

Research Article

## Molecular Cloning of Leukocyte Surface Molecules by Retrovirus-Mediated Expression Cloning System

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### Abstract

**Objective:** To clone cDNA encoding leukocyte surface molecules recognized by four newly generated monoclonal antibodies (mAbs) by retrovirus-mediated expression cloning system.

**Methods:** A novel retrovirus-mediated expression cloning system were employed. KG1a cDNA library constructed in retroviral vector pBabeMN-Z were transfected into Phoenix packing cells to produce ecotropic viruses. The produced retroviruses were transduced into BW5147 cells. The BW5147 transductants expressing the desired leukocyte surface molecules were sorted by immunomagnetic bead sorting using mAbs MEM-257, MEM-258, MEM-259 and MEM-263. The sorted cells were grown and stained with various mAbs by indirect immunofluorescence and analysis by flow cytometry.

**Results:** We have successfully isolated cDNA encoding the molecules recognized by mAbs MEM-257, MEM-258, MEM-259 and MEM-263. To identify the cloned molecules, MEM-257-, MEM-258-, MEM-259 and MEM-263-expressing transductants were stained with known mAbs specific for all CD molecules. The results revealed that molecules recognized by MEM-257, MEM-258, MEM-259, and MEM-263 were CD43, CD46, CD63 and CD44, respectively.

**Conclusion:** The retrovirus-mediated expression cloning system were used, in this study, to clone cDNA encoding leukocyte surface molecules. We found that this cloning system is a very effective method for cloning of leukocyte surface molecule when specific antibody is available. *Bull Chiang Mai Assoc Med Sci* 2002; 35: 36-44.

**Keywords:** Retroviral cloning system, Leukocyte surface molecule, Cluster of differentiation, CD

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## บทคัดย่อ: การโคลนยีนกำหนดการสร้างโมเลกุลบนผิวเซลล์เม็ดเลือดขาวโดย Retrovirus-Mediated Expression Cloning System

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**วัตถุประสงค์:** เพื่อโคลน cDNA ที่กำหนดการสร้างโมเลกุลบนผิวเซลล์เม็ดเลือดขาวที่ทำปฏิกิริยาจำเพาะ กับ โมโนโคลนอล แอนติบอดีที่ผลิตขึ้นมาใหม่โดย retrovirus-mediated expression cloning system.

**วิธีการ:** ผู้วิจัยได้นำวิธี retrovirus-mediated expression cloning system มาใช้ในการศึกษา โดยได้ทำการสร้าง cDNA library จากเซลล์มะเร็งชนิด KG1a และใส่เข้าไปในรีโทรไวรัสเวกเตอร์ชนิด pBabeMN-Z จากนั้นทำการเหนี่ยวนำเวกเตอร์ดังกล่าวเข้าไปใน Phoenix packaging cell เพื่อผลิตรีโทรไวรัส นำรีโทรไวรัสที่ได้ไป transduce เข้าไปในเซลล์ BW5147 และทำการคัดเลือกเซลล์ BW5147 ที่แสดงโมเลกุลบนผิวเซลล์เม็ดเลือดขาวที่สนใจโดยใช้โมโนโคลนอล แอนติบอดี 4 ชนิดคือ MEM-257, MEM-258, MEM-259 และ MEM-263 ร่วมกับวิธี immunomagnetic bead sorting นำเซลล์ที่ได้มาเลี้ยงและย้อมด้วยโมโนโคลนอล แอนติบอดีชนิดต่างๆ โดยวิธี immuno-fluorescence และตรวจวิเคราะห์ด้วยวิธี flow cytometry

**ผลการทดลอง:** ผู้วิจัยสามารถแยก cDNA ที่กำหนดการสร้างโมเลกุลที่จำเพาะกับโมโนโคลนอล แอนติบอดี MEM-257, MEM-258, MEM-259 และ MEM-263 ที่นำมาศึกษา และเพื่อพิสูจน์โมเลกุลบนผิวเซลล์เม็ดเลือดขาวที่ถูกโคลนได้ จึงนำเซลล์ BW5147 ที่แสดงออกโมเลกุล MEM-257, MEM-258, MEM-259 และ MEM-263 ที่ผิวเซลล์มาย้อมกับโมโนโคลนอล แอนติบอดีที่จำเพาะต่อ CD molecules ชนิดต่างๆ ผลการทดลองพบว่าโมเลกุลที่จำเพาะต่อโมโนโคลนอล แอนติบอดี MEM-257, MEM-258, MEM-259 และ MEM-263 คือ โมเลกุลบนผิวเซลล์เม็ดเลือดขาวชนิด CD43, CD46, CD63 และ CD44 ตามลำดับ

**สรุป:** ในการศึกษาครั้งนี้ ผู้วิจัยได้นำ retrovirus-mediated expression cloning system มาโคลนยีนที่กำหนดการสร้างโปรตีนบนผิวเม็ดเลือดขาว โดยพบว่าวิธีการที่นำมาศึกษานี้มีประสิทธิภาพสูงมากในการโคลนยีนที่กำหนดการสร้างโปรตีนบนผิวเม็ดเลือดขาว เมื่อมีแอนติบอดีจำเพาะอยู่ วารสารเทคนิคการแพทย์ เชียงใหม่ 2545;

35: 38-44.

**คำรหัส:** การโคลนยีนโดยระบบรีโทรไวรัส, โมเลกุลบนผิวเซลล์เม็ดเลือดขาว, Cluster of differentiation (CD)

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## Introduction

The immune system plays a major role in maintaining the homeostasis of health. It has involved in protection the body from pathogens and from mutated and/or oncogenic cells. Leukocytes are cells, which play a major role in the immune system. They recognize and dispose the invasive pathogens as well as tumor cells. To attain their full functional potential, cell-cell interaction and ligand-receptor interaction are required.<sup>1-3</sup> In the recent years, several studies were performed to achieve a better understanding of leukocyte communications and demonstrated that leukocyte surface molecules are responsible for cell interactions.<sup>2</sup>

Leukocytes express a large number of surface molecules. The discoveries of monoclonal antibodies against leukocytes have become a major tool in determination and characterization of their structure and functions. By Human Leukocyte Differentiation Antigen (HLDA) Workshop, leukocyte surface molecules are named systematically by assigning them as cluster of differentiation (CD) antigen.<sup>2,4</sup> At the present, many leukocyte surface molecules are identified<sup>5</sup>, however, intensive researches are still required to functional characterize the defined molecules and determine new leukocyte surface molecules.

A variety of approaches have been adopted to identify and functionally characterize leukocyte surface molecules with roles in mediating cellular interactions in immune system. Monoclonal antibody that

directed against leukocyte surface molecule has provided a means of identifying and characterizing the function of many membrane proteins. Moreover, a number of expression cloning systems<sup>6-8</sup> have been developed to enable the isolation of cDNA encoding molecule of interest. The sequence of the cDNA cloned provides information for amino acid sequences, the primary structure of encoded molecule which lead to the prediction of the possible function of the molecule. The isolated cDNA can also be used to express the encoded proteins in cells or even in transgenic animals, so that reconstitute experiments for functional analysis can be attempted *in vitro* and *in vivo*.

In the present study, we describe a new approach, retrovirus-mediated expression cloning system, for isolating of cDNA encoding leukocyte surface molecules. By this method, we have isolated cDNA encoding CD43, CD44, CD46 and CD63 molecules. The retroviral cloning system was demonstrated to be a very effective method for cloning of cDNA encoding leukocyte surface molecules.

## Materials and methods

### Cell lines and monoclonal antibodies

An ecotropic retrovirus packaging cell line, Phoenix cells, developed by Nolan *et al.*<sup>9</sup>, were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL; Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Gibco) and 40 µg/mL gentamicin and

2.5 µg/mL amphotericin B in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. The BW 5147 mouse thymoma cells and KG1a cell line were cultured in RPMI-1640 (Gibco) medium supplemented with 10% FCS (Gibco) and antibiotics at 37°C, 5%CO<sub>2</sub> atmosphere.

Monoclonal antibodies (mAbs) MEM-257, MEM-258, MEM-259 and MEM-263 directed against leukocyte surface molecules as well as the mAb against human α-fetoprotein AFP-01 were generated by Dr. V. Horejsi at the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic.

### Molecular cloning of leukocyte surface molecules by retrovirus-mediated expression cloning system

#### Transfection and transduction of target cells

A KG-1a cDNA library constructed in the retroviral expression vector pBabeMN-Z (Fig. 1), recently established in the laboratory of H. Stockinger, was transfected into Phoenix packaging cells by DEAE-Dextran transfection. Briefly, Phoenix cells at 50% confluence were harvested by trypsinization, then, 3x10<sup>7</sup> Phoenix cells were added to a cocktail of 50 ml DMEM with 1%NuSerum, 200 µg/mL DEAE-Dextran, 25µM Chloroquine

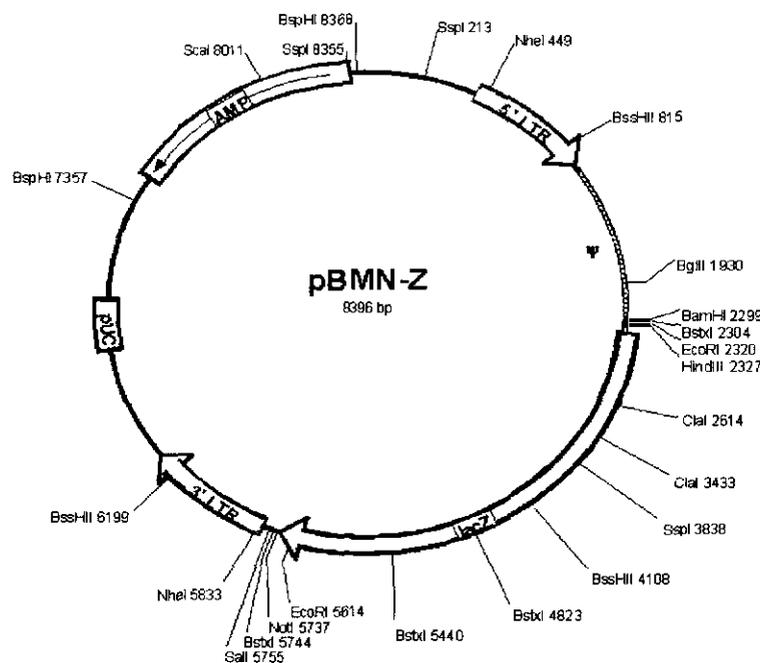


Figure 1. Structure of the pBabeMN-Z vector. Ψ; packaging signal; Amp; ampicillin-resistant gene; LTR; long terminal repeat.

diphosphate and 60  $\mu\text{g}$  of the pBabeMN-Z retroviral library. The packaging cells were kept in suspension for 2 hours at 37°C, washed once and cultivated in a 175 cm<sup>2</sup> flask in DMEM with 10% FCS at 37°C. Twenty-four hours post transfection, the medium was exchanged by adding 10 ml DMEM with 10% FCS. After additional 48 hours cultivation at 32°C the virus containing supernatant was collected, supplemented with 10  $\mu\text{g}/\text{mL}$  hexadimethrene bromide and added to 10 mL RPMI 1640 with 10% FCS containing  $1 \times 10^6/\text{mL}$  BW5147 target cells. Twenty-four hours post infection medium was changed to RPMI with 10% FCS.

#### Immunomagnetic bead sorting and enrichment of library-infected BW cells

To isolate and enrich BW5147 cells expressing the desired leukocyte surface molecule, a protocol involving the use of immunomagnetic beads was carried out. Infected BW5147 cells ( $4 \times 10^7$ ) were washed with 1% BSA in PBS and incubated with a mixture of four mAbs, MEM-257, MEM-258, MEM-259 and MEM-263, for 30 min on ice. After another washing step, the cells were incubated with goat-anti mouse IgG microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. After washing, cells were resuspended in 500  $\mu\text{L}$  of MACS sorting buffer (0.5% BSA/2mM EDTA in PBS) and loaded onto MS<sup>+</sup> separation columns (Miltenyi Biotec) for positive selection of transduced cells. The positive fraction was

maintained in RPMI 1640 medium supplemented with 10% FCS and then expanded for further rounds of MACS sorting. After 5 days cultivation, isolated BW5147 cells were subjected for the second round of immunomagnetic bead sorting but using individual mAb as isolation antibody. The positive fraction was then expanded in RPMI 1640 medium supplemented with 10% FCS and subjected for the third round of immunomagnetic bead sorting.

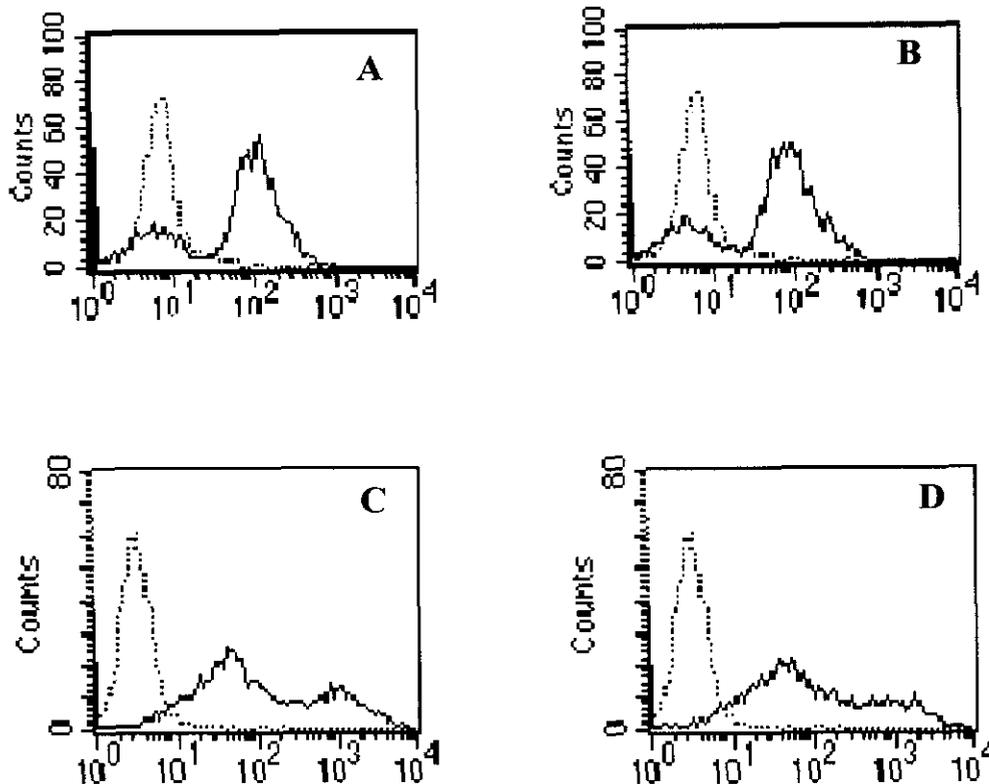
To obtain single clone of positive target cells, the limiting dilution technique was performed. Infected BW5147 cells were counted and single cell was seeded into 96-well plate. Cells were expanded in RPMI 1640 medium supplemented with 10% FCS and surface antigen expression was assessed by indirect immunofluorescence and flow cytometry.

#### Indirect immunofluorescence analysis

Cells were pre-incubated for 30 min at 4°C with 10% human AB serum before staining to block nonspecific FcR-mediated binding of mAb. Thereafter, 50  $\mu\text{L}$  of cells ( $1 \times 10^7/\text{mL}$ ) were stained with primary antibody or an irrelevant antibody for 30 min, at 4°C. After washing cells with phosphate buffered saline containing 1% bovine serum albumin and 0.02% sodium azide (1% BSA-PBS-Na<sub>3</sub>N<sub>3</sub>), binding of primary antibody was visualized by using FITC-conjugated sheep F(ab')<sub>2</sub> anti-mouse immunoglobulins antibodies (Immunotech/ Coulter Corporation, Miami, FL).

After washing cells three times with 1%BSA-PBS-NaN<sub>3</sub>, the membrane fluorescence was

analyzed under fluorescence microscope or flow cytometer.



**Figure 2** Immunomagnetic bead sorting of transduced BW5147 cells expressing leukocyte surface molecules of interest. After immunomagnetic bead sorting, the sorted cells were stained with mAb MEM-257 (A), MEM-258 (B), MEM-259 (C) and MEM-263 (D). The solid lines represent cells stained with MEM mAbs and dashed lines represent cells stained with AFP-01 as negative control.

**Results**

MEM-257, MEM-258, MEM-259 and MEM-263 are mAbs against human leukocyte surface molecules. In an attempt to clone cDNA encoding molecules specific for these mAbs, the recent described retrovirus-mediated expression cloning system was employed. By this clone strategy, KG1a cDNA library constructed in retroviral vector pBabeMN-Z were transfected into Phoenix packing cells to produce ecotropic viruses. The produced

retroviruses were transduced into BW5147 cells. The BW5147 transductants expressing the desired leukocyte surface molecules were sorted by immunomagnetic bead sorting using the mixture of mAbs MEM-257, MEM-258, MEM-259 and MEM-263 in the first round. The positive cells were expanded and subjected for the second and third rounds of immunomagnetic bead sorting by using individual mAb MEM-257, MEM-258, MEM-259 and MEM-263. After the third round

sorting, the isolated cells were then stained with each mAb by indirect immunofluorescence. As shown in figure 2, the sorted cells were positive with the corresponding mAb. These results indicated that we have succeeded in cloning of cDNA encoding molecules recognized by mAbs MEM-257, MEM-258, MEM-259 and MEM-263.

In order to know what are the molecules that are recognized by mAbs MEM-257, MEM-258, MEM-259 and MEM-263, the MEM-257-, MEM-258-, MEM-259- or MEM-263-expressing BW5147 clones obtained from limiting dilution were stained with all known CD monoclonal antibodies by indirect immunofluorescence and flow cytometry. We found that MEM-257-, MEM-258-, MEM-259- or MEM-263-expressing BW5147 cells were positive with mAbs CD43, CD46, CD63 and CD44, respectively (data not shown). These results indicated that molecules recognized by mAb MEM257, MEM258, MEM259 and MEM263 are CD43, CD46, CD63 and CD44, respectively.

## Discussion

Leukocyte surface molecules are of important molecules in cell communication and cell functions. Full characterization of leukocyte surface molecules, therefore, leads to a better understanding of the immune mechanisms. To fully characterize leukocytes surface molecules, molecular characterization is indispensable. The sequence of the cDNA encoding the molecule

of interest provides information for its primary structure. Examination of the primary structure is a feasible method by which to clarify the possible function of the molecule. In addition, the cloned cDNA can be used to generate recombinant encoded proteins. The recombinant proteins are further used for biochemical characterization and functional analysis.<sup>10</sup>

Recently, retroviral vector for the construction of cDNA libraries has developed and was used for molecular cloning of cDNA encoding cell surface molecules.<sup>8,11-15</sup> The principle of the retrovirus-mediated expression cloning system is that a cDNA library is constructed in retroviral vector and used to generate retroviruses in retrovirus packaging cells. The retroviral vector consist only of the essential  $\Psi$  (psi) packaging sequence to initiate virion assemble and all the retroviral protein coding genes (*gag*, *pol*, and *env*) are replaced by the cloned cDNA insert. To produce viral particles from constructed library, the viral structural proteins are supplied by packaging cell line. After introduction of the constructed cDNA library into packaging cells, viral particles that carried cDNA from the library are produced. The generated viruses are then transduced into target cells. The retroviruses enter target cells by binding to target cell through a host-cell receptor. The virus then reverse transcribes and integrates into host genome. A full-length viral transcript is initiated in the 5' LTR and end in the 3' LTR. However, the viral transcript typically does not

encode any of the protein required to make a viral capsid. It usually encodes a gene in the library. Therefore, the transduced target cells are stable express proteins of interest. Target cells expressing protein of interest are then isolated and the cDNA of interest in the target cell genome will be cloned by PCR based technique.

One of the important advantages of the retroviral cloning system over the previous described conventional COS cell expression cloning system is that a single infection can give rise to cells that stably express the introduced gene.<sup>11, 14</sup> Moreover, this method allows expansion and sorting of individually infected cells expressing particular cell surface proteins. Therefore, once infected, the cells expressing the surface molecules of interest can be easily selected by multiple immunomagnetic bead sorting.

In an attempt to clone cDNA-encoding protein recognized by four mAbs against human leukocyte surface molecules, the retroviral cloning system was carried out. By our procedure, after transduction of target cells, mouse BW5147 cells, the transductants expressing protein of interest were sorted by immunomagnetic bead. In the first round we used pool mAbs of interest for staining transduced cells. This sorting would pick up the mixture of cells which expressed proteins recognized by any mAb used. Then the sorted cells were subjected for the second and the third rounds sorting but using individual mAb in

separated tube. Therefore, the cells expressing protein specific to individual mAb were isolated. By our strategy, we could isolate cDNA encoding all molecule recognized by the four mAbs.

In summary, in the present report, we described an efficient procedure for cloning of leukocyte surface molecules by retrovirus-mediated expression cloning system. By this procedure, we could isolate cDNA encoding CD43, CD46, CD63 and CD44 within one cloning step. Our results suggested that this cloning strategy is an effective method for cloning of molecule of interest when the specific antibody is available.

#### Acknowledgements

We would like to thank Dr. Garry Nolan for providing the retroviral vector pBabeMN-Z and the Phoenix<sup>TM</sup> packaging cell line, Dr. Václav Horejsí for providing mAbs. We would like to thank Dr. Hannes Stockinger for training of the retroviral cloning system. This work was supported by the Thailand Research Fund (TRF), the Royal Golden Jubilee Ph.D. program of Thailand (RGJ), Austrian Science Fund and the Competence Center for Biomolecular Therapeutics (BMT) in Austria.

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