Development of Monoclonal Antibodies Suitable for Rabies Virus Antibody and Antigen Detection

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Abstract The control of an infectious viral disease as rabies is made easier by rapid and accurate diagnosis. Successful rabies prophylaxis is dependent upon the active immunization with vaccine along with passive administration of rabies virus neutralizing antibodies which together clear the virus before widespread infection of central nervous system occurs. The present study aimed at the development of monoclonal antibodies (MAbs) suitable for rabies virus antibody and antigen detection. For the production of rabies specific MAbs, immunization of Swiss albino mice with a commercially available vaccine was done and Polyethylene glycol mediated fusion of spleenocytes with myeloma cells was performed. The positive clones were selected on the basis of distinct reactivity by cell Enzyme linked immunosorbent assay and fluorescence in Indirect Fluorescent antibody test. The positive clones obtained were subjected to single cell cloning by limiting dilution method. The reactive clones were further titrated and employed for virus titration and virus neutralization. The neutralizing activity was evaluated using Fluorescence Activated Cell Sorter technique. Three MAb clones showed a distinct percent inhibition in the presence of positive serum. One of the MAb clone No. 5C3 was relatively more specific in detecting rabies antibodies and also found suitable for competitive ELISA to assess the antibody level in vaccinated subjects.

Keywords Antigen · Monoclonal antibody · Rabies virus · Enzyme linked immunosorbent assay

Introduction

Rabies is one of the oldest, dreadful and highly contagious diseases known to mankind since early civilization dating back 5,000 years and is prevalent in all parts of world except Australia, New Zealand, Britain, Japan and Scandinavia. The disease is transmitted through the bite of the rabid animal. Rabies is a severe and fatal viral disease of the central nervous system of warm blooded animals, including man [32]. Rabies virus belongs to Genus: *Lyssavirus*, of the Family: *Rhabdoviridae* under the Order: *Mononegavirales* [23]. The virus is bullet shaped, its genome is non-segmented, minus-stranded RNA molecule of 11,932 nucleotides long [31]. Viral genome has five major genes, which code for five major viral proteins namely Nucleoprotein (N), Non-structural protein (NS or M1), Matrix protein (M2), Glycoprotein (G), and Polymerase (L) [7]. Among these proteins, the G protein is involved in cellular reception and is the only antigen that induces virus-neutralizing antibodies. Variability in the sequence of this protein appears to be responsible for the serotypic differences among Lyssaviruses [22].

In India, rabies is endemic except the Lakshdweep, Andaman and Nicobar, Dadra and Nagar Haveli. States like Manipur, Meghalaya, Sikkim, Arunachal Pradesh, Mizoram and Nagaland have reported occasional cases of rabies while substantial deaths have been reported in all other states of India [26]. Rabies exposure has profound worldwide medical and economic implications with as many as 4 million people per year receiving post-exposure treatment to prevent rabies [15]. Worldwide, it is estimated

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that approximately 55,000 persons die of rabies each year [7, 34] and about 20,000 deaths are reported every year in India [14, 30]. MAbs developed against rabies virus has been successfully used for passive immunization [3], and are relatively safer than rabies immunoglobulin (RIG) [19]. The present study was planned with the objective to develop monoclonal antibodies suitable for rabies virus antibody and antigen detection; and to monitor the level of antibody response in immunized subjects.

Two fusions were carried out using a commercially available antigen for immunizing mice and (1) production of 24 MAbs are hereby reported; (2) Virus neutralizing ability of the selected MAbs was done using FACS; and (3) the subsequent use of MAbs to cell ELISA, Indirect ELISA, FAT, FACS and Competitive ELISA was carried out.

Materials and Methods

Cell Lines

Vero cells, BHK-21 cells and myeloma cells used in the present study were made available in the author's laboratory. Vero cells (African Green Monkey Kidney cells) between 170th to 190th passages were propagated in EMEM (Earle's minimum essential medium) containing 10 % FBS (Fetal bovine serum). For maintenance, EMEM containing 2 % FBS was used. This cell line was used for screening with cell ELISA, Indirect ELISA and FACS. BHK-21 (Baby Hamster Kidney) clone 13 cells between 120th to 150th passages were used as the substrate for the growth of Pasteur virus (PV) strain of rabies virus. BHK-21 cells were propagated in Glasgow Minimum Essential Medium (GMEM) containing 10 % FBS. For maintenance, GMEM containing 2 % FBS was used. Myeloma cells [sp2/O, derived from X63-Ag8X Balb/c mice [2] were revived from deep freezer, propagated and maintained in Iscove's Modified Dulbecco's Medium (IMDM) containing 20 % FBS under 5 % carbon dioxide tension in a desiccator under humid condition. These myeloma cells were used as fusion partners with sensitized spleen lymphocytes.

Virus

Pasteur virus (PV) strain of rabies virus is a fixed virus strain and is one of the common vaccine strains adapted to grow in BHK-21 clone 13 cells and Vero cell system. PV strain of rabies virus having a titre of 10^7 TCID₅₀/ml (Tissue culture infectivity dose₅₀/ml) after propagation in cell system was used. Rabies virus aliquots were stored at -70°C .

Experimental Animal

Swiss albino mice of age group 4–6 weeks (16–20 g) were used for immunization for hybridoma production.

Production of Monoclonal Antibodies

Production of monoclonal antibodies (MAbs) was performed as per the protocol [8, 20, 27] with some modifications which included use of Swiss albino mice for immunization and Cell ELISA for screening. A commercially available cell culture vaccine with potency ≥ 2.5 I.U. was used for immunization of Swiss albino mice to produce hybridoma against rabies virus. Two Swiss albino mice were immunized using rabies vaccine. The immunizing dose was 0.2 I.U. and three intraperitoneal inoculations were made: the first (day 0) with Freund's complete adjuvant (FCA) for priming, the second (day 15) and third (day 22) with Freund's incomplete adjuvant (FIA) for boosting. Subsequently 3 days before fusion, the mice were immunized with three continuous intravenous injections without any adjuvant (day 37, 38 and 39). One mouse was sacrificed on 40th day of fusion.

The peritoneal macrophages (feeder cells) were collected from a Swiss-albino mouse and seeded in three 24-well cell culture plates using IMDM containing 20 % FBS, 3 days before the fusion. Peritoneal macrophages were collected after washing the peritoneal cavity of the Swiss albino mouse in sterile IMDM. The collection tube was kept on ice to avoid adherence of the macrophages on the walls of the collection tube. The feeder cells were centrifuged at 1,000g for 10 min. The pellet containing mouse peritoneal macrophages was treated with chilled RBC lysis buffer (0.83 % ammonium chloride solution) in order to remove contamination of red blood cells. These cells were suspended in 100 ml of IMDM having antibiotics and 20 % FBS. The cells were seeded in 24 well cell culture plates at the rate of 1 ml/well.

Myeloma cells and spleen lymphocytes were prepared for fusion as described earlier [28]. Fusion was performed using 50 % polyethylene glycol (PEG M.Wt.-1500) (500 μl PBS + 500 μl PEG) in PBS (phosphate buffer saline) strictly at 37°C . Cells were seeded in 24 well tissue culture plates. Growth of hybridoma was observed regularly under a selection medium containing aminopterin. The culture plates were screened well to well at appropriate stage of growth. A cell-ELISA assay system was employed for screening of rabies specific MAbs in the hybridoma supernatants using acetone fixed rabies virus infected cells [24, 25]. Fluorescent antibody test (FAT) was also employed for screening of positive clones. Single cell cloning and sub-cloning of rabies virus positive hybridoma was performed using limiting dilution method [8, 20].

Hybridoma clones derived from single cells were selected, expanded and preserved at -70°C .

Rabies Virus Titration in BHK-21 Cells

The titration of rabies virus (PV strain) in BHK-21 cells was done by co-cultivation. Virus dilution was made using deep-well plate in which serial $\log_{10}$ dilutions in chilled 2 % EMEM was done. BHK-21 cells were taken at the cell concentration of $4-6 \times 10^5$ cells per ml in 10 % EMEM and added in a 96 well cell culture plate. From the deep-well plate, 100 μl of the $\log_{10}$ diluted virus samples were added to the cells in quadruplicates in the same order from 10^{-1} to 10^{-7} , using a multi-channel pipette. After 48 h of incubation at 37°C , the old medium was replaced using pre-warmed 2 % EMEM and kept for incubation. After 72 h of incubation at 37°C , spent medium was aspirated completely and cell monolayer was fixed with 100 μl of chilled acetone (80:20 in PBS) per well and kept at -20°C for 1 h. While aspirating acetone completely from each well, care was taken not to damage the cell monolayer and the plate was air dried (20–30 min in laminar airflow). After air drying, the plate was blocked using 200 μl of blocking buffer (Gelatin-1 %, FCS-0.5 % and Tween 20-0.1 % in PBS) and incubated at 37°C for 1 h or $+4^{\circ}\text{C}$ overnight. Then the blocking buffer was discarded and 100 μl of monoclonal antibody [1:2 dilution in blocking buffer] was added and plate was incubated at 37°C for 1 h. Plate was washed 3 times with wash buffer (0.01 % Tween-20 in 0.0025 M PBS). 100 μl of rabbit anti-mouse HRPO (horseradish peroxidase) conjugate was added per well (1:2,500 dilution).

in blocking buffer) and the plate was covered with aluminium foil and incubated at 37°C for 1 h. 100 μl of substrate-chromogen mixture (4 μl of 3 % hydrogen peroxide per ml of pre-warmed OPD (ortho-phenylene diamine) was added per well and the plate was covered with aluminium foil and observed for colour development. Thereafter the reaction was stopped by adding 100 μl of 1 M Sulphuric acid per well and reading was taken at 492 nm in an ELISA reader. Based on the ELISA reading, virus titre was calculated using Reed and Muench formula [21].

MAB Titration Based on Cell ELISA

For detection of titre of monoclonal antibodies in cell ELISA, BHK-21 cells were infected (by co culture method) with 0.1 TCID₅₀/well of PV strain virus in microtitre plates. These cells were given one media change at 48 h with EMEM containing 2 % FCS and were fixed after 72 h with chilled acetone/PBS in the ratio of 80/20 for 1 h at

-20°C . After air drying, the plate was blocked using 200 μl of blocking buffer and incubated at 37°C for 1 h or $+4^{\circ}\text{C}$ overnight. Then the blocking buffer was discarded and 100 μl of monoclonal antibody (1:2 dilution in Blocking buffer) was added in two fold dilution of hybridoma culture supernatant (up to 1: 4,048) and plate was incubated at 37°C for 1 h. Plate was washed 3 times with wash buffer. 100 μl of rabbit anti-mouse HRPO conjugate was added per well (1:2,500 dilution in blocking buffer) and the plate was covered with aluminium foil and incubated at 37°C for 1 h. Rest of the steps were same as that in rabies virus titration.

Fluorescence Activated Cell Sorter (FACS) Technique

The neutralizing ability of MABs was investigated using FACS technique following the protocol described earlier [4] with some modifications like, use of chilled acetone for fixing of cells and use of less quantity of anti-nucleocapsid FITC (Fluorescein iso thiocyanate) conjugate as reported earlier [1]. The protocol followed for determining the neutralizing ability of various reactive clones using FACS technique is given below in brief. BHK-21 cells were propagated in EMEM containing 10 % FBS in a 24 well cell culture plate and incubated at 37°C for 24 h. 500 μl of reactive MAB supernatant (1:2 dilution) and 10,000 TCID₅₀ of PV strain of rabies virus in EMEM were mixed in a sterile 24 well plate incubated for 2 h and then transferred to 24 well containing BHK-21 cell monolayer and further incubated for 48 h at 37°C . After 72 h, the supernatant was discarded completely. 300 μl of Trypsin-Versene (pre-warmed at 37°C) was added per well, when cells detached from surface 700 μl of GMEM-10 % was added and cells were pipetted to get single cells. Cells were collected in an Eppendorf tube (2 ml) and centrifuged at 2,000 rpm for 10 min and the cell pellet was given a washing in 1 ml of phosphate buffer at 2,000 rpm for 10 min. The cell pellets were dispersed gently to get single cells and cells were fixed in chilled acetone at -20°C for 1 h. Then the cells were centrifuged at 2,000 rpm for 10 min and acetone was discarded completely using separate tips for each dilution. The cell pellets were dispersed to single cells and were given a washing with PBS at 2,000 rpm for 10 min and again the pellets were dispersed manually to get single cells. Rabies anti-nucleocapsid FITC conjugate (1:20) diluted in phosphate buffer at pH 7.2 was added at the rate of 20 μl per Eppendorf tubes and incubated at 37°C for 30 min in a humid chamber. Cells were again washed with 1 ml Phosphate buffer (pH 7.2) and were dispersed manually and resuspended in 400 μl of Phosphate buffer and the samples were subjected for FACS technique. Results of the FACS technique were recorded for analysis.

Antigen Preparation for Competitive ELISA

To perform MAb titration and competitive ELISA, PV strain antigen of rabies virus was prepared as per protocol previously described [27, 28]. Briefly BHK-21 cells and PV strain virus cultivated at a multiplicity of infection 0.1 TCID₅₀/ml in EMEM containing 10 % FBS. The monolayer was replenished with fresh media having 2 % FBS every 48 and 72 h. At least two media changes were made till the culture was harvested. Culture harvest was inactivated with β -propiolactone in alkaline medium (pH 8.5 made by adding sterile filtered 7.5 % NaOH) and kept at 37 °C for 24 h. Then it was shifted to −20 °C for 24 h. Chilled culture after thawing was subjected to polyethylene glycol (PEG-6000) precipitation using 8 % (w/v) PEG 6000 in the presence of 2.3 % (w/v) sodium chloride and kept overnight at 4 °C. The precipitate was centrifuged at 4800 rpm for 45 min. The pellet was dissolved in TNE (Tris, sodium chloride and EDTA buffer) buffer in 1:10 of the original volume; this PEG concentration was repeated twice to concentrate the desired material 100 times. The resulting antigen was used for standardization of Indirect ELISA and competitive-ELISA to detect rabies virus antibody at an appropriate dilution.

Titration of Prepared Antigen Using Indirect ELISA

Titration of antigen was performed using Indirect ELISA as per standard protocol. Based on the ELISA reading, MAb titre for various positive clones was calculated using Reed and Muench formula [21].

Pattern of Inhibition of MAb Binding to Antigen in Competitive ELISA

Pattern of inhibition of MAb binding to antigen in the presence of eight clones was studied using competitive ELISA as per method reported earlier [28]. Seventy one field samples of unknown status to rabies virus antibodies were tested. The ELISA plates were coated with 1:5 dilution of rabies virus antigen (50 μ l/well) in carbonate bicarbonate buffer and plates were incubated at 37 °C for 1 h under regular shaking. Unbound antigen in the plate was washed 3 times with wash buffer. All the wells of the plates received 40 μ l of blocking buffer (Tween 20-0.1 % in PBS). Additionally 10 μ l of blocking buffer was added in monoclonal control (Cm). Aliquots of 10 μ l of serum samples were added to all the wells of the plate in duplicate sets followed by addition of 50 μ l of MAb. The plates were incubated at 37 °C for 1 h and then washed three times. 100 μ l of rabbit anti-mouse HRPO conjugate was added per well (1:2,500 dilution in blocking buffer) and the plate was covered with aluminium foil and incubated at 37 °C

for 1 h and then washed thrice. Rest of the steps were performed as mentioned earlier. Pattern inhibition (PI) was calculated using formula:

$$PI = 100 - \frac{(O.D.(test) \times 100)}{O.D.(Cm)}$$

Results

Production of Monoclonal Antibodies

Swiss albino mice were immunized with rabies vaccine as per the immunization protocol with some modification [27, 28]. Fusion of myeloma cells with sensitized spleenocytes was performed at the end of immunization schedule. Two fusions were performed in all. The fused cells were grown in HAT (Hypoxanthine aminopterin thymidine) selection media containing aminopterin at 37 °C under 5 % CO₂ tension. The growing clones (bunch of 8–10 cells) became visible after fourth day of fusion.

Screening of Hybridoma

After 14 days of fusion supernatant from different clones were collected and screened using cell ELISA and Indirect FAT.

Cell-ELISA

Hybridoma culture supernatants were screened using cell ELISA with PV strain of rabies (0.1 TCID₅₀/well) virus infected BHK-21 cells. The culture supernatants that gave significantly high absorbance A₄₉₂ values were selected for further investigation. Out of 64 clones screened 7 clones were found to be highly reactive after first fusion experiment (Fig. 1a). In second fusion 17 clones were found to be positive from 96 hybridoma clones supernatant screened (Fig. 1b).

Fluorescent Antibody Test (FAT)

Indirect FAT using micotitration plates (micro FAT) was performed on rabies virus infected BHK-21 cells for screening of hybridoma clones using rabies anti-nucleo-capsid FITC conjugate and observed under a fluorescent microscope. Some of the hybridoma clones with distinct fluorescence indicating the site of virus replication were selected. The pattern of fluorescence using specific monoclonal antibodies is indicated in Fig. 2. The distinct cytoplasmic fluorescence was noted for clones 1A2, 1B6 and 2C5, and less but similar fluorescence in clones 1B4 and 1A5 as compared to background fluorescence.

Fig. 1 Reactivity of hybridoma culture supernatant of first fusion (a) and second fusion (b) in cell-ELISA with PV strain of rabies virus infected BHK-21 Clone 13 cells

Single Cell Cloning and Subcloning

Clones with distinct reactivity in FAT and cell ELISA were subjected to single cell cloning and subcloning. Cells derived from a single cell (highest limiting dilution) with a distinct reaction of culture supernatant in ELISA were expanded and preserved in deep freezer (-70°C).

Titration of Monoclonal Antibodies

Reactive hybridoma supernatants were subjected to cell ELISA for titration of antibody to be used as diagnostic reagents. For this two fold dilution of MABs were titrated using virus infected cells at 0.1 MOI (Multiplicity of infection). Corresponding dilution of MABs, 75 % absorbance (A_{492}) of the plateau/saturation of titration curve, was taken as its titre. The titration curve of selected monoclonal antibodies is shown in Fig. 3. The titre for

clones 3A1, 3F1, 3D3, 4C3, and 5D3 was 8 and for clones 3D2, 3B3 and 5C3 it was 16. Reactive clones with high titre were used in competitive ELISA.

Infectivity Titration of Rabies Virus

PV strain of rabies virus was titrated with selected MAB using 10-fold virus dilution in BHK-21 cells using fixed quantity of hybridoma supernatant in cell ELISA. The dilution of virus which showed distinct absorbance as compared to virus blank well was taken as positive (Fig. 4). Virus titre was calculated in the presence of clones Rab-48, 3A1, 3F1, 3D2, 3E1, 3A3 and 3B3 using Reed and Muench formula. The mean titre in presence of all these MABs was found to be $10^{6.5}$ TCID₅₀/well.

Investigation of Neutralizing Ability of Reactive MAB

Virus neutralization test (VNT) was performed using 10^4 TCID₅₀ of virus/well with 1:2 dilutions of MABs. BHK-21 Clone 13 cells were trypsinized and fixed with acetone at 48 h post infection in 24 well plate and subjected to FACS using Rabies anti-nucleocapsid antibody conjugated with FITC. Pattern of distribution of cells with virus specific fluorescence in FACS technique indicating degree of neutralization of rabies virus with MAB is shown in Fig. 5. The degree of fluorescence was compared with positive control having rabies virus and without virus as negative control. Graphical presentation of no. of cells showing fluorescence is shown in Fig. 5. Clone 5C3, 3D3, 4C3, 3F1 and 3B3 showed less percent of cells with fluorescence and neutralizing activity against the virus above 95 %.

Detection of Rabies Antibody in Vaccinated Individuals/Animals

The MAB for antibody detection was selected from available set of MABs. The MAB for antibody detection and the inhibition pattern to antigen in the presence of known positive serum was selected on the basis of its

Fig. 2 Indirect FAT (fluorescent antibody test) showing BHK-21 cells infected with 10^{-1} dilution of PV strain of rabies virus

Fig. 3 Titration curve of different monoclonal antibodies

Fig. 4 Reactivity pattern of selected MAbs in cell-ELISA with variable quantity of virus infected BHK-21 cells

high ELISA signal. Clone 5C3 was found to be the most appropriate on the basis of these parameters (Fig. 6). The inhibition of MAb binding to antigen in the presence of seventy one test serum samples which include 4 human serum samples of known status and 67 dog serum samples of unknown status to rabies was studied in competitive ELISA using clone 5C3. Out of 4 human samples tested 3

were found to be positive and 12 out of 67 dog serum samples were found to be positive for antirabies antibody (Fig. 7).

Discussion

The control of an infectious disease is made easier if there is an effective vaccine and efficient diagnosis. Timely diagnosis is important as immunity to rabies virus induced by natural infection or current vaccines is not potent at removing disease causing rabies virus from brain tissues [9]. The use of MAb can be a promising tool for specific diagnosis of rabies and for monitoring the antibody level in vaccinated animals and human beings. Selection of appropriate monoclonal antibodies can provide desired sensitivity, specificity and convenience to the diagnosticians. The advent of pathogen specific monoclonal antibodies in combination with VNT, FAT, RFFIT and ELISA greatly ease the task of specific antigen and antibody detection in clinical samples. Though the use of such large array of tests have been very promising but availability of suitable monoclonal antibodies may

Fig. 5 FACS technique application for investigation of the neutralizing ability of various MABs

Fig. 6 Inhibition pattern of binding of selected MABs to antigen in a competitive ELISA in the presence of both positive and negative serum samples

Fig. 7 Screening of 71 serum samples for the presence of rabies virus antibody with MAB 5C3. Serum samples above cut off were considered positive for rabies virus antibody

explore the possibility of developing an alternative test such as competitive-ELISA/blocking-ELISA which could be more convenient for seromonitoring of target population to evaluate immune response. Also rabies virus neutralizing MABs of mouse origin are reliable alternatives to human and equine rabies immunoglobulin (HRIG and ERIG)

[18]. Development and application of cell ELISA, Indirect ELISA, FAT, RFFIT and FACS technique for quantitation of vaccine virus and screening of serum samples from field for seromonitoring of vaccinated animals was studied in order to assess the quality of vaccine. The study proved that use of vaccine antigen for immunization of Swiss albino mice was efficacious. The specificity of the hybridoma clones to rabies virus was established by cell ELISA. Monoclonality of a clone was considered only when all the wells of a microtitre plates with growing cells gave a positive reaction with Indirect ELISA. Enzyme immunoassay is the most convenient test for screening hybridoma supernatants because nanogram amounts of antibody can be detected and upto 100 samples can be tested at the same time [12]. Enzyme immunoassay has been used to detect rabies antibody in hybridoma culture fluids and also to differentiate street and laboratory strains of rabies virus [29]. Hybrid clones have been screened for production of antirabies antibody by radioimmunoassay (RIA) [33]. During the present investigation set of hybridoma clones with distinct reactivity as compared to background absorbance (A_{492}) value were selected for further diagnostic application. In the present study 24 hybridoma clones were found to be highly reactive after screening in two fusion experiments (Fig. 1a, b), these results were comparable to the kind of screening by EIA as performed earlier. [12]. FAT has proven to be a fast and reliable diagnostic tool for the routine diagnosis of rabies [6], so the confirmatory mouse inoculation test [11] was abandoned in many laboratories. The direct FAT is employed as diagnostic technique because of its sensitivity, accuracy and speed as recommended by WHO [16]. Indirect FAT using microtitration plates (MicroFAT) was also employed for preliminary screening of reactive hybridoma clones obtained from first fusion. MicroFAT was performed

on rabies virus infected BHK-21 cells and observed under fluorescent microscope. The fluorescence was observed because of intracytoplasmic replication of virus. Some of the hybridoma clones with distinct fluorescence indicated the site of virus replication called “viral factories”. The results indicate that these reactive clones could be used for antigen detection in FAT (Fig. 2).

Culture supernatant from the reactive hybridoma clones were also subjected for antibody titration in cell ELISA to be used as diagnostic reagent. In order to further evaluate MABs, infectivity titration of rabies virus was performed with selected set of MABs. In the presence of clones Rab-48, 3A1, 3F1, 3D2, 3E1, 3A3, 3B3 and 3D3 it was also possible to calculate the virus titre using Reed and Muench formula which came out to be $6.5 \log_{10}$ TCID₅₀ virus/well (Fig. 4). A distinct dose dependent absorbance was observed with selected set of MABs. This indicated the suitability of these MABs in the detection of rabies virus in BHK-21 cells. The findings also suggest that use of cell ELISA may replace mouse inoculation test for infectivity titration of rabies vaccine virus. However, both the tests should correlate well, before cell ELISA is used routinely.

Flowcytometry has emerged as a method of choice for automated analysis of cells in suspension [10] and also been described for monitoring of rabies infection in BHK-21 and C6 cell lines [1, 4]. During the present study, FACS technique was employed for the investigation of neutralizing ability of MABs against rabies virus. Conventional Virus neutralization test was performed with MABs in 1:2 dilution using 10^4 TCID₅₀/well of rabies virus antigen. Proportion of distribution of cells with fluorescence indicated the degree of neutralization of rabies virus with MAB. The percentage of cells showing fluorescence was compared with positive control having only virus without any antibody and mock infected cell control. Cells were infected with virus and antibody mixture in order to assess infectivity of cells after fixed time interval and subjected to FACS analysis (Fig. 5). The hybridoma clones which showed complete or partial neutralization were considered as virus neutralizing clones.

A diagnostic technique which is simple, rapid, specific, sensitive and economical is preferred for intense sero-monitoring of disease. Competitive ELISA is one of the simple tests in comparison to VNT for screening of antibodies to various viruses. Competitive ELISA had been a key test to establish neutralizing ability of monoclonal antibody [13].

Extensive studies were carried out to produce rabies virus specific MABs. The most appropriate clone found on this basis was 5C3 since its percent inhibition was relatively high in the presence of positive serum. At the same time a known negative serum could not inhibit binding of

this MAB to antigen significantly. This indicated its suitability in competitive ELISA (Fig. 6). In the present study, 71 serum samples of human and canine origin of known and unknown status for rabies virus antibodies were tested with MAB 5C3. This MAB detected a proportion of rabies virus positive serum indicated by inhibition of MAB binding indicated by no colour formation (Fig. 7). These findings correlated well with the observation as performed earlier with ELISA using Glycoprotein antigen taking positive and negative control serum for standardization and establishment of a rabies test for assessing the efficacy of oral fox vaccination campaigns [5]. Capture ELISA based on monoclonal antibodies to rabies nucleoprotein (N) and glycoprotein (G) was developed to detect immune complexes to rabies N and G proteins [17]. The main objective of development of competitive ELISA was to replace mouse neutralization test as a method of choice for detection of antibodies to rabies. Competitive ELISA developed in the present study using MAB 5C3 is expected to be an ideal test for assessing antibody response against rabies virus in the serum of vaccinated objects.

The findings of competitive ELISA in comparison with FACS technique showed that clone 5C3 is the most suitable neutralizing clone alongwith 3D2 and 3A1. The pattern of result of FACS shows that it can replace the MAB based competitive ELISA and RFFIT to establish the neutralizing ability of MAB. This technique can really help in selecting potent neutralizing clones to be used as diagnostic reagents. Competitive ELISA and cell ELISA developed in present studies may be an alternative system for seromonitoring of rabies virus antibodies. The binding of MAB to rabies virus was reduced in presence of rabies virus positive sera (post vaccination). Thus competitive ELISA can replace mouse neutralization test and cell ELISA and thus can be a good alternate test for estimation of virus titre in place of mouse inoculation test though these tests have to be validated on a large scale before replacing the use of mouse in any of the above mentioned tests. Present studies justified that MAB 5C3 is suitable for competitive ELISA. Findings from the present investigation indicate that MAB from clone 5C3 is a suitable candidate for exploring large scale diagnostic application. This MAB has proven its suitability for the assessment of virus quantity in BHK-21 cells using cell-ELISA and FAT. The MAB has also been found to be suitable for assessment of antibody response using limited number of serum samples in competitive-ELISA.

Further investigations are required in the direction of characterization of selected MAB for isotype and antigenic specificity. The diagnostic techniques developed also should be compared with the gold standard test such as mouse inoculation and mouse protection assay as recommended by WHO [11].

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