Original Article

Evaluation of Cell Mediated Immunity following Rubella Vaccination Using Lymphocyte Proliferation

Jafari E1, Mohammadi A2, Arabzadeh SAM1, Esna-Ashari F2*, Sadigh ZA2, Shokri GhR3, Shahbazi R2, Foroughi A2

1. Microbiology&Virology Department, Kerman University of Medical Sciences, Kerman, Iran.
2. Human viral vaccines Department, Razi Vaccine and Serum Research Institute, Karaj, Iran.

Abstract

Background and Aims: Rubella is predominantly a childhood disease that is endemic throughout the world and when rubella outbreaks occur, they are accompanied by birth defects following congenital rubella syndrome. Immunity to rubella virus as a teratogenic agent has an important role for prevention of these serious congenital defects. Lymphocyte proliferation assay is a way for investigation of human cell-immunity and its ability against rubella infection.

Materials and Methods: The blood samples were obtained in sodium heparin tubes. Ficoll was added to separate lymphocytes. The cells were cultured with RPMI 1640 medium with 15% calf serum in microplates and incubating at 37°C in 3-5% CO2. Mitogens including Phytohemagglutinin and rubella hemagglutinin antigen (derived Takahashi strain) were added, separately. Then a fluorescent nucleotide was added. On day 10th-11th the wells stained and observed.

Results: Lymphocytes stimulated with the mitogens were observed directly with an inverted microscope. Their aggregation and growth were detected after two days. Also lymph proliferation was shown using labeled nucleotide comprising a new fluorophore, by fluorescent microscopy. Response to full particle of attenuated virus was better than antigens derived from different parts of the virus.

Conclusion: Comparison of the data with previous studies on proliferation of specific lymphocytes in response to rubella vaccination confirms our results. Thus cell-immunity to rubella infection was activated timely, in individuals who were vaccinated against rubella virus approximately 10 years before or exposed to it, but the intensity of responses to different antigens varied in each subject.

Keywords: Rubella; Vaccination; Cell-Mediated Immunity; Fluorescence Microscopy

Introduction

Rubella virus (RV) is an enveloped positive-single stranded RNA virus of the genus Rubivirus in Togaviridae family that is transmitted by aerosol via the respiratory tract. The RV virion contains a RNA genome enclosed within an icosahedral capsid composed of multiple copies of a basic protein, C and surrounded with a lipid bilayer in which viral glycoproteins E1 and E2 are inserted (1).

Rubella is predominantly a childhood disease that is endemic throughout the world. Primary replication occurs in the nasopharynx. The incubation period of rubella is 14 to 21 days, with most patients developing a rash 14 to 17 days after exposure. During the first week after

*Corresponding author: Esna-Ashari F, Ms.C. Human viral vaccines Department, Razi Vaccine and Serum Research Institute, Karaj, Iran. Email: fesna2002@yahoo.com
exposure, there are no symptoms but in the second week, lymphadenopathy may be noted. Later in the second week, virus appears in the blood and there may be a mild self-limiting fever, malaise and conjunctivitis (1, 2). When rubella outbreaks happen, in consequence of maternal infection during the first trimester of pregnancy, congenital Rubella syndrome (CRS) occurs. This disease accompanied by birth defects such as cataracts, cardiac abnormalities and mental retardation (3, 4).

Immunity to rubella virus as a teratogenic agent has an important role to prevent these serious congenital defects and is conventionally determined by measuring specific immunoglobulin G (IgG). To design better vaccination strategies, it is essential to define the critical immunological mechanisms for effective immunity to rubella vaccine. Virus specific T cells play a prominent role in viral immunity particularly in the elimination of infected cells even in the absence of antiviral antibodies. In other words, investigation of rubella cell immunity is a confident way for following up active protection in time past (2, 5).

In the current study, we examined humoral and cellular immune responses to rubella vaccination by rubella-specific lymphoproliferation and measuring antibody levels.

Methods

Blood donors
Blood was obtained, with informed consent from 12 healthy individuals that some of them were vaccinated from one to ten years ago and the others were exposed to rubella infection (without determined history of vaccination).

Isolation of peripheral blood mononuclear cells (PBMC)
Blood sample (6 ml) was collected from each person, in sterile plastic tubes containing sodium heparin(Golden Vac). It was mixed well and diluted with sterile RPMI 1640 medium (Gibco) without serum 1:1. Then the diluted blood was carefully poured onto a 4 ml Ficoll solution (Kronberg/Taunus) and centrifuged at 2500rpm for 30 minute for separating buffy coat layer and then washed twice in sterile RPMI 1640 medium in 2000rpm for 5 min to separate lymphocytes from platelets. The viability of isolated cells was determined by the trypan blue exclusion test (6). The remaining of blood (2ml) was incubated at 4°C overnight and centrifuged for serum separation. Each serum sample was separately stored in deep freeze (20°C).

Mitogens
Phytohemagglutinin (PHA, Baharafshan) at concentration of 100 μg / ml, rubella hemagglutinin antigen (1:128), In-house standard (IhS) including live particle (Takahashi strain: 10^3 CCID50/ml) [7] were used.

Lymphocyte proliferative assay
The lymphocytes (100μL/well) were cultured in (100 μl/well) RPMI 1640 supplemented with 2% bicarbonate, 15% irradiated calf serum (Razi Institute), 0.3% KN (kanamycin-neomycin) and 1% Tricin at a concentration of 2 X 10^5/ml on a 96-well flat-bottom microplate in duplicate at 37°C in 3-5% CO_2_. At each plate, medium without cells was added to 4 wells as a negative control. After 3 days mitogen and rubella antigens were added, separately (50μl/well) in each micro plate at quadruplicate. After 24-48hr incubation fluorescent nucleotide (Alexa Fluor Dyes, Invitrogen) was added to positive wells from each microplate.

Fluorescent staining
On day 10th -11th following the stimulation, the cultures were halted after 1hrs incubation with colchicine (0.2μg/ml, Baharafshan) at room temperature (RT), KCL (0.075M) was added for 15min. Then the wells were fixed with ethanol-acetate (3:1). After air drying, they were stained with Hoechst 33258 using a modification of the S.A. Latt method (8). The slides were screened after 60-minute incubation (with the dye H33258) in dark condition at RT then examined fluorescence microscopy.

Serological test
Hemagglutination Inhibition (HI) and ELISA test were done on the serum samples. The EUROIMMUN Anti-Rubella Virus ELISA
(IgG) was used to measure antibody titer against rubella virus (9, 10).

Results

Proliferative activity of lymphocytes in vitro

Lymphocytes were observed directly with inverted microscopy. The conventional fluorescence microscopy was used to analyze the in vitro proliferation of lymphocytes labeled with an intracellular fluorophore. Lymphocytes from all donors showed a significant increase of cell proliferation with regard to mitogenic stimulation by PHA (Fig. 1-b) and antigenic stimulation by Rub-IhS (Fig. 1-c) and Ag HA (Fig. 1-d) at day 5-6 upon stimulation and the greatest observed stimulation ratio belonged to PHA and RubIhS. However the responses between individuals were different, the degree of responses had direct correlation with fluorescent intensity (Fig. 2, Table 1).

Antibody response

The survey on humoral immunity of two group showed in individuals who were exposed to rubella virus or were vaccinated 1 year earlier, antibody titers remained seropositive (1:32), but in individuals who were vaccinated 10 years before, the rubella virus antibodies declined (HI titer lower than 1:16) (Table 2).

Discussion

To design better vaccination strategies, it is essential to define the critical immunological mechanisms for effective immunity to measles, mumps, and rubella vaccines. Recent data demonstrate the importance of cell mediated immunity (CMI) in controlling and protecting against viral diseases (11, 12). For example, measles virus specific CD8+ cytotoxic T cells

Fig. 1. Lymphocyte proliferation within microplate. (a) Negative control, (b) Positive control (PHA), (c) RubIhS and (d) Ag HA.
Table 1. Proliferative activity of lymphocytes exposed to different mitogens.

<table>
<thead>
<tr>
<th>Samples</th>
<th>PHA Pw</th>
<th>PHA FI</th>
<th>IhS Pw</th>
<th>IhS FI</th>
<th>HA Ag Pw</th>
<th>HA Ag FI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8/8</td>
<td>++++</td>
<td>7/8</td>
<td>+++</td>
<td>6/8</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>7/8</td>
<td>++++</td>
<td>6/8</td>
<td>+++</td>
<td>7/8</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>8/8</td>
<td>++++</td>
<td>7/8</td>
<td>+++</td>
<td>7/8</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>8/8</td>
<td>++++</td>
<td>7/8</td>
<td>+++</td>
<td>6/8</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>7/8</td>
<td>++++</td>
<td>7/8</td>
<td>+++</td>
<td>5/8</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>6/8</td>
<td>++++</td>
<td>4/8</td>
<td>++</td>
<td>3/8</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>7/8</td>
<td>++++</td>
<td>6/8</td>
<td>+++</td>
<td>6/8</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>6/8</td>
<td>++++</td>
<td>4/8</td>
<td>++</td>
<td>2/8</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>8/8</td>
<td>++++</td>
<td>7/8</td>
<td>+++</td>
<td>7/8</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>8/8</td>
<td>++++</td>
<td>7/8</td>
<td>+++</td>
<td>7/8</td>
<td>++</td>
</tr>
<tr>
<td>11</td>
<td>8/8</td>
<td>++++</td>
<td>7/8</td>
<td>+++</td>
<td>7/8</td>
<td>++</td>
</tr>
<tr>
<td>12</td>
<td>7/8</td>
<td>++++</td>
<td>6/8</td>
<td>+++</td>
<td>5/8</td>
<td>++</td>
</tr>
<tr>
<td><strong>Total %</strong></td>
<td>7.3/8</td>
<td>6.2/8</td>
<td>5.6/8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pw: Positive Wells, FI: Fluorescent Intensity
+: 10-25%, ++: 25-50%, +++: 50-75%, ++++: 75-100%. Samples 1-3 were exposed to Rubella infection, samples 4-8 and 9-12 were vaccinated 1 year and 10 years prior to this experiment, respectively. For each sample, eight wells were stimulated with different mitogens and labeled nucleotide were added to positive wells which showed cell growth, to determine proliferative responses.

Table 2. Qualitative test results obtained from two serological assays HI & ELISA

<table>
<thead>
<tr>
<th>Samples</th>
<th>Anti-Rubella (IgG)</th>
<th>HI Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EUROIMMUN</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>40(IU/ml)</td>
<td>1:32</td>
</tr>
<tr>
<td>2</td>
<td>43(IU/ml)</td>
<td>1:32</td>
</tr>
<tr>
<td>3</td>
<td>39(IU/ml)</td>
<td>1:32</td>
</tr>
<tr>
<td>4</td>
<td>51(IU/ml)</td>
<td>1:32</td>
</tr>
<tr>
<td>5</td>
<td>50(IU/ml)</td>
<td>1:32</td>
</tr>
<tr>
<td>6</td>
<td>87(IU/ml)</td>
<td>1:32</td>
</tr>
<tr>
<td>7</td>
<td>33(IU/ml)</td>
<td>1:16</td>
</tr>
<tr>
<td>8</td>
<td>13(IU/ml)</td>
<td>1:8</td>
</tr>
<tr>
<td>9</td>
<td>27(IU/ml)</td>
<td>1:16</td>
</tr>
<tr>
<td>10</td>
<td>16(IU/ml)</td>
<td>1:8</td>
</tr>
<tr>
<td>11</td>
<td>11(IU/ml)</td>
<td>1:8</td>
</tr>
<tr>
<td>12</td>
<td>10(IU/ml)</td>
<td>1:4</td>
</tr>
</tbody>
</table>

In ELISA: Negative range = 8 (IU/ml), Cut-off =10 (IU/ml), Positive range ≥11 (IU/ml)

Immune antibody titer of HI test for Rubella is considered to be ≥ 1: 8. Cut off: the upper limit of the reference range of non-infected persons.

proliferate and activate in the peripheral blood with measles rash onset, suggesting a central role in viral clearance (13, 14). Further, individuals who are infected with human immunodeficiency virus (HIV) and those with impaired cell-mediated immunity have a higher morbidity and mortality rate with measles co-infection compared to those who are immunologically intact (15, 16), demonstrating the importance of CMI in measles virus elimination. Similarly, Lovett and colleagues (17) and Hyöty and colleagues (18)
demonstrated that induction of specific cellular immunity in response to rubella and mumps is necessary for recovery from disease and long-term protection.

The lymphocyte proliferation assay (LP) and the chromium-release assay have been widely used in the past for investigation of CMI. Since estimation of CMI not only is critical for effective immunity and long term protection against viral infections but also is the best for evaluation of vaccine efficacy and post marketing surveillance, we evaluated a surrogate measure of CMI, lymphoproliferative response, across the Rubella components of MMR-Razi through 12 blood sample in appropriate vaccinated individuals or exposed to the particle. Further an evaluation of the correlation within CMI responses and ELISA to this component of MMR-RAZI was done 1&10 years after vaccination.

In vitro lymphocyte proliferation assays can be used to analyze immune reactions and to reach a better understanding of cell-cell interactions during the course of immunological events. In general, radioactive tracers such as $[3\text{H}]$ thymidine are added to lymphocyte preparations and incorporated into newly synthesized DNA during cell division and proliferation (19). The radioactive uptake can be measured using a beta counter and correlates with the amount of cell proliferation. However working with radioactive substances and expensive counting equipment in the method, has motivated a quest for easier alternatives.

In this study two different measurement systems for analyzing immunity against rubella vaccine Takahashi strain have been done and compared. For evaluating CMI, Fluor chrome based method using Alexa flour dye was installed in human viral vaccine department of RAZI institute. The results of the various experiments were compared and analyzed in order to investigate whether non-radioactive Fluor chrome assays could be substituted as the previous radioactive $[3\text{H}]$ thymidine labeling method or not. In parallel HI and ELISA was done on same samples for judgment of rubella humoral immunity.

This study showed that a proliferation of lymphocyte in response to rubella antigen appears both in 1&10 year after vaccination (Table 1, Fig. 2). This result reveal that in group study after PHA which is a nonspecific stimulator, highest observed stimulation ratio in all of the samples belonged to RubIhS. It’s confirmed the presence of rubella CMI in all of cases against TAKAHASHI strain of RAZI vaccine, although the serologic evidence revealed that there is seropositivity in $>91.6\%$ of samples (Table 2). It means 8.3% of sample showed sero negativity meanwhile was CMI positive.
All cases that vaccinated last year were still seropositive (100%). The other vaccinated people to 85% were still seropositive, after 10 years but their mean HI titer had dropped to 1:8 (Table 2). In a sensitive population, rubella outbreaks may be explosive. But a history of having been exposed to a rubella outbreak does not necessarily indicate immunity to rubella as showed in. In the other hand there are immune persons (CMI positive) that in humoral assay showed negative results (Table 2, Fig 3).

Approximately 5% of RV-vaccinated people do not seroconvert and are therefore regarded as non-responders (2). However, with regard to vaccination against other viral diseases it is reported that persistent sero negative people develop CMI and can therefore be classified as immune (2). There are only few reports about CMI in RV. Toyoda and colleagues (20) found a generally good correlation between antibody levels and the expression of interleukin-2 receptor alpha on T lymphocytes cultured with RV antigen. However, in several individuals the correlation was poor. Ovsyannikova and co-workers (21, 22) showed that it is genetically determined whether a vaccinated person develops humoral immunity or CMI to RV. A correlation between the proliferation index of antigen-stimulated lymphocytes and antibody response to RV was not detected (2).

In rubella virus diagnostics, no routine test for the assessment of CMI exists. Conventionally the correlate of immunity to RV is a hemagglutination inhibition titer ≥1:32 or an antibody level ≥15 IU/ml (2). In future, we aim to determine if a correlative and predictive intra class relationship exists between CMI responses to individual virus, the measles, mumps, and rubella components of the MMR – RAZI vaccine by lymphoproliferation assay in compare of a relatively new method, the interferon-gamma (IFNγ)-ELISpot (23).

Acknowledgements

The authors would like to thank Razi Vaccine and Serum Research Institute for providing facilities and financial support and the staff of Human viral vaccines department for their help in progression of this research.

References


