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Antigen-Specific Lymphocyte Proliferation Assay and Virus Neutralization Test for Measurement of Measles-Specific Immunity in 15-19 Years Old High School Students in Tehran, Iran

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Abstract: Limited information is available concerning the role of measles-specific cell mediated immunity as a correlate of long-term protection from measles infection. Although serological responses are determined in epidemiological studies and high antibody titer is a good indicator of protection, the role of Cell-Mediated Immunity (CMI) has to be defined more clearly. In this study, Lymphocyte Proliferation (LP) and Viral Neutralization Test (VNT) were used in order to measure measles-specific cellular and humoral immune responses of 100 high school students in Tehran. From total number of subjects studied, 33 were girls and 67 were boys and all were in good health. Of these, 77 had protective neutralizing measles antibody titers and 23 did not have such titer. The results of LP showed that 89 subjects had protective cellular immune responses and 11 did not. A quantitative relationship between humoral and cellular immune responses was not observed. These findings suggest that measles-specific protective CMI is measurable for longer time in comparison to humoral immunity. These data suggest that LP responses may be better sustained than antibody titers in some children.

Key words: Measles, lymphocyte proliferation, viral neutralization, high school students

INTRODUCTION

Measles is a highly contagious viral disease that remains the leading vaccine-preventable cause of child mortality worldwide (Moss *et al*., 2004) and is considered as a major health problem world wide with nearly 45 million new cases and one million deaths occurring each year (Nossal, 2000). Frequent incidence of measles disease in Europe, North America and some other countries including Iran has been reported (Khodabandeh Loo, 2003; Ohsaki *et al*., 2000; Papania *et al*., 2000). In the context of increased efforts by World Health Organization (WHO, 2005) and other agencies to achieve a reduction in mortality due to measles there is an on-going need for reliable laboratory assays to assess measles immunity (Cohen *et al*., 2006). In addition, to design better vaccination strategies, it is essential to define the critical immunological mechanisms necessary for effective immunity to measles vaccines (Dhiman *et al*., 2005). The immunological determinants, which lead to permanent immunity against measles virus, have been recently evaluated. Previous studies have shown that acute measles infection leads to a life-long immunity in the host. In contrast, it is now clear that the immunization with attenuated measles vaccine does not induce life-long immunity in every individual (Klinge *et al*., 2000; Tischer and Gerike, 2000). Previous data demonstrated that cellular immunity is necessary for recovering from measles and might be sufficient for long-term immunity, although neutralizing anti-measles antibodies can be protective in high titers (Ohsaki *et al*., 2000).

In this study, however, Lymphocyte Proliferation (LP) and Virus Neutralization Test (VNT) have been used to evaluate the specific immune responses of the subjects to measles virus. The results can be used to evaluate the correlates of long-term immunity against measles virus and to provide a background for extensive epidemiological studies to survey the performance of the measles eradication programs.

MATERIALS AND METHODS

Subjects: One hundred young adults (33 girls and 67 boys) ages 15-19 high school students from Tehran were randomly enrolled in this study. This research was performed with the permission of the Education Department of Tehran and informed consent was obtained at the time of blood collection.

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Sample handling: All samples were kept at room temperature and processed within 8 h of collection. Uncoagulated blood was centrifuged (300 g for 10 min) and the serum was removed, aliquoted and frozen at -70°C till the performance of VNT. Heparinized blood was resuspended in twice the original volume of D-PBS (Sigma) and Peripheral Blood Mononuclear Cells (PBMCs) were isolated using Ficoll-Paque (Pharmacia, NJ). Lymphocyte proliferation was performed on fresh PBMCs.

Measles virus: In this study, we utilized the attenuated measles virus strain AIK-C (RAZI Vaccine and Serum Production Institute, Karaj, Iran). The virus titer was increased by repeated subculture on Vero cells. After the observation of CPE, the supernatant was used as antigen by repeated freeze-thaw of the culture flasks followed by centrifugation at 400 g for 10 min. For VNT, the supernatant containing $10^{4.25}$ p mL^{-1} measles virus was used. For LP test, the supernatant was centrifuged one more time at 29,000 g for 2 h at 4°C. The resulting viral precipitate was repeatedly freeze-thawed, added to culture medium, aliquoted and stored at -70°C till use. This antigen was heat inactivated at 56°C before use in LP assay. The optimal concentration of antigen used in LP test was 9.4 μg p mL^{-1}.

Viral neutralization test: The VNT was carried out as previously described (Roodbari *et al*., 2003). Vero cells growing as monolayers in 96-well tissue culture plates (Falcon) were inoculated with measles virus-serum mixtures containing increasing dilutions of each heat-inactivated serum sample (50 μL of ½ to 1/128 dilutions) and a constant amount of virus (50 μL of virus at 100 $TCID_{50}$). Before inoculation, the virus-serum mixture was incubated for 1 h at 37°C. Fifty micrometer of each virus-serum mixture was added to wells of microplates and after an adsorption period of 1 h at 37°C, 200 μL of EMEM (Sigma) was added. Each serum dilution was examined in duplicate. The tests were read when in virus control wells more than 75% of cells showed cytopathic effect, which usually took 4-5 days.

Lymphoproliferative assay: The lymphoproliferative assay was performed as previously described (Sabahi *et al*., 1995). PBMCs were separated from whole blood by Ficoll-Hypaque gradient density centrifugation and washed twice with RPMI medium containing L-glutamine (Sigma). The cells were suspended at final concentration of 10^6 p mL^{-1} in RPMI supplemented with 20% heat-inactivated autologous serum. One hundred microliter of this cell suspension (10^5) was added in triplicate to sterile Falcon flat-bottom microtest plate wells. Predetermined concentration of measles (9.4 μg p mL^{-1}) was added to appropriate chambers. PHA-M (Sigma), at optimal concentration of 3 μg p mL^{-1} was used. The microtiter plates were incubated in a 37°C humidified incubator containing 5% CO_2 for 3 days with PHA and for 4 days with measles antigen and then pulsed with 1 μCi of titrated thymidine (Amersham, England) in 50 μL volume. The plates were harvested 18 h later in a microharvester and counted in a scintillation counter. Final results were reported as stimulation index (SI).

Statistical analysis: Mc Nemar test ($p = 0.002$) and student t-test were used for comparisons between tests.

RESULTS

Measles virus neutralization test: The VNT was performed on 100 sera. In most studies, subjects with 1:8 or higher dilutions of the serum capable of CPE prevention are considered as immune (Neuman *et al*., 1985). In this study, 23 of the subjects had lower antibody titers and are therefore regarded as susceptible.

Measles specific lymphoproliferative test: Lymphoproliferation to measles antigens was readily detected in the high school students. Stimulation indices (SI≥3) were observed in 89/100 (89%) of the children and 11/100 (11%) had lower SI and were considered as susceptible.

The correlation of VNT with LP: To evaluate the two tests, a two-dimensional table was depicted (Table 1). As is shown in the Table 1, LP showed 89 immune and 11 susceptible subjects, while in neutralization test only 72 were immune and 23 were susceptible. From the 23 subjects reported to be sensitive by neutralization test, 12 (7.52%) were immune by LP. Table 1 also demonstrates that 100% of those immune based on the antibody criterion were also positive based on SI. Finally two criterion of SI and antibody titer were compared in the two sex groups. The descriptive results showed that the mean SI is 4.1 in males and 3.1 in females, which was shown to

Table 1: Comparison of humoral (VNT) and Cellular (Lymphoproliferative) responses in 100 children tested

	Stimulation prediction		
Ab prediction	Susceptible	Immune	Total
Susceptible	11	12	23
Immune	0	77	77
Total	11	89	100

VNT values greater than 1:8 have been shown protected against measles virus infection. SI≥3 are considered to be significant

be significant by student t-test ($\alpha = 0.05$) ($p<0.001$). However, there was no difference between the two groups regarding the antibody test.

DISCUSSION

The evaluation of human immune response to viral infections and vaccination has been focused on measurement of serum antibodies (Moss *et al*., 2004). High antibody titers are protective against a wide range of viral infections. Enteroviruses are among the viruses against which the antibody alone seems to be sufficient in protection of the body against the infection and recovery from the disease (Lopez *et al*., 2000). Serological tests are useful in the study of measles epidemiology and high antibody titers in circulation are a good index for protection against measles. However, in evaluation of the immunity of individuals to measles, the humoral response may not be completely responsive (Roberts and Tobias, 2000).

Recent data demonstrate the importance of cell mediated immunity (CMI) in controlling and protecting against viral diseases (Wong-Chew *et al*., 2003; Pukhalsky *et al*., 2003). For example, measles virus specific CD8+ cytotoxic T cells proliferate and activate in the peripheral blood with measles rash onset, suggesting a central role in viral clearance (Permar *et al*., 2003). 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 (Al-Attar and Reisman, 1995), demonstrating the importance of CMI in measles virus elimination. Similarly, several data demonstrated that induction of specific cellular immunity in response to rubella and mumps is necessary for recovery from disease and long-term protection (Dhiman *et al*., 2005).

In the current study, we enrolled 100 healthy individuals (15-19 years old) previously vaccinated with measles vaccine to characterize their cellular and humoral immunity by evaluating measles-specific antibody and measles-specific lymphoproliferation levels. Based on the criteria defined in the literature (Lopez *et al*., 2000), 89% were diagnosed to be immune with regard to cellular responses, while only 77% were considered to be immune with regard to humoral responses. Interesting to mention that from 23 subjects diagnosed to be sensitive by VNT, 12 (52%) were diagnosed to be immune by LP.

Although it was observed that the lymphoproliferative response to measles virus persists in 89% of present subjects. However, the interpretation of an immune response based on a cut-off of $SI \geq 3$ (Lopez *et al*., 2000), while commonly accepted, is somewhat arbitrary, as there is no gold standard with which we can define the sensitivity or implications of its positivity for quality or durability of vaccine-induced protective immunity.

However, the contribution of CMI to long term protection against measles following vaccination is poorly understood because studies on protection against these diseases have been based solely on antibody levels. Further, antigen specific CMI is extremely difficult to detect and measure. Present data are in agreement with a study by Lopez *et al*. (2000) which examined long-term CMI to measles post-MMR-II immunization in children. Lopez *et al*. (2000) detected significant measles antigen-specific lymphoproliferative response in ~64% of their subjects 5-13 years after MMR-II vaccination.

In addition, low and unidentifiable antibody titers is strongly related to sensitivity to various viruses, numerous reports show that antibody alone is not sufficient to recover from acute measles or to preserve long time immunity against measles infection (Lopez *et al*., 2000; Tsai *et al*., 1999). Nanan and Rauch (2000) reported that in persons with no history of measles disease, antibody titer against measles virus were unidentifiable. Interestingly, the lymphocytes of these persons strongly proliferated in response to measles virus antigen. These studies increase the likelihood that the subjects studied by us have low antibody titers but show adequate lymphocyte propagation response.

This study demonstrates that measles-specific lymphoproliferative responses can be detected in 89% of children. We do not yet know the sensitivity of CMI assay we used in this study or the implications of a positive proliferative response for either the durability or quality of vaccine-induced protection. However, the observation that measles vaccination can induce strong measles-specific lymphoproliferation without generating significant amounts of antibody in some children raises important questions about the role of CMI in the definition of vaccine success and failure and the laboratory correlates of protective immunity against measles.

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