		1.6	–	40	–	40	–	40	–	–	–	–	–	–

[a] Immunizations were performed on femal rabbits, 0.5 ml subcutaneously . Titers given represent the mean of two animals.

TABLE 2

Immunological evaluation of rubella virus hemagglutinin rosettes and virosomes[a] by ELISA assay

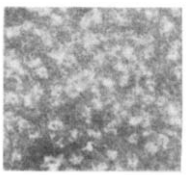
	Protein concentration (μg HA)	2 Weeks	4 Weeks	6 Weeks	8 Weeks
Hemagglutinin rosettes	120	160	80	80	80
	60	160	80	80	80
	30	80	40	40	40
	15	80	40	40	40
	7.5	40	40	20	20
	3.5	40	40	40	–
Virosomes:	120	320	160	160	80
made from preformed	60	320	80	80	80
liposomes	30	160	160	80	80
	15	160	80	80	80
	7.5	80	40	40	40
	3.5	80	40	40	40
Virosomes:	60	80	80	40	40
made from dried	30	80	40	40	20
lipid films	15	40	20	–	–
	7.5	–	–	–	–
	3.5	–	–	–	–

[a] Immunizations were performed on female rabbits, 0.5 ml subcutaneously. Titers given represent the mean of two animals.

DISCUSSION

Our results suggest that the antigenic sites responsible for the induction of hemagglutination inhibiting antibodies (HAI) require a structural integrity that is found in intact virions (live of inactivated) and that we have sofar been unable to reconstitute with our in vitro preparation. Rubella virus hemagglutinin (HA) efficiently induces HAI antibodies when it is in its native configuration in the viral membrane as shown by our results and those of Schmidt et al. [19] using 1000 $TCID_{50}$ of rubella virus. Inactivation of the virus by formalin [2] or β-propiolactone (Table 1) does not destroy these antigenic sites, although a small reduction in immunogenicity occurs. Viral membrane or cellular membrane fragments containing viral proteins retain some sites responsible for the induction of HAI antibody as shown by experiments using small non-infectious particles. Schmidt et al. [19], working with rabbits, had to use five injections of 1 ml HA ($\geqslant$128 HA units) to obtain HAI antibody titers of 1 : 64. In our own work we had to use 100 μg HA, which is 600 times the amount needed with intact virus particles. HA particles produced by disruption with Tween 80/ether were less efficient than the small non-infectious

TABLE 3

ANTIGENS	POLYPEPTIDE COMPOSITION	MOLECULAR WEIGHT	MORPHOLOGY
LIVE VIRUS	VP_1, VP_2-VP_3	30-35 x 10^6	
INACTIVATED VIRUS	VP_1, VP_2-VP_3	30-35 x 10^6	
ROSETTES (26S)	VP_2-VP_3	0.85 x 10^6	
VIROSOMES	VP_2-VP_3	2-20 x 10^6	

particles: Väänänen and Vaheri [21] had to use three doses of 2500 (HA units) in aluminum phosphate adjuvant to raise HAI antibody titers of 1 : 128. With two doses of 100 μg HA with complete Freund's adjuvant we were able to obtain similar results. This is 1200 times more antigen than whole virus particles and needs the help of a good adjuvant. Structures observed by elctron microscopy were not rosettes as defined by our previous studies [15] but amorphous material of variable size. We feel that the Tween 80/ether treatment does not completely solubilize the membrane proteins but leaves partially delipidated aggregates of variable size as shown by Väänänen and Vaheri [21] and by our own data. Ultracentrifugation of complete infectious rubella virus through Nonidet P40 detergent [3] overlaying a sucrose gradient permitted retention of a few HAI antigenic sites because the contact with the detergent was very short. Two subcutaneous injections of 100 μg of the resulting particles with complete Freund's adjuvant were needed to induce HAI antibody titers of 1 : 64. This is also an approximate 1000-fold increase over the amount of antigen needed with whole virus. Complete delipidation of the viral membrane proteins (rosettes) by treatment with Triton X-100 and exchange with β-D-octylglucoside completely destroyed the capacity of the HA to induce HAI antibodies (Tables 1 and 2) even at concentration of 120 μg HA. Addition of Freund's complete adjuvant did not help in eliciting HAI antibody formation, even if animals were given a booster dose 2 weeks after the first immunization.

In the case of Semliki Forest virus, the antigenic site for the induction of neutralizing antibodies is retained on the rosette forms and is not dependent on structural integrity

[2]. These can induce protective antibodies in mice after two injections of 0.1 μg protein or one injection of 1 μg without adjuvant. Virosomes of Semliki Forest virus membrane proteins did not have higher immunogenicity than the isolated proteins [11]. Another example of antigenic site differences resembling our experience was reported with two small RNA viruses: foot-and-mouth disease virus and poliovirus [10]. It was shown that one of the structural polypeptides of foot-and-mouth disease virus can induce neutralizing antibodies, whereas none of the individual polypeptides of poliovirus could induce neutralizing antibody.

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