Study of the teratogenicity of the vaccine strain of the Rubella virus «Orlov-V» (*Matonaviridae: Rubivirus: Rubella virus*) in experience on rhesus macaques

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Introduction. Rubella virus has pronounced teratogenic properties that can cause generalized and persistent intrauterine infection of the fetus. As a result, the control of the loss of teratogenicity inherent in «wild-type» virus strains is a necessary stage of a preclinical study of the vaccine strain for a live attenuated rubella vaccine.
The purpose of the study is to comprehensively study the teratogenic properties of the vaccine strain of rubella virus «Orlov-V» in the experiment on rhesus macaques.
Material and methods. Seronegative to rubella virus female rhesus macaques in early pregnancy at the age of 4–7 years (n = 13) were used in the experiment. Animals of the experimental group (*n* = 9) received single immunization intramuscularly with a preparation from the «Orlov-V» strain. The control group of the monkeys (*n* = 3) were immunized with a commercial vaccine containing Wistar RA27/3 strain. The female of the control group (*n* = 1) was injected with a solvent used in the rubella vaccine. Study of possible teratogenic properties of vaccine strains of rubella virus was carried out using a complex of clinical, immunological, pathomorphological and virological methods. Clinical observations were made within 3 months after the monkeys' birth. Determination of antibody titers in the blood serum of immunized monkeys was performed in HI test on the 28th–30th day after infection. The ELISA method was applied to determine IgM antibodies in the blood serum of newborns within the first month of life. Detection of rubella virus RNA was performed by PCR with electrophoretic detection of amplicons.
Results. No markers of congenital rubella infection were found in infants born from monkeys vaccinated during the pregnancy. It is shown that PCR can be an informative method to confirm the absence of teratogenic properties of vaccine strains of rubella virus.
Discussion. The obtained data demonstrated that vaccine strains of the «Orlov-V» rubella virus and Wistar RA27/3 have lost their teratogenic properties. The possibility of using an alternative strategy for preclinical assessment of specific safety of antiviral vaccines including a complex of clinical, immunological, pathologic and virological methods instead of the classical pathologic method is discussed.
Conclusion. The results obtained in this study showed the absence of teratogenic properties and high immunogenic activity of the vaccine strain of rubella virus «Orlov-V».

***Keywords**: teratogenicity; rubella virus; attenuated vaccine strain; rhesus macaque monkeys; polymerase chain reaction.*

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ORIGINAL RESEARCH

Изучение тератогенности вакцинного штамма вируса краснухи «Орлов-В» (*Matonaviridae: Rubivirus: Rubella virus*) в опыте на обезьянах макак-резус

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Введение. Вирус краснухи обладает выраженными тератогенными свойствами, способными вызывать генерализованную внутриутробную инфекцию плода персистирующего характера. В связи с этим необходимым этапом доклинического изучения вакцинного штамма для живой аттенуированной вакцины против краснухи является контроль утраты тератогенности, присущей диким штаммам вируса.
Цель исследования: комплексное изучение тератогенных свойств вакцинного штамма вируса краснухи «Орлов-В» в опыте на обезьянах макак-резус.
Материал и методы. В эксперименте использовали серонегативных к вирусу краснухи самок вида макак-резус (*Macacca rhesus*, *Macaca mulatta*) на ранних сроках беременности в возрасте 4–7 лет (*n* = 13). Животных опытной группы (*n* = 9) однократно внутримышечно иммунизировали препаратом из штамма «Орлов-В». Обезьян группы сравнения (*n* = 3) иммунизировали коммерческим препаратом, содержащим вакцинный штамм Wistar RA27/3. Самке контрольной группы (*n* = 1) вводили растворитель вакцины против краснухи.
Изучение возможных тератогенных свойств вакцинных штаммов вируса краснухи проводили с использованием комплекса клинических, иммунологических, патоморфологических и вирусологических методов. Клинические наблюдения осуществлялись в течение 3 месяцев после рождения детёнышей. Определение титров антител в сыворотке крови иммунизированных макак-резусов выполняли в реакции торможения гемагглютинации (РТГА) на 28–30 сутки после заражения. Метод иммуноферментного анализа (ИФА) использовали для определения антител класса IgM в сыворотке обезьян первого месяца жизни. Выявление РНК вируса проводили методом полимеразной цепной реакции (ПЦР) с электрофоретической детекцией продуктов амплификации.
Результаты. Установлено отсутствие маркёров врождённой краснушной инфекции у особей, родившихся от привитых во время беременности обезьян. Показано, что метод ПЦР может являться информативным тестом, подтверждающим отсутствие тератогенных свойств вакцинных штаммов вируса краснухи.
Обсуждение. Полученные данные охарактеризовали вакцинные штаммы вируса краснухи «Орлов-В» и Wistar RA27/3 как утратившие тератогенные свойства. Обсуждается возможность использования альтернативной по отношению к классическим патоморфологическим методам стратегии доклинической оценки специфической безопасности противовирусных вакцин с применением комплекса клинических, иммунологических, патоморфологических и вирусологических методов.
Заключение. Результаты, полученные в рамках настоящего исследования, показали отсутствие тератогенных свойств вакцинного штамма вируса краснухи «Орлов-В».

Ключевые слова: *тератогенность; вирус краснухи; аттенуированный вакцинный штамм; обезьяны макак-резус; полимеразная цепная реакция.*

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Introduction

Because of high teratogenicity of the rubella virus and the attenuated vaccine strain present in the live vaccine, pregnancy is considered a contraindication to the administration of the rubella vaccine [1, 2]. Several studies demonstrate that rubella virus vaccine strains can pass through the placenta [3, 4].

In Great Britain, a total of 515 women were examined; all of them were vaccinated for different reasons against rubella during their pregnancy; however, their children

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born after the vaccination did not display any symptoms of congenital rubella syndrome (CRS), though 10.6% of the women were diagnosed with an intrauterine infection of the fetus [5]. In 2003, in Iran, during the examination of infants born from mothers who were vaccinated during their pregnancy (during the large-scale immunization against measles and rubella), 5 infants were found to have IgM antibodies in their umbilical cord blood; however, no infant was diagnosed with birth defects similar to those observed in CRS [6]. According to Mangtani P., Evans S.J.W. *et al.*, the theoretical risk of fetus involvement resulting from vaccination of the pregnant women against rubella is estimated from 0 to 0.6%, i.e. at much lower levels than the risk associated with CRS development resulting from the woman's primary infection during the first 12 weeks of the gestation period [7]. Therefore, some scientists concluded that inadvertent vaccination during pregnancy should not entail an indication for its abortion [1, 7–9].

However, strains for live rubella vaccines intended for parenteral administration to people should be thoroughly studied in terms of any risk of teratogenic effect [10]. Undoubtedly, the teratogenic potential of new immune-biological medicinal products (IBMP) can be studied only through experiments on the adequate laboratory model [11].

Laboratory primates are the best model for studying the rubella virus. The virus is pathogenic for rhesus monkeys (*Macaca mulatta; Macaca rhesus*), which are sensitive to intranasal, intramuscular, and intravenous infection. The pathogenesis of the disease is similar to the pathogenesis observed in people in terms of such parameters as the duration of the incubation period and virus shedding, the presence of viremia, the dynamics of generation of virus-specific antibodies [12]. The experimentally proven development of viremia in rhesus monkeys infected by the rubella virus strain [13] implies that the pathogen can pass through the placenta, which, in its turn, means that the model can be used in for monitoring of any potential teratogenic effect of vaccine strains.

The Orlov-V vaccine strain [14] was restored at the Saint Petersburg Pasteur Research Institute of Epidemiology and Microbiology from the initial Orlov strain, which had been kept for many years at –20 °C; the restoration required additional passages in primary rabbit kidney cell culture (PRK), a tissue substrate used for attenuation of the original virus. Such processes may affect the integrity of the main biological properties of the viral particles comprising the heterogeneous population of the original pool of the virus-containing culture liquid (VCL). Therefore, thorough safety monitoring during the application, including the control of the teratogenic properties loss inherent in wild-type strains, is seen as an essential stage of preclinical studies of vaccine strains for a live attenuated vaccine against rubella. Such experiments should include a reference preparation – a single-target vaccine, preferably a commercial type [10].

The study was aimed to explore teratogenic properties of the Orlov-V vaccine strain of the rubella virus through an experiment on rhesus monkeys.

Materials and methods

The vaccine strains used in the study were represented by Orlov-V rubella virus vaccine strains (39 passages in PRK cell culture) and Wistar RA27/3 strains (in the live attenuated vaccine manufactured by Microgen, Moscow, Russia). The laboratory animals were represented by 13 female rhesus monkeys, 4–7 years of age, during early pregnancy (the first third – the first half). The female monkeys were divided into 3 groups: 9 females were immunized with the Orlov-V strain preparation; 3 females were immunized with the reference preparation (the Wistar RA27/3 vaccine strain); 1 female was given a placebo (the solvent for rubella vaccine).

The animals were handled in strict compliance with the Rules for Keeping and Maintenance of Nonhuman Primates (GOST 33216-2014).

The females seronegative for rubella were immunized by a single intramuscular injection of 0.5 ml of the preparation containing the studied strains. The vaccine dose corresponded to 1 vaccine dose for people (not less than 1000 TCD_{50}).

Potential teratogenic properties of the vaccine strains of the rubella virus were studied by using a combination of clinical, immunological, and pathomorphological methods.

The clinical observation of the infants was conducted daily. The observation results were registered twice: immediately after birth and 90 days after birth. The monkey infants were examined for motor activity (the range and strength of movements in limbs, the ability to resist removal from the cage and forced unbending/bending of limbs); muscular activity: (clinging to and holding on the mother on their own); the sucking reflex. The degree of skeletal development was checked one time through X-ray examination. The weight of the infants was regularly checked during the observation period. 90 days after birth, the infants were examined and assessed for the following functions: motor functions (the coordination of movements was assessed by the ability of the animals to make coordinated movements by using their limbs when grabbing an item). The muscle tone was checked by palpating limb muscles in the relaxed state and during bending/unbending. The infants were also checked for any signs of tremor, hyperkinesia; vision and hearing acuity; eating patterns and appetite, bowel movement, and weight gain dynamics.

The detection of antibody titers in blood serum of the immunized pregnant monkeys was conducted in the HAI assay in 28–30 days after the immunization by using the dry antigenic rubella diagnosticum for HAI assay, manufactured by the Saint Petersburg Pasteur Research Institute of Epidemiology and Microbiology following the user manual for the reagent.

IgM immunoglobulins were detected in blood serum of the first-month-of-life infants born to females immunized during pregnancy. IgM antibodies were detected by using the Vector-Rubella-IgM-strip ELISA test kit (Novosibirsk, Russia) in accordance with the user manual. All the serum samples were codified and were examined concurrently.

An autopsy examination, together with a morphological and histological study of internal organs, was conducted on 2 newborns who died in birth. The examination of the monkeys' tissues and organs was performed at the Pathomorphological Laboratory at the Research Institute of Medical Primatology. The histological study (with hematoxylin and eosin as well as Nissl staining methods) was conducted on the brain, lungs, liver, kidneys, pancreas gland, and spleen of the newborns as well as on the placenta of the females.

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The PCR test and electrophoresis for detection of amplification products were used to detect any presence of the rubella virus RNA in the sample tissues from the newborns that died in birth, in the placenta and umbilical cord blood. RNA was extracted from the examined material with the help of the RIBO-sorb reagent kit (InterLabService LLC, Moscow, Russia); the reverse transcription reaction was conducted by using the REVERTA-L kit (InterLabService LLC, Moscow, Russia); the amplification of complementary DNA (cDNA) was performed by using the AmpliSens Rubella virus-EPh reagent kit (Central Research Institute of Epidemiology, Rospotrebnadzor, Moscow, Russia). All the reaction steps were completed in compliance with the standard procedure and in accordance with the user manual. The amplification was performed in the programmable TP4-PCR-01-Tertsik thermostat (DNA-Technology LLC, Protvino, Russia).

Results

The pregnancy of 11 out of 13 females ended in the delivery of full-term babies; in 2 cases (1 – a female immunized with the Orlov-V strain-based preparation; 1 – a female immunized with the Wistar RA27/3 strain-based preparation), the pregnancy ended in stillbirth caused by a birth injury.

During 90 days after they were born, the infants were daily examined by the veterinary physician who checked them for presence or absence of any developmental delay, clinical symptoms of thew central nervous system, respiratory, hearing, and vision disorders. No live-born and breastfed infant presented any developmental defects or anomalies. The infants were actively developing; their behavior, weight, hearing, vision, and bone structure were within the normal range. The X-ray examination of the skeleton detected rickets-like changes in 2 of them (one from the Orlov-V group, (ID 40066, and one from the placebo group, ID 39978); however, these changes were not virus-specific and could be explained by the food received by the animals.

Antibody titers in HI test in serum of monkeys immunized and not immunized against rubella

Monkey No.	Strain used for immunization	Antibody titers in HI test
34086	«Orlov-V»	1 : 320
33605	«Orlov-V»	1 : 160
37085	«Orlov-V»	1 : 640
37174	«Orlov-V»	1 : 1280
37686	«Orlov-V»	1 : 320
32771	«Orlov-V»	1 : 160
37721	«Orlov-V»	1 : 640
33276	«Orlov-V»	1 : 1280
37988	«Orlov-V»	1 : 640
31939	Wistar RA27/3	1 : 640
37301	Wistar RA27/3	1 : 320
33992	Wistar RA27/3	1 : 640
36518	Placebo	< 1 : 10

To identify a possible relationship between the death of 2 newborns and the vaccination of their mothers during pregnancy, their organs and tissues as well as the females' placentas, were examined pathomorphologically and virologically. No virus-specific damage of organs and tissues, in dead-born infants was found. The histologic pattern of the brain and peripheral organs corresponded to the normal range. Therefore, in both cases, the death was not caused by the maternal immunization against rubella during pregnancy.

For comparison purposes, the virology study included the autopsy material of the infant who did not participate in the experiment and who died of alimentary dystrophy caused by the absence of breastfeeding (the mother's agalactia) (ID 32771).

No rubella virus RNA was detected in any of the examined samples (**Figure**), which implies that vaccine strains of the virus did not penetrate the placenta and, therefore, there was no intrauterine infection of the fetus in each of the studied deaths of the infants.

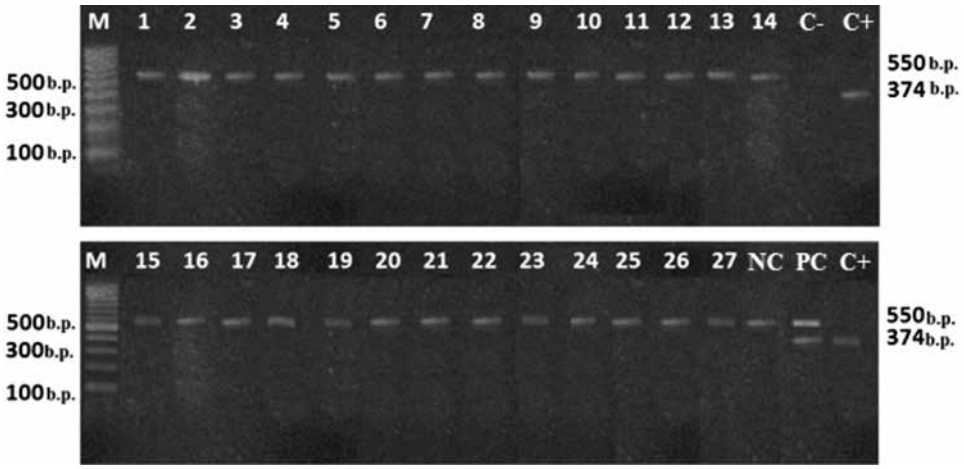
The examination of the blood serum of the females that were given a single dose of the rubella vaccine during pregnancy showed 100% seroconversion, meaning that all the experimental animals responded the injection of vaccine preparations by specific high-titer antibodies. In the meantime, upon the completion of the observation, no specific antibodies were detected in the serum of the monkey from the placebo group (**Table**).

Miller et al. demonstrated that the frequency of detection of IgM antibodies in blood serum of children with intrauterine infection of rubella etiology reached maximum rates during the first month of life when it was 100% [15]. Based on their data, the infants' sera were examined for the presence of an IgM fraction of anti-rubella antibodies by collecting blood samples during the first 30 days after the infants were born. Blood sera from 2 people were examined as positive and negative control samples (in addition to the control samples incorporated in the test system). One of them was obtained from a patient with the laboratory-confirmed rubella; the other sample was obtained from a patient who was tested negative for IgM antibodies to the rubella virus. In none of the cases, the blood serum from the infants born from the mothers immunized during pregnancy was detected as having anti-rubella IgM.

Discussion

Considering the fact that the rubella virus can pass through the placenta and cause multiple systemic defects of the fetus, monitoring of the loss of teratogenic properties in vaccine strains for live rubella vaccines should be seen as an essential and important stage in preclinical studies of such preparations. The Orlov-V vaccine strain of the rubella virus was selected as a model in this study. It was obtained by attenuation of a wild-type virus through serial passages in the rabbit kidney cell culture. The Wistar RA27/3 strain, which is present in all currently available commercial vaccines against rubella, was used as a reference preparation.

The experiment provided data on the clinical observation of infants born to the rhesus monkeys vaccinated during pregnancy. All of the 11 infants were examined by the veter-



Detection of rubella virus RNA in clinical material by PCR with electrophoretic detection of amplicons in 2% agarose gel.

Note. M – DNA size marker; 1–27 – clinical samples; K− – negative PCR control of cDNA amplification; K+ – positive PCR control of cDNA amplification; ОК – negative RNA extraction control; ПК – positive RNA extraction control, пн – nucleotide pairs.

inary physician and found clinically healthy; the first month of the daily clinical observation did not demonstrate any delay in the development of conditioned reflexes in any of the infants. The regular weight check did not reveal any delay in their physical development. In the next 3 months of observation, no pathological conditions were found in any of the experimental animals.

Taking into account that increased permeability of bone tissues is one of the most frequent symptoms of congenital rubella (CR) [16], the X-ray examination was conducted to check the infants for any deviations in the skeletal development from the physiological age-specific normal range. No bone lesions typical of CR were found in any of the infants. The rickets-like changes that were found in 2 animals (1 was immunized with the Orlov-V strain and 1 was immunized with the solvent of a vaccine against rubella) in growth areas of the most quickly growing bones were diagnosed by the radiologist as non-specific.

The virology examination of the placenta tissues (with the PCR test) was conducted in 3 cases to check the tissues for the presence of rubella virus RNA. The viral RNA was not detected in any of the examined placenta samples (2 were obtained from the Orlov-V group and 1 – from the Wistar RA27/3 group) and in any sample of umbilical cord blood (1 sample from the Orlov-V group).

The thorough and comprehensive pathomorphological examination of tissues and organs of the infants, who died in birth (1 – from the Orlov-V group; 1 – from the Wistar RA27/3 group), also did not detect any virus-specific defects of organs and tissues.

At the final stage of the experiment, the females' blood sera were examined in the HAI assay for the presence of anti-rubella antibodies; the infants' sera were examined for the presence of an IgM fraction of antibodies.

The results of the HAI assay demonstrate the development of the pronounced immune response to the vaccination with preparations containing Orlov-V and Wistar RA27/3 strains, and prove the fact of the animals' immunization.

Unlike anti-virus IgG antibodies, which are transferred from the mother to the fetus and provide the fetus with passive anti-virus immunity during the first months of life, IgM immunoglobulins do not pass through the placenta [17]. Their presence in the infants' blood is indicative of developing an intrauterine infection. This phenomenon was convincingly proved by Miller et al. in cohort studies involving children born with intrauterine infections (IUI) of rubella etiology [15]. Therefore, according to the WHO classification, congenital rubella can be diagnosed only when IgM rubella antibodies are detected in an infant's blood, even though clinical symptoms may be not displayed [18]. Thus, if such immunoglobulins were detected in the blood of the monkey infants born to the mothers immunized against rubella, they would be seen as unquestionable evidence of retained teratogenic properties of the vaccine virus. On the other hand, their absence proves convincingly that the strains used in this study do not cause intrauterine infection of the fetus. Most importantly, the results were obtained without scarification of infants, following the principles of humane treatment of laboratory animals.

It should be noted that the PCR used for detection of the rubella virus RNA in the autopsy material, placenta, and umbilical cord blood can be seen as a reliable marker for assessment of potential teratogenic effects [19] of the tested preparations during their preclinical studies.

Conclusion

During the study of any potential teratogenic effect of the rubella virus vaccine strains by using experimental rhesus monkeys, no evidence that would be indicative of an intrauterine infection of the fetus in the females immunized during pregnancy was discovered. Thus, the study results demonstrated that teratogenic properties were absent both in the Orlov-V vaccine strain of the rubella virus and in the Wistar RA27/3 strain used as the reference preparation and commonly used for commercial vaccines against rubella. The study demonstrated the informative value of PCR as the test proving the specific safety of vaccine strains for live virus vaccines during their preclinical studies.

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