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CALCULATING RABIES VIRUS NEUTRALIZING ANTIBODIES TITRES BY FLOW CYTOMETRY

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SUMMARY

The determination of the rabies neutralizing antibody (VNA) response after immunization against rabies is an acceptable index of the efficacy of a vaccine and a successful treatment. Several tests have been developed in attempt to improve the assessment of VNA, from mice inoculation to cell-culture fluorescence inhibition tests. All of them, however, present special difficulties in terms of reading or accuracy. The present study describes a neutralization test performed in cell-culture appraised by flow cytometry (FC). Serial dilutions of the serum samples were mixed *in vitro* with rabies virus before the addition of BHK-21 cells. After 24h-incubation, cells were released by trypsin treatment, fixed and permeabilized with a p-formaldehyde solution and stained with a rabies virus nucleocapsid protein-specific antibody conjugate. The percentage of virus infection inhibition caused by specific antibodies present in the serum were evaluated in a Beckton & Dickinson FACSCalibur® flow cytometer. A correlation curve between the IU/ml content and the percentage of infective inhibition was built with a reference serum and the VNA titers of serum samples were obtained by extrapolation. Titers obtained by FC and standard test showed an effective pairing results ($p < 0.01$), with a correlation coefficient (r) = 0.7. These results permit to envisage the FC as a suitable technique to evaluate VNA in sera from immunized animals and likely in human serum samples. Nevertheless, new studies comparing FC to gold-standard techniques are required for determining the FC values of Sensibility and Specificity .

KEYWORDS: Rabies virus; Flow cytometry; Neutralizing antibodies.

INTRODUCTION

The determination of an antibody response after immunization against rabies is an accepteble index of the efficacy of vaccine and successful treatment in both animals and humans. Among the different antibodies elicited after immunization, those specific for the virus glycoprotein (neutralizing antibodies) are considered the most important to provide protection (WIKTOR *et al.*, 1973). Several *in-vitro* tests have been developed to assess neutralizing antibodies, including those based on fluorescent focus inhibition (SMITH *et al.*, 1973; ZALAN *et al.*, 1979) which are the most widely used. The Brazilian National Reference Institute for rabies is Pasteur Institute (São Paulo), which uses the simplified fluorescent inhibition test - SFIMT - (FAVORETTO *et al.*, 1993) in its serological routine. In addition, several variations of Enzyme-linked immunosorbent assay (ELISA) have also been proposed by several authors as a possible alternative to cell-culture tests (ATANASIU *et al.*, 1977; ELMGREN & WANDELER, 1996; ESTERHUYSEN *et al.*, 1995; NICHOLSON & PRESTAGE, 1982; PERRIN *et al.*, 1986; PIZA *et al.*, 1999). Flow Cytometry, a methodological analysis approach based upon refracted light, has been used for detecting antibodies to different viruses such as HCMV (McHUGH *et al.*, 1986 and 1988), HSV (SCILLIAN *et al.*, 1989) and HIV (SLIGH *et al.*, 1989). We have recently reported the use of Flow Cytometer for rabies virus detection (BORDIGNON *et al.*, 2002) and the present study describes the standardization of a Flow Cytometry technique for detection and quantification of rabies VNA. Results are compared to those obtained by SFIMT and ELISA.

MATERIALS AND METHODS

CELL CULTURE: BHK-21 cells (C-13) (ATCC CCL10) were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Sigma #D-8900), supplemented with 2 mM glutamine, 10% (v/v) fetal calf serum (Sigma #F-2442) and 1 X antibiotic/antimycotic solution (Sigma #A-5955) at 37 °C. Confluent cell monolayers were trypsinized with 0.25% Trypsin-EDTA solution (Sigma #T-4049).

VIRUS: The fixed rabies virus (PV) was supplied by the Rabies Diagnosis Laboratory of the Instituto Pasteur, São Paulo, Brazil. The work dilution of virus was established by incubating 5 x 10^4 cells in 100 µl with a serial two-fold dilutions of the virus suspension (50 µl). One cell-culture infective dose ($CCID_{50}$) was then established as being the reciprocal of the dilution (1:80) which infected about 50% of the cells. One $CCDI_{50}$ was used in all experiments.

SERUM SAMPLES: The 104 heat-inactivated dog serum samples used in this study were kindly supplied by the Serology Section of

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Instituto Pasteur (São Paulo, Brazil). An International Reference Serum (Copenhagen, Denmark) containing 30 IU/ml was used in parallel in all experiments.

ELISA: The immunoassay used was that previously described by PIZA *et al*. (1999*)*, adapted by RODRIGUES da SILVA *et al*. (2000), excepting for the dog serum samples and the anti-Dog IgG conjugate (Sigma #A6792) which were diluted at 1:200 and 1:10,000, respectively. Antibody titres were expressed as equivalent units (EU/ml).

FLUORESCENCE INHIBITION MICROTEST: Neutralizing antibodies were measured by the simplified fluorescence inhibition microtest - SFIMT (FAVORETTO *et al*., 1993), currently used at Pasteur Institute of São Paulo. Serum titers (expressed in IU/ml) were obtained by comparison of the results of unknown sera and standard serum, as described by SMITH *et al*., 1996.

FLOW CYTOMETRY

Virus neutralization in cell culture for FC analysis: One hundred microliters of an optimal pre-determined serum sample dilution (1:25) were added to 24-well plates and mixed with 1 $CCDI_{50}$ of PV virus (100 µl). A titration of the International Reference Serum, pre-diluted to contain 20 IU/ml, was always performed in parallel. Serum samples and virus were then incubated for 45 min (37 °C, 5% CO_2) and 5 x 10^5 BHK-21 cells/well were then added. Plates were incubated for additonal 24 h. Cell monolayers were disrupted by incubating with 500 µl of trypsin-EDTA solution for 5 min. The detached cells were recovered and resuspended in 2.0 ml MEM supplemented with 10% FCS. The cells were washed twice with PBS containing 0.05% Tween-20 and centrifuged at 3,000 rpm in a bench centrifuge at room temperature.

Cell fixation and staining: After detached and washed, cells were resuspended with 1 ml solution of 4% (v/v) solution of p-formaldehyde and 1 ml of a 1:10 FACS lysis fluid (Beckton Dickinson #349202) dilution in water. After 10 min incubation at room temperature, the cells were washed twice again as described before. The pellet from the last wash process was resuspended in 100 µl of a 1:20 dilution of rabies virus nucleocapsid protein-specific antibody conjugate solution (Sanofi, France) in PBS containing 0.2% of Evan Blue, and incubated for 45 min in the dark at 37 ºC. After this last incubation, the cells were centrifuged and washed as before. The final pellet was resuspended in 1 ml of ISOTON II® (Coulter) and stocked at 4 ºC (no longer than 24h) until flow cytometry analysis. Uninfected cells and infected cells incubated with negative serum, submitted to the same procedure as above were used as negative and positive control, respectively.

Flow cytometry analysis: A Beckton Dickinson FACSCalibur® flow cytometer equipped with a 15 mW 488nm air-cooled argon-ion laser was used to analyze the cell infection kinetics. The forward scatter (FSC) detector was set at E00 with a threshold value of 200, and the side scatter (SSC) at 370 V. An area containing all cellular events was defined as R1. A total of at least 10,000 events were recorded for each sample at R1 area. For fluorescence analysis, the intensity of the green fluorescence (FITC - FL1 detection) was obtained from the R1 area. The FL1 was set at 325 V. Such voltage placed the negative control cells bellow 10^1, containing at least 97% of these cells. Markers M1, between 10^0 and 10^1, and M2, between 10^1 and 10^4, were determined. M2 values define the percentage of cells considered as infected (positive for intracellular viral particles).

Calculations of VNA titers: The maximum rate of infection (100%) was obtained when cell suspension were infected with rabies virus with a negative control serum. Based upon the rates of infection obtained with the serial dilutions of reference serum, a standard curve was drawn correlating the percentage of infection inhibition and the contents of IU/ml of the serum dilutions, as shown in Fig. 1. The values of EU/ml of the other 104 serum samples were obtained by extrapolation.

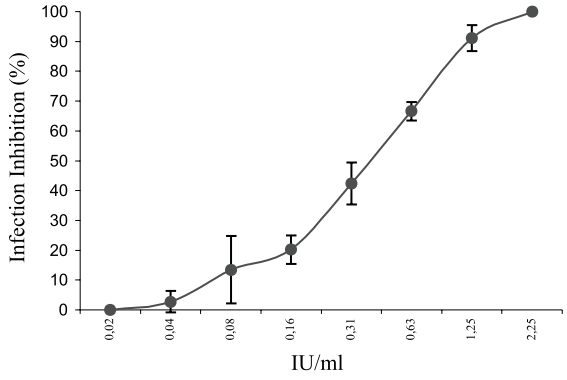

Fig. 1 - Standard curve of infection inhibition. Serial twofold dilutions of an international reference serum (30 IU/ml - Copenhagen, Denmark) were mixed to $1CCID_{50}$ of rabies virus (PV) prior to addition of BHK-21 cells. After incubation for 24 h, cell monolayers were disrupted by trypsin, fixed with p-formaldehyde and stained with a rabies virus nucleocapsid protein-specific antibody conjugate. Flow cytometry analysis was carried out in a Beckton Dickinson FACSCalibur® flow cytometer. The percentage of inhibition of infection provoked by each dilution of the reference serum was obtained by comparing with positive infected control cells. Results represent the mean ± SD of four independent experiments.

STATISTICAL ANALYSIS: Results obtained by the different techniques were compared by the non-parametric Wilcoxon rank test and Spearman correlation test.

RESULTS

Optimization of technical parameters: The VNA titers of 104 dog serum samples were evaluated by flow cytometry and results were compared to those obtained from SFIMT and ELISA. A checkerboard titration employing a negative and the positive reference sera was initially performed to determine the best number of cells/well, the best dilution of sera and virus concentration to be used in flow cytometry analysis. The best results were obtained employing 5 x 10^5 cells/well, 1:25 serum samples dilutions and 1 $CCDI_{50}$ (1:80) of rabies virus. Even minute modifications of those parameters profoundly affected the final results (data not shown).

Standard curve of infection inhibition: Fig. 1 shows the percentage of inhibition of viral infection of BHK-21 brought about by the decreasing dilutions (or increasing IU/ml-concentration) of the reference standard serum in four independent experiments. Serial twofold dilutions of reference serum ranging from undiluted up to 1:16 (corresponding 30 to 1.88 IU/ml, respectively) completely abrogated viral infection. Even when the reference serum was diluted to contain 0.16 IU/ml, a significant reduction (20%) of viral infection could be observed.

Correlation between FC X SFIMT and FC X ELISA: In contrast to the reference serum which was used in a serial two-fold dilutions in all experiments, dog serum samples were always tested in a single

dilution, 1:25. The VNA titer of each serum sample, obtained by flow cytometry, was compared to conventional techniques. As shown in Fig. 2a, Flow cytometry and SFIMT results showed a high statistical correlation (Wilcoxon rank test: $p < 0.001$; Spearman correlation coefficient $r = 0.7734$; $p < 0.001$).

When VNA titers obtained by flow cytometry was compared to those obtained by ELISA, there was a lower statistical correlation between the results (Wilcoxon rank test: $p < 0.001$; Spearman correlation coefficient $r = 0.5928$; $p < 0.001$) as shown in Fig. 2b.

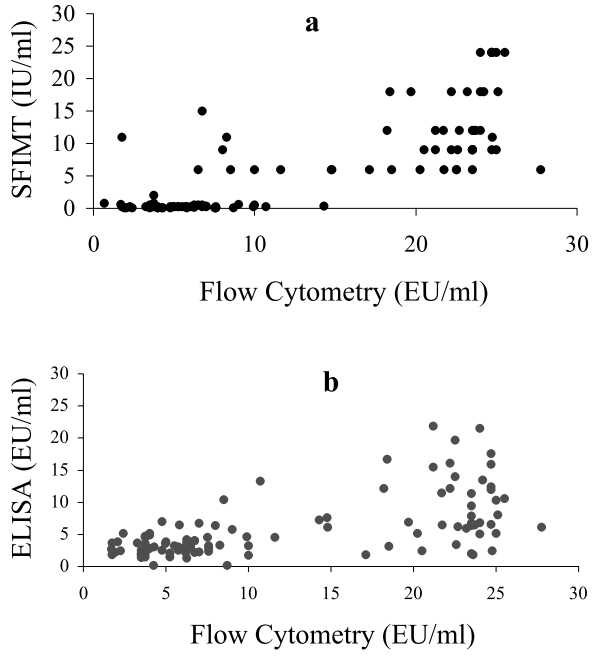

Fig. 2 - Correlation between FC X SFIMT and FC X ELISA. One-hundred and four dog serum samples were titrated by flow cytometry, SFIMT and ELISA. **a**. Correlation between FC and SFIMT (Wilcoxon rank test: $p < 0.001$; Spearman correlation coefficient $r = 0.7734$; $p < 0.001$). **b**. Correlation between FC and ELISA (Wilcoxon rank test: $p < 0.001$; Spearman correlation coefficient $r = 0.5928$; $p < 0.001$).

DISCUSSION

The present study describes a new approach for appraising rabies virus neutralizing antibodies based upon flow cytometry. This method have been used for detection of a range of different viruses (see McSHARRY, 1994, for review) and we have recently adapted it for monitoring rabies infection in BHK-21 and C6 cell lines by fixed (PV) or wild rabies virus (WRS) strains (BORDIGNON *et al.*, 2002). In addition to detecting and quantifying virus-infected-cells, antibodies to viruses have also been detected and quantified by flow cytometric analysis, such as to HCMV (McHUGH *et al.*, 1986 and 1988), HSV (SCILLIAN *et al.*, 1989) and HIV (SLIGH *et al.*, 1989). Those authors used microspheres coated with viral antigens to detect specific antibodies in serum samples. In the present study, however, the measurement of VNA was performed indirectly, since their presence was disclosed by inhibition of infection.

Since rabies virus nucleocapsid structures are intracytoplasmic, permeabilization of the cells at the time of fixation was necessary to allow antibody conjugate enter the cells. This was achieved by resuspending cells in a solution containing p-formaldehyde in FACS lysis fluid. In order to evaluate the humoral immune response against rabies virus in vaccinated humans or animals, the WHO Expert Committee on Rabies recommends that only neutralizing antibodies be measured, since this kind of antibody has extensively been shown to be protective (WIKTOR *et al.*, 1973; DIETZSCHOLD *et al.*, 1987). For that reason, in all tests available for detecting VNA, dilutions of heat-inactivated serum are previously incubated with a fixed amount of rabies virus before being inoculated in a susceptible cell-culture or experimental animals (WEBSTER & DAWSON, 1935; WIKTOR & CLARCK, 1973; SMITH *et al.*, 1973; ZALAN *et al.*, 1979). The neutralizing power of the serum is then evaluated by residual virus infectivity observed on those systems. The most widely used cell-culture techniques are those based on observation of reduction of infected-cell foci by direct fluorescence method (WIKTOR & CLARCK, 1973; SMITH *et al.* 1973; ZALAN *et al.*, 1979), and the technique described by SMITH *et al.* (1973) is a reference world-wide. In most of those assays the serum-neutralization end-point titer is defined as the dilution factor that neutralizes 50% to 100% of the challenge inoculum. The titers of antibody in the test serum samples (IU/ml) are obtained by comparison with the titer of a reference standard which must be included in each test. In all tests, the reading are made by optical reading in fluorescence microscopes which turns the work highly arduous and rises the chance of errors when a great number of serum samples are routinely processed.

The flow cytometric method for VNA titration here described uses an analytic automatized procedure for reading. After constructing a standard curve of virus infection inhibition, based on the ability of the reference serum in neutralizing rabies virus, values of any serum test may be obtained by extrapolation. The procedure showed to be very reproducible, as it can be observed by the small standard deviation values from four independent experiments showed in Fig. 1. When the results of VNA titration of 104 dog serum samples obtained by FC were compared to those obtained by SFIMT and ELISA, the values showed a positive correlation ($p < 0.01$) with *r* values of 0.7 and 0.6, respectively. As it can be seen in Fig. 2, although FC and SFIMT had a positive correlation, a complete concordance between the methods was not possible. The disagreement was more evident with serum samples that exhibit lower VNA titers in SFIMT, which otherwise could indicate a higher sensitivity of FC. Nevertheless, no specific tests for determining specificity and sensitivity of FC was carried out. For that, it would be necessary a number of negative and positive serum samples. Since the serum samples were obtained from dogs which attend a Regional Vaccination Campaign, we did not know the exact immunization status of the animals. LYNG (1994) had also reported observations about occasional unexplainable differences between relative potencies of a serum obtained by two unrelated methods.

The lower correlation between FC and ELISA (Fig. 2) was easily explainable since the latter technique detect not only neutralizing antibodies but also anti-RNP antibodies.

The study here presented has shown evidences that Flow Cytometry may be used for titration of rabies VNA. Our results permit to envisage the FC as an automated and sensitive technique to evaluate VNA in sera not only from immunized animals but most likely in human serum samples too.

RESUMO

Detecção e quantificação de anticorpos anti-rábicos neutralizantes pela técnica de citometria de fluxo

A titulação de anticorpos neutralizantes contra o vírus rábico (AcN) pós-imunização é um parâmetro aceito como indicador de eficácia da vacina e do sucesso do processo de imunização. Este estudo descreve um teste de neutralização realizado em cultura de células, analisado através da técnica de citometria de fluxo. Diluições seriadas de amostras de soro foram misturadas *in vitro* com vírus rábico e adicionados a células BHK-21. Após incubação de 24 h, as células foram individualizadas por tratamento com tripsina, fixadas e permeabilizadas com p-formaldeído e coradas com conjugado específico. A porcentagem de inibição da infecção viral causada pelos anticorpos específicos presentes nas amostras de soro foi determinada em citômetro de fluxo *Beckton & Dickinson FACSCalibur*® e comparada com um soro de referência internacional. Os títulos de AcN foram determinados por extrapolação. Os níveis de correlação (*r*) entre os títulos obtidos com CF e SFIMT foi de 0.7712 ($p < 0,001$), e entre CF e ELISA de 0,6702 ($p < 0,001$). Os resultados indicam que a CF pode ser utilizada na análise de títulos de anticorpos neutralizantes em amostras de soro, com a vantagem de ser um método automatizado. No entanto, devem ser realizados novos estudos, comparando a CF com as técnicas consideradas como padrão ouro, para que os valores reais de Sensibilidade e Especificidade sejam determinados.

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