CVJ CLUJ VETERINARY JOURNAL

Communication

Assessing two strategies for production of murine ascites with anti-SARS-CoV-2 monoclonal antibodies

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Abstract: Studies were conducted to improve the production of murine ascites with monoclonal antibodies that recognize SARS-CoV-2 proteins. BALB/c mice were primed with 0.5 mL of mineral oil into the abdominal cavity. Seven days after priming, mice were divided in two groups: the group 1 was inoculated intraperitoneally with $2x10^6$ cells/mL of MAb-secreting hybridomas against the nucleocapsid and spike proteins of SARS-CoV-2; the group 2 was injected simultaneously with the same inoculum of hybridoma cells and mineral oil, 18 days after priming. No disturbances or suffering signals were observed in mice from both groups, suggesting that double administration of mineral oil did not produce significant distress with respect to the single dose used for priming, and that none of the hybridoma cell lines were particularly aggressive for the inoculated mice. Ascites was collected in 90.48% and 97.68% of mice from groups 1 and 2, respectively. Ascites was not collected in 7.42% of all mice. The main cause was they never developed ascites tumors but no solid tumors were observed either. The volume of ascitic fluid per mouse was increased significantly in mice from group 2, and there were no significant differences between groups in terms of the concentration of IgG in clarified ascites. According to these results, to obtain higher amounts of MAb the strategy applied in group 2 should be used, since it showed the best results in the development of ascites tumors and it significantly increased the volume of ascites fluid per mouse. This could allow the use of fewer animals for ascites production, which is an ethical and economic benefit.

Keywords: ascites; hybridoma; mice; mineral oil

1. Introduction

At the end of 2019, a new coronavirus began to spread in China to become the pandemic that has cost the most human lives: the SARS-CoV-2. All efforts of healthcare personnel and scientists have had to focus on its rapid diagnosis and treatment. In the strategies to reduce the viral transmission, monoclonal antibodies (MAbs) have become reliable tools, especially for immunoassay techniques used for diagnosis, which are cost-effective, sensitive, rapid and selective [1].

There are basically two main phases in the production of MAbs: the selection of MAb-producing hybridoma cells, generated by the fusion of antibody-producing lymphoid cells from an immunized mouse and murine myeloma cells, and the propagation of selected hybridoma clones, in *vivo* or in *vitro*. The *in vivo* production of MAbs has been carried out by injecting the hybridoma cells into the mice abdominal cavity. The propagation of these cells in the ascitic fluid offers a rapid and economical route to the production of MAbs [2]. *In vitro* production techniques have been developed over the years as alternatives to *in vivo* production of MAbs, but many of these techniques have not been practical due to their high cost, requirements for specialized equipment or their propensity to become contaminated [3]. MAb production in transgenic plants is also a promising technology, since they are considered inexpensive and facile production platforms for recombinant MAbs [4], but it still not solves the great demand of these molecules. Consequently, the total replacement of the ascites method is not yet possible, much less in the current pandemic context, when the rapid and effective production of MAbs is needed for the diagnosis and control of viral transmission.

Received: 20.10.2021
Accepted: 26.11.2021
Published: 31.12.2021

DOI: 10.52331/cvj.v26i3.31

Under these circumstances, medium or large-scale productions of MAbs are required. Medium-scale production is demanded to make 0.1-1.0 g quantities for diagnostic or developing assays [3]. Large-scale production requires an extensive inoculation program with a large number of mice, mostly when a single mouse is inoculated with a single antigen. Hence, from an ethical and economic perception, it would be necessary to acquire efficient and high throughput strategies to maximize the MAb production and to reduce the number of animals used [5].

The Center for Genetic Engineering and Biotechnology of Sancti Spiritus (CIGBSS), Cuba, has been in charge of generating several MAb-secreting hybridomas against the nucleocapsid and spike proteins of SARS-CoV-2 virus. These antibodies must be produced to satisfy the increasing demands of the enzyme-linked immunosorbent assay (ELISA) applied currently for detecting SARS-CoV-2 antigen. The present work aimed to improve the production of murine ascites containing anti-SARS-CoV-2 MAbs, by assessing two strategies for the administration of mineral oil for BALB/c mice: a single injection, as priming agent, 7 days before the inoculation of hybridoma cells and the simultaneous injection of mineral oil and hybridoma cells to mice previously primed with mineral oil. The capability of mice to develop ascitic tumors, the volume of ascites per mouse and the IgG concentration in clarified ascites were monitored to select the best strategy for production of ascites with anti-SARS-CoV-2 MAbs.

2. Materials and Methods

2.1 Hybridoma cell lines

For the production of anti-SARS-CoV-2 MAbs in murine ascites, there were used six hybridoma cell lines generated by CIGBSS, Cuba. Three of them secrete MAbs that recognize the SARS-CoV-2 nucleocapsid protein (CBSSNCOV.2, CBSSNCOV.3 and CBSSNCOV.10) and the others recognize the receptor binding domain (RBD) of the SARS-CoV-2 spike protein (CBSSRBD-S.1, CBSSRBD-S.2 and CBSSRBD-S.3).

2.2 Mouse priming and inoculation

BALB/c male and female mice of 22 ± 1 and 24 ± 1 g of weight, respectively, were used for ascitic fluid production. They were maintained in Eurostandard type II cages (268 mm x 215 mm x 141 mm) at (22-25) °C and 50-65% relative humidity. All animals were primed with 0.5 mL of mineral oil (Zahori, Mexico) in the abdominal cavity. Each hybridoma inoculum was prepared at a dilution of 2 x 10^6 cells per milliliter of RPMI-1640 medium (Sigma, Hybri-Max). Before the inoculation of cells, the mice were divided into two groups: the group 1 was inoculated intraperitoneally with 1 mL of cell suspension seven days after priming, and the group 2 was inoculated analogously to group 1 but 18 days after priming and simultaneously with 0.5 mL of mineral oil.

The mice were observed at least twice daily, including weekends, by personnel familiar with the clinical signs related to the production of ascites, to assess the degree of abdominal distention and to monitor health and well-being.

All the experimental procedures were approved by the Ethical Committee on Animal Experimentation of the Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba).

2.3 Ascites harvest

The extraction of ascites was carried out in two times: 10 and 12 days after the inoculation of the hybridoma cells. Before the ascites extraction, the abdomen of each mouse was cleaned with 70% ethanol. The abdominal paracentesis was performed in the right side of the inguinal-abdominal region with an 18-gauge needle, inserted at a 35-45° angle. The ascites fluid was harvested in 50 mL Corning tubes with 150 µL of 8% EDTA. The ascites was clarified by centrifugation at 1125 x g for 30 min at (22-25) °C and filtered through 0.45 µm glass wool.

2.4 Quantification of IgG in clarified ascites

The quantification of murine IgG in clarified ascites was performed by a sandwich ELISA. Polystyrene 96-well microtiter plates (Costar 3590 High Binding) were coated with 10 µg/mL goat anti-mouse IgG polyclonal antibody in 100µL/well coating buffer (10mM carbonate-bicarbonate buffer, pH 9.6) and incubated 2 h at 37°C. Wells were washed twice with 380µL/well washing buffer (0.05% Tween-20) and blocked with 200µL/well blocking buffer (phosphate-buffered saline, pH 7.2, and 1% nonfat milk). This and the two subsequent steps were carried out 1 h at 37°C. The plate was washed once and 100 µL of ascites diluted 1:20 0000 with blocking buffer were added to wells. The calibration curve was prepared in the range of 150 ng/mL to 2.34 ng/mL using the CBSSPSA.4, supplied by CIGBSS as standard MAb. Plates were washed three times and 100µL of peroxidase

(HRP)-conjugated goat anti-mouse IgG diluted 1:8 000 with blocking buffer were added per well and incubated. After four washes, 100µL/well 5.5mg/mL o-phenylenediamine dihydrochloride with 0.015% hydrogen peroxide in 0.1M citrate-phosphate buffer, pH 5.0, were added and incubated 20 min at (22-25) °C in the dark. The reaction was stopped with 100µL/well of 2M sulfuric acid, and the absorbance was measured at 492 nm in a microplate reader (Labsystems Multiskan® Plus, Finland).

2.5 Statistical analysis

The data of ascites volume per mouse and IgG concentration in clarified ascites, obtained from each group of mice, were analyzed by means of independent samples t-test (α=0.05) using the Statistical Package for Social Science, version 15.0.

3. Results

Mice developed the gradual swelling of the abdomen that accompanies the accumulation of ascites fluid over a period of 7 days after the injection of hybridoma cells. The daily observation did not detect evidence of distress in the animals of both groups under study: their coats were clean and smooth, they maintained food and water consumption and the activity into the cages was normal in all of them.

The first extraction of ascites was performed 10 days after the inoculation of the cells. At that time, the abdominal distention was moderate since ascites fluid volumes did not exceed 20% of the baseline body weight of mice. Each mouse was tapped two times. After the second and last tap, the following clinical irregularities were perceived in some animals of both groups: hunched posture, piloerection and decreased activity.

Table 1 shows the number of mice used in both ascites producing groups and the number of mice from which it was possible to collect ascites in two taps. There were included 889 mice in the study, 29.13% of them received the simultaneous injection of mineral oil and hybridoma cells after being primed with mineral oil. In this group, 97.68% of the mice developed ascitic tumors and it was possible to collect ascites from them, while from the group that received a single dose of mineral oil the ascites was collected in a smaller number of mice (90.47%). Ascites was not collected in 7.42% of all mice; the main cause was they never developed ascites tumors but no solid tumors were observed either. Within this percentage, the minority fraction, 9.09%, corresponded to the group 2. The observations performed by personnel familiar with the clinical signs associated with the production of ascites, did not report differences in the behavior and appearance of the mice, related to the hybridoma cell lines that were inoculated, which means that none of these lines was particularly aggressive to the well-being of mice.

Table 1. Distribution of the mice inoculated with hybridoma cell lines and the mice that produced ascites in both groups under study.

Hybridoma cell lines	No. mice injected		No. mice collected	
	Group 1	Group 2	Group 1	Group 2
CBSSNCOV.2	130	10	124	10
CBSSNCOV.3	100	130	93	126
CBSSNCOV.10	120	50	117	50
CBSSRBD-S.1	150	25	120	23
CBSSRBD-S.2	50	24	39	24
CBSSRBD-S.3	80	20	77	20

Group 1: Mice were inoculated intraperitoneally with $2x10^6$ cells of each hybridoma suspended in 1 mL of RPMI-1640 medium, 7 days after priming with 0.5 mL of mineral oil.
Group 2: Mice were inoculated simultaneously with the same inoculum of hybridoma cells and 0.5 mL of mineral oil, 18 days after priming with 0.5 mL of mineral oil.

The volume of ascites per mouse was calculated by dividing the total volume of ascites harvested by the number of mice collected in each group. The values ranged from 1.38 to 4.90 mL per animal. The independent samples t-test demonstrated that both groups under study were significant different in terms of the volume of as-

cites per mouse. Simultaneous injection of mice with mineral oil and hybridoma cells increased these volumes in all cases with respect to the mice injected with a single dose of mineral oil (Figure 1). The mean ascites volume per mouse in this group was 1.75-fold higher than the estimated mean for group 1.

Fluctuations in the IgG concentration quantified in clarified ascites, both within and between groups, were observed (Table 2). Nevertheless, the analysis of the data from both groups following an independent samples t-test, showed no significant differences in the mean IgG concentration in clarified ascites between both groups (t=1.115; p=0.291). In addition, an approximation of the MAb mass yield per mouse was also similar, 17.64 mg for group 1 and 16.48 mg for group 2.

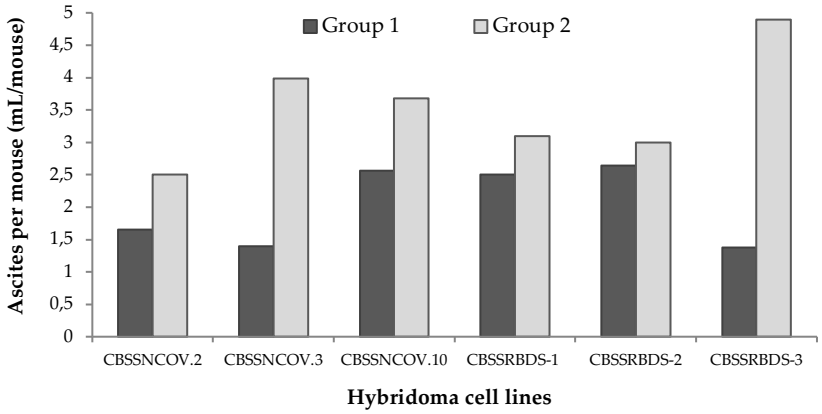

Figure 1. Volume of ascites produced per mouse in both groups under study. All mice were primed with 0.5 mL of mineral oil into the abdominal cavity. Group 1: Seven days after priming, mice were inoculated intraperitoneally with $2x10^6$ cells of each hybridoma suspended in 1 mL of RPMI-1640 medium. Group 2: Eighteen days after priming, mice were inoculated simultaneously with the same inoculum of hybridoma cells and 0.5 mL of mineral oil. The independent samples t-test showed that the volume of ascites per mouse was significant different between the groups (t= -3.747; p= 0.004).

Table 2. Volume of clarified ascites and resultant quantity of IgG from each hybridoma cell line inoculated in both groups of mice.

Hybridoma cell lines	Clarified ascites volume (mL)		IgG in ascites (mg/mL)	
	Group 1	Group 2	Group 1	Group 2
CBSSNCOV.2	215	25	9.06	8.56
CBSSNCOV.3	187	280	7.19	9.05
CBSSNCOV.10	300	82	9.95	6.28
CBSSRBD-S.1	300	72	2.49	5.81
CBSSRBD-S.2	103	72	13.04	3.64
CBSSRBD-S.3	265	98	6.83	3.67

Group 1: Mice were inoculated intraperitoneally with $2x10^6$ cells of each hybridoma suspended in 1 mL of RPMI-1640 medium, 7 days after priming with 0.5 mL of mineral oil.
Group 2: Mice were inoculated simultaneously with the same inoculum of hybridoma cells and 0.5 mL of mineral oil, 18 days after priming with 0.5 mL of mineral oil.

4. Discussion

In this work we assayed two strategies for production of murine ascites containing anti-SARS-CoV-2 MAbs using BALB/c mice. Since sensitive techniques have not been developed to measure signs that might indicate the presence of pain or distress [6], one of the most important tasks in the present study was the daily observation of the mice involved, including the examination and palpation of the injection site, the evaluation of abdominal distention and the detection of all possible side effects of the injected mixture. Some hybridoma cell lines can produce clinical signs in mice indicating distress, such as anorexia, hunched posture, hypothermia, rapid breathing and decreased activity [6]. But none of the six hybridoma cell lines used in this work caused worrisome adverse effects as a sign of an aggressive cell line, and this was an important ethical advantage.

Regarding the timing of priming agent administration in relation to the inoculation of the hybridoma cells, it is common practice to perform priming and several days later to inject the hybridoma cell-suspension into the peritoneal cavity of the mouse. This leads to the development of a tumor, the accumulation of ascitic fluid and the abdominal distention [2, 6]. However, it is not usual the simultaneous administration of the hybridoma cells with an additional dose of the priming agent. In the present study, the objective of the second administration of the mineral oil was to increase the throughput in the production of ascites. Mineral oil is thought to act by inducing granulomatous inflammation and interfering with peritoneal lymphatic drainage, thus increasing the volume of ascites produced [7]. We consider that the purpose was achieved because it was possible almost to double the volume of ascites per mouse in the group that received the second injection of mineral oil. This procedure could cause animal distress, but it did not occur in the mice included in this work. Some authors have evaluated the effects of priming agent injection on mouse well-being and ascites production, using parameters related with the mouse activity and food and water consumption [8]. In our study, the observation of mice at least twice a day by qualified personnel, did not inform significant evidences of distress or pain in the animals, since they maintained their normal activities and external appearance until the second tap. The volume of ascitic fluid did not cause gross abdominal distention even when a double volume of the priming agent was administered; moreover, the abdominal tap was done before fluid accumulation became excessive and distressful, following the recommendations that it not exceed 20% of the baseline body weight of the mouse [6].

Ascites yields can be improved also by performing several harvests; nevertheless, the well-being of mice must be strictly observed. The number of taps should be restricted according to animal welfare and characteristics of the hybridoma being used. Some hybridomas seem to cause little distress and various taps could be permissible [9], but the prolongation of tapping time increases the pathological abnormalities in the mice due to solid tumor growth within the peritoneal cavity and the accumulation of ascites [10]. Various guidelines and reports have required that the abdomen may be tapped no more than twice before the mouse is euthanized for final harvest of ascites [2, 8, 11]. The present study was careful with this aspect, since ascites pressure was relieved before abdominal distention was great enough to cause discomfort or disturb the normal activity of the animals, and only two taps were performed to obtain ascites, despite the fact that the hybridomas inoculated did not cause adverse effects in mice.

A crucial finding was that there were no significant differences in the concentration of IgG in ascites and in the mass yield of MAb per mouse between the groups analyzed. According to these results, in order to obtain a greater mass of MAb, the strategy applied in the group of mice that significantly increased the volume of ascites fluid per mouse must be taken into account: the simultaneous injection of hybridoma cells and mineral oil eighteen days after priming. Furthermore, the lowest percentage of mice that never developed ascites tumors was found in this group. Consequently, this strategy could allow the usage of fewer animals for the production of ascites. It is clear that the number of mice to be used will be determined by the total amount of MAbs needed [10]; but, certainly, the reduction of the amount of animals required for the MAbs production offers important ethical and economic advantages.

Medium-scale production of MAbs is demanded to make 0.1-1.0 g quantities for diagnostic or developing assays [3]. Applying the strategy used in group 2 it was possible to produce more quantities than 1.0 g of each MAb. For example, mice included in this group produced approximately 4.0 g of CBSSNCOV.3 and more than 1.0 g of CBSSNCOV.10. Both MAbs are being used in the ELISA developed in our country for the diagnosis of SARS-CoV-2 by detecting viral antigen, and they are constantly demanded for immediate diagnosis as urgent necessity of current epidemiological scenario. Therefore, with the simultaneous injection of hybridoma cells and mineral oil in previously primed mice, it is possible to guarantee the production of required quantities of both MAbs with a minimum number of animals involved.

The MAbs against the RBD of the SARS-CoV-2 spike protein (CBSSRBD-S.1, CBSSRBD-S.2 and CBSSRBD-S.3) could play an important role in subunit vaccines development. For this reason, it is necessary to optimize all the productive stages to ensure the availability of these MAbs for future assays, and this work also contributes to this.

5. Conclusions

The double administration of mineral oil, for priming and for the inoculation of hybridoma cells, does not produce additional stress in the mice. None of the six anti-SARS-CoV-2 MAb-secreting hybridoma cell lines, which were injected to mice, are harmful to the animal well-being. The simultaneous inoculation of the hybridoma cells with the mineral oil to the primed mice improves the efficiency of ascites tumor formation and significantly increases the volume of ascites per mouse. This inoculation strategy will optimize the production of ascites with anti-SARS-CoV-2 MAbs, involving fewer animals, which translates into ethical and economic benefits.

Author Contributions: Conceptualization, J.J.P.P. and M.P.B; methodology, J.J.P.P., R.B., D.D., A.D., C.T., R.P., J.D. and I.P.; validation, S.P.; formal analysis, C.H.; writing—original draft preparation, M.P.B. and J.J.P.P.; writing—review and editing, R.B., A.D., D.D. and M.P.B.; supervision, R.B. and E.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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