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Short communication

Utilization of leucine methyl ester for the generation of hybridomas producing monoclonal antibodies specific to tumor-associated antigens

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Abstract. In an attempt to obtain monoclonal antibodies specific to tumor-associated antigens, C3H/He mice were immunized with syngeneic MM2 tumor cells, and the primed spleen cells were fused with P3-X63-Ag8.653 myeloma cells. The outgrowth of hybridomas, however, was extremely low and monoclonal antibodies were not obtained. The reason for the low hybridoma growth was studied. It was found that MM2 cells used as the immunogen, the fusion partner myeloma cells and the resulting hybridomas shared at least one tumor-associated antigen, namely Q5 antigen. Because of this common antigen, cytotoxic cells, presumably cytotoxic T lymphocytes, which were lytic to the hybridomas, were induced during the culture for generation of the hybridomas. Removal of lysosome-rich cells, including cytotoxic T lymphocytes, from the primed spleen cells before the fusion by treatment with leucine methyl ester, a lysosomotropic agent, drastically improved the outgrowth of hybridomas. By this method, seven stable hybridoma clones producing monoclonal antibodies specific to tumor-associated antigens were obtained. Two of the seven clones were found to secrete monoclonal IgM species, which reacted with the extra-cellular region of the Q5 antigen. This procedure will be an option when production of monoclonal antibodies specific to cell-surface antigens is intended and outgrowth of hybridomas is unexpectedly low.

Key words: Hybridoma – Leucine methyl ester – Tumor-associated antigen – Monoclonal antibody

Introduction

We have reported that MM2 mouse mammary tumor cells express the Q5 gene product (Q5 antigen, one of the non-classical histocompatibility class I antigens [7]) and other antigens that are not expressed in normal adult C3H/He mice from which the MM2 cells were derived, that the Q5 antigen is expressed commonly on the surface of various experimental tumor cells of mice, and that Q5-specific antibody of the IgD class was detected in the serum of C3H/He mice obtained after transplantation and regression of MM2 tumor cells [6, 8]. We tried to generate hybridomas producing monoclonal antibodies (mAb) specific to the Q5 and other tumor-associated antigens expressed on the MM2 cells by fusing P3-X63-Ag8.653 myeloma cells (X63 cells) and MM2-primed lymphocytes obtained from MM2-immune C3H/He mice. We had difficulty in doing so because outgrowth of only an unexpectedly limited number of hybridomas was observed when the standard method was employed. Since the Q5 antigen was detected on the surface of most of the established experimental tumor cells of mice, we suspected that X63 cells and the X63-derived hybridomas also expressed the Q5 antigen, that Q5-specific cytotoxic T cells, lytic to the Q5-positive hybridomas, were generated from the Q5-sensitized lymphocytes during the culture with X-63 cells after the fusion, and that removal of the cytotoxic cells might result in better growth of the hybridomas. Here we report the results of our attempt to prove this assumption by eliminating cytotoxic cells from the sensitized lymphocytes using leucine methyl ester (LeuOMe) [1, 9].

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Materials and methods

Mice, tumor cells and immunization. Male C3H/He mice, about 8–10 weeks of age, were used. C3H/He mice lack the expression of any of the lymphocyte-specific non-classical histocompatibility Qa/TL antigens including the Qa-2 and the Q5 antigens [5, 6]. MM2 ascites tumor cells, derived from a mammary carcinoma of a C3H/He mouse, were used to immunize C3H/He mice. As reported previously, MM2 ascites tumor shows regression after transplantation and simple removal of the tumor cells from the hosts [3]. The regressor mice were immunized repeatedly by biweekly intraperitoneal inoculation of 5×10^6 MM2 cells (MM2-immunized mice).

Cell fusion and culture. Spleen cells were obtained from the MM2-immunized mice 4 days after the third immunization as well as from

the MM2-regressor mice 1 week after the removal of the tumor cells. The cell fusion was carried out by a standard method. Briefly, 1×10^8 spleen cells after removal of red blood cells [2] were fused with 2×10^7 X63 cells using polyethylene glycol 1500 (Boehringer Mannheim GmbH, Germany) and suspended in Dulbecco's modified minimal essential medium (DMEM) containing 100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine (HAT) and 10% fetal calf serum (FCS) at a concentration of 3×10^6 cells/ml. In each well of a flat-bottomed 96-well plate (Corning, N.Y.), 3×10^5 cells were seeded. The plates were incubated at 37° C in a CO_2 incubator. The number of wells that showed growth of hybridomas was counted 2 weeks after the start of the culture. Recloning of hybridomas was carried out by limiting dilution.

Treatment of cells with LeuOMe. LeuOMe was purchased from Sigma (St. Louis, Mo.). Spleen cells, after removal of red blood cells, were incubated in 2.5 mM LeuOMe in DMEM at a concentration of 1×10^7 cells/ml for 40 min at 37° C in a CO_2 incubator. The cells, after the incubation, were washed three times with DMEM and then used for the generation of hybridomas.

Demonstration of cytotoxic activity. Spleen cells, obtained from C3H/He mice, after depletion of red blood cells, were mixed with intact X63 cells at a ratio of 5:1 and suspended in DMEM containing 10% FCS and HAT at a concentration of 3×10^6 cells/ml. A 2-ml sample of the cell suspension was placed in each well of a 12-well plate. The cells were cultured for 5 days at 37° C in a CO_2 incubator, harvested and used as effector cells in the cytotoxicity assay. The assay was carried out by incubating various numbers of the effector cells with 1×10^4 ^{51}Cr-labelled X63 cells at 37° C for 18 h in 0.4 ml of DMEM supplemented with 10% FCS. After the incubation, the radioactivity released from the cells into the medium, and that remaining in the cells, was determined and the cytotoxic activity was expressed in terms of percentage specific lysis as described previously [4].

Detection of mAb by ELISA. Hybridoma culture supernatants were screened for antibodies reactive to MM2 cells by enzyme-linked immunosorbent assay using MM2-immobilized plates (cell ELISA). Each well of flat bottomed 96-well plates for ELISA (Nunc, Denmark) were treated with 50 µl of 0.1 mg/ml poly-L-lysine (Sigma) solution in phosphate-buffered saline (PBS) at 4° C overnight and then washed twice with PBS. A 50-µl sample of MM2 cell suspension (2×10^6 cells/ml) in DMEM was placed in each well and the plates were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 0.025% glutaraldehyde solution in PBS was added (100 µl/well). The plates were kept at room temperature for 30 min and washed three times with PBS. The wells were then filled with 1% bovine serum albumin solution in PBS and kept for 2 h at room temperature. After washing three times with PBS, 50 µl hybridoma culture supernatant was added to a well and the plates were kept for 90 min at room temperature. The supernatant was removed and the wells were washed three times with PBS. Then 50 µl 1000× diluted sheep anti-(mouse immunoglobulin) antibody conjugated with horse radish peroxidase (Amersham, UK) was added to each well and the mixture was kept for 1 h at room temperature. The wells were then washed three times with PBS. The presence of mAb reactive to MM2 cells was detected by coloration after 100 µl ABTS peroxidase substrate and 100 µl 0.02% hydrogen peroxide solution (Kirkegaad & Perry Laboratories Inc., Gaithersberg, Md.) had been added to each well.

Screening for mAb reactive to the Q5 antigen was carried out by an ELISA using the 96 well plates coated with a fusion protein, constructed of the extra-cellular region of the Q5 protein, and the lacZ protein produced as described previously [6]. The reactive antibodies were detected by the peroxidase-conjugated anti-(mouse immunoglobulin) sheep antibody described above.

Production of mAb of the IgD class was detected by an ELISA as described previously [8] using the 96-well plates coated with sheep anti-(mouse IgD) serum, and peroxidase-labelled rat mAb specific to mouse IgD (Meiji Institute of Health Science, Kanagawa, Japan).

Membrane immunofluorescence method. Detection of cell surface Q5 antigen was carried out by flow cytometry as described previously [6] using a Qa-2-specific mAb 141–15.8 (American Type Culture Collection, Rockville, Md.), which cross-reacts with the α3 domain of the Q5 antigen, and the fluorescein-isothiocyanate (FITC)-labelled $F(ab')_2$ fraction of goat antibody specific to mouse immunoglobulins (Tago Inc., Burlingame, Calif.). The FITC-labelled cells were analyzed using a FACS III apparatus (Becton Dickinson & Co., Mountain View, Calif.). Qa-2-specific mAb 34–1.2, which did not cross-react with the Q5 antigen, was also used. Treatment of the cells with phosphatidylinositol(PtdIns)-specific phospholipase C was carried out as described previously [6].

Determination of immunoglobulin subclasses. This was carried out by a double-diffusion method using a mouse monoclonal typing kit (The Binding Site, Birmingham, UK) and anti-(mouse IgD) sheep serum (ICN ImmunoBiologicals, Lisle, Ill.).

Table 1. Detection of Q5 antigen on X63 cells, X-63 hybridomas and MM2 cells by flow cytometry

Cells	Log (mean fluoresence intensity) in the presence of the following first mAb	
	None (control)	mAb 141-15.8
X63	80.4	183.2
Hybridoma 1	98.1	162.7
Hybridoma 2	105.3	169.2
MM2	102.1	143.5
BW5147	75.2	133.3
C3H/He spleen cells	46.8	47.6

Results

Cytotoxic activity of spleen cells cultured for hybridoma generation.

It has been reported that Q5 antigen expressed on the surface of tumor cells cross-reacted with Qa-2-specific mAb 141–15.8 but not with mAb 34–1.2 [6]. On the other hand, cell-surface Qa-2 antigen (the Q7 gene product) reacted with both mAb. It has also been found that the cell-surface Q5 antigen is resistant to PtdIns-specific phospholipase C treatment whereas the Qa-2 antigen is released from the cell surface by the treatment [6]. As shown in Table 1, X63 cells and the derived hybridomas, as well as MM2 cells, reacted with the mAb 141–15.8. Under identical conditions, Q5 antigen was detected on BW5147 cells, which were proved to express Q5 antigen [6], whereas it was not detected on C3H/He spleen cells, which were shown to lack expression of the antigen. From the above criteria, it was concluded that the antigen detected by mAb 141–15.8 on these cells was the Q5 antigen (data not shown). The Q5 antigen was detected on the surface of all hybridomas arbitrarily chosen from a stock of X63 hybridoma clones, from which two representative examples are shown here. Since the Q5 antigen is expressed also on MM2 cells used for in vivo immunization of the $Q5^-$ C3H/He mice, there was a possibility that cytotoxic lymphocytes, presumably cytotoxic T cells, specific to the Q5 antigen, and perhaps those specific to other tumor-associated antigens, could be induced by culturing the spleen cells from the MM2-im-

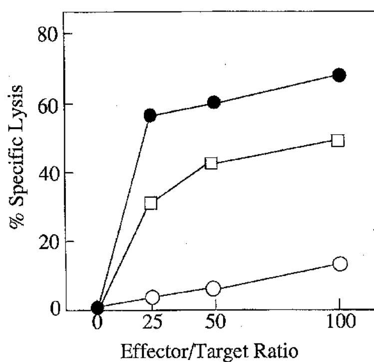

Fig. 1. Induction of cytotoxic activity during culture of MM2-regressor C3H/He spleen cells with X63 cells under conditions mimicking those used for hybridoma generation. The spleen cells were obtained from the regressor mice 1 week after the removal of the MM2 cells and the cytotoxic activity was assayed in the absence (●) and in the presence (□) of mAb 141–15.8. Cells treated with LeuOMe were also used (○). X-63 cells were used as the target cells of the cytotoxicity reaction. Values show means of duplicate determinations

Table 2. Generation of hybridomas by fusion of X63 cells and spleen cells from MM2-immunized or MM2-regressor C3H/He mice with or without LeuOMe pretreatment

Cells and pretreatment with LeuOMe	Number of wells seeded	Number of hybridoma-positive wells[a]
MM2-immunized mouse spleen cells		
–	288	32 (11.1%)
+	288	192 (66.7%)
MM2-regressor mouse spleen cells		
–	1904	2 (0.1%)
+	1175	914 (77.8%)
Normal mouse spleen cells		
–	117	15 (12.8%)
+	95	13 (13.7%)

[a] The number of wells with hybridoma growth was counted 2 weeks after the onset of the culture

mune C3H/He mice with X63 cells in vitro. Figure 1 shows that cytotoxic lymphocytes that were lytic to X63 cells were actually induced under conditions that mimicked those used for generation of the hybridomas using spleen cells obtained from the MM2-regressor mice. When spleen cells from the MM2-immunized mice were used, more pronounced cytotoxic activity was induced. From the spleen cells with cytotoxic activity, eight cell clones with cytotoxic activity were established by culturing in the presence of interleukin-2 and repeated antigenic stimulation with mitomycin-C-treated X63 cells. All of these clones were found to bear T cell receptor of the α/β type and to be $CD3^+$, $CD4^-$ and $CD8^+$. The cytotoxic activity was also partly inhibited by addition of mAb 141–15.8 to the cytotoxicity reaction mixture. These results indicate that the immune spleen cells after culture with X63 cells contained cytotoxic T lymphocytes with specificity to the Q5 antigen. Similar cytotoxic activity was detected when X63 hybridomas were used as the target cells (data not shown). The induction of the cytotoxic activity was abrogated when the MM2-primed lymphocytes were treated with LeuOMe before culturing with X63.

Effect of LeuOMe on the hybridoma generation.

Table 2 shows the results of hybridoma generation. When spleen cells from the MM2-immunized mice were used, 11.1% (32/288) of the wells showed outgrowth of the hybridomas. The number increased to 66.7% (192/288) when the spleen cells were pretreated with LeuOMe. Among the resulting 192 hybridoma-positive wells, 9 wells contained hybridomas producing mAb that were found to react with MM2 cells by cell ELISA. From the cells in these 9 wells, seven stable hybridoma clones producing mAb reactive to MM2 cells were established. They consisted of four IgM-producing clones, one IgG1-producing clone, one IgG2a-producing clone and one IgG2b-producing clone. On the other hand, none of the 32 hybridoma-positive wells in the experiment without LeuOMe treatment contained cells producing MM2-reactive antibodies. Among the seven MM2-reactive mAb, two mAb of the IgM class were found to react with the Q5-lacZ fusion protein. This result shows that these mAb recognize the extra-cellular region of the Q5 antigen. The exact antigen specificities of other mAb obtained are still to be determined.

Spleen cells from the MM2-regressor mice were used for trials to generate MM2-reactive mAb of the IgD class. The percentage of hybridoma-positive wells in the total wells was negligible (2/1904) when LeuOMe treatment was not employed, whereas the number increased to 77.8% (914/1175) after pretreatment with LeuOMe. Among the resulting 914 positive wells, cells in 2 wells produced IgD. From these 2 wells, two IgD-producing hybridoma clones were established. These mAb of the IgD class did not react with MM2 cells, however. Since the concentration of MM2-specific IgD in the serum of MM2-regressor mice was quite low, it will be necessary to screen a substantially larger number of hybridoma clones to obtain such an mAb.

In control experiments, spleen cells from non-immunized mice were fused with X63 cells. Without LeuOMe pretreatment, growth of hybridomas was detected in about 13% of the wells, and the enhancing effect of LeuOMe pretreatment was not observed. This result shows that the enhancing effect of LeuOMe treatment is caused by removal of cells induced by the immunization.

Discussion

LeuOMe is a lysosomotropic agent and is toxic to lysosome-rich cells such as large granular lymphocytes, monocytes, cytotoxic T cells and a population of $CD8^+$ T cells. It has been reported that in monocytes, LeuOMe disrupts lysosomes and is subsequently converted to Leu-LeuOMe, which acts directly on the susceptible cells and kills them [9]. LeuOMe does not affect B cells and helper T cells. Borrebaeck et al. utilized LeuOMe for the production of human mAb using peripheral blood lymphocytes by in vitro immunization [1]. In this case, it was thought that

treatment with LeuOMe removed cells that exerted a nonspecific suppressive effect on B cell function.

In the present communication, we report that cytotoxic lymphocytes were induced during culture for hybridoma generation when cells used as the immunogen and the fusion partner lymphoma cells shared common antigens. Such a case may not be rare when the generation of mAb specific to common tumor-associated antigens is attempted. Removal of the cytotoxic cells by treatment with LeuOMe resulted in a drastic improvement in hybridoma growth. This procedure will also be effective when production of mAb specific to some allogeneic cell-surface antigens is attempted and the fusion-partner lymphoma cells express the antigen. It will be an option to try when the production of mAb specific to cell-surface antigens is attempted and outgrowth of hybridomas is unexpectedly low.

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References

1. Borrebaeck CA, Danielsson L, Moeller SA (1988) Human monoclonal antibodies produced by primary in vitro immunization of peripheral blood lymphocytes. Proc Natl Acad Sci USA 85: 3995
2. Boyle W (1968) An extension of the ^{51}Cr-release assay for the estimation of cytotoxins. Transplantation 6: 7614
3. Kimura Y, Tanino-Komeiji T (1966) Survival of host mice and induced resistance to transplantable ascites tumors. Jpn J Exp Med 36: 371
4. Kiyohara T, Tanino T, Egawa K (1982) T cell-dependent nonspecific cytotoxicity induced by culture of mouse spleen cells against natural killer-insensitive tumor cells. Jpn J Exp Med 52: 201
5. McKenzie IFC, Potter T (1979) Murine lymphocyte surface antigens. Adv Immunol 27: 179
6. Seo N, Okazaki T, Nakanishi-Ito C, Tanino T, Matsudaira Y, Takahashi T, Egawa K (1992) Expression of the Qa-2^{k} phenotype encoded by the Q5^{k} gene on the surface of tumor cells derived from H-2^{k} mice. J Exp Med 175: 647
7. Steinmetz M, Winoto A, Minard K, Hood L (1982) Cluster of genes encoding mouse transplantation antigens. Cell 28: 489
8. Tanino T, Seo N, Okazaki T, Nakanishi-Ito C, Sekimata M, Egawa K. (1992) Detection of allogeneic Qa/TL and Ly specificities on murine tumor cells with IgD in tumor-regressor mouse serum. Cancer Immunol Immunother 35: 230
9. Thiele D, Lipsky PE (1986) The immunosuppressive activity of L-leucyl-L-leucine methyl ester: selective aberration of cytotoxic lymphocytes. J Immunol 136: 1038