

Preparation and Characterization of a Neutralizing Monoclonal Antibody against Poliovirus Type 1 (Mahoney Strain)

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ABSTRACT

Background and Objectives: Poliomyelitis remains a major public health problem in developing countries, which signify the need for extensive diagnostic and prevention research. The aim of the present study was to design monoclonal antibodies (MABs) against poliovirus type I with biomedical, diagnostic and therapeutic applications.

Methods: B-cells were isolated from a mouse challenged with polio antigen injection. The B-cell were fused with myeloma tumor cells. After evaluation and screening of approximately 250 hybridoma colons by ELISA, 35 colons with the highest antibody titer and no cross-reactivity were selected and subsequently cloned by limiting dilution. Finally, three colons capable of secreting MABs against epitopes of poliovirus type I were used for MAB production. Next, the MABs were characterized by antibody assays, isotyping, epitope analysis (western blot), cross-reactivity test, stability test, sterility test and mycoplasma test.

Results: The results indicated that the MABs were of IgG1 kappa chain, had good stability and no cross-reactivity. In western blot, a band at 26 kDa which is associated to VP3 neutralization protein was observed.

Conclusion: These serotype-specific MABs can be potentially used for identification of type I poliovirus for research, diagnostic and prevention purposes.

Keywords: Monoclonal antibody, Hybridoma, Poliomyelitis, Poliovirus.

INTRODUCTION

Of the three serotypes of poliovirus, serotype 2 was eradicated in 1999, while the other two serotypes (1 and 3) remain endemic (1). Poliovirus type 1 (PV1) is the most common poliovirus serotype (2). PV1 is currently localized to Pakistan, Afghanistan, Iraq, Syria, Nigeria, Somalia, Equatorial Guinea, Cameroon, Ethiopia and Kenya (3). Inactivated polio vaccine (IPV) and live attenuated polio vaccine (OPV) are prepared from Mahoney of Brunenders (PV1), MEF-1/Lansing (PV2) and Saukett/Leon (PV3) (4). To control diseases caused by the virus, in addition to use of potent and safe vaccines, gaining a better understanding of the structure and mechanisms involved in immune recognition, neutralization of viral infection and epidemiology of the strains prevalent in the community is essential (5). In countries where poliomyelitis is controlled, it is crucial to rapidly determine whether strains from isolated incidents of paralytic poliomyelitis are vaccine-like or non-vaccine-like (6). It is possible to differentiate serotypes by antisera prepared from laboratory animals (7). Cross-adsorbed monospecific antisera against poliovirus have been long used for intratypic sero-differentiation and quality control of polio monovalent and trivalent vaccines. Since the development of hybrid cell lines (hybridoma) by Kohler and Milstein, monoclonal antibodies (MAbs) have been replacing conventional antisera because of their numerous advantages (8). MAbs are indefinitely renewable and capable of detecting a single protein epitope, making them more desirable for various applications (9). In hybridoma technology, B-cells are fused with myeloma cells, which results in antibody production. This process has been selected for its ability to grow in tissue culture and for an absence of antibody synthesis (10). The production of MAbs has become commercialized in some laboratories but is not still available in developing countries, mainly due to the high cost of establishing an immunoclone, non-availability of BALB/c mice and difficulty in production of hybridomas (11). With the progress of monoclonal neutralizing antibodies, neutralization of poliovirus was taken into consideration. This study aimed to first design MAbs against PV1 and then to characterize them by the Kohler and Milstein method with

some modifications. These MAbs could be used for studying the epitopes of poliovirus and neutralizing each serotype in trivalent polio vaccine quality control tests.

MATERIALS AND METHODS

Reference virus and antigen preparation
Sabin oral vaccine strain stock as sabin I (LS-c, 2ab) obtained from the Razi Vaccine and Serum Research Institute was propagated in MRC-5 (ATCC-CCL-171) in the absence of fetal bovine serum (FBS) for mice inoculation (12). After elimination of the monolayers, supernatant was clarified by ultracentrifugation (Hettich, East Westphalia-Lippe, Germany) and concentrated (25X) with PEG 6000 (6, 13). Infection titer of the non-concentrated and concentrated supernatant was calculated as $10^{7.10}$ CCID₅₀/ml and $10^{8.67}$ CCID₅₀/ml, respectively in Hela cell line (ATCC-CCL-2) by a validated method (14). Total protein concentration of antigen was 0.77mg/ml in the Lowry method.

Indirect ELISA and serum neutralization (SN) test

An indirect ELISA method was modified for evaluation of antibody titer in mice sera. A mouse with the highest antibody titer was selected for hybridoma clone screening. Serial dilutions of antigen (1/50-1/8000) and antibody (1/1000-1/64000) were applied in a checkerboard of the ELISA and the optimum dilution of polio antigen (1/300) was selected for coating of ELISA microplate. For the SN test, 100 CCID₅₀/ml of virus (challenge dose) were reacted with 2-fold dilutions (1/80-1/640) of the hybridoma supernatant to be tested on Vero cell line (ATCC-CCL-81). The neutralization endpoints were read after 4-5 days (15), and the SN titer was calculated 1.85 for the selected mouse.

Mouse immunization

Female BALB/c mice were inoculated subcutaneously with mixed and homogenized concentrated virus and an equal amount of complete Freund's adjuvant, followed by booster injections with an equal amount of incomplete Freund's adjuvant three and six weeks after the first injection. Three days before fusion, the same viral antigen was diluted in PBS (1:3, at pH 7.4) without adjuvant, and then injected directly into vein of the mouse with the highest antibody titer (13).

Fusion of myeloma and B-cells

After sacrificing the mouse and removing the spleen, spleen cells were counted ($9 \times 10^7/\text{ml}$) and fused with non-secretor mouse myeloma cells ($2.5 \times 10^7/\text{ml}$) strain SP2 /0 – Ag 14 (Svanova Institute, Sweden) (spleen : myeloma cells ratio : 3.6) using polyethylene glycol 1500 (Sigma-Aldrich) (16, 17). The fused cells were distributed in a 96-well plate ($1.4 \times 10^5/\text{well}$) coated by feeder layer (macrophage cells collected from peritoneal fluids, following intraperitoneal inoculation of sodium glycolate) 72 hours before fusion. When the first hybridoma clone was detected, the media were replaced with hypoxanthine thymidine instead of hypoxanthine-aminopterin-thymidine (Biotech Resources, 1995-7) (13).

Hybridoma screening and cloning

Seven to 14 days after the fusion, the wells were observed microscopically for the presence of hybridoma clones. After two weeks, the supernatant of growing hybridomas was tested daily for a month using ELISA. In each hybridoma-screening test, PV1, 2 and 3 were tested individually for evaluation of cross-reactions between serotypes. The selected hybridomas were tested using SN test for detection of neutralizing antibody.

MAb production

For maintenance of antibody secretion, antibody-positive hybridomas were cloned twice by limiting dilution in a microplate with feeder layer. After one week, the wells containing a clone were identified, and the secretive activities were evaluated by ELISA. Clones with the highest optical density and the lowest cross-reaction with others serotypes were transferred to a 24-well plate (17). MAbs were produced by continued reduction of FBS (18).

MAb characterization

The MAbs were tittered against PV1 based on the designed ELISA. The MAbs' class, subclass and type of light chain were determined using Isostrip kit (Roche, Germany). The antibody epitope was analyzed by western blot (protein immunoblot) using all three serotypes. The produced MAbs were concentrated and purified by a 100 kDa filter membrane (Amicon) in 4000 g at 25 °C. Total protein concentration in MAbs was determined by the Lowry method before and after concentration with media containing 5% serum and without serum (Opti-MEM, Gibco). For detection of cross-reactivity with other

serotypes (2 and 3), the MAbs were tested by ELISA and SN test. In western blot, MAbs of serotypes 1 and 2 showed simultaneous parallel reactions due to the high cross-reactivity. Stability of MAbs was tested after being tittered after placement at 50 °C for 30 minutes and after three times of freeze-thawing. Moreover, sterility test was performed for detection of possible fungal or bacterial contamination in the MAbs (11).

RESULTS

The first hybridoma clone was detected seven days after the fusion and a day later, the SP2 cells were eliminated. Overall, we achieved 250 hybridoma clones. The best hybridoma clones in ELISA were subjected to the SN test. Four clones producing neutralizing antibody had no cross-reactivity with PV2 and 3, but the rest of the clones cross-reacted with PV2. The selected hybridomas were cloned by a two-step limiting dilution method. Three hybridoma clones with the highest antibody titer and the lowest cross-reactivity were selected and cultured in low percentage of FBS. Finally, the supernatant was collected and characterized. As shown in table 1, the antibodies were found to be IgG1 with kappa chain in antibody isotyping. On electrophoresis of PV1, 2 and 3 by SDS-PAGE, viral proteins including VP1, VP2 and VP3 showed 33 kDa, 30 kDa and 26 kDa bands, respectively (Figure 1).

In western blot, the MAbs showed a reaction against PV1 by creating a band at 26 kDa, which is related to the VP3 neutralization viral protein. However, such reaction was not observed in the cases of PV2 and PV3.

Moreover, the type 2 MAbs reacted only to PV2 in western blot (Figure 1). The clones' supernatant (15 ml) was concentrated 40 times by ultracentrifugation until reaching a final volume of 370 μl . Protein content of the antibodies in initial concentration (X) was 4.03, 3.08 and 3.56 mg/ml with FBS and 1.23, 1.02 and 1.08 mg/ml without FBS, respectively. The protein content of the antibodies at concentration of 40X was 62.39, 49.78 and 54.63 mg/ml with FBS and 22.18, 17.46 and 19.15 mg/ml without FBS, respectively (Table 1).

The results of ELISA showed that the clones had no cross-reactivity with PV2 and 3. These results were confirmed in the SN test. In

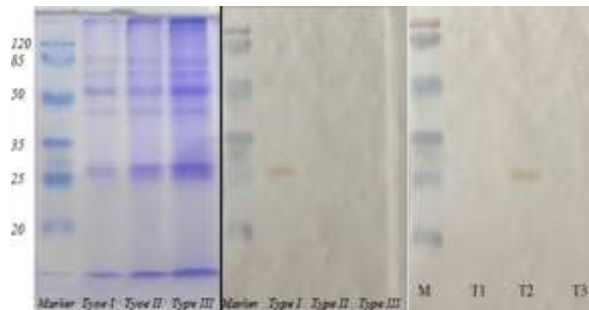
addition, stability study showed no significant difference between the initial titer and the one placed at 50 °C and subjected to freeze-thaw

cycling. Moreover, all MAbs in this study were free of bacterial, fungal and mycoplasma contamination.

Table 1- Summary of MAbs evaluations and titers of selected MAbs compared with PV2 and 3.

MAb	Titration								Antibody isotyping	Epitope analysis	Protein (concentrated)		
	Pure	Type 1				Type 2	Type 3	+FBS			-FBS		
6H2F5B3	3.060	1/1000	1/2000	1/4000	1/8000	1/16000	1/32000	0.238	0.064	IgG ₁ κ	26 KDa	62.39	22.18
7A7C9F5	2.958	2.880	2.375	1.805	1.518	1.113	0.749	0.306	0.449	IgG ₁ κ	26 KDa	49.78	17.46
7A7C9E2	3.12	2.960	2.723	2.218	1.792	1.316	0.889	0.129	0.07	IgG ₁ κ	26 KDa	54.63	19.15

Figure 1-. SDS-PAGE analysis of the poliovirus serotypes (left) and western bolt of 7A7C9F5 type 1 colon (center) and 3B7F2C3 type 2 colon (right).



DISCUSSION

Poliomyelitis is an infectious disease caused by poliovirus. For the control of this disease, in addition to use of potent and safe vaccines in comprehensive vaccination programs, it is essential to gain a better understanding of the mechanisms responsible for immune recognition, neutralization of viral infection, and epidemiology of the strains prevalent in the community (5, 19). Iran has managed to eradicate the wild-type poliovirus in 2000 via OPV, but the disease is still endemic in Iran's neighboring countries (1, 20). Cross-adsorbed monospecific antisera against polioviruses have been long used for intratypic sero-differentiation and quality control of trivalent polio vaccines. Since the development of hybridoma technology, MAbs have replaced conventional antisera due to numerous advantages (21). This technology has paved the way for production of high-quality MAbs (22) that could be used for rapid characterization of poliovirus isolates, studying the epitopes of poliovirus, neutralization of viral serotypes in trivalent polio vaccine quality control tests, and in epidemiological research (23). Most studies in

this field were conducted after the first successful production of human MAbs by the fusion of patients' spleen cells with myeloma tumor cells in 1980 (24). In an early study, two hybridomas (H3 and D3) capable of secreting monoclonal neutralizing antibodies against PV1 were produced (25). In a study by Gupta et al., pure hybridoma cell lines designated T1VD1CSH1 (type I), T2VB3C5F6 (type 2) and T3RD4B6G6 (type 3) were grown in culture and in LACA strain mice (26). In another study, pooled MAbs had 100-fold greater activity against high-titer Sabin PV3 than rabbit antisera (27). In another study, monoclonal-based IgM capture ELISA was developed for each serotype and for detection of responses to trivalent OPV in 224 infants (28). The objective of this study was to develop and characterize MAbs that could neutralize PV1 for research, diagnosis and prevention purposes. Of 215 hybridoma colons, 35 with the highest antibody titer and lowest cross-reactivity with PV2 and 3 (in screening assays) were selected. The selection of an appropriate screening assay is very critical in hybridoma production. Generally,

the screening assay is chosen based on the antigen characteristics, the laboratory conditions, and the MAb's intended applications. However, regardless of the method used, screening assay should be fast, cost-effective, reliable and simple; otherwise, it would miss the opportunity for harvesting the desired hybridoma cells (29). In this study, ELISA and SN test with a modified protocol were designed as the hybridoma screening assays. In another study, sensitivity of MAbs and ELISA was compared for identification of vaccinated children and those with poliomyelitis (30).

Clones with the highest and most stable antibody titer against PV1 were selected for MAb production via continuous in vitro culture of hybridoma cells in scaled down serum. The MAbs were characterized by titration, isotyping, epitope analysis, protein assay, cross-reactivity test, stability test, sterility test and mycoplasma test. The results revealed that the MAbs were of IgG1 kappa chain, and in western blot, a partial band was detected in the antigenic location of VP3 (26 kDa, neutralization protein). The protein assay showed that in culture of colons in serum-free media, the amount of immunoglobulin had a direct correlation with concentration of antibody. Another study has shown that the VP4 epitopes are conserved between the poliovirus serotypes (31, 32). The results of the MAbs SN test indicated no cross-reactivity

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with PV2 and 3. These antibodies had suitable stability in high temperature and after the freeze-thawing process. These serotype-specific antibodies have great potential for identification of PV1 isolates derived from the Sabin vaccine. However, further studies are necessary for producing MAbs against other serotypes of poliovirus.

CONCLUSION

After evaluation and screening of 250 hybridoma clones by limiting dilution cloning, three clones capable of secreting MAbs against epitopes of PV1 were used for MAbs production. The MAbs (IgG1 kappa chain) have good stability and does not cross-react with other serotypes. We believe that these type-specific MAb antibodies could be invaluable for identification and rapid characterization of poliovirus.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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