VACCINATION AGAINST RUBELLA
Aspects on vaccines and determination of immunity

By

LENA GRILLNER

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This thesis is based on the following papers which will be referred to in the text by the given Roman numerals:

I. Grillner, L., Hedström, C-E., Bergström, H., Forssman, L., Rignér, A. and Lycke, E. Vaccination against rubella of newly delivered women. Scand J Infect Dis 5: 237-241, 1973.

II. Grillner, L. and Forssman, L. Post-partum rubella vaccination, anti-D immunoglobulin, and blood transfusion. Br Med J 4:47, 1974.

III. Grillner, L. Neutralizing antibodies after rubella vaccination of newly delivered women: A comparison between three vaccines. Scand J Infect Dis 7: 169-172, 1975.

IV. Grillner, L. Immunity to intranasal challenge with rubella virus two years after vaccination. A comparison between three rubella vaccines. Submitted for publication.

V. Strannegård, Ö., Grillner, L., and Lindberg, I-M. Hemolysis-in-gel test for the demonstration of antibodies to rubella virus. J Clin Microbiol 1: 491-494, 1975.

VI. Grillner, L. and Strannegård, Ö. Evaluation of the hemolysis-in-gel test for the screening of rubella immunity and the demonstration of recent infection. Submitted for publication.

CONTENTS

INTRODUCTION

Rubella virus. Rubella virus, isolated in 1962 in cell culture by Weller and Neva and by Parkman et al, is an RNA virus. It has been described by Best et al (1967) as pleomorphic, but generally spherical, with a central nucleoid of 30 nm diameter within an envelope 60 - 70 nm wide. Lipid is an essential part of the envelope and the infectivity of the virus is destroyed by ether. Rubella virus multiply in the cytoplasm, and are released by budding. Hemagglutinin, one or more complement-fixing antigens, and two precipitinogens, theta and iota are structural components of the purified intact virion. Only one serological type of rubella virus has been described. On the basis of its physical and chemical characteristics rubella virus has been classified as belonging to the Alpha virus genus of the Toga virus family.

Rubella antibody tests. The isolation of rubella virus in cell cultures was rapidly followed by the development of techniques to detect serum antibodies to the virus by neutralization tests (Parkman et al 1964). Neutralizing (NT) antibodies develop after rubella infection and persist probably life-long. The existence of NT antibodies could be shown to protect against clinical infection, thus indicating immunity (Schiff et al 1965). In 1967 the hemagglutinating ability of rubella virus was demonstrated and antibodies to the virus could be detected by hemagglutination-inhibition (HI) tests (Stewart et al 1967, Halonen et al 1967). Pre-treatment of serum with either kaolin (Stewart et al 1967, Halonen et al 1967), heparin and $MnCl_2$ (Feldman 1968) or dextransulfate and $CaCl_2$ (Liebhaber 1970) is necessary for removal of the nonspecific inhibitors normally present in serum. The HI technique is now in general use for diagnosis of rubella and for determination of immunity since it is more rapid and easier to perform than the NT test. However, the presence of NT antibodies is considered a more reliable indication of immunity. Testing for complement-fixing antibodies is of value only for the diagnosis of recent infection. Immunofluorescence techniques are available for determination of antibodies (Lennette et al 1967), but are not in general use. In 1975 a new technique, hemolysis-in-gel (HIG), was described for detection of rubella antibodies (paper V, Skaug et al 1975).

Rubella infection. At the International Congress of Medicine in London in 1881 rubella was distinguished from scarlet fever and measles and described as a distinct and specific entity (Squire 1881). Veale desribed in 1866 30 cases from an epidemic and proposed the name rubella. The postnatal rubella disease is characterized by mild upper respiratory symptoms, fever, glandular swelling and rash. The most common complication of rubella is arthralgia and arthritis which occur in more than 50% of adult women but less frequently in children and men (Banatvala 1972). Encephalitis and thrombocytopenia are other, less common, complications of the disease. The clinical symptoms are variable and subclinical infections are common. A rate of 2:1 for subclinical to apparent infection was found in an epidemic by Horstmann et al (1970b).Thus, a correct diagnosis can only be established by serological tests.

The disease was considered as a harmless exanthematous disease of childhood until 1941 when Gregg described the association of rubella infection during early pregnancy and congenital cataract and heart disease. The characteristic feature of congenital rubella is the multiplicity of defects seen, the most frequent being ocular, cardiac and hearing defects, microcephaly, mental and growth retardation and the chronic persistence of fetal infection. Neonatal manifestations of the rubella syndrome are failure to thrive, thrombocytopenic purpura, hepatitis with jaundice and hepato- and splenomegaly (Cooper et al 1969). The risk of fetal damage is highest during the first eight weeks of pregnancy, with a sharp decline in incidence after the 20th week, although evidence of deafness and mental retardation after infection between the 20th and 24th weeks of pregnancy was found by Hardy et al (1969). Dudgeon (1970) quoted an overall incidence of damage of 30-40% for rubella infection during the first trimester. During a large epidemic of rubella in the United States in 1964 1% of all pregnancies were rubella-damaged and more than 25 000 children were born with congenital rubella (Cooper 1972). This epidemic emphasized the rubella problem and initiated intensive research work aimed at the development of rubella vaccines.

Rubella vaccines. The first effective vaccine strain, HPV-77 was developed by Meyer et al (1966) and had been attenuated by 77 passages in green monkey kidney (GMK) cells. This strain was further attenuated by five passages in duck embryo cells, HPV-77 DE-5, and was in preli-

minary trials shown to induce a satisfactory serological response with an acceptable incidence of side-effects (Buynak et al 1968). The HPV-77 strain was also passaged in dog kidney cell cultures, HPV-77 DK-12, but has recently been withdrawn from the market because of a high incidence of joint symptoms.

The Cendehill strain was developed by Huygelen and Peetermans (1967) and was used after the 51st passage in primary rabbit kidney cells.

The RA 27/3 strain, finally, was developed at the Wistar institute in Philadelphia (Plotkin et al 1967) and the 29th passage level in Wistar 38, human diploid cells, was used as vaccine. All three vaccine strains are infectious and immunogenic by the subcutaneous route but, in addition, the RA 27/3 strain was shown to be immunogenic also after intranasal administration (Plotkin et al 1968).

Vaccination programs. At least, two different programs are in use for the prevention of rubella during pregnancy. In the United States the aim of active immunization has been to reduce the incidence of rubella, thus to protect pregnant women from infection in the community. This program involves the vaccination of all children between the ages of 1 and 11 years in order to induce so-called "herd immunity". In the United Kingdom and many other European countries, including Sweden, the principal aim of vaccination has been to ensure that women are individually immune to rubella when reaching childbearing age by the vaccination of adolescent girls.

These two different programs also include recommendations for the vaccination of certain categories of adult women who have been shown to be susceptible to rubella by prior serological testing. Since live attenuated vaccine virus may exhibit the teratogenic properties of rubella virus, vaccination of adult women can only be performed if the woman is not pregnant at the time of vaccination and will not become pregnant for at least two months afterwards. Thus, mass vaccination of adult women will be difficult to perform since even with the present limited programs there are several reports of inadvertent vaccination in pregnancy, some of which have been associated with recovery of vaccine virus from fetal tissue and products of conception (Phillips et al 1970, Vaheri et al 1972, Wyll and Herrmann 1973, Fleet et al 1974). To circumvent this problem vaccination during the post-

partum period of susceptible women has been used and is recommended in Sweden as a complement to the vaccination of adolescent girls.

Background to the present study. Up to 1972, when the present study started, no obvious differences between the three available vaccines in seroconversion rates and transmissibility to contacts had been demonstrated. However, there were indications that differences in antibody titers after vaccination existed (Dudgeon et al 1969, Fogel et al 1971, Tobin 1971), with a higher HI antibody response induced by the RA 27/3 strain in comparison with the Cendehill strain. Neutralizing antibodies had only been studied in small groups and had not included all three vaccines (Fogel et al 1971, Tobin 1971).

Side-effects were found more often in adult women than in adolescent girls and children (Weibel et al 1972). In adult women joint symptoms had been shown to occur in as many as 40% after vaccination with the HPV-77 strains (Weibel et al 1969) and 10% after immunization with the Cendehill strain (Horstmann et al 1970 a). Direct comparative trials of the Cendehill and RA 27/3 vaccines in adult women (Dudgeon et al 1969, Fogel et al 1971, Tobin 1971) sometimes showed little difference in the incidence of joint symptoms while in others RA 27/3 gave somewhat more joint reactions. However, larger comparative studies in adult women including all three vaccine strains were lacking both concerning antibody titer response and side-effects.

The duration of immunity induced by rubella vaccination is not satisfactorily known. As the main group in many vaccination programs consists of children and adolescent girls the immunity induced by vaccination should have a duration of at least 20 years to protect against fetal infection during a future pregnancy. Another cause of concern about the impact of rubella vaccination was the high reinfection rate, 50-80%, found in vaccinees both after challenge (Meyer et al 1968, Schiff et al 1969, Wilkins et al 1969) and after exposure in rubella epidemics (Wilkins et al 1969, Chang et al 1970, Horstmann et al 1970 b, Davis et al 1971). Reinfections are known to occur in individuals with naturally acquired immunity but at a much lower rate, 3-10%, (Meyer et al 1968, Horstmann et al 1969, Parkman 1969). These findings emphasized the importance of a vaccine which induced an immune response as close as possible to that of natural infection (Horstmann 1971).

Rubella vaccination of adult women on a large scale necessitates the use of a safe and rapid serological technique for the determination of rubella immunity. In the HI test the nonspecific inhibitors normally present in serum cause some problems and lead to uncertainty about the value of low antibody titers. In 1974 Beale reported a single radial hemolysis technique for detection of antibodies to hemagglutinating viruses and this method had been applied to influenza virus (Russel et al 1975) but seemed also applicable to rubella.

AIM OF THE PRESENT INVESTIGATION

One purpose of the present study was to compare the three available rubella vaccines considering immunogenicity and adverse reactions after vaccination of newly delivered women by the investigation of

1. the serological response eight weeks and two years after vaccination.
2. the immunity to intranasal challenge two years after vaccination.
3. the incidence of side-effects after vaccination.

Due to the fact that vaccination was carried out during the postpartum period it was also important to consider

4. the possibility for transmission of vaccine virus to the babies of vaccinated mothers.
5. the influence of anti-D immunoglobulin and blood transfusion on the serological response.

Finally, one purpose of the study was

6. to evaluate a new technique, hemolysis-in-gel, for determination of immunity to rubella.

MATERIALS AND METHODS

Patients

During 1972 newly delivered women in Göteborg, who had no or low HI antibody titers, were offered vaccination against rubella. Three groups of patients were included in the study. One, a non-immune group, consisted of women with pre-immunization HI antibody titers of 10 or less. Of the two other groups one was composed of vaccinees with pre-immunization titers of 20 and the other consisted of women with titers of 40 or more. All pregnant women were tested for immunity against rubella at their first visit to an antenatal clinic or to their private physician. The vaccination was performed within four days postpartum at the obstetric department. To avoid a new pregnancy contraceptives were used for three months following vaccination. Blood samples were obtained from 872 women before vaccination and then six weeks or later (mean eight weeks) after the vaccination. 143 rubella vaccinated women were followed up by taking a third blood sample after two years and 99 of these women were revaccinated intranasally. The result of the revaccination was tested by a new blood sample after another six weeks.

The incidence of side-effects after vaccination was evaluated in 949 women by means of a questionnaire and in a number of cases by clinical examination. Also, blood samples were obtained from 63 babies of vaccinated mothers during routine examination at the infant welfare clinics, usually at the age of two to eight months.

Vaccines

Three different vaccines were used throughout the study: the Cendehill (Cendevax, Smith, Kline and French laboratories), HPV-77 DE-5 (Meruvax, Merck, Sharp and Dohme), and RA 27/3 (Almevax, Burroughs Wellcome) vaccine strains. The vaccines contain live attenuated rubella virus and differ from each other with regard to the type of cells and passage level used for attenuation.

All three vaccines were, after reconstitution, administered subcutaneously in a dose of 0.5 ml, containing at least 10^3 TCID 50. For revaccination by the intranasal route the RA 27/3 strain was used and each intranasal dose contained at least 10^4 TCID 50 of the attenuated virus strain. The intranasal vaccination was performed with the patient in the supine position with her head unsupported and hyperextended and 0.25 ml of the reconstituted vaccine was dropped into each nostril. After administration of the dose this position was maintained for at least one minute.

Serology

Serum samples. Sera were inactivated and stored at -20°C until tested. All sera from one and the same patient were always run in parallel.

Definitions. Using HI or the NT test an at least fourfold increase in titer between paired samples was considered significant. Seroconversion was defined as a titer difference of two or more titration steps between pre and post-immunization serum samples. For evaluation of results with the HIG test see paper VI.

Reinfection or booster response was defined as a significant titer rise in HI antibodies between paired serum samples.

Mean titers are given as geometric mean (GMT) for HI, as median titers for NT and as the mean diameter (mm) of hemolytic zones in the HIG test.

Hemagglutination-inhibition test. The HI technique developed by Halonen et al (1967) was used with slight modifications. Pigeon erythrocytes were employed and sera were adsorbed on kaolin (Schmidt 1970). An HI titer of 20 or more was considered to indicate immunity.

Neutralization test. Conventional technique was used for NT antibody assay and the procedure is described in detail in paper III. Twofold serum dilutions from 1/2 to 1/16 were tested for inhibitory effect on the cytopathic changes of rubella virus on RK_{13} (rabbit kidney cell line) tube cultures (Furesz 1969). To enhance the neutralizing capacity 4% unheated guinea-pig serum was added to the diluent used for sera (Leerhøy 1968a). A NT titer of 2 was considered to indicate immunity to

rubella.

Hemolysis-in-gel test. The HIG test is based on the principle that erythrocytes coated with antigen will be lysed by complement in the presence of specific antibodies. The following procedure was used for performance of the test. (Details are given in papers V and VI).

Erythrocytes (RBC), collected in Alsevers solution, were washed 3-6 times in phosphate buffered saline (PBS) before incubation with antigen. Pigeon erythrocytes were used in most experiments, but in addition sheep, goose, ox or human group O RBC were tested. For coating of RBC 300 hemagglutinating units (HAU) of rubella HA antigen (Wellcome laboratories) were used per 25 µl of a 50% RBC suspension. Antigen-coated RBC were mixed thoroughly with 1.6% agarose (Behringwerke) or 1.5% Indubiose A-37 (L´Industrie Biologique Francaise) at a temperature of 47°C and poured onto Petri dishes placed on a level surface. After solidification 3 mm holes were punched out in the gel and 5 µl of serum or serum dilutions was added to the holes. After diffusion of serum at +4°C normal guinea-pig serum was poured onto the plates which were incubated at 37°C for 2 hours. Unless otherwise stated, a diffusion time of 24 hours was used. The diameter of the hemolytic zones was measured with precision calliper to the nearest 0.1 mm. Two perpendicular diameters were measured for each zone and the mean of these values was recorded. A diameter of 6 mm or more was considered to indicate immunity to rubella.

Statistical methods

Standard methods were used for calculation of mean values and standard deviations. Values of $P < 0.05$ were considered to be significant. The chi-square method was used to test differences. Statistical comparison between mean titers in paper I was performed by using the method trend in a contingency table. Regression lines (paper VI) were calculated from results of 6 replicates for each antibody concentration by the method of least squares.

RESULTS

Serological response after rubella vaccination

872 women were vaccinated with the Cendehill, HPV-77 DE-5, and RA 27/3 vaccines. The vaccinees were grouped according to their immune status and 511 were found to lack rubella HI antibodies whereas 361 had HI titers of 20 or more before vaccination. The serological responses induced by the three different vaccines were compared. In all women HI antibodies were determined before vaccination and after eight weeks and 143 women were retested after two years.

NT antibodies were determined in serum samples from 111 women after eight weeks and from 70 women two years later. The antibody response in 55 women was, in addition, evaluated by the HIG technique.

Hemagglutination-inhibition antibody response (papers I and IV). In susceptible women the seroconversion rates were 91% in Cendehill, 92% in HPV-77 DE-5 and 96% in RA 27/3 vaccinees (Table I). The differences in seroconversion rates observed with the three vaccines were not statistically significant. The mean titers seen after vaccination of non-immune women were 32 in the Cendehill, 39 in the HPV-77 DE-5 and 51 in the RA 27/3 group. These differences were significant ($P < 0.01$), suggesting that the RA 27/3 vaccine was more immunogenic than HPV-77 DE-5, the latter being in turn more effective than the Cendehill strain. In the two groups of immune women less than 10% responded to vaccination with a significant titer rise. In these vaccinees a doubling of the pre-immunization titers was as a rule seen.

Table I. HI antibody response to rubella vaccination in seronegative women

Vaccine strain	No of women	Seroconversion No	Seroconversion %	Geometric mean titer
Cendehill	210	192	91	32
HPV-77 DE-5	182	168	92	39
RA 27/3	119	114	96	51

In the majority of the 143 vaccinees studied the HI titers after two years were within ± one titration step of the titers found eight weeks after vaccination. The mean titers in this group of women were 32 for Cendehill, 36 for HPV-77 DE-5 and 59 for RA 27/3 vaccinees at eight weeks postvaccination compared to 33, 56 and 48, respectively, two years later. Although no general decrease in antibody titers could be demonstrated it should be observed that three women in the Cendehill group had HI titers less than 10 after two years. All three had previously responded to vaccination by formation of HI antibodies.

Neutralizing antibody response (paper III). In a group of 111 women, who had responded with seroconversion according to the HI test, only 56% of Cendehill compared to 79% of HPV-77 DE-5 and 95% of RA 27/3 vaccinees had developed NT antibodies eight weeks after vaccination (Table II). However, two years later, when 70 women were retested, 82, 94 and 100%, respectively, of the vaccinees had NT titers of 2 or more. Statistical comparisons revealed that the differences in NT antibody response seen at eight weeks and two years postvaccination between RA 27/3 and Cendehill vaccinees were significant ($P < 0.05$). Between the RA 27/3 and the HPV-77 DE-5 groups a significant difference was found only in sera collected after eight weeks. This was also the case when HPV-77 DE-5 and Cendehill vaccinees were compared.

The NT antibody titers induced by vaccination were generally low. The median titer for the Cendehill group was 2 compared to 4 in the other two groups. After two years the median titers had increased to 4, 8 and 8, respectively, but the difference in response between Cendehill vaccinees and the two other groups persisted. In a comparison between HI and NT antibody titers it could be shown that in 20 out of 61 women

Table II. Neutralizing antibody response 8 weeks and 2 years after rubella vaccination

	8 weeks			2 years		
Vaccine strain	No pos / No tested	%	Median titer	No pos / No tested	%	Median titer
Cendehill	24/43	56	2	27/33	82	4
HPV-77 DE-5	23/29	79	4	16/17	94	8
RA 27/3	37/39	95	4	20/20	100	8

a significant titer rise in NT antibodies was demonstrable during the follow-up period while only 6 of these women showed a fourfold rise in HI antibodies ($P < 0.001$).

Antibody response determined with the hemolysis-in-gel test (paper VI). Fifty-five women, vaccinated against rubella and tested by HI and NT before vaccination and eight weeks and two years afterwards were also studied by the HIG test. All had shown seroconversion by development of HI antibodies at eight weeks after the vaccination. Fifty-two women (95%) were then found to be positive in HIG compared to 72% demonstrating NT antibodies. Two years after vaccination all except one women had antibodies demonstrable by HIG while 8% still lacked NT antibodies. In two women the HI titers had by then decreased to less than 20.

Immunity to intranasal challenge

A high reinfection rate has been reported in Cendehill and HPV-77 DE-5 vaccinees (Wilkins et al 1969, Chang et al 1970, Horstmann et al 1970 b, Davis et al 1971) but a lower rate was indicated in RA 27/3 vaccinees (Liebhaber et al 1972). Reinfection occurs in 3-10% of naturally immune individuals and is probably of importance for maintenance of immunity (Horstmann 1971). However, since reinfection during pregnancy has been associated with transmission to the fetus (Northrop et al 1972, Eilard and Strannegård 1974) the high reinfection rate seen in vaccinees has caused concern. The present study offered a possibility to compare the resistence to reinfection induced by the three vaccines (paper IV).

Two years after the primary vaccination a challenge by intranasally administered virus was performed. As challenge virus the RA 27/3 strain was used. HI antibody titers were determined before and six weeks after challenge. Ninety-nine women were included in the study and of these 38 had received the Cendehill, 29 the HPV-77 DE-5 and 32 the RA 27/3 vaccine. A response to challenge with a significant titer rise was taken as an indication of susceptibility to reinfection.

In the Cendehill group 53% were found to be susceptible compared to 24% in the HPV-77 DE-5 (Table III). In contrast to these results only 9% of RA 27/3 vaccinees demonstrated a significant titer rise after challenge. The difference in susceptibility to challenge between

Table III. HI antibody titer response after intranasal challenge with the RA 27/3 strain two years after rubella vaccination

Vaccine	No of women	% titer rises	GMT	
			Before challenge	After challenge
Cendehill	38	53	33	117
HPV-77 DE-5	29	24	55	99
RA 27/3	32	9	46	59

RA 27/3 vaccinees and Cendehill was statistically significant ($P < 0.001$) as also was that between the latter and the HPV-77 DE-5 group ($P < 0.03$). As shown in Table III, intranasal challenge did not significantly influence the mean titer in the RA 27/3 group while in the other two groups marked increases were demonstrable after revaccination. The susceptibility to intranasal challenge was found to be related to the antibody level induced by primary vaccination. Thus, the mean titer after two years, before challenge, was 25 in the susceptible group compared to 54 in the group of women who did not show any booster response upon challenge.

Side-effects of rubella vaccination

Side-effects were studied in 949 women of whom 280 had received the RA 27/3, 332 the Cendehill and 337 the HPV-77 DE-5 vaccine (paper I).

The reactions seen after vaccination were rubella-like symptoms like fever, glandular swelling, sore throat, rash and joint symptoms including both arthritis and arthralgia. Fever, glandular swelling, sore throat and rash appeared between the 7th and the 12th day after vaccination and usually had a duration of three to four days. The onset of joint symptoms was generally later than that of other side-effects and in most cases they were first noticed two weeks, in some vaccinees as late as four weeks, after vaccination. The duration was also longer but did not usually exceed one week. However, in the HPV-77 DE-5 group a few women experienced persistent arthritis for as long as two months and one of them still has recurrent joint symptoms after three years.

One or more reactions were experienced by 34% of the vaccinees in the RA 27/3 group, by 23% in the Cendehill group and by 44% in the HPV-77 DE-5 group. The differences between the groups are all statistically significant ($P < 0.03$). Local reactions at the site of injection were rarely seen except in the RA 27/3 group, in which 18% of the vaccinees noticed erythema and swelling compared to 3% and 2% in the other two groups ($P < 0.001$). Fever and glandular swelling occurred in 3 to 9% of the vaccinees and no differences between the groups were found. Sore throat, rash and joint symptoms, on the other hand, occurred significantly more often in the HPV-77 DE-5 group ($P < 0.005$) and were seen in 17, 20 and 32%, respectively, of the vaccinees compared to between 7 and 11% in the other two groups. A comparison between the RA 27/3 and the Cendehill group revealed no certain differences except for local reactions and the total number of side-effects.

However, since side-reactions could be expected to occur more often in seronegative than in previously immune women the incidence of joint symptoms and rash was evaluated according to the serological pre-immunization status. As may be seen from Table IV, these two side-effects were more common among susceptible women and the differences were statistically significant for all except joint symptoms in the Cendehill group ($P < 0.05$). A comparison between the latter and the RA 27/3 group revealed a higher incidence of joint symptoms, but not rash, among RA 27/3 rubella-susceptible vaccinees ($P < 0.005$). The number of reactions in the RA 27/3 group was on the other hand significantly lower than in the seronegative HPV-77 DE-5 group ($P < 0.001$).

Table IV. Incidence of rash and joint symptoms in seronegative and immune women after rubella vaccination.

Vaccinees	Side-effect	Cendehill		HPV-77 DE 5		RA 27/3	
		No	%	No	%	No	%
Seronegative	Rash	19/195	10	53/191	28	17/125	14
	Joint symptoms	14/195	7	80/191	42	22/125	18
Immune	Rash	3/102	3	5/99	5	3/102	3
	Joint symptoms	3/102	3	7/99	7	2/102	2

Serological response in babies of vaccinated mothers

Sixty-three babies of rubella-susceptible women vaccinated postpartum were tested at the age of two to eight months for the presence of HI antibodies (paper I). None of the children demonstrated an antibody response, indicating that no immunization through transmission of vaccine virus had occurred. Many possibilities of exposure existed since two-thirds of the babies had been breast-fed for at least three months.

Influence of anti-D immunoglobulin and blood transfusion on the serological response to vaccination

To evaluate if blood transfusion and administration of anti-D immunoglobulin could influence the serological response to rubella vaccination 659 obstetric reports from rubella-vaccinated women were examined. In addition to the material presented in paper II another four women who had received blood transfusion during delivery were included.

Twelve women had received one or more units of blood in connection with the delivery and 58 women had received anti-D immunoglobulin. The seroconversion rates were 97% for the controls, i.e. the 589 women who had not received transfusion or anti-D prophylaxis, and 100% for the anti-D group. In the transfusion group only six out of twelve (50%) responded to vaccination with seroconversion. This difference was statistically significant ($P < 0.001$).

The mean titers after vaccination in women showing seroconversion varied between 34 and 38 and no significant difference between the groups could be detected. In two of the women in the transfusion group HI antibodies were found in blood samples obtained after the transfusion but before vaccination. In both cases no HI antibodies were demonstrable eight weeks after vaccination, indicating that the HI titers before immunization reflected passively transferred antibodies.

Evaluation of a new test, hemolysis-in-gel, for determination of antibodies to rubella

The hemolysis-in-gel technique is based on the principles of the sing-

le radial immunodiffusion method of Mancini et al (1965). In the HIG test the antigen is bound to erythrocytes incorporated in agarose gel. Specific antibodies will lyse the erythrocytes in the presence of complement. The technique could be expected to be sensitive and simple to perform and thus well suited for both determination of immunity and diagnosis of rubella infection. The HI test, generally used, is rather simple to perform, but requires titration of sera and absorption to remove nonspecific inhibitors and erythrocyte agglutinins.

In the present study a HIG technique suitable for demonstration of rubella antibodies was developed (paper V). The relationship between the antibody concentration and the size of the hemolytic zone was determined and the sensitivity of this technique for detection of differences in antibody concentrations was evaluated (paper VI). The results obtained with the HIG technique were correlated to results of HI and NT tests.

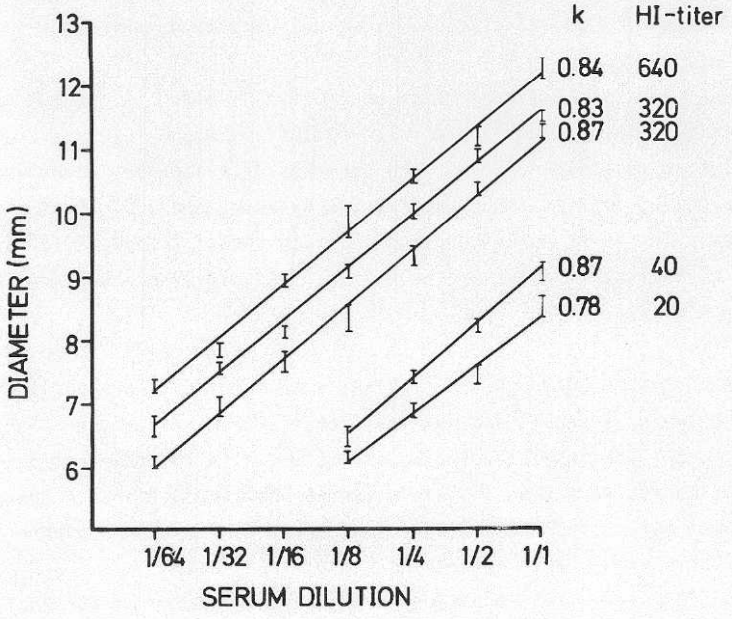

Fig 1. Regression lines for five different serum samples tested in twofold dilutions. The mean diameter and the standard deviation for 6 replicates are indicated. The slope value (k) of the regression line and the hemagglutination-inhibition (HI) titer for each serum sample are given in the figure. (From Fig 2 paper VI).

Relationship between antibody concentration and size of zone of hemolysis. A linear relationship was found between the diameter of the zone of hemolysis and the log antibody concentration after a diffusion time of 24 and 48 hours. Five sera with different HI titers were tested in twofold dilutions and the regression lines were calculated from the results of 6 replicates per serum dilution (Fig 1). As shown in the figure, the regression lines are almost parallel and a good correlation between the HI antibody titer and the HIG diameter was found. According to these regression lines, twofold and fourfold titer rises should be possible to detect. The sensitivity of HIG for detecting differences in rubella antibody concentrations was further evaluated. Twenty-five paired sera with a twofold difference in antibody concentration were tested in triplicate. In addition 78 paired samples with a fourfold difference were tested as a single specimen and these paired samples were also assayed in HI. All tests were performed with coded samples. It could be shown (Table V) that twofold and fourfold titer differences could be detected with high accuracy in the HIG test. When the 78 paired samples were assayed in HI a tendency towards higher titer increases than tested was observed (Table V). This finding may be related to the use of an automatic diluter in the test.

Statistical analysis of one of the regression lines showed that a difference in diameter of 0.62 mm between two serum samples after a diffusion time of 24 hours gave a probability of 0.95 for a positive difference in antibody concentration. The probability level for a difference of 0.82 mm was found to be 0.99. This indicates that all differences in diameter obtained for fourfold differences in antibody concentration and all except one for twofold differences may be considered significant (Table V). When tested by HI, on the other hand, 2 of the 78 paired samples gave no titer difference and 10 of these only a twofold increase in titer, a difference which is usually not considered significant.

HIG in comparison with HI and NT. A good correlation was found between HI and HIG when 137 serum samples were tested. None of 22 sera with HI titers less than 20 gave a hemolytic zone diameter $\geq$ 6 mm and only one serum was found to be positive in HI but negative in HIG. In addition, 93 serum samples with low (HI 20-40) or no antibody content as determinated by HI were tested by HIG as well as NT. In 33 of these samples antibodies were demonstrable by all three methods. Of 60 sera with HI

Table V. Differences in diameters (HIG) and titers (HI) for paired dilutions of serum samples with twofold and fourfold differences in antibody concentration. Serum samples were tested under code.

		HIG				HI		
Diff in antibody conc	No of paired sera	Mean diff in diam (mm) ± SD	Diff in diam (mm)+ < 0.6	0.6 - 0.9	≥ 1	Diff in titer+ ≤ 2-fold	4-fold	≥ 8-fold
2-fold	25	0.8 ± 0.2	1	17	7	N.D.	N.D.	N.D.
4-fold	78	1.9 ± 0.5		3	75	12++	46	20

\+ Figures indicate no of paired sera showing the indicated differences in diameter or titer.

++ 2 paired sera did not show any increase in titer.

N.D. Not done

titers of 10 or less, 57 were also negative in HIG and 58 in NT. Two of the HI negative sera contained antibodies which were demonstrable by both HIG and NT. Finally, one serum was positive in HIG, had an HI titer of 10 and was negative in NT.

Acute and convalescent-phase sera from 55 clinical cases of rubella were tested in HIG and HI. Seroconversions were demonstrated by the HI method in 53 cases and all these paired samples also changed from negative to positive reaction in HIG. In two patients no titer increases could be shown by either HIG or HI. However, the acute-phase sera were obtained late after the onset of rash and significant titer rises were shown in complement-fixation tests.

Twenty of the acute-phase sera contained HI antibodies which could not be demonstrated by the HIG test. Immunoglobulin separation performed by sucrose density gradient centrifugation revealed that the fractions containing antibodies of the immunoglobulin M (IgM) class did not give hemolysis in the HIG test although antibodies could be demonstrated in these fractions by the HI method. Even when a diffusion time of 48 hours was used no IgM antibodies could be shown. Thus, a relative inability of the HIG test, performed according to the presently adopted procedure, to detect rubella antibodies of the IgM class was found.

DISCUSSION

Comparison between the vaccines

The aim of rubella vaccination is to prevent congenital rubella by creating an immunity as close as possible to that induced by natural infection. Reinfection probably plays an important role for the maintenance of the immunity following natural infection (Horstmann 1971) and usually serves as a booster, with the development of a qualitatively and quantitatively better immunity. However, cases have been reported in which reinfection has been associated with clinical symptoms (Strannegård et al 1970, Forrest et al 1972, Wilkins et al 1972) and possible transmission to the fetus (Northrop et al 1972, Eilard and Strannegård 1974), suggesting that viremia has occurred. Even though reinfection with viremia may be a rare event, the high rate of reinfection seen in vaccinees shortly after immunization causes concern since the duration of vaccine-induced immunity is unknown. The susceptibility to reinfection is related to low antibody levels and differences in immunologic properties between the vaccines have been suggested (Plotkin et al 1973). The present study offered an opportunity to compare the three commonly used vaccines in the same trial regarding immunogenicity, adverse reactions and subsequent immunity to reinfection.

Rubella vaccination of newly delivered women induced an HI antibody response in 91 to 96% of the vaccinees without any significant differences between the vaccine strains used. HI antibody titers after vaccination are in general four to eightfold lower than those seen after natural infection. In comparative studies between the Cendehill and the RA 27/3 vaccines higher HI antibody titers have been shown after immunization with the RA 27/3 strain (Dudgeon et al 1969, Fogel et al 1971, Tobin 1971). The Cendehill vaccine has been reported to give lower HI titers than the HPV-77 DE-5 vaccine (Dudgeon et al 1969) but in a study by Isacson et al (1971) no certain differences were found. However, the present study revealed statistically significant differences in HI antibody titers between all three vaccines, confirming that the RA 27/3 strain is more immunogenic than the HPV-77 DE-5 vaccine, which is in turn more effective than the Cendehill strain.

The differences noted in mean titers are important since a slight decline in antibody titers is usually noted in follow-up studies two to seven years after vaccination (Liebhaber et al 1972, Farquhar 1973, Schiff et al 1974, Hilleman 1975) as well as after natural infection. Davis et al (1971) reported that less antigenic vaccine strains showed the greatest loss of definite levels of HI antibodies after one to three years. In the present study the mean titers were well maintained in the three groups during the follow-up period. However, three women in the Cendehill group had HI titers below 10 after two years and Schiff et al (1974) have reported that 3.9% of Cendehill-vaccinated children had no detectable antibodies 4 1/2 year after immunization.

The comparison between HI and NT antibody titers performed in the present study suggested that the maximum level of NT antibodies appeared later than that of HI antibodies. This observation is in accordance with the findings of Leerhøy (1968b) in naturally infected individuals. The better neutralizing capacity demonstrated after two years may be explained by differences in avidity of antibodies produced early and late after vaccination. The rise in NT antibody titers seen after two years could not be due to a booster response to natural infection since no simultaneous rise in HI antibody titers was demonstrated.

Vaccination with the RA 27/3 and HPV-77 DE-5 strains induced a neutralizing antibody response which was superior to that induced by the Cendehill vaccine. At eight weeks postimmunization RA 27/3 vaccinees also demonstrated a better response compared to the HPV-77 DE-5 group. In a comparison between the Cendehill and the RA 27/3 strain Fogel et al (1971) reported similar differences six to eight weeks after vaccination but when some of the vaccinees were retested after seven months no differences could be demonstrated. Carlsson et al (1974) found NT antibodies in 95% of adult women 12 weeks after vaccination with the Cendehill, HPV-77 DE-5 and RA 27/3 vaccines. No differences were found between the vaccine groups but sera were only tested undiluted.

The NT antibody titers we have seen after vaccination were in general low. The lowest median titers were seen in the Cendehill group at both eight weeks and two years postvaccination while no differences were found between the other two groups. Other comparative studies between the Cendehill and RA 27/3 vaccines have also demonstrated lower NT antibody titers after Cendehill vaccination (Tobin 1971, Plotkin et al

1973).

The quantitatively better HI and NT antibody response observed in RA 27/3 vaccinees was found to correlate with a lower susceptibility to intranasal challenge two years postimmunization. Among Cendehill vaccinees a significantly higher reinfection rate was observed while HPV-77 DE-5 vaccinees demonstrated a susceptibility rate in between that of RA 27/3 and Cendehill vaccinees. Most challenge studies have involved rather small groups and have usually not included comparison of all three vaccine strains. The findings of the present comparative study are in agreement, however, with results of the other non-comparative studies. Thus, a high reinfection rate has been observed among Cendehill and HPV-77 DE-5 vaccinees both after exposure to wild rubella virus and after challenge with intranasally administered virus (Wilkins et al 1969, Chang et al 1970, Horstmann et al 1970b, Davis et al 1971). In contrast, a lower reinfection rate has been demonstrated after RA 27/3 immunization (Liebhaber et al 1972, Plotkin et al 1973). In another comparative study in children, Ogra et al (1973) found that a booster antibody response occurred most often among Cendehill and HPV-77 DE-5 vaccinees after challenge. Children immunized with RA 27/3 by the subcutaneous route were more susceptible to reinfection than those who received the vaccines intranasally or were naturally immune.

The lower reinfection rate among RA 27/3 vaccinees is also related to the qualitatively better immune response elicited by this vaccine. Thus, both precipitating iota antibodies and secretory IgA antibodies are regularly found after both intranasal and subcutaneous RA 27/3 immunization, whereas these antibodies are rarely detected after vaccination with the Cendehill and HPV-77 DE-5 vaccines (Le Bouvier and Plotkin 1971, Ogra et al 1971). The presence of iota antibodies has been claimed to correlate directly with the appearence of resistance to reinfection (Horstmann et al 1970b, Liebhaber et al 1972), and secretory IgA antibodies are obviously important for protection against viral infections of respiratory mucosa.

Another qualitative difference between the vaccines is their ability to induce a booster response by revaccination. The RA 27/3 rubella vaccine which can be administered intranasally, has been successfully used to boost vaccine-induced immunity (Plotkin et al 1973, paper IV) while the use of subcutaneously administered rubella vaccines has met with only

partial success (Wyll et al 1971, Chang et al 1973). It is reasonable to assume that this type of vaccination may be a valuable supplement to boost waning immunity in adult women who have been vaccinated during childhood or adolescence.

As a rule, a linear relationship exists between the immunogenicity and the reactogenicity of a vaccine. However, the RA 27/3 strain seems to be an exception to this rule. In the present study susceptible RA 27/3 vaccinees experienced joint symptoms and rash significantly less often than HPV-77 DE-5 vaccinees. In the latter group the side-effects induced by vaccination were also more severe and of longer duration than in the other two groups. In a few women vaccinated with the HPV-77 DE-5 vaccine the joint symptoms persisted for up to two months and one woman still has recurrent arthritis after three years. Lerman et al (1971) reported the same symptom in one woman during at least one year following HPV-77 DE-5 immunization. Intermittent arthritis has also been described in children following immunization with the HPV-77 DK-12 vaccine (Thompson et al 1973). Otherwise, adverse reactions are rare in children and there seems to be little differences in incidence and severity between the three available vaccine strains (Andzhaparidze et al 1970, Wallace and Isacsson 1972). The Cendehill vaccine has been associated with few and mild side-effects in adult women (Dudgeon et al 1969, Fogel et al 1971, Tobin 1971) and in the present study joint symptoms occurred significantly less frequently compared to in RA 27/3 and HPV-77 DE-5 vaccinees.

A new Japanese vaccine, To-366, has recently been used in a comparative trial (Best et al 1974). The To-366 strain was found to induce an HI antibody response comparable to that observed with the RA 27/3 and HPV-77 DE-5 strains with a low incidence of adverse reactions. The Japanese vaccine may be of interest for further evaluation since, it has been claimed to be less teratogenic in rabbits than other rubella virus strains (Kono et al 1969).

Rubella vaccination during the postpartum period

Vaccination during the postpartum period is associated with some particular problems. There is a small but definite risk of conception within two months postpartum (Baldwin and Freestone 1971, Sever 1971) and thus,

contraceptives should be used also when newly delivered women are immunized.

Although transmission of vaccine virus to contacts has not been proven to occur (Dudgeon et al 1969, Halstead and Diwan 1971, Scott and Byrne 1971) babies to vaccinated mothers are prone to be exposed because of the close contact between mother and child. However, in agreement with Boué et al (1969) and Tobin (1971) we could not demonstrate any immunization of the babies indicating that probably no transmission of virus had occurred.

Administration of anti-D immunoglobulin and transfusion of blood could be supposed to influence the serological response to vaccination. There was no evidence that anti-D immunoglobulin interfered with the effectiveness of the vaccination in accordance with the findings of Grünberger et al (1972) and Maroni and Munzinger (1975). Blood transfusion, on the other hand, reduced the seroconversion rate significantly and it is therefore essential that the result of vaccination in this group of women is controlled after eight weeks.

About 5-7% of vaccinees fail to respond to vaccination but all failures are not due to blocking of vaccine virus from passively transferred antibodies. Inadvertent inactivation of the vaccine may occur if the manufacturers recommendations for storage and reconstitution are not followed. However, the vaccinated women who fail to respond, believe themselves to be protected against rubella and may therefore neglect to seek medical advice and examination on exposure. These risks could be prevented by a routine control of the results of immunization after vaccination of adult women.

Determination of rubella antibodies by hemolysis-in-gel, hemagglutination-inhibition and neutralization tests.

After natural rubella infection HI and NT antibodies usually appear within a few days after onset of rash and then persist probably lifelong. The presence of NT antibodies is generally accepted as a more reliable indication of immunity than HI antibodies. However, the NT test is time-consuming to perform and not well suited for determination of immunity in a large scale. The HI test, on the other hand, is rather

simple to perform but the presence of nonspecific inhibitors of hemagglutination in sera sometimes causes an uncertainty about the diagnostic value of low HI titers.

In the present study another test, HIG, was introduced for the demonstration of antibodies. The HIG technique was found to be sensitive and to give reproducible results in agreement with the findings of Russel et al (1975) and Skaug et al (1975). The test is in contrast to the HI method independent within certain limits of the concentration of antigen (paper V). However, the choice of erythrocytes seems to be important. Pigeon RBC were more sensitive than sheep erythrocytes and also gave fewer nonspecific hemolytic reactions. Although nonspecific lysis is a rather rare phenomenon, when pigeon RBC is used, it seems to necessitate the use of control plates, i.e. plates with uncoated erythrocytes. Instead of pigeon cells chicken RBC can be used in the test as shown by Skaug et al (1975) who obtained results with a good correlation to the results of the rubella HI test.

In the single radial immunodiffusion method of Mancini et al (1965) a linear relationship is obtained between the area and the antibody concentration when equilibrium is reached, i.e. when antibodies are no longer available to react with the antigen in the gel. For antibodies of the immunoglobulin G class this will not be achieved within a diffusion time of five to six days. However, before this time a linear relationship exists between the diameter of the zone and the log of the antibody concentration (Fahey et al 1965). This was also found to be valid for the HIG test, where the diffusion time usually could not be extended beyond 48 hours due to the instability of the erythrocytes. It was shown that fourfold and even lower titer increases could be detected with a high degree of accuracy after a diffusion time of 24 hours. An increase of this time up to 48 hours gave higher slope values and thus larger differences in diameter. However, the standard deviation increased and the accuracy of the test is probably not favoured by a diffusion time longer than 24 hours.

Seroconversions after natural infections were easily detected by HIG seven to ten days after onset of rash. The only limitation hitherto observed seems to be its inability to detect rubella-specific IgM antibodies. This has also been shown to be valid for influenza (Russel et al 1975) but is not yet satisfactorily explored. Because of the in-

ability to detect IgM antibodies by HIG the HI test may be preferred for the demonstration of very early titer rises i.e. three to four days after onset of rash. The same is valid when the early response to vaccination is studied. In the present study 3 out of 55 women had no demonstrable antibodies according to HIG but their sera were all positive in HI eight weeks after vaccination.

A good correlation was found between the results of HIG and NT when sera with low or no HI antibody titers were tested. Thus, since the HIG test is rapid, simple to perform an unaffected by nonspecific inhibitors it is well suited for screening of immunity in a large scale. It may be concluded that the HIG test seems to be a good alternative to HI for determination of rubella immunity as well as for the serological diagnosis of rubella.

SUMMARY AND CONCLUDING REMARKS

Three rubella vaccines (Cendehill, HPV-77 DE-5 and RA 27/3) were used for immunization of adult women postpartum. Serological responses and adverse reactions were studied and the three vaccines were compared. The susceptibility to intranasally administered challenge virus two years after vaccination were investigated.

Differences in serological response and adverse reactions were observed. The best HI and NT antibody response, at eight weeks postvaccination, were seen after immunization with RA 27/3. The immunity induced by this vaccine gave the best protection against intranasal challenge, reinfection, two years later. The HPV-77 DE-5 vaccine gave the largest number of side-effects, which in addition were more severe and of a longer duration than those observed after vaccination with the two other strains. In Cendehill vaccinees few and mild side-effects were noticed but also the lowest HI and NT antibody response. After two years a significantly greater susceptibility to intranasal challenge was found in this group. The differences in immunogenicity between the vaccines are particularly important to consider since the duration of vaccine-induced immunity is unknown.

Previous administration of anti-D immunoglobulin had no effect on the serological response whereas a previous blood transfusion significantly decreased the seroconversion rate. It, thus, seems particularly important to control the result of the vaccination in the latter group of vaccinees. In the study we could not demonstrate any serological signs of a possible transmission of vaccine virus to the babies of vaccinated mothers.

A new method, the HIG technique (hemolysis-in-gel) was found to be well suited for determination of immunity with a good correlation to results of HI and NT tests. The technique has the advantage of being independent of nonspecific inhibitors and does not require titration of sera. Seroconversions and fourfold and even twofold differences in antibody concentrations were detected by HIG with a high degree of accuracy. Thus, the HIG test may as well be used for the diagnosis of rubella

infection. One limitation of the test hitherto observed is its inability to detect rubella-specific IgM antibodies, at least with the presently adopted procedure.

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