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Original Article

Citation: Pharm Biomed Res 2019; 5(3):15-19.

Production and Characterization of a Monoclonal Antibody Neutralizing Poliovirus Type 2

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Received: May 21, 2019**, Accepted:** Aug 18, 2019

Abstract

Monoclonal Antibodies (mAbs) are used for biomedical research, diagnosis, treatment, production, and the quality control of biological products. mAbs are also very helpful in poliovirus research studies because it is still one of the major public health problems in developing countries. The main objective of this study was the production of mAbs against Poliovirus Type 2 (PV2) to be prepared and respond to the re-emergence of this virus. After fusion of immunized B cells prepared from mice with myeloma tumor cells and screening of about 250 hybridoma colonies, 22 with the highest antibody titer and without cross-reaction with others types were selected and cloned by limiting dilution. In the end, two colonies capable of secreting mAbs against epitopes of PV2 were used to produce mAbs. The mAbs were characterized by antibody assays, isotyping, and epitopes analysis using western blotting test, the cross-reactivity with other types, as well as stability, sterility, and mycoplasma tests. The results indicated that the produced mAbs had high specificity, sensitivity and stability against PV2 without any cross-reactivity and were of IgG1 Kappa chain with similar bands at 26 kDa during electrophoresis associated with viral protein VP3 neutralization. These mAbs were specific in serum neutralization tests for PV2 vaccine strain, and therefore, they are potentially valuable for routine polio research, diagnosis, isolation, production, and control of poliovirus vaccines.

Keywords: Monoclonal antibody, Hybridoma, Cloning, Polio type 2, Virus

Introduction

Poliovirus that causes poliomyelitis is represented by three relatively distinct serotypes: type 1 (PV1), type 2 (PV2), and type 3 (PV3). PV2 is the most common form encountered in nature. Nowadays, poliovirus is highly localized to countries in Asia (Pakistan and Afghanistan) and Africa (Nigeria, Niger, and Chad) (1, 2). Inactive Polio Vaccine (IPV) and Oral Polio Vaccine (OPV) are prepared from three strains of Mahoney (PV1), MEF-1/Lansing (PV2), and Saukett/Leon (PV3) (3).

Since PV2 was declared eradicated in the world, IPV is used for the immunity by priming population against this type of poliovirus. To control poliovirus, except for potent and safe vaccines, it is essential to analyze the structure and mechanisms responsible for immune recognition and neutralization of viral infection and epidemiology of polio strains prevalent in the region (wild or vaccine-derived polioviruses) (4). In countries where poliomyelitis is under control, it is critical to determine whether the rare cases of paralytic polio are vaccine-like or non-vaccine-like strains (5), considering that PV2 has been eradicated in Iran like many other countries. It is possible to differentiate poliovirus serotypes by antisera prepared in laboratory animals (6). Cross-absorbed monospecific antisera against polioviruses have been used for many years for intratypic serodifferentiation and the production and quality control of monovalent and trivalent poliovirus vaccines. The interest in the issue of poliovirus neutralization has been recently increased when neutralizing monoclonal antibodies (mAbs) produced and became available. After the development of hybridoma technology by Kohler and Milstein's in 1975

for the production of mAbs, these antibodies were replaced with conventional antisera because of their reliable results (7). This technology is based on forming hybridomas (hybrid cell lines) by fusing an antibody-producing B cell with a myeloma cell that is selected for growing ability in tissue culture and antibody chain synthesis absence (8-10). Although mAbs have been produced and commercialized in some laboratories, they are still not available in many laboratories of the developing countries because of the high cost and the high number of effective factors in hybridoma production (11).

In this regard, in this study, we produced and characterized an mAb which neutralizes PV2 with modifications by immortalization of antigen-specific mouse B cell and hybridoma technology. This mAb could be used to characterize poliovirus isolates rapidly for detection of isolated circulating polioviruses as vaccine or wild strains, and for the study of poliovirus epitopes and neutralizing each serotype in quality-control testing of trivalent polio vaccine to be produced in case of the re-emergence of serotype 2.

Materials and methods

Virus strain and antigen: The Sabin oral vaccine strain stock as Sabin 2 (LSc, 2ab) for mouse inoculation was propagated in MRC-5 (ATCC-CCL-171) (12). After the destruction of the monolayers, the supernatant was clarified by centrifugation at 1000 g and concentrated (25X) with polyethylene glycol (PEG) 6000 (13, 14). Infection titer of the non-concentrated and concentrated supernatants was estimated in Hela cell line (ATCC-CCL-2) by validated method (15), and the amount of protein was calculated by Lowry's method.

Mouse immunization: Female BALB/c mice were inoculated subcutaneously with a mixture of undiluted and concentrated virus and an equal amount of Freund's complete adjuvant, followed by booster injection by an equal content of Freund's incomplete adjuvant 3 and 6 weeks after the first injection. Three weeks later, the mice with the highest antibody titer and received intravenous booster inoculation of the same viral antigen was diluted in phosphate-buffered saline (pH=7.4) in a 1:3 ratio injected directly into a tail vein without using adjuvant (13, 14).

Indirect ELISA and Serum Neutralization (SN) tests designing: A modified-indirect enzyme-linked immunosorbent assay (ELISA) was used for detection of antibody titer in the selected mouse sera with the highest antibody titer and hybridoma clone screening. Serial dilutions of antigen (1:50 to 1:8000) and antibody (1:2000 to 1:128000) were applied in the checkerboard of ELISA, and the optimum dilution of polio antigen was selected for coating ELISA microplate. For the serum neutralization test (SN), 100 $CCID_{50}$ of virus (challenge dose) were reacted with two-fold dilutions (1:80 to 1:640) of the hybridoma supernatant to be tested in Vero cell line (ATCC-CCL-81), and the neutralization endpoints were recorded after 4 to 5 days (15).

The fusion of myeloma and B cells: The spleen cells of mice were fused with the non-secretor mouse myeloma cell strain Sp2/0Ag 14 (Svanova Institute, Sweden) by using PEG 1500 (16, 17). The 96-well microplates were coated with macrophage cells that were collected from peritoneal fluids following intraperitoneal inoculation of sodium glycolate to the BALB/c mice as a feeder layer, and 72 hours later, the cells were distributed in the plates with hypoxanthine-aminopterin-thymidine medium (HAT) (Biotech Resources,1995-7).

Hybridoma screening and cloning: When the first hybridoma colony was detected, the media was replaced with hypoxanthine thymidine (HT) instead of HAT (13, 14). After two weeks, the supernatants of growing hybridomas were tested by ELISA daily up to one month. In each hybridoma screening test, serotypes of poliovirus were tested individually for the evaluation of cross-reactions between these serotypes. The selected hybridomas were tested by the SN method for detection of neutralizing antibodies.

For maintenance of antibody secretion, positive antibody hybridoma was cloned twice by limiting dilution. After one week, the wells with one colony were identified, and the secretion activities were evaluated by ELISA. The colonies with the highest OD and the lowest cross-reaction with other serotypes were transferred to the 24-well microplates (17, 18).

Monoclonal antibody production and characterization: Based on the ELISA titer, the colonies with the highest activity were selected and cultured. The mAbs were produced by continued reduction of Fetal Bovine Serum (FBS) (5). Then the mAbs were allocated and frozen.

The mAbs were tittered against PV2 by the designed ELISA. Their class, subclass, and the type of light chain were determined by Isostrip Kit (Roche, Germany). The antibodies epitope was analyzed by Western blotting (protein immunoblot) using all of the three virus serotypes. The produced mAb was concentrated and purified by 100 kDa filter membrane (Amicon) in 4000 g at 25 °C. Then, the amount of protein was determined by Lowry's method before and after its concentration by media with 5% serum and with no serum (Opti-MEM). For detection of cross-reactivity with other polio types (1 and 3), the mAbs were tested by the previously mentioned ELISA and SN methods. The mAbs were tittered after maintaining for 30 minutes at 50 °C and three times of freeze-thawing for stability compared to the control group. The sterility test was performed for detection of possible fungal or bacterial contaminations (11).

Results

Virus titration: The infection titer of the non-concentrated virus was calculated $10^{7.55}CCID_{50}$/ml vs. $10^{8.69}CCID_{50}$/ml for concentrated supernatant.

The amount of protein in the concentrated supernatant was estimated 0.71 mg/ml.

Evaluation of mouse immunization: The microplate was coated with 1:300 dilution of PV2 antigen. Then, the antibody titers were assayed in the collected sera. The mouse No.5 with the highest antibody titer was selected for fusion based on the designed ELISA results. The SN titer for the chosen mouse was obtained 1.223.

Monoclonal antibody production: The number of mouse spleen cells were 1.7×10^8/ml, and for the myeloma cells it was 0.45×10^8/ml. So, the ratio of spleen cells to myeloma cells was 3:7. Overall, 2.15×10^8/ml cells were used in fusion, and after that, the cells were distributed to eight microplates (2.7×10^6/well). The first hybridoma colony was detected 12 days after fusion and a day later; the SP2 cells were demolished. In this study, 250 hybridoma colonies were achieved. The results indicated that the three colonies producing neutralization antibody had no cross-reactivity with PV1 and PV3, while others had cross-reactions with PV1.

Monoclonal antibody characterization: The supernatant of three hybridoma colonies with the highest antibody titer and the lowest cross-reactivity was collected and characterized in the following order: The antibodies were Immunoglobulin G (IgG1) with kappa chain (Figure 1).

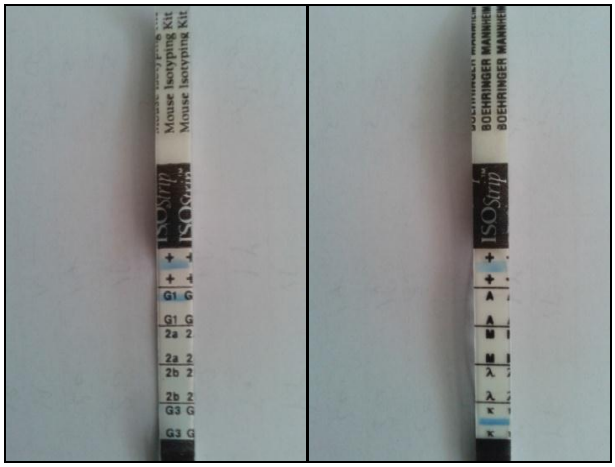

Figure 1 Isotyping of 3B7F2C3 colony, IgG1 (Left) and Kappa chain (Right)

After electrophoresis of poliovirus type 1, 2 and 3 by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), the viral proteins of VP1, VP2, and VP3 produced bands of 33, 30, and 26 kDa, respectively (Figure 2, left). The mAbs were showed the reaction against PV2 in western blot test because there was a band in the range of 26 kDa that was related to VP3 as neutralization viral protein in the antigenic location of electrophorese band, while there was no band with PV1 and PV3 (Figure 2, right). By ultracentrifuge of the 15-mL

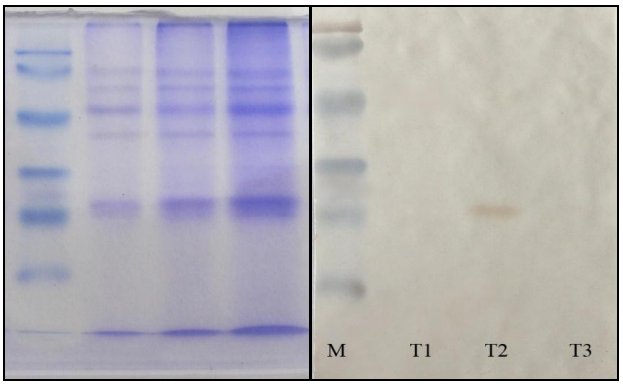

Figure 2 Poliovirus types electrophoresis (Left) and western bolting for 3B7F2C3 colony (kDa) (Right)

colonies' supernatant, the final volume was reported 370 µL. So, a 40-X concentration factor was achieved.
The protein amount of two antibodies in X concentration with FBS was 4.97 and 3.08 mg/ml and without FBS was 1.07 and 1.09 mg/ml. In 40-X concentration, it was 64.57 and 48.27 mg/ml with FBS and 18.31 and 21.23 without FBS mg/ml (Table 1).
The ELISA result of mAbs showed that the colonies had no cross-reaction with PV1 and PV3. These results were confirmed by the SN test (Table 2). The stability result of mAbs showed no significant difference between the initial titers and the titer after placing between at 50 °C and freeze-thawing. All of the mAbs in this study were free of bacterial, fungal, and mycoplasma contaminations.

Discussion

The role of OPV in global polio eradication is crucial (19). Although Iran planned to eradicate the poliovirus in 2000 by using OPVs, the outbreak in neighboring countries affected the program. Also, despite the eradication of the poliovirus type 2 in Iran, the reappearance of this serotype is possible.
For several years, cross-absorbed monospecific antisera for polioviruses have been used for intratypic serodifferentiation of serotypes, production, and quality control of polio vaccines. After the advent of hybridoma technology, mAbs are replacing conventional antisera because of their several advantages (20). Hybridoma technology has long been a suitable platform for monoclonal antibodies production with high quality (21). The mAbs can be used to characterize poliovirus isolates rapidly for detection of circulating polioviruses vaccines from wild strains, studying the poliovirus epitopes, neutralization of each serotype in trivalent polio vaccine quality-control testing as well as in epidemiological studies (22). After the first success in human mAbs production in 1980 by the fusion of human spleen and myelomas cells, many studies have been conducted in this area (23). In one of the first studies, two hybridomas (H3 and D3) secreting monoclonal neutralizing antibody to PV1, were established (24). In a study by Gupta et al. (25), pure hybridoma lines designated three types, including T1VD1CSH1, T2VB3C5F6 and T3RD4B6G6 were grown in LACA strain (mice) and cell cultures.

Table 1 The summary of results on monoclonal antibodies evaluations

mAb	Titration									Ab	Epitope analysis	Protein (Concentrated)	
	Type 2							Type1	Type3	Isotype	Western blot KDa	+FBS	-FBS
	Pure	1/1000	1/2000	1/4000	1/8000	1/16000	1/32000						
3B7H5E7	2.970	2.704	2.422	2.218	1.776	1.360	0.876	0.238	0.231	IgG_1 κ	26	64.57	18.31
3B7F2C3	2.858	2.620	2.345	1.763	1.534	1.106	0.757	0.246	0.214	IgG_1 κ	26	48.27	21.23

In Horie et al. (14) study, pooled mAbs had a neutralizing activity against high-titer PV3 Sabin about 100-fold greater than that of rabbit antisera. In another study, monoclonal-based IgM capture ELISAs were designed for each poliovirus serotype and for detecting responses to trivalent OPV compared with a neutralization assay (26).

Table 2 The results of the SN test

mAb	Type 1	Type 2	Type 3
3B7H5E7	-	+	-
3B7F2C3	-	+	-

The objective of this study was to prepare and characterize mAbs that could differentiate vaccine-like and non-vaccine-like isolates by continued reduction of FBS. This method produced an mAb with high sensitivity and specificity. From 250 hybridoma colonies, 22 colonies with the highest PV2 antibody titer and the lowest cross-reaction with PV1 and PV3 in screening assays were selected. The most crucial part of hybridoma production is the selection of an appropriate screening assay. The screening assay is selected based on the characteristic of the antigen, the laboratory conditions and the facility and application of the mAb in the laboratory (27). In this regard, the ELISA and SN tests with special formula were designed as hybridoma screening assays. The ELISA, based on monoclonal antibody-binding inhibition for the evaluation of vaccinated kids and poliomyelitis sera, showed 100% sensitivity for tracing anti-PV1, 98.3% for anti-PV2, and 96.5% for anti-PV3 antibodies and the specificity was 93.1%, 100%, and 92.7%, respectively compared to the results of SN test (28).

Two colonies of 3B7H5E7 and 3B7F2C3 with the best and stable antibody titer against PV2 were selected for mAb production by in vitro continuous culture of hybridoma cells with scale-down of serum and characterized by eight tests of titration, isotyping, mAb epitope analysis, concentration and purification, protein assay, cross-reactivity test, stability test, sterility and mycoplasma tests. These tests indicated that all mAbs were IgG1 with kappa chain and a party band in a range of 26 kDa as to VP3 (neutralization protein) was formed in the antigenic location of electrophoresis band under western blotting test. The protein assay showed that the culture of colonies by media without serum, had a real amount of immunoglobulin and the immunoglobulin was increased by concentration. Since the epitopes on viral protein VP4 were conserved between poliovirus serotypes (29,30), which may account for the predominantly cross-reactive poliovirus-specific T-cell response and the same antibody binds to PV1 and PV2 through distinct specific interactions, evaluation of mAbs cross-reactivity by SN test is very critical in using antibodies. Hence, the result indicated that the mAb had no cross-reaction with poliovirus type 1 and 3. These antibodies had suitable stability at high temperature and freeze-thawing condition.

Conclusion

Concerning the use of poliovirus monoclonal antibodies for biomedical research, diagnosis, treatment, and production of biological products, the results of mAbs characterization indicated that they had good stability without cross-reactivity and were of IgG1 kappa chain. Also, in western blotting test, the same bands at 26 kDa associated with VP3 neutralization protein were formed during electrophoresis. This type of mAb, which was specific for vaccine strain in neutralization tests, had significant potential for detection of PV2 isolates. Based on the mAb characterization tests, the produced mAbs in this study can be used in polio research, isolation, diagnostic studies, production, and quality control of polio vaccines.

Funding

This paper was supported by Razi vaccine and serum research institute, karaj, Iran.

Authors' Contributions

Sina Soleimani collected the data, set up the tests, performed the interpretation of the results and wrote the manuscript; Morteza Kamalzadeh set up the tests and performed the practical experiments; Mohsen Lotfi contributed to the performing of the experiments.

Conflicts of Interests

The authors declare no conflict of interest.

Acknowledgments

This work is the result of a research project No. 2-18-18-89052, which was conducted in Razi Vaccine and Serum Research Institute, Karaj, Iran in 2010 (before getting banned from working on PV2). The authors would like to thank the staff of Human Viral Vaccine Department and Quality Control Department of Razi Vaccine and Serum Research Institute for their cooperation.

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