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Development and evaluation of a competitive ELISA for estimation of rabies neutralizing antibodies after post-exposure rabies vaccination in humans

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Summary
Objectives: Currently three tests are approved for the estimation of neutralizing antibodies after rabies vaccination: the mouse neutralization test (MNT), the rapid fluorescent focus inhibition test (RFFIT), and the fluorescent antibody virus neutralization (FAVN) test. Performance of these tests requires a lot of expertise and is generally carried out in reference laboratories and, hence, they are not available to many people. The aim of the present study was to develop and evaluate a competitive ELISA (C-ELISA) for estimation of neutralizing antibodies in order to make this testing more widely available.
Methods: The C-ELISA was designed based on competition between a murine neutralizing monoclonal antibody (Mab) and the antibodies in serum of vaccinated people. The test was initially standardized using known negative and known positive serum samples for determining the optimal dilution of the Mab as well as the cut-off value (%) for ascertaining the level of inhibition. Nine hundred and ninety serum samples were tested from 250 people who had been administered purified chick embryo cell vaccine (PCECV). Serum samples were collected on days 0, 14, 30 and 90 post-vaccination, and were tested by C-ELISA.
Results: All the serum samples that were positive by RFFIT were also positive by C-ELISA. The titers obtained with C-ELISA were marginally higher than the RFFIT titers, but a significant correlation was noted between the two tests ($r = 0.897$). None of the negative controls were detected to be positive for rabies antibodies by either of these tests. Therefore the C-ELISA was found to be 100% specific and sensitive in comparison to RFFIT. Further, the initial rise and fall of antibody titers on different days post-vaccination was comparable for both tests.

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Conclusions: The C-ELISA described herein can be used to quantify rabies neutralizing antibody levels after vaccination. This test is simple and can be conveniently used under field conditions for monitoring seroconversion after post-exposure rabies vaccination. Moreover it does not require handling of infectious virus by the end user.

Introduction

Rabies is a fatal zoonotic viral infection of the central nervous system that is transmitted by the bite of an infected animal. According to a World Health Organization (WHO) estimate, 50 000 human deaths due to rabies are reported worldwide every year, the majority of them being in the developing countries of Asia and Africa.[1] Amongst these 50 000 cases, India alone accounts for 20 000.[2] More than 95% of cases of human rabies in India are due to dog bites. The disease is also re-emerging as an important public health problem in North America where several cases of human rabies have occurred due to exposure to bats.[3] Recently some cases of human rabies due to exposure to bats have also been reported from Brazil.[4]

In India more than 4 million people receive post-exposure vaccination annually. In January 2005, use of the nerve tissue vaccine was discontinued and presently the vaccines used are purified chick embryo cell rabies vaccine (PCECV), purified vero cell rabies vaccine (PVRV) and purified duck embryo vaccine (PDEV). All these vaccines protect the individual by producing neutralizing antibodies to rabies glycoprotein (G). WHO has recommended a titer of 0.5 IU/ml of serum as an accepted level of seroconversion.[5]

Monitoring antibody titers are required in certain circumstances, i.e., elderly people and the malnourished and immunocompromised, and also when clinical trials are conducted for new vaccines or new vaccination schedules. Currently three tests are approved by WHO for determining the levels of rabies neutralizing antibodies: the mouse neutralization test (MNT),[6] the rapid fluorescent focus inhibition test (RFFIT)[7] and the fluorescent antibody virus neutralization (FAVN) test.[8] These tests are time consuming, require expertise, are expensive and are generally carried out in reference laboratories and, hence, are not widely available. Therefore there is a need to develop and standardize simple techniques such as ELISA for the measurement of antibodies. ELISA tests developed earlier by Perrin et al. do not actually measure neutralizing antibodies.[9] However, it is possible to measure the levels of neutralizing antibodies by using the principle of competitive ELISA, where the antibodies in the test serum are allowed to compete with biotin/enzyme-labeled neutralizing monoclonal antibodies with a known titer. Based on this principle Sugiyama et al. have developed a competitive ELISA (C-ELISA) as a simple, rapid and inexpensive alternative to the virus neutralization test and have used this test to determine the overall immune status of rabies-vaccinated domestic dogs in Japan.[10] However, this principle has not been used widely for measuring humoral immunity in people receiving post-exposure rabies vaccination. In this study we have developed a C-ELISA for estimating the neutralizing antibody titer in vaccinated people and evaluated our results with RFFIT, which is an approved test for estimating rabies-neutralizing antibodies.

Materials and methods

Serum samples

In this assay, serial serum samples (n = 990) obtained from 250 individuals who had received a course of post-exposure vaccination with PCECV were evaluated. Their antibody titers on day 0 (n = 250), day 14 (n = 250), day 30 (n = 250) and day 90 (n = 240), were evaluated. These samples were obtained from the antirabic treatment center, Kempegowda Institute of Medical Sciences (KIMS), Bangalore. Negative controls consisted of 50 serum samples obtained from age and gender-matched people who had not received any rabies vaccine. In addition, a panel of 50 positive serum samples, previously tested positive by RFFIT, was used to validate the results of the C-ELISA.

Monoclonal antibodies (Mabs)

In a recently completed study, we produced and characterized Mabs to rabies virus (CVS 11 strain) in our laboratory by using established procedures.[11] We were able to generate 50 hybridomas to G protein of which 11 Mabs showed a high virus neutralizing titer for CVS in RFFIT. We chose one of these Mabs (2C5E8) in designing the C-ELISA. This Mab had a neutralizing titer of 1:10 000 000 and belonged to isotype of IgG, subtype of IgG2a. The Mab recognized a conformational epitope as revealed by a native polyacrylamide gel electrophoresis. This Mab was biotinylated using an established procedure described earlier.[12] The optimal dilution of the Mab to be used in the competition assay as well as the percentage inhibition to be used as a cut-off value was determined by using a panel of known high positive (n = 50), low positive (n = 50) and negative sera (n = 50).

Preparation of antigen for C-ELISA

The CVS strain of rabies virus was grown in BHK-21 cells. Cell-culture supernatants containing high titers (10^7 FFD_{50} (50% fluorescent focus forming dose)) of rabies virus were clarified by low-speed centrifugation at 500 *g* for 30 min, to remove cellular debris. After clarification and inactivation with beta-propiolactone (BPL, 1:4000) the virus was partially purified by ultracentrifugation at 70 000 *g* and 4 °C for 2 h using an ultracentrifuge (Sorvall Discovery, 100S). The pelleted virus was resuspended in phosphate-buffered saline (PBS) to a final volume of 1/100 of the original and stored at −70 °C until required.

Rapid fluorescent focus inhibition test (RFFIT)

This was performed as per the WHO advocated procedure with some modifications. Instead of tissue culture chambers