Non-mitogen containing conditioned medium for hybridoma production and single cell cloning

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Summary

Background: **The hybridoma technique is the standard method for production of monoclonal antibodies of interest. However, the newly formed hybridomas and cells at low density often grow poorly or die. This is the major obstacle to production of monoclonal antibodies.**

Objective: **The aim of this study was to establish a method for preparation of conditioned medium in the absence of mitogen for promoting the growth of hybridomas after cell fusion and during single cell cloning.**

Methods: **Culture supernatants were obtained from the cultures of BW5147 mouse thymoma cells without mitogen stimulation. Novel conditioned mediums were investigated for their ability to support hybridoma single-cell cloning and hybridoma production.**

Results: **We demonstrated that these conditioned mediums could support hybridoma single-cell growth, both for stable and newly generated hybridomas, at a level equal to the commercial conditioned medium BM-Condimed H1. The conditioned medium was most effective at a final concentration of 20% with the basal medium supplemented with 10% fetal calf serum. The novel conditioned medium could also be effectively employed for generation of hybridomas secreting various monoclonal antibodies. Interestingly, fibroblast overgrowth in the post-fusion wells was markedly reduced when using the novel conditioned medium, as compared to the commercial BM-Condimed H1, likely due to the absence of mitogen in the conditioned medium.**

Conclusion: **We describe the method for production of a novel conditioned medium for hybridoma technology. This method is simple, needing no special technology or sophisticated equipment. The novel conditioned medium is, therefore, recommended for use in place of expensive commercial conditioned medium for hybridoma technology, especially in resource-limited countries.** ***(Asian Pac J Allergy Immunol 2012;30:114-22)***

Key words: *conditioned medium, hybridoma, single cell cloning, monoclonal antibody, BW5147 mouse thymoma cells*

Introduction

Since its discovery by Kohler and Milstein in 1975, hybridoma technology has become widely adopted for the production of monoclonal antibody (mAb).[1] To produce mAb by the standard hybridoma technique, spleen cells of an antigen immunized mouse are fused with myeloma cells. The fused cells are then plated onto tissue culture plates. The hybridomas are generated and screened for their secreted antibodies. Each original antibody positive well often contains more than one hybridoma clone, only one of which is producing the desired antibody. The non-producers, however, tend to overgrow the producer, leading to failure of production of the focal mAb. To minimize the overgrowth by the non-antibody secreting cells, hyrbidoma single cell cloning needs to be performed as soon as the desired hybridomas are identified. However, post-fusion newly generated hybridomas and hybridomas that are grown at low density often grow poorly or die. This is due to the fact that

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growing mammalian cells, including hybridomas, requires various bioactive autocrine and paracrine growth factors.[2-4] Supplements of such factors can thus support cell growth. Feeder layer cells to provide factors that enhance hybridoma growth have been widely used in the past.[5-9] However, feeder cells in hybridoma culture have several serious drawbacks. Preparation of feeder cells is labor intensive and imposes time constraints on hybridoma preparation. During cultivation, feeder cells metabolize nutrients, necessitating frequent changes of medium. Dividing cells in the feeder culture may overgrow and kill the desired hybridomas. In addition, feeder cells also present a potential source of microbial contamination.

During the last two decades, various substitutes for feeder layers have been considered. Conditioned media produced from various cell types and containing the necessary factors for supporting hybridoma growth have been described.[10-15] Some of the conditioned media have become commercial products. Although the commercial conditioned media can be used as growth supplements for murine hybridomas, they are very expensive. In addition, the commercial conditioned media are prepared from culture supernatants of cell lines stimulated with mitogens, e.g. phorbol myristate acetate (PMA). These conditioned media, therefore, contain mitogen in addition to the growth factors. In some cases, in using of hybridoma culture supernatant as the source of mAb, the mitogen may affect the relevant biological assay, leading to misinterpretation of the results. Moreover, upon hybridoma production, the presented mitogen induces fibroblasts in the spleen cells to proliferate[16-18] and finally overgrow and kill the newly formed hybridomas. To overcome these problems, an effective conditioned medium, containing no mitogen, for supporting growth of freshly fused hybridomas and of single-cell during cloning would be required.

In the present study, we describe a method for production of a novel conditioned medium in the absence of mitogen for promoting growth of hybridomas. This conditioned medium can replace the expensive commercial conditioned mediums,

Table 1. Support of hybridoma single-cell growth by BW conditioned medium.

Condition	**Size (Day5)			**Size (Day7)			**Size (Day10)			**Size (Day14)			***Single clone
	S	M	L	S	M	L	S	M	L	S	M	L	(day 14)
Exp. 1													
Medium only	30	-	-	39	-	-	26	-	-	21	2	-	23
BW conditioned medium (18 h)*	34	-	-	33	-	-	29	2	-	15	15	-	30
BW conditioned medium (40 h)*	25	-	-	25	-	-	19	6	-	3	16	6	25
BM-Condimed H1	26	-	-	28	-	-	15	11	-	2	13	11	26
Exp. 2													
Medium only	22	-	-	24	-	-	18	-	-	10	3	-	13
BW conditioned medium (18 h)*	22	-	-	22	-	-	17	2	-	11	6	1	18
BW conditioned medium (40 h)*	30	-	-	32	-	-	27	4	-	12	14	1	27
BM-Condimed H1	26	-	-	25	1	-	19	6	-	3	14	6	23
Exp. 3													
Medium only	29	-	-	29	-	-	29	-	-	28	1	-	29
BW conditioned medium (18 h)*	25	-	-	25	-	-	19	6	-	11	8	6	25
BW conditioned medium (40 h)*	24	-	-	24	-	-	12	12	-	3	17	4	24
BM-Condimed H1	40	-	-	40	-	-	15	22	3	8	16	15	39

A stable hybridoma clone Thal N/B was subjected to single cell cloning by limiting dilution using 10%FCS-IMDM supplemented with 50% BW conditioned medium or 10% BM-Condimed H1 or no supplement. Three independent experiments were performed. The size and number of hybridoma containing wells were determined at day 5, 7, 10, and 14 of cultivation by an inverted microscope.

*BW5147 thymoma cell line was cultured for 18 hours (18 h) or 40 hours (40 h). The culture supernatants were harvested and used as conditioned medium.

**Number of wells containing hybridoma single clone at various clone sizes. The size of a hybridoma single clone was scored as described in materials and methods. S; small, M; medium, L; large

*** Number of wells containing hybridoma single clone at day 14 of cultivation. There was no statistical difference in he number of wells containing hybridoma single clone at day 14 compare between BW conditioned medium (40 h) and BM-Condimed H1 by using Mann-Whitney U test T test (P =0.83).

thus reducing the cost of monoclonal antibody production.

Methods

Preparation of conditioned mediums

BW5147 mouse thymoma cells, kindly provided by Prof. Hannes Stockinger (Department of Molecular Immunology, Medical University of Vienna, Austria), were cultured at $1x10^6$ cells/ml of Iscove's Modified Dulbeco's Medium (IMDM; GIBCO BRL, Grand Island, NY, USA) containing 10% fetal calf serum (FCS), gentamycin 40 mg/l and fungizone 2.5 mg/l (10% FCS-IMDM) at 37 °C in a 5% CO_2 incubator. After 18 or 40 hours of cultivation, the culture supernatants were harvested. Cells and debris were removed by centrifugation at 550 g for 5 minutes. The supernatant was filtered through a 0.1 µm membrane filter (Pall Corporation, Ann Arbor, MI, USA). The obtained supernatants (named BW conditioned medium) were aliquoted and stored at -20 °C until use.

Hybridoma single cell cloning

Hybridomas were counted and cell concentrations were adjusted to 4 cells, 2 cells and 1 cell per 150 µl in 10% FCS-IMDM, 10% FCS-IMDM supplemented with various concentrations of BW conditioned medium, or 10% FCS-IMDM supplemented with 10% commercial BM-Condimed H1 (Roche, Penzberg, Germany). Then, 150 µl of each cell suspension were added in to a 96-well tissue culture plate (Costar, Corning Incorporated, Corning, NY, USA). The cells were cultured at 37°C in a 5% CO_2 incubator. At days 5, 7, 10 and 14 of cultivation, the sizes of the generated hybridoma clones and numbers of wells containing single hybridoma clones were determined under an inverted microscope**.** The clone size was scored as one of three levels, small (S), medium (M) and large (L) as described below.

S: clone size less than 1/8 of well; M: clone size between 1/8-1/4 of well; L: clone size more than 1/4 of well.

Hybridoma production

BALB/c mice were intra-peritoneally immunized with tested antigens three times at two week intervals. After the third immunization, mice were boosted with the same antigens. Five days after boosting, spleen cells were aseptically prepared and fused with myeloma cells (P3-X63Ag8.653) by a standard hybridoma technique using 50% polyethylene glycol (PEG; Sigma-Aldrich, St-Louis. Mo, USA). After cell fusion, cells were resuspended in HAT selective medium (Sigma-Aldrich) supplemented with 10% BM-Condimed H1 or 20% BW conditioned medium to obtain a cell concentration of 3 x 10^5 cells/ml. 100 µl of cell suspension were seeded into each well of 96-well plates (Costar) using 5 plates for each supplement. The plates were cultured at 37°C in a 5% CO_2 incubator. After 5 days of cultivation, 150 µl of HT medium (Sigma–Aldrich) supplemented with BM-Condimed H1 or BW conditioned medium were added into each corresponding well. The plates were further incubated at 37°C in a 5% CO_2 incubator for hybridoma generation. The generated hybridomas

Table 2. Support of hybridoma single-cell growth by various concentrations of BW conditioned medium.

Condition	**Size (Day5)			**Size (Day7)			**Size (Day10)			**Size (Day14)			***Single clone (day 14)
	S	M	L	S	M	L	S	M	L	S	M	L	
10% BW conditioned medium*	25	-	-	28	-	-	23	8		11	15	4	30
20% BW conditioned medium*	34	-	-	34	-	-	17	18	2	11	10	13	34
50% BW conditioned medium*	25	-	-	26	-	-	12	12	3	4	18	5	27
BM-Condimed H1	27	-	-	28	-	-	5	14	7	1	13	11	25

A stable hybridoma clone Thal N/B was subjected to single cell cloning by limiting dilution using 10%FCS-IMDM supplemented with 10%, 20%, 50% BW conditioned medium or 10% BM-Condimed H1. The size and number of hybridoma containing wells were determined at day 5, 7, 10, and 14 of cultivation by an inverted microscope.

*BW5147 thymoma cell line was cultured for 40 hours. The culture supernatants were harvested and used as conditioned medium.

**Number of wells containing hybridoma single clone at various clone sizes. The size of a hybridoma single clone was scored as described in materials and methods. S; small, M; medium, L; large

*** Number of wells containing hybridoma single clone at day 14 of cultivation.

were monitored by an inverted light microscope. The numbers of hybridoma containing wells were counted. Supernatants collected from the hybridoma containing wells were examined for the presence of specific antibody by ELISA or immunofluorescent technique.

Lysed whole blood immunofluorescence staining

50 µl of K_3EDTA blood were incubated at 4 °C with 50 µl of tested hybridoma culture supernatants. After 30 minutes incubation, cells were then washed twice with PBS containing with 1% bovine serum albumin (BSA) (Sigma-Aldrich) and 0.02% sodium azide (1% BSA-PBS-0.02% NaN_3). The FITC labeled anti-mouse immunoglobulin conjugate (Chemicon Australia Pty. Ltd., Melbourne, Australia) was added and incubated for another 30 min at 4 °C. Then, 1 ml of BD FACS™ lysing solution (Becton Dickinson, Franklin Lakes, NJ, USA) was added and allowed to stand at room temperature in the dark for 10 minutes. Cells were washed three times with 1% BSA-PBS-0.02% NaN_3 and fixed with PBS containing 1% paraformaldehyde. The stained cells were then analyzed by a flow cytometer (FACSort; Becton Dickinson).

ELISA

Antigens (20 µg/ml) were coated onto 96-well ELISA plates (Costar) using carbonate/bicarbonate coating buffer pH 9.6 at 50 µl/well and incubated at 4 °C overnight. After that the plate was blocked with 60 µl of 2% BSA-PBS at 37°C for 1 hour. 50 µl of hybridoma culture supernatant were added to each well and incubated at 37 °C for 1 hour. Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins antibody (DAKO, Glostrup, Denmark) was added to each well and incubated at 37 °C for 1 hour. Thereafter, the plate was washed and TMB substrate (Invitrogen, Carlsbad, CA, USA) was added. The reaction was stopped by 1M HCl and the absorbance was measured at 450 nm.

Results

Utilization of BW conditioned medium on hybridoma single cell cloning

In the initiation phase, we determined whether the novel conditioned medium, BW conditioned media, could be used to support single-cell growth during hybridoma cloning. In this experiment, a stable hybridoma clone producing anti-hemoglobin mAb, Thal N/B, was used as a study model. The Thal N/B hybridoma cells were subjected to single cell cloning using a basal medium, IMDM, consisting of 10% FCS supplemented with BW conditioned media obtained from 18-h and 40-h BW5147 cultivation and compared to the commercial conditioned medium, BM-Condimed H1, or to no supplement. The number of wells containing hybridoma single clone and the clone size were determined at day 5, 7, 10, and 14 of cultivation. As shown in Table 1, in three individual experiments, the size of the hybridoma single clone using BW conditioned media and BM-Condimed H1 supplementations were scored range from small

Table 3. Support of newly formed hybridoma single cell growth by various concentrations of BW conditioned medium.

Condition	**Size (Day5)			**Size (Day7)			**Size (Day10)			**Size (Day14)			***Single clone (day 14)
	S	M	L	S	M	L	S	M	L	S	M	L	
10% BW conditioned medium*	26	-	-	26	-	-	22	5	-	11	6	8	25
20% BW conditioned medium*	25	-	-	25	-	-	21	4	-	5	15	2	22
50% BW conditioned medium*	22	-	-	22	-	-	18	5	-	7	13	1	21
BM-Condimed H1	22	-	-	22	-	-	20	2	-	9	9	3	21

A newly formed hybridoma clone MT3 was subjected to single cell cloning by limiting dilution using 10%FCS-IMDM supplemented with 10%, 20%, 50% BW conditioned medium or 10% BM-Condimed H1. The size and number of hybridoma containing wells were determined at day 5, 7, 10, and 14 of cultivation by an inverted microscope.

*BW5147 thymoma cell line was cultured for 40 hours. The culture supernatants were harvested and used as conditioned medium.

**Number of wells containing hybridoma single clone at various clone sizes. The size of a hybridoma single clone was scored as described in materials and methods. S; small, M; medium, L; large

*** Number of wells containing hybridoma single clone at day 14 of cultivation

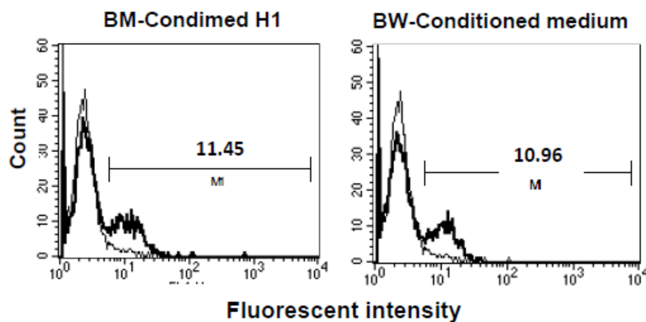

Figure 1. Activity of monoclonal antibody produced by hybridomas supplemented with BW conditioned medium and BM-Condimed H1. Culture supernatants of hybridoma producing anti-lymphocytes mAb clone MT3, after single cell cloning using 10% FCS-IMDM supplemented with BW conditioned medium or with BM-Condimed H1, were evaluated for their antibody reactivity by lysed whole blood immunofluorescence staining and analyzed by flow cytometry. The lymphocyte populations were gated and analyzed for membrane fluorescence using a flow cytometer. Thick lines represent the immunofluorescence profiles of the cells stained with hybridoma culture supernatants obtained from the indicated conditioned media. Thin lines represent background fluorescence of conjugate control. The mean fluorescent intensity (MFI) of the positive cells are indicated.

to large, in relative to the period of cultivation. At day 14, more than 50% of the growing cells were graded as medium to large in size. In contrast, without conditioned medium supplement, the generated hybridomas were almost all arrested at a small size (Table 1). These results indicated that the BW conditioned medium could support single-cell growth during hybridoma single cell cloning at the same level as the BM-Condimed H1. In comparing the BW conditioned media obtained from 18-h and 40-h cultivation of BW5147, the 40-h BW conditioned medium had slightly better growth of hybridomas than the 18-h conditioned medium (Table 1). Using 40-h conditioned medium, more medium and larger clone sizes were observed, compared to those using 18-h conditioned medium. At day 14 of cell cloning, the number of wells containing hybridoma single clone obtained by the generated BW conditioned medium and commercial BM-Condimed H1 were not significantly different (Table 1). Our results suggest that the BW conditioned medium can be used as a supplement for hybridoma single cell cloning.

We further determined the optimal concentration of BW conditioned medium for supporting hybridoma growth. The Thal N/B hybridoma cells were subjected to single cell cloning using various concentrations of 40-h BW conditioned medium. 20% BW conditioned medium supplement showed better results compared to those for 10% and 50% supplements (Table 2).

As the experiments described above were carried out using a stable hybridoma clone, we further confirmed whether BW conditioned medium could support the growth of newly generated hybridomas as well. A hybridoma clone named MT3, which was generated immediately after cell fusion, was subjected to single cell cloning using BW conditioned medium and BM-Condimed H1. Both conditioned media could support the growth of newly generated hybridomas. At day 14, the majority of the hybridoma clones were scored as medium to large in size (Table 3). The number of wells containing hybridoma single clones obtained by using BW conditioned medium and BM-Condimed H1 were not significantly different (Table 3). In addition, 20% BW conditioned medium supplementation was confirmed to be the optimal concentration. Taken together, our results indicated that BW conditioned medium can be used to support the growth of both stable and newly formed hybridomas. The BW conditioned medium can be employed in hybridoma single cell cloning in the hybridoma technique.

We then explored the question of whether the BW conditioned medium has any effect on the antibody production of the grown hybridomas. To address this question, after single cell cloning, culture supernatants of the obtained hybridoma MT3, using BW conditioned medium and commercial BM-Condimed H1, were evaluated for antibody reactivity. Culture supernatants obtained from both conditioned media showed the same positivity patterns (Figure 1). The results indicated that the novel BW conditioned medium could be used to support the growth of hybridomas without altering the antibody production.

Employment of the BW conditioned medium in the hybridoma technique for production of monoclonal antibodies

Since the BW conditioned medium had been shown to provide factors that promote growth of

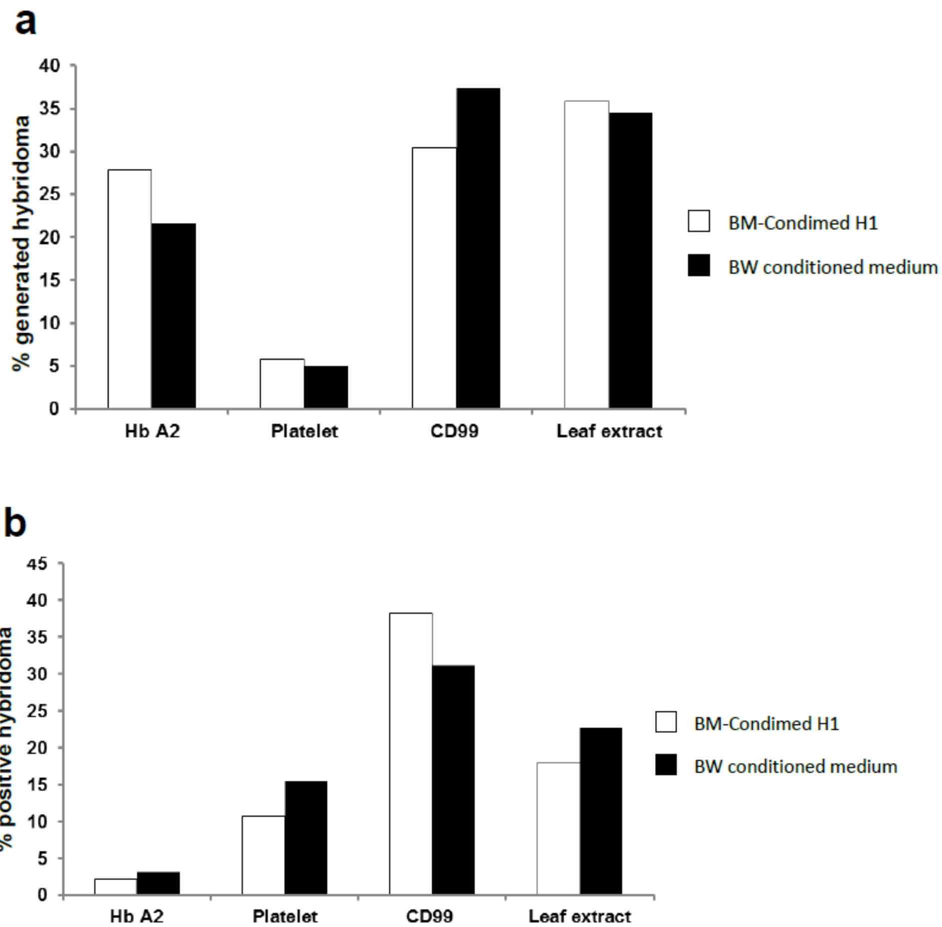

Figure 2. Comparison of BW conditioned medium and BM-Condimed H1 in the generation of hybridomas from mice immunized with HbA_2, platelets, CD99 and sugarcane crude leaf extract. Mice were immunized with the indicated antigens. Spleen cells of the immunized mice were fuse with myeloma cells by standard hybridoma technique using BW conditioned medium and BM-Condimed H1 supplements. After cell fusion, the fused cells were spread onto 96-well tissue culture plates for total 5 plates for each supplement. Total numbers of hybridoma containing wells were determined and converted to % hybridoma containing wells (a). Supernatants of the generated hybridomas were screened for the specific antibody. The numbers of antibody positive wells were determined and converted to % antibody positive wells (b).

hybridomas, we studied the possibility of using the generated BW conditioned medium in the hybridoma technique for production of mAbs. We studied production of mAbs to 4 different antigens, including $HbA_{2,}$ platelet, CD99 and sugarcane crude leaf extract. Spleen cells from antigen immunized mice were fused with myeloma cells. After cell fusion, HAT and HT medium prepared using BW conditioned medium or BM-Condimed H1 as supplements, were separately added to the fused cells. Cells were then spread onto 96-well plates. The numbers of hybridoma containing wells obtained from both conditioned medium supplementations were determined. As shown in Figure 2a, for all antigens used, the % of hybridoma containing wells obtained in BW conditioned medium and BM-Condimed H1 supplements were not significantly different. Culture supernatants from hybridoma containing wells were screened for antibody activity. Hybridomas producing the mAb of interest could be obtained using BW conditioned medium in amounts not significantly different from

the commercial BM-Condimed H1 medium (Figure 2b.). It is important to note that in the post-fusion hybridoma wells, fibroblasts were markedly reduced when using BW conditioned medium compared to the BM-Condimed H1 medium. This is very advantageous, as it reduces the chance of fibroblast overgrowth of the generated hybridomas. We, therefore, conclude that the BW conditioned medium could be used as a supplement for generation of hybridomas for mAb production.

Discussion

Currently, commercial conditioned media are normally used to replace feeder layer cells in the hybridoma technique. In general, the available commercial conditioned media can support hybridoma growth. However, one major concern in using the commercial supplements is its high cost, which consequently leads to the high expenditure on mAb production. In our routine mAb production, a commercial conditioned medium, BM-Condimed H1 purchased from Roche, was effective in hybridoma production. BM-Condimed H1, as described by the company, is prepared from the culture supernatant of a mouse lymphoma cell line EL4 stimulated with PMA [19]. This conditioned medium contains a complex mixture of growth factors for supporting the growth of hybridoma cells and also the rest of PMA. Besides its high cost, other disadvantages have been observed. Upon hybridoma production, overgrowth of fibroblasts in post-fusion wells is constantly observed and prevents successful mAb production. As PMA can induce fibroblast proliferation [16-18], fibroblast overgrowth in the post-fusion wells is possible due to the effect of PMA present in the conditioned medium. To minimize the overgrowth by the fibroblasts, hybridoma single cell cloning has to be performed as soon as the desired hybridomas are identified. Delay in doing this may result in the death of hybridomas producing the mAb of interest. Moreover, in some experiments where mAb in the form of hybridoma culture supernatant is employed to determine the effect of mAb on cellular functions, the PMA present may affect the assay system. In the present report, we aim to establish a method for preparing conditioned medium in the absence of mitogen in order to replace the expensive commercial conditioned medium. The conditioned medium necessarily contains a complex mixture of growth factors and cytokines that have a marked stimulatory effect on the growth of hybridoma cells after cell fusion and during single cell cloning.

A mouse thymoma cell line, EL4, produced and released several cytokines that support hybridoma growth upon PMA activation.[19,20] The EL4 cell line has therefore been employed in the production of commercial hybridoma conditioned media, e.g. BM-Condimed H1. In an attempt to produce conditioned medium free of mitogens or other stimulating agents, we searched for another mouse thymoma cell line to supplant the EL4 cells. The mouse thymoma line BW5147 has been demonstrated to constitutively produce various cytokines upon culturing without mitogen stimulation.[21] This study therefore selected the BW5147 cells for conditioned medium production. BW5147 cells were cultured for 18 and 40 hours and culture supernatants were harvested and tested for their effect on single cell cloning of both stable and newly formed hybridomas. IMDM medium, consisting of 10% FCS and conditioned with supernatant from BW5147 cells, could be successfully employed in hybridoma single cell cloning with equal effectiveness to that of commercial BM-Condimed H1 supplement. The 40-h culture-conditioned medium was shown to be optimal for supporting single-cell growth of both freshly formed hybridomas and stable hybridoma clones. Supplementation at 20% was shown to be the most effective concentration. It was also demonstrated that 20% BW conditioned medium supports the growth of hybridomas without altering their antibody reactivity. As observed for other thymoma cells,[19] supporting hybridoma growth with the BW conditioned medium is likely due to the production of various cytokines which support B cell differentiation and growth of the BW5147 cells,[21] As the BW conditioned medium was produced in the absence of any mitogen, enhancing of hybridoma growth appears to be BW5147 cell mediated. We therefore conclude that the prepared BW conditioned medium can be substituted for the relatively expensive commercial conditioned medium in hybridoma single cell cloning. Another benefit of using the BW conditioned medium is that the mAb produced by hybridomas using this supplementation method can be used for biological characterization, without concern about the presence of mitogen.

Since the BW conditioned medium was demonstrated to provide growth factors that promote growth of hybridomas cultured at low cell density,

we investigated whether this conditioned medium could be used as a supplement for production of mAb using the hybridoma technique. The novel BW conditioned medium was successfully used as a growth supplement for production of mAbs in all cell fusions. The number of hybridomas formed by BW conditioned medium supplement was at the same level as those using commercial BM-Condimed H1, with corresponding equality in the numbers of antibody-producing clones. The results, as predicted, indicate that BW conditioned medium contains growth factors that not only promote growth of hybridomas cultured at low cell densities but also promote newly formed hybridoma growth. In our routine experiments for production of mAbs using BM-Condimed H1 we have always observed some overgrowth of fibroblasts in post-fusion wells. However, fibroblast overgrowth was reduced when using the BW conditioned medium. Although the reason for this is still not well understood, we speculate that PMA is the cause.[10,19] As the BW conditioned medium was prepared in the absence of any mitogen, the BW conditioned culture supernatants lacked PMA for induction of fibroblast proliferation.

As the purpose of this study was to produce a relatively inexpensive conditioned medium, we analyzed and compared the cost of BW conditioned medium and BM-Condimed H1. The cost for 100 mL of BW conditioned medium was approximately 50 US$. In contrast, 100 mL BM-Condimed H1 purchased from Roche is 750 US$. The method for production of the BW conditioned medium is quite simple and needs no special techniques or sophisticated equipment. We have now implemented the BW conditioned medium in our routine work and are convinced that the BW conditioned medium can be used as a supplement for mAb production. The use of this home-made conditioned medium can lower the cost of the production of monoclonal antibodies.

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