4. เป็นวิทยากรบรรยายเรื่อง Expression of protein in mammalian cells ในการประชุมวิชาการเรื่อง การผลิตโปรตีนโดยใช้ระบบสังเคราะห์ในเซลล์เจ้าบ้านแบบต่างๆ วันที่ 17-21 สิงหาคม 2541 อาคารสำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ กรุงเทพฯ
5. เป็นวิทยากรบรรยายเรื่อง DNA Vaccine ในการประชุมวิชาการเรื่อง The First National Seminar on Pharmaceutical Biotechnology วันที่ 23-25 มิถุนายน 2542 โรงแรมอมิตี้กรีนฮิลล์ เชียงใหม่
6. เป็นวิทยากรบรรยายเรื่อง Production of polyclonal and monoclonal antibodies to human leukocyte surface molecules and their use for immunophenotyping and functional analysis ในการประชุมวิชาการThe 5th Asia-Pacific Biochemical Engineering Conference 1999 วันที่ 15-18 พฤศจิกายน 2542 โรงแรม ภูเก็ตออคาเดีย ภูเก็ต
7. เป็นวิทยากรบรรยายเรื่อง DNA Vaccines ในการประชุมวิชาการเรื่อง New Drug and Novel Concepts วันที่ 22-26 พฤศจิกายน 2542 โรงแรมโลตัสปางวนแก้ว เชียงใหม่

(4) ในเชิงวิชาการ

ทุนวิจัยนี้ทำให้เกิดการพัฒนาการเรียนการสอนในภาควิชาฯ โดยผลงานวิจัยส่วนหนึ่งได้นำมาใช้ในการเรียนการสอนทั้งระดับปริญญาตรีและโท มีนักศึกษาปริญญาตรีจำนวน 5 คนทำภาคนิพนธ์และนักศึกษาปริญญาโท 1 คนทำวิทยานิพนธ์ โดยอาศัยโครงการวิจัยนี้

นอกจากนี้โครงการวิจัยนี้ยังส่วนร่วมในสร้างนักวิจัยใหม่ โดยโครงการวิจัยนี้ มีผู้ช่วยนักวิจัยมาร่วมงานตั้งแต่เริ่มโครงการวิจัยจนถึงปัจจุบัน จำนวน 2 คน ซึ่งผู้ช่วยนักวิจัยได้มีความรู้ ความคิดอย่างมากทางด้านงานวิจัย ซึ่งเชื่อว่าผู้ช่วยนักวิจัยเหล่านี้จะสามารถเป็นนักวิจัยที่ดีในอนาคต

ผลที่ได้จากโครงการวิจัยนี้ ทำให้ผู้วิจัยสามารถนำเอาเทคนิค DNA immunization หรือ DNA vaccination มาใช้ในการผลิตแอนติบอดีต่อโปรตีนชนิดต่างๆ โดยสามารถนำมาผลิตได้ทั้ง polyclonal antibody และ monoclonal antibody ความรู้นี้ได้นำไปประยุกต์ใช้กับการผลิต monoclonal antibody ต่อ Dengue virus protein อยู่ในขณะนี้

3. **อื่น ๆ**

3.1 การนำเสนอผลงานในที่ประชุมวิชาการ จำนวน 6 ครั้ง

(1) แสงเดือน มูลสม, นิรมล โตกระสินธุ์วิตร์, **วัชระ กสิณฤกษ์**. การผลิตโมโนโคลนอลแอนติบอดีต่อ CD14 โปรตีน. การประชุมวิชาการโฟลไซโตเมทรีประจำปี ครั้งที่ 6, ยะลา, 22-26 มีนาคม 2542.

(2) Moonsom S, Tokrasinwit N, Kasinrerk W. Generation of CD14 expressing COS cells by eukaryotic expression system and production of monoclonal antibody against CD14 protein for identification of monocytes. 17th Annual Health Sciences Meeting, Chiang Mai, 20 July 1999.

(3) **Kasinrerk W**, Tokrasinwit N. Inhibititon of PHA-induced cell proliferation by polyclonal CD4 antibodies generated by DNA immunization. International *Conference on Development and Clinical Progress of DNA Vaccines*. Langen, Germany, 6-8 October 1999.

(4) **Kasinrerk W**, Tokrasinwit N, Moonsom S, Stockinger H. CD99 monoclonal antibody induces homotypic cell aggregation of Jurkat cells through protein tyrosine kinase and protein kinase C-dependent pathways. 2nd FIMSA Congress. Bangkok, Thailand, 23-27 January 2000.

(5) แสงเดือน มูลสม, พรทิพย์ ไพจิตจินดา, **วัชระ กสิณฤกษ์**. การผลิตโมโนโคลนอลแอนติบอดีต่อ CD45 โมเลกุลและการติดฉลาก FITC เพื่อใช้ในการตรวจวิเคราะห์เม็ดเลือดขาว. การประชุมวิชาการโฟลไซโตเมทรีประจำปี ครั้งที่ 7, ขอนแก่น, 20-23 มีนาคม 2543.

(6) Moonsom S, Knunkeawla P, **Kasinrerk W**. Production of monoclonal antibody to CD54 protein by DNA immunization. The 18th Annual Health Sciences Meeting. Chiang Mai, Thailand, 8 June 2000.

3.2 รางวัลผลงานวิจัย

ได้รับรางวัล The 1999 Taguchi Prize for Outstanding Research Achievements by Young Scientist in the Field of Biotechnology

ภาคผนวก

Reprint / Manuscript จำนวน 4 เรื่อง:

(1) Kasinrerk W, Tokrasinwit N. Inhibititon of PHA-induced cell proliferation by polyclonal CD4 antibodies generated by DNA immunization. Immunol Letters 1999; 67:237-242

(2) Moonsom S, Khunkeawla P, Kasinrerk W. Production of polyclonal and monoclonal antibodies against CD54 molecules by intrasplenic immunization of plasmid DNA encoding CD54 protein. 2000 (in press).

(3) Moonsom S, Kasinrerk W. Production of anti-CD14 monoclonal antibodies by using CD14 expressing COS cells as immunizing antigen. Asian Pac J All Immunol 2000; 18: 53-61.

(4) Kasinrerk W, Tokrasinwit N, Moonsom S, Stockinger H. CD99 monoclonal antibody induces homotypic cell aggregation of Jurkat cells through protein tyrosine kinase and protein kinase C-dependent pathways. Immunol letters 2000; 71: 33-41.

Production of polyclonal and monoclonal antibodies against CD54 molecules by intrasplenic immunization of plasmid DNA encoding CD54 protein

Watchara Kasinrerk, Seangdeun Moonsom[a], Panida Khunkeawla[a]

Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand

[a] S. Moonsom and P. Khunkeawla are researchers supported by The Thailand Research Fund (TRF) and The National Science and Technology Development Agency of Thailand (NSTDA), respectively.

Corresponding author: Dr. Watchara Kasinrerk, Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand. Tel. : (..6653)946043; Fax: (..6653)946043

E-mail: watchara@chiangmai.ac.th

Keywords: CD54; DNA immunization, intrasplenic immunization, monoclonal antibody

Abstract

DNA immunization, in theory, is of great interest as a source of specific antibodies against different antigens. In an attempt to produce polyclonal and monoclonal antibodies against cell surface molecules by using the DNA immunization strategy, intramuscular and intrasplenic routes of DNA injection were compared. Two to Five, but not a single, intramuscular DNA immunizations induced anti-CD54 and anti-CD147 antibody production. In contrast, a single intrasplenic immunization of CD54-encoding DNA could induce anti-CD54 antibody production. To produce monoclonal antibody (mAb), spleen cells obtained from an intrasplenic CD54-encoding DNA immunized mouse were fused with myeloma cells using the standard hybridoma technique. A hybridoma secreting specific mAb to CD54 was established. The generated mAb reacted to CD54 protein expressed on transfected COS cells and various cell types, the same as using standard CD54 mAb MEM-111. Our results demonstrated that direct immunization of antigen-encoding DNA into spleen is an effective route for production of both polyclonal and monoclonal antibodies to cell surface molecules. This finding is very useful for the production of antibodies to cell surface molecules where the protein antigen is not available or difficult to prepare, but cDNA encoding the corresponding protein is available.

1. Introduction

Immunization with plasmid vectors encoding protein antigens derived from pathogens or tumor cells, termed DNA immunization or DNA vaccine, has been reported to induce protective immunity in several animal models [1-3]. The theoretical advantages of DNA immunization over conventional vaccines including: [1] the induction of both antibody and cell-mediated immune responses, [2] the relative ease of production and [3] the ability to express proteins intracellularly for the induction of cytotoxic T cell (CTL) responses. In addition, the DNA immunization strategy has been used as a tool for production of the hyperimmune globulin products [4-7].

Monoclonal antibodies directed against leukocyte surface molecules are powerful reagents for biochemical and functional characterizations of the corresponding proteins. To generate a mAb to a molecule where the protein antigen is not available or difficult to prepare, but cDNA encoding the corresponding protein is available, DNA immunization is therefore very useful. Few reports have demonstrated the use of this strategy for production of mAbs to molecules of interest [8-9]. However, in our attempts to produce mAbs to leukocyte surface proteins by intramuscularly immunization of antigen-encoding DNA, unsuccessful experiments were obtained (unpublished observations). Direct immunization of protein antigen into spleen (i.e., intrasplenic immunization) has been reported as an effective method for the production of polyclonal and monoclonal antibodies [10-20]. Direct immunization of plasmid DNA into spleen may, therefore, be a possible alternative route for production of both polyclonal and monoclonal antibody.

In the present report, we compared 2 plasmid DNA immunization routes, intramuscular and intrasplenic, for the production of polyclonal antibodies to cell

surface molecules. It was demonstrated that a single DNA immunization by intrasplenic route could induce antibody production, whereas, a single-dose intramuscular immunization could not. Furthermore, by using intrasplenic immunization, an anti-CD54 mAb could be generated by DNA immunization.

2. Materials and Methods

2.1. Cells and antibodies

Human monocytic cell line, U937 cells, were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 40 μg/ml gentamicin and 2.5 μg/ml amphotericin B in a humidified atmosphere of 5%CO_2 at 37°C. COS7 cells were cultured in MEM (Gibco) containing 10% FBS and antibiotics. Purified CD54 mAb MEM-111 (IgG2a)[21] were kindly provided by Dr. Vaclav Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic). FITC-conjugated rabbit F(ab')2 anti-mouse immunoglobulin antibodies was purchased from Dako (Glostrup, Denmark).

2.2 Preparation of plasmid DNA

cDNA encoding CD54 protein inserted into an eukaryotic expression vector pCDM8 (designated CD54-DNA) was a kind gift from Dr. Hannes Stockinger, University of Vienna, Vienna, Austria. cDNA encoding CD147 protein, named CD147-DNA, was generated in our Department [22]. For large scale preparation, the plasmid DNA were transformed into *E. coli* MC1061/p3. The plasmid DNA were then isolated from transformed *E. coli* by Qiagen chromatography columns (Qiagen, Hilden, Germany). The plasmid DNA obtained were subsequently resuspended in PBS. The concentration and purity of DNA preparation were determined by OD260/280 reading. DNA were stored at –20°C, until injected into mice,.

The isolated plasmid DNA were proved for expression of the corresponding proteins by using the COS cell expression system and indirect immunofluorescent staining of the transfected COS cells with specific mAbs.

2.3 DNA immunization

For intramuscular immunization, BALB/c mice were injected one or five times at 2-week intervals by intramuscular route at the hind legs (100 μg of DNA/dose). For intrasplenic immunization, mice were anesthetized with diethylether. The skin and peritoneum on the left side of the body was open to expose the spleen. Fifty microliters (100 μg) of plasmid DNA were injected into the spleen. The peritoneum and skin were then closed by fine sutures. Blood samples were collected from the immunized mice by tail bleeding at 2-week intervals. Sera were separated and stored at –20°C.

2.4. Hybridoma production

Spleen cells were collected from CD54-DNA intrasplenic immunized mouse and fused with P3-X63Ag8.653 myeloma cells by the standard hybridoma technique using 50% polyethylene glycol. After HAT medium selection, culture supernatants were firstly analyzed for antibody reactivity against CD54 expressing U937 cells using indirect immunofluorescent assay. The positive supernatants were then differentially screened for CD54 specificity using CD54 transfected COS cells. The positive hybridomas were subcloned by limiting dilution. The isotype of antibodies was determined by using the isotyping enzyme-linked immunosorbent assay kit (Sigma, St. Louis, MO).

2.5. Immunofluorescence analysis

To determine antibodies in the sera, indirect immunofluorescence was carried out. To block nonspecific Fc-receptor-mediated binding of antibody, U937 cells were pre-incubated for 30 min at 4°C with 10% human AB serum before staining. Blocked cells were then incubated for 30 min at 4°C with tested sera. After washing, cells were

incubated with FITC-conjugate for 30 min. Membrane fluorescence was analyzed on a FACSCalibur (Becton Dickinson, Sunnyvale, CA) flow cytometer.

The reactivity of anti-CD54 mAb with peripheral blood cells was assessed by indirect immunofluorescence using the lysed whole blood staining method. EDTA-blood was incubated with tested mAb for 30 min at 4°C. After washing, cells were incubated with FITC-conjugate for another 30 min at 4°C. RBCs were lysed by using RBC lying solution (Becton Dickinson). Membrane fluorescence was then analyzed by using a flow cytometer (FACScallibur, Becton Dickinson). For flow cytometry analysis, individual populations of blood cells were gated according to their forward and side scatter characteristics.

2.6 DEAE-dextran transfection of COS cells

Plasmid DNA were transfected into COS cells by the DEAE-dextran transfection method. Briefly, $1x10^6$ COS cells were transfered to 6 cm tissue culture dishes (NUNC, Roskilde, Denmark) on the day before transfection. Cells were incubated with 2 ml of MEM containing 250 μg/ml DEAE-dextran (Sigma), 400 μM chloroquine diphosphate (Sigma) and 2 μg DNA for 3 h at 37°C. Supernatant was removed and cells were treated with 10% DMSO in PBS for 2 min at room temperature. Cells were then cultured in MEM containing 10% FBS overnight, washed once, and re-cultured with the same medium for another 2 days to allow expression of the corresponding proteins.

3. Results

3.1. Production of polyclonal antibodies by immunization of plasmid DNA encoding CD54 and CD147 protein

In order to produce antibodies to human leukocyte surface molecules by injection of antigen-encoding plasmid DNA, intramusular immunization and direct immunization of plasmid DNA into spleen were used as inoculation routes. In this study, plasmid DNA encoding CD54 and CD147 protein, termed CD54-DNA and CD147-DNA respectively, were prepared by using Qiagen chromatography column. The plasmid DNA obtained were able to express the corresponding proteins on transfected COS cell surfaces (data not shown). Mice were intramuscularly immunized with CD54-DNA or CD147-DNA 5 times at two-week intervals or with a single injection. In some mice, CD54-DNA were inoculated by single intrasplenic immunization. Sera collected at two-week intervals from each mouse were firstly screened for the presence of anti-CD54 or anti-CD147 antibodies by staining U937 cells. As shown in figure 1, sera from all 5-doses intramuscularly immunized mice (8/8) reacted to U937 cells. In contrast, none of the sera from single-dose intramuscularly immunized mice (0/4) reacted to U937 cells. Interestingly, sera obtained from 2 out of 4 single-dose intrasplenic immunized mice showed positive reactivity (Fig. 2).

To confirm that the generated antibodies after plasmid DNA immunization were specific for CD54 and CD147 protein, CD54-DNA and CD147-DNA transfected COS cells were stained with the positive sera. As predicted, sera obtained from CD54-DNA immunized mice reacted to CD54 transfectants, but did not react to mock transfectants (data not shown). Sera obtained from CD147-DNA immunized mice bound CD147 transfectants, but did not react to mock transfectants (data not shown).

Pre-immune sera of each mouse did not react to any transfectants. These results indicated that DNA immunization can be used to produce polyclonal antibodies to cell surface proteins. Direct immunization of plasmid DNA into spleen can induce antibody response after only a single DNA immunization.

3.2. Production of monoclonal antibodies to CD54 protein by intrasplenic immunization of plasmid DNA

To produce monoclonal antibody to CD54 protein, spleen cells from a CD54-DNA intrasplenic immunized mouse, which produced anti-CD54 antibodies, were fused with myeloma cells using the conventional hybridoma technique. Hybrids that produced anti-CD54 mAb were determined by indirect immunofluorescence using CD54 transfected COS cells as antigen. By this screening, an anti-CD54 mAb, designated MT54 (IgG2a isotype), which strongly bound to CD54 transfectants, but not to mock transfectants, was obtained. To confirm that MT54 is an anti-CD54 mAb, the reactivity of this mAb to various cell lines and peripheral blood leukocytes was studied and compared to standard anti-CD54 mAb, MEM-111 [21]. All cell lines tested including the human monocytic cell line U937, the erythroid/myeloid cell line K562, the B cell line Daudi and the T cell lines Molt4, Jurkat and Sup T1 were positive with MT54 (Fig. 3). With peripheral blood leukocytes, the mAb MT54 bound to lymphocytes and monocytes and reacted weakly to neutrophils (n=5)(Fig. 4). All of these staining patterns were similar to those obtained by using mAb MEM-111 (Fig. 3 and 4). Data obtained from the COS cell transfectant experiments together with the cellular distribution patterns, indicated that the generated mAb MT54 is an anti-CD54 mAb. Our results demonstrated that direct immunization of plasmid DNA into spleen is a possible alternative route for the production of both polyclonal and monoclonal antibodies to cell surface molecules.

4. Discussion

Production of monoclonal antibodies to leukocyte surface molecules by DNA immunization is very useful for producing specific antibody where the protein antigen is absence but antigen-encoding DNA is available. In our previous experiments, intramuscular immunization of plasmid DNA encoding various human leukocyte surface molecules, including CD4, CD14, CD45, CD54 and CD147, induced antibody responses [5-7 and unpublished observations]. In theory, spleen cells from antibody-producing mice can be used in the hybridoma technique. However, in our previous fusion experiments, none hybrid produced specific antibody was generated from the DNA intramuscularly immunized mice. Instead of intramuscular immunization, an optimal DNA immunization route must be taken into consideration for the generation of hybrid produced monoclonal antibody of interest.

Injection of protein antigen directly into lymphoid organs such as lymph node or spleen offers some strong theoretical advantages over other injection routes [13, 23]. In this type of immunization, the immunogen is concentrated in one region that is specialized in dealing with it. Intrasplenic immunization was introduced by Nilsson [13] as an extremely efficient procedure, especially useful for low amounts of antigen. This immunization route has been further used for polyclonal and monoclonal antibody production purposes [10-20]. In the present study, intrasplenic and intramuscular routes were, therefore, compared for the production of antibodies to cell surface proteins after antigen-encoding DNA immunization.

In this study, mice immunized with plasmid DNA encoding CD54 and CD147, generated specific antibodies after two to five DNA inoculations (Fig. 1). In contrast, no mice that obtained a single dose intramuscular immunization generated an antibody. Intramuscular injection of plasmid DNA has been widely used with DNA

vaccines. In most cases of DNA intramuscular immunization, high titers of antibodies have been found against the expressed protein. Some investigators demonstrated that a single intramuscular immunization of plasmid DNA induced antibody responses [24-26], however, booster(s) was required in several reports including this one [27-30]. These differences appear to be due to the nature of the particular antigen, the expression vector used and may be due to the skill of the individual administering the DNA [29,31]. In contrast to intramuscular immunization, only a single DNA intrasplenic injection resulted in the production of serum antibodies (Fig. 2). These results indicate the possibility of using intrasplenic immunization for the induction of antibody responses by DNA immunization. Enhancement of antibody responses by intrasplenic immunization is likely to be related to that the injected plasmid DNA are directly transfected splenocytes, including antigen presenting cells. The antigens are then expressed and concentrated in the spleen, where the immune responses are initiated. Antigen presenting cells are thought to play at least three distinct roles in DNA immunization: (1) MHC class II-restricted presentation of antigens secreted by neighboring, transfected cells, (2) MHC class I-restricted "cross" presentation of antigens released by neighboring, transfected cells, and (3) direct presentation of antigens by transfected antigen presenting cells themselves [32]. Therefore, immunization of DNA into spleen allows direct delivery of antigens to the spleen and the induction of antibody responses is occurred. In protein immunization, it was clearly easier to obtain an antibody response using minute amounts of antigen by intrasplenic route than by the intraperitoneal or intravenous route [33]. Spitz et al, reported the production of antibodiess after a single intrasplenic injection of either soluble antigen or of allogeneic cells [10]. In agreement with protein immunization, this study demonstrated that injection of CD54-encodingDNA into spleen induced

antibody response better than that intramuscular route, as the antibodies could be detected after a single intrasplenic immunization. While this manuscript was in preparation, the use of intrasplenic immunization of plasmid encoding carcinoembryonic antigen (pCEA) for induction of immune responses was reported by White et al [34]. Intrasplenic administration of pCEA could induce specific antibody responses and partial immunoprotection against tumor challenge [34]. This and our findings support future investigations of DNA vaccination strategies that specifically promote the uptake of plasmid by splenocytes.

Spleen cells from a CD54-DNA intrasplenic immunized mouse were fused with myeloma for generation of monoclonal antibody. A hybridoma cell line stably secreting specific mAb to CD54 was established. The generated mAb reacted to CD54 protein expressed on transfected COS cells and various cell types as well as using a standard CD54 mAb MEM-111. Our results show that, rather than polyclonal antibody production, intrasplenic immunization can be used to produce monoclonal antibody by DNA immunization.

Intrasplenic immunization of protein antigens has been demonstrated to be an efficient procedure for the induction of antibodies [13]. Antibody responses were also obtained when the immunogen was immobilized on nitrocellulose or Sepharose beads [11,32]. As described here, the intrasplenic route was used for production of both polyclonal and monoclonal antibodies using plasmid DNA as immunizing agent. Intrasplenic immunization seems to be an appropriate route for raising polyclonal and monoclonal antibodies against cell surface proteins by DNA immunization. However, many constraints have limited the use of this immunization route. Intrasplenic immunization is not easy to perform, especially in small animals such as mice, since major surgical procedures are required and animal mortality and morbidity can not be

underestimated. This immunization route should be carried out only under the guidance of a trained animal worker or veterinarian.

Acknowledgments

The authors wish to thank Dr. Vaclav Horejsi and Dr. Hannes Stockinger for providing mAbs to CD54 protein and CD54-encoding DNA, respectively. This work was supported by the National Science and Technology Development Agency of Thailand and the Thailand Research Fund.

References

[1] J.B. Ulmer, J.J. Donney, S.E. Parker, et al., Science 259 (1993) 1745-1749.

[2] J.J. Donney, J.B. Ulmer, J.W. Shiver, M.A. Lui, Ann. Rev. Immunol. 15 (1997) 617-648.

[3] E. Manickan, K.L. Karem, B.T. Rouse, Critical Rev. Immunol. 17 (1997) 139-154.

[4] W.H. Robinson, S.S. Prohaska, J.C. Santoro, et al., J. Immunol. 155 (1995) 4739-4748.

[5] W. Kasinrerk, N. Tokarsinwit, Y. Piluk, Asian Pacific J. Allerg. Immunol. 14 (1996) 99-105.

[6] W. Kasinrerk, N. Tokarsinwit, K. Changtumroung, Asia Pacific J. Mol. Biol. Biotech. 5 (1997) 123-129.

[7] W. Kasinrerk, N. Tokarsinwit, Immunol. Lett. 67 (1999) 237-242.

[8] M.A. Barry, M.E. Barry, S.A. Johnston, Biotechniques 16 (1994) 616-620.

[9] C. Ulivieri, D. Burroni, J.L. Telford, C.T. Baldari, J. Biotechnol. 51 (1996) 191-194.

[10] L. Spitz, M. Spitz, R. Thorpe, E. Eugui, J. Immunol. Methods 70 (1984) 39-43.

[11] B.O. Nelsson, P.C. Svalander, A. Larsson, J. Immunol. Methods 99 (1987) 67-75.

[12] A.J. Gearing, R. Thorpe, L. Spitz, M. Spitz, J. Immunol. Methods 27 (1988) 305-343.

[13] B.O. Nelsson, A. Larsson, Immunol. Today 11 (1990) 10-12.

[14] M. Jin, L. Johasson, P. Sundstrom, B.O. Nilsson, Mol. Reprod. Devel. 37 (1994) 446-451.

[15] E.M. Fusi, A.M. Gasparri, M. Pelagi, et al., Amer. J. Reprod. Immunol. 33 (1995) 122-130.

[16] M. Jin, A. Larsson, B.O. Nilson, Mol. Reprod. Dev. 43 (1996) 47-54.

[17] N. Tumosa, J.R. Baker, Vis. Neuosci. 13 (1996) 671-682.

[18] NR, Gidlund Damasceno, J.A. Lindoso, D.S. Abdalla, H. Goto, Braz. J. Med. Biol. Res. 29 (1996) 1625-1628.

[19] S.P. Schrimpf, U. Hellman, L. Carlsson, et al., Prosate 38 (1999) 35-39.

[20] T.S. Hu, Y.C. Qian, Y.G. Yang, Y.L. Hu, X.M. Qu, S.L. Yang, Hybridoma 19 (2000) 95-99.

[21] K. Imai, M. Tsujisaki, T. Hareyama, Y. Hinoda, in: T. Kishimoto, H. Kikutani, A.E.G.Kr.v.d. Borne, et al. (Eds.) Leukocyte typing VI, Garland Publishing, 1997, pp. 409.

[22] W. Kasinrerk, E. Fiebinger, I Stefanova, et al., J. Immunol. 149 (1992) 847-854.

[23] M.B. Sigal, Y.N. Sinha, W.P. VanderLaan, Methods Enzymol. 93 (1983) 3-12.

[24] M. Major, L. Vitvitski, M.A. Mink, et. al., J. virology 69 (1995) 5798-5805.

[25] W. Bohm, A. Kuhrober, T. Paier, et al, J. Immunol. Methods 193 (1996) 29-40.

[26] A. Noll, N. Bucheler, E. Bohn, et al., Eur. J. Immunol. 29 (1999) 986-996.

[27] D. Gray, H. Skervall, Nature 336 (1988) 70-73.

[28] B. Wang, J Boyer, V. Srikantan, et el., DNA Cell Biol. 12 (1993) 799-805.

[29] H.L. Davis, Curr. Opin. Biotechnol. 8 (1997) 635-646.

[30] J. Hinkula, C. Svanholm, S. Schwartz, et al., J Virol. 71 (1997) 5528-5539.

[31] Y. Nishimura, A. Kamei, S. Uno-Furuta, et al., Vaccine 18 (1999) 675-680.

[32] A. Takashima, A. Morita, J. Leukoc. Biol. 66 (1999) 350-356.

[33] A. Larsson, B.O. Milsson, Scan. J. Immunol. 27 (1988) 305-309.

[34] S.A. White, A.F. LoBuglio, R.B. Arani, et al., J. Gene Med. 2 (2000) 135-140.

Figure Legends

Figure 1. Antibody response in mice intramuscularly immunized with CD54-encoding DNA (A) or CD147-encoding DNA (B). Groups of mice were immunized on days 0, 14, 28, 42 and 56 (solid lines) or single immunization at day 0 (dashed lines). Sera from blood drawn periodically were analyzed by indirect immunofluorescence and flow cytomery using CD54 and CD147 expressing U937 cells as antigens.

Figure 2. Antibody response in mice intrasplenic immunized with CD54-encoding DNA. Mice were single immunized on days 0 and sera from blood drawn periodically were analyzed by indirect immunofluorescence and flow cytomery using CD54 expressing U937 cells as antigens.

Figure 3. Immunofluorescence analysis of the reactivity of mAb MT54 with U937 (A), K562 (B), Daudi (C), Molt4 (D), Jurkat (E) and Sup T1 (F). Solid lines represent the immunofluorescence profiles of cells stained with indicated mAb and dashed lines represent background fluorescence of the negative control mAb.

Figure 4. Immunofluorescence analysis of the reactivity of mAb MT54 with lymphocytes (A), monocytes (B) and neutrophils (C). Solid lines represent the immunofluorescence profiles of cells stained with indicated mAb and dashed lines represent background fluorescence of the negative control mAb.

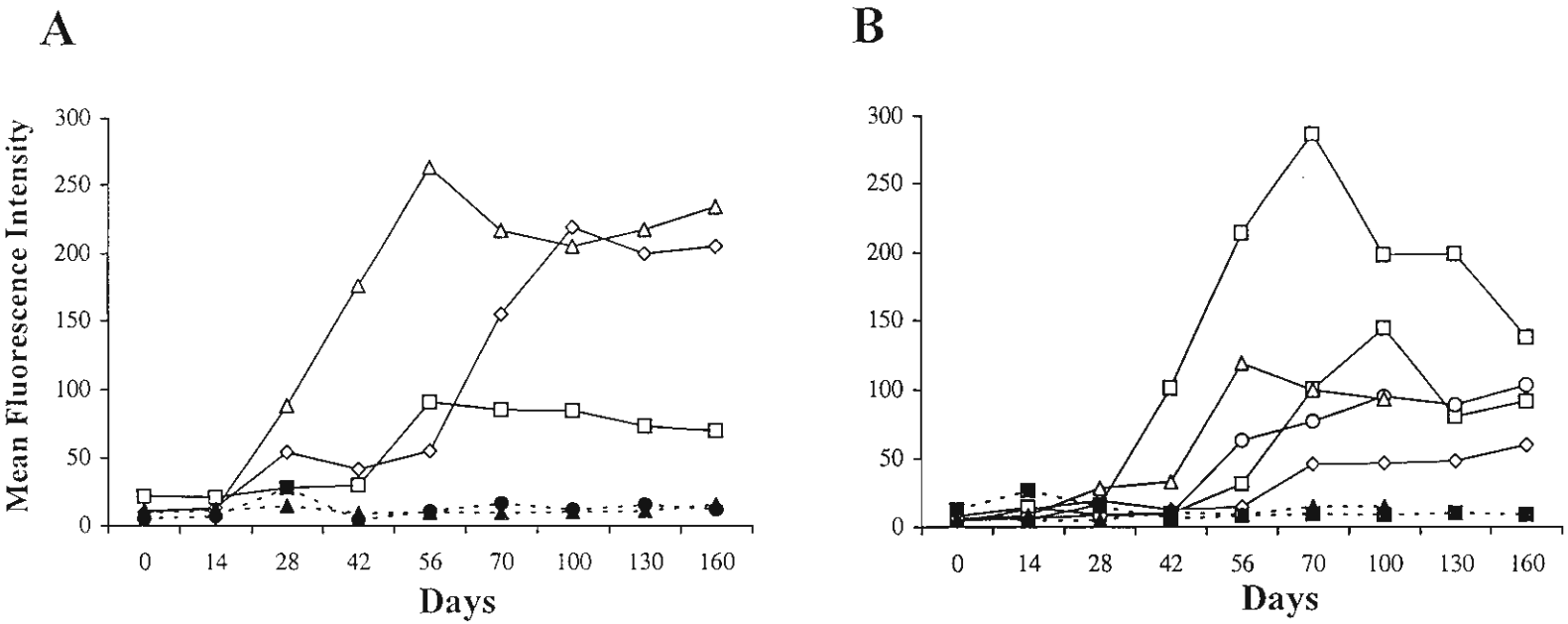

A
B
Mean Fluorescence Intensity
300
250
200
150
100
50
0
0
14
28
42
56
70
100
130
160
Days

Fig. 1

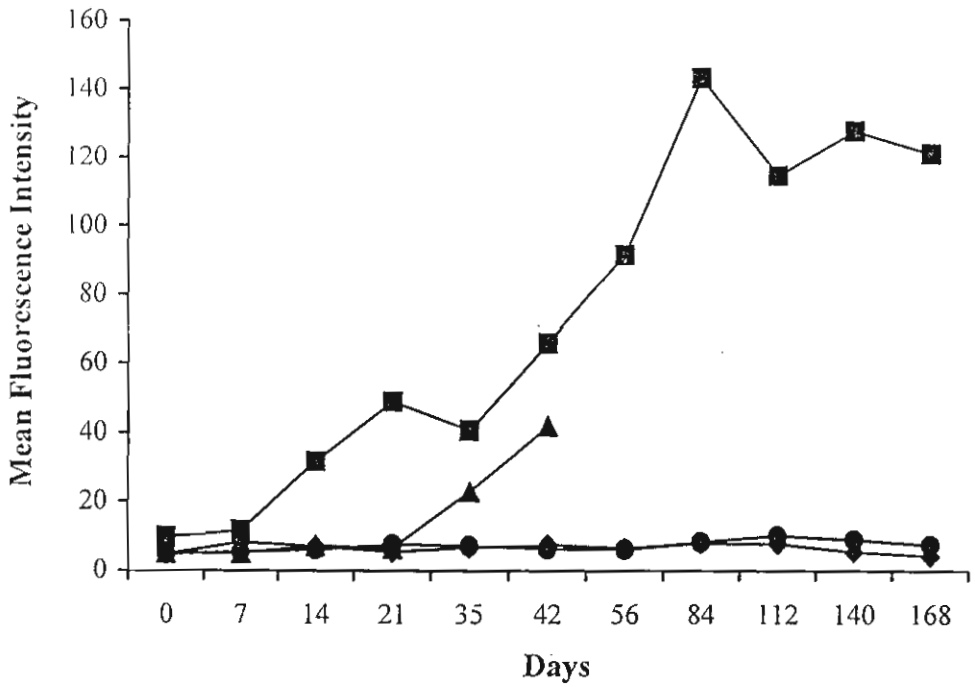

Mean Fluorescence Intensity
160
140
120
100
80
60
40
20
0
0
7
14
21
35
42
56
84
112
140
168
Days

Fig. 2

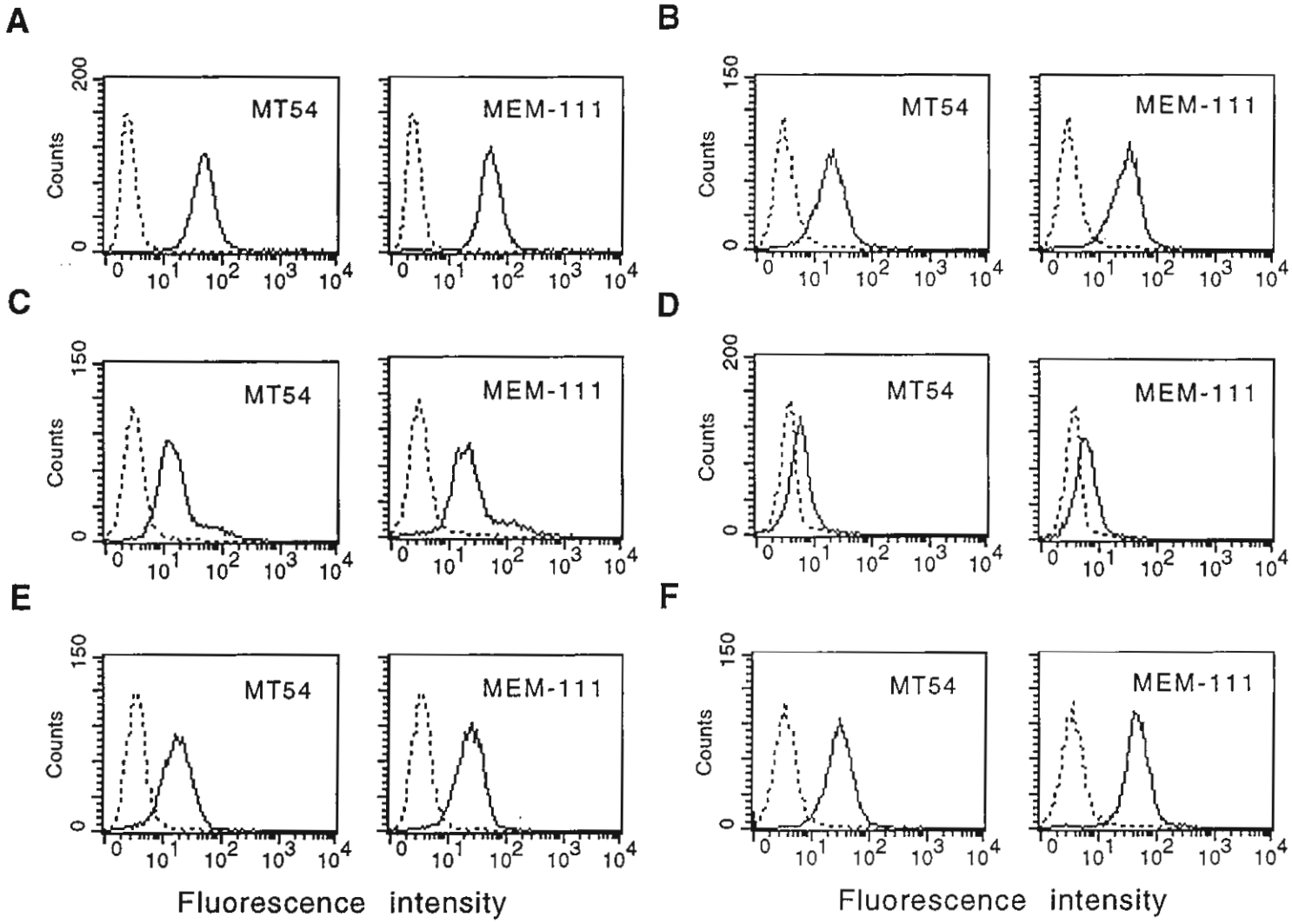


Fig 3

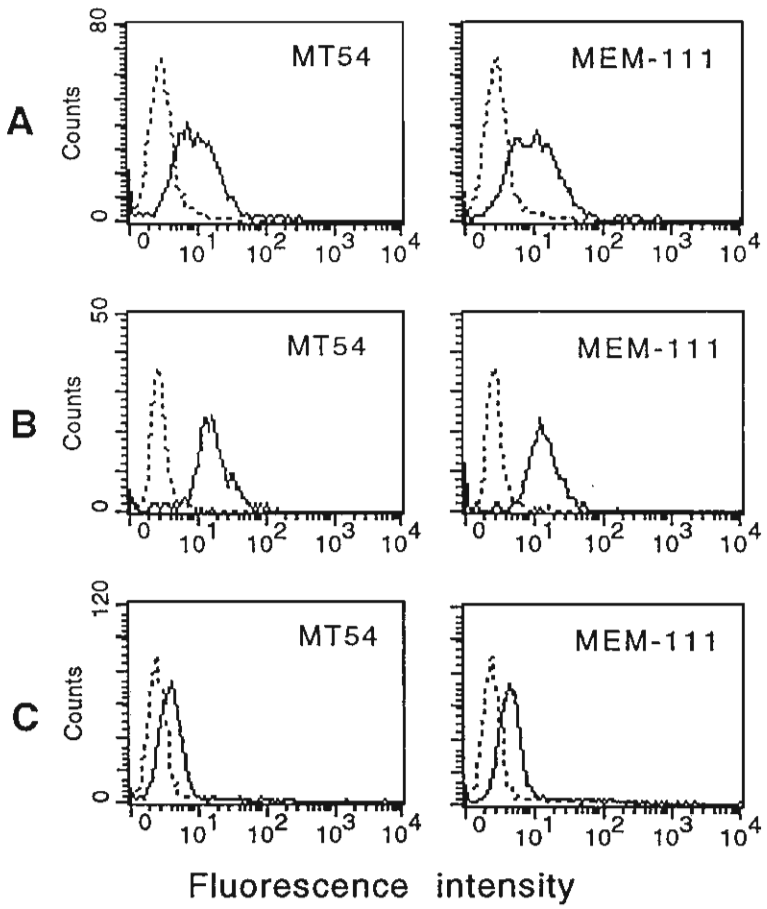

A
B
C
Counts
MT54
MEM-111
80
50
120
0
10^1
10^2
10^3
10^4
Fluorescence intensity

Fig. 4

ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2000) 18: 53-61

Production of Anti-CD14 Monoclonal Antibodies Using CD14 Expressing COS Cells as Immunizing Antigen

Seangduen Moonsom and Watchara Kasinrerk

SUMMARY **CD14 is a leukocyte surface molecule expressed on monocytes but not on lymphocytes. Recently, CD14 molecule was demonstrated to function as a receptor for endotoxin. CD14 specific monoclonal antibody (MAb), therefore, can be used to identify monocytes and study the host defense mechanism to bacterial endotoxin. To produce MAb against CD14 protein, in this study cDNA encoding CD14 protein and COS cell expression systems were used to prepare CD14 expressing COS cells. The CD14 transfectants were then used as antigen for mouse immunization. The spleen cells of the immunized mouse were then fused with myeloma cells by conventional hybridoma technique. By using this strategy, 5 hybridroma clones secreting antibody specific for CD14 molecule were generated within one fusion. The generated CD14 MAbs were strongly positive with monocytes, weakly positive with neutrophils but negative with lymphocytes. In addition, the generated CD14 MAb blocked the binding of lipopolysaccharide (LPS) to the CD14 molecules. These CD14 MAbs could be used to enumerate peripheral blood monocytes as well as using referent CD14 MAb. We, therefore, introduce an alternative method for preparation of antigen for production of monoclonal antibody. This type of antigen is a very effective antigen for the production of monoclonal antibodies against cell surface molecules.**

Monocytes belong to the mononuclear phagocytic system, which is a cell lineage that originates in bone marrow and eventually transforms into tissue macrophages. The stages of development are: committed stem cell, monoblast, promonocyte, monocyte and macrophage.[1] Differentiation which gives rise to monocytes takes place in the bone marrow. The newly formed monocytes leave the bone marrow within 24 hours. They remain in the blood for about 36-104 hours and then leave the circulation to become tissue macrophages which may live for many months.[2] Monocytes and macrophages play an important role in both natural immunity and acquired immunity.[3,4,5] They perform many of their functions in host defense prior to the development of specific immunity, including phagocytosis and cytokine production. These cells also function as both accessory and effector cells in acquired immune responses. They display foreign antigens on their surface in a form that can be recognized by antigen-specific T lymphocytes and express proteins and secrete various cytokines that promote T cell activation. Thus, monocytes/macrophages function as accessory cells in lymphocyte activation. After T lymphocyte activation, antigen-stimulated T lymphocytes secrete lymphokines that activate monocytes/macrophages. Such activated mononuclear phagocytes are more efficient at performing phagocytic, degradative and cytocidal functions than unstimulated cells, and are thus better able to destroy phagocytosed anti-

From the Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand
Correspondence: Watchara Kasinrerk

gens. In the humoral immune responses, foreign antigens, such as microbes, become coated, or opsonized by antibody molecules and complement proteins. Monocytes and macrophages bind and phagocytose opsonized particles much more avidly than uncoated particles. Thus, mononuclear phagocytes also participate in the elimination of foreign antigens by humoral immune responses.

To identify monocytes, simple staining of cells with Giemsa or Romanovsky stains was used in routine laboratory work. However, small monocytes may be difficult to distinguish from lymphocytes. Monocytes also can be identified by using cytochemical methods. The most widely used cytochemical methods for identifying monocytes are reactions for nonspecific esterases, NSE.[6,7] However, NSE is present not only in all cells of the mononuclear phagocytic lineage but also in many other cell types including some T lymphocytes.[6] In recent years, various monoclonal antibodies specific to monocytes have been generated.[8,9] These monoclonal antibodies were used to stain peripheral blood cells by immunofluorescent technique and the stained cells were analyzed by flow cytometry. According to both size and granularity and the reactivity of the stained monoclonal antibody, monocytes present in the blood sample were easily identified by flow cytometer.[10]

The CD14 antigen is a glycosyl-phosphatidylinositol-linked single chain surface membrane glycoprotein with the molecular weight of 53-55 kDa.[11] It is strongly expressed on monocytes, macrophages, and weakly on neutrophils.[11] However, it is absent from T cells, null cells, red blood cells and platelets. Therefore, antibody to CD14 antigen was routinely used for identification of monocytes by flow cytometry.[10,12] Recently, the CD14 molecule was demonstrated to function as a receptor for endotoxin (lipopolysaccharide; LPS).[13,14] LPS binds to a serum protein, LBP (LPS-binding protein) which facilitates the binding of LPS to the CD14 molecule.[13,14,15] When LPS binds to CD14 expressed by monocytes or neutrophils, the cells become activated and release cytokines such as TNF, IL-6 and IL-8[13,15,16,17,18,19] and up-regulate cell surface molecules, including adhesion molecules.[14,20] CD14 specific MAbs, therefore, can be used to identify monocytes and study the host defense mechanism to bacterial endotoxin.

To produce monoclonal antibody against CD14 protein, in general, CD14 expressing cell lines or freshly isolated monocytes were used as antigen for mouse immunization and the spleen cells of immunized mouse were fused with myeloma for the generation of specific monoclonal antibody.[16,21,22,23] Alternatively, in the present study, we used the COS expression system[24] to generate CD14 expressing COS cells. The CD14-COS cells were used as immunizing antigen for a generation of CD14 MAbs. By this technique, 5 hybridomas producing CD14 MAbs were generated within one fusion. The produced CD14 MAbs strongly react with human monocytes, weakly react with granulocytes, but do not react with lymphocytes. These CD14 MAbs can be used to enumerate monocytes as well as referent CD14 MAb.

MATERIALS AND METHODS

Plasmid DNA and antibodies

cDNA encoding CD14 protein, which was constructed into an eukaryotic expression vector, pCDM8 (designated CD14-DNA), was kindly donated by Dr. H. Stockinger, University of Vienna, Vienna, Austria. cDNA encoding CD147 protein, designated M6-DNA, was cloned in our laboratory.[25] Referent CD14 MAb, MEM18, was kindly provided by Dr. Horejsi, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Praha, Czech Republic. PE-labeled CD14 (Leu-M3) MAb and FITC-labeled CD45 (HLe-1) MAb were purchased from Becton Dickinson (San Jose, CA). FITC-conjugated sheep F(ab')2 anti-mouse immunoglobulins antibodies were purchased from Immunotech (Immunotech/Coulter Corporation, Miami, FL).

DNA preparations

For large scale preparation of CD14-DNA, the plasmid DNA were transformed into competent *E. coli* MC1061/p3 and the resulting bacteria were grown with vigorous shaking in 250 ml LB broth per 1-liter flask. After overnight cultivation, cells were harvested and lysed by the alkaline lysis procedure according to the QIAGEN protocol (QIAGEN, Hilden, Germany). DNA were then purified by QIAGEN ion exchange column (QIAGEN). The concentration and purity of DNA preparation was determined by OD260/280 reading.

Expression of CD14 protein on COS cells

To express the CD14 protein on COS cells, the CD14-DNA was transfected into COS cells using the modified DEAE-Dextran transfection method.[25] Briefly, 1 x 10^6 COS cells were transferred to 6 cm tissue culture dishes (NUNC, Roskilde, Denmark) on the day before transfection. Cells were transfected in 2 ml of MEM containing 250 μg/ml DEAE-Dextran, 400 μM chloroquine diphosphate *and 2 μg DNA. After 3 hours* at 37°C, the transfection mixture was removed and the cells were treated with 10% DMSO in PBS for 2 minutes at room temperature. COS cells were then cultured overnight in MEM containing 10% FCS, washed once, and re-cultured with the same medium for another 2 days to allow expression of the encoded proteins.

Production of monoclonal antibody to CD14 protein

A BALB/c mouse was intraperitoneally immunized three times at two-week intervals with 1 x 10^7 CD14-DNA transfected COS cells. A booster immunization was followed two-weeks after the third immunization by intravenous injection of 1 x 10^6 CD14-DNA transfected COS cells. The animal was sacrificed 3 days after the booster and the spleen was removed. Spleen cells were then fused with myeloma cells X63-Ag8.653 using 50% PEG. After that, cells were resuspended with HAT medium containing 10% BM combimed medium (Boehringer Mannheim, Germany) and distributed into 960 wells of 96 well-plates. Two weeks later, hybridomas were identified by an inverted microscope. Cell culture supernatants from hybridoma-containing wells were screened for antibody against CD14 protein. The positive clone was re-cloned two rounds by limiting dilution.

Screening for CD14 specific monoclonal antibody

Hybridoma cell culture supernatants were firstly analyzed by indirect immunofluorescence using peripheral blood mononuclear cells (PBMCs) as antigens. The stained cells were analyzed by *flow cytometry. The supernatants* which had shown positive reactivity with monocytes but negative or weakly positive with lymphocytes were screened further for antibody specific to CD14 protein by the same technique but using CD14-DNA transfected COS cells as antigens and analyzed by a fluorescent microscope. In all experiments, COS cells transfected with cDNA encoding unrelated protein (CD147) were used as negative control.

Indirect immunofluorescence analysis

The specificity of antibody against CD14 protein was assessed by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulin antibodies (Immunotech). PBMCs were firstly isolated from heparinized blood of healthy donors by density gradient centrifugation over Ficoll-Hypaque solution (Sigma, St. Louis, MO). PBMCs were then washed 3 times with PBS and adjusted to 1 x 10^7 cells/ml with 1% BSA-PBS-0.02% sodium azide. To block the non-specific Fc receptor mediated binding of the antibodies, cells were incubated for 30 minutes at 4°C with 10% human AB serum before staining. Fifty microliters of blocked cells were then incubated for 30 minutes at 4°C with hybridoma culture supernatants or MAb. After washing twice, cells were incubated with the FITC-conjugate for another 30 minutes. Membrane fluorescence was analyzed by a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA). Individual populations of blood cells were gated according to their forward and side scatter characteristics.

Determination of isotypes of monoclonal antibody

The isotypes of MAbs were determined by capture ELISA (Sigma) in accordance with the recommended protocol. Goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM were used as capture antibodies, and peroxidase conjugated rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) were as used as conjugate. The reactivity was visualized by using 3',3',5',5'-tetramethylbenzidine (TMB) as substrate.

Inhibition of referent CD14 monoclonal antibody binding by generated CD14 monoclonal antibodies

PBMCs were pre-incubated with supernatant containing CD14 MAbs or irrelevant MAb for 30 minutes on ice. PE-labeled CD14 MAb Leu-M3 (Becton Dickinson) or FITC-labeled CD45 MAb HLe-1 (Becton Dickinson) was then added to the pre-stained cells, and incubated for another 30 minutes. Membrane fluorescence was analyzed by a flow cytometer.

The percent inhibition of fluorescence intensity was calculated from the mean fluorescence intensity (MFI) of the sample in the presence and absence of the first unlabeled MAb.

Inhibition of LPS binding to CD14 molecule by generated CD14 monoclonal antibody

CD14 MAb, MT14/3, was purified from ascites by using anti-mouse IgG coated sepharose column (Zymed Laboratory Inc., San Francisco, CA) in accordance with the recommended protocol.

To study the effect of generated CD14 MAb on LPS binding, PBMCs were incubated with 50% autologous plasma for 30 minutes at 4°C. PBMCs were then preincubated with purified MT14/3 MAb or OKT3 (anti-CD3 MAb; Ortho Diagnostic System Inc., Raritan, NJ) for 30 minutes on ice. FITC-labeled LPS (Sigma) was then added, and incubated for another 60 minutes. The membrane fluorescence of monocytes was analyzed by a flow cytometer.

RESULTS

Preparation of plasmid DNA encoding CD14 protein and expression of CD14 on COS cell membrane

Plasmid DNA encoding CD14 protein, named CD14-DNA, were transformed into *E. coli* MC 1061/p3. The plasmid DNA were then isolated from the transformed bacteria by QIAGEN ion exchange chromatography and the DNA yields were determined by the OD 260/280 reading after completion of all the purification steps. The OD 260/280 ratios of isolated plasmid DNA was 1.82, indicating the purity of the isolated DNA.[26] The yield of isolated CD14-DNA was 2.8 mg/liter of starting bacteria.

To verify whether the isolated CD14-DNA can be expressed to the encoded protein in eukaryotic cells, the isolated DNA was transfected into COS cells and analyzed for CD14 protein expression by indirect immunofluorescence using referent CD14 MAbs, MEM-18 and Leu-M3. As shown in Table 1, COS cells which were transfected with CD14-DNA showed a very strong positive reaction with both MEM-18 and Leu-M3, but negative with an irrelevant CD147 MAb.[27] The percentage of CD14 expressing COS cells obtained from this CD14-DNA transfection was approximately 60-70%. These results indicated that the CD14-DNA and the transfection method used effectively induced CD14 protein expression on COS cell membrane.

Production of monoclonal antibodies to CD14 protein using CD14 expressing COS cells as antigen

To generate hybridoma producing CD14 MAb, we first prepared CD14 expressing COS

Table 1 Expression of CD14 protein on CD14-DNA transfected COS cells

COS transfectants	Immunofluorescent reactivity		
	Anti-CD14 MAb		Anti-CD147 MAb
	MEM-18	Leu-M3	M6-1D4
CD14 COS	+	+	-
M6 COS	-	-	+

COS cells were transfected with CD14-DNA or M6-DNA by DEAE-Dextran transfection technique. The transfected cells were stained with CD14 MAbs, MEM-18 and Leu-M3 or CD147 MAb, M6-1D4 Immunofluorescent reactivity was analyzed by a fluorescence microscope.

Table 2 Flow cytometric immunophenotyping of monocyte population, comparison of results between MT14/1, MT14/2, MT14/3, MT14/4 and referent anti-CD14 MAb MEM-18

Donor no.	Monoclonal antibody				
	MT14/1	MT14/2	MT14/3	MT14/4	MEM-18
1	95	95	95	95	96
2	95	95	97	94	97
3	92	95	97	97	98
4	94	94	92	96	96
5	91	94	96	92	94

cells by transfection of COS cells with CD14-DNA. After 3 days, approximately 70% of the transfected COS cells were strongly positive with CD14 MAb, MEM-18. These CD14-expressing COS cells were then used as antigen for mouse immunization. Spleen cells of an immunized mouse were fused with myeloma cells as per the conventional hybridoma technique. A total of 612 hybridoma clones was identified from 960 wells. By indirect immunofluorescent technique, cell culture supernatants from 12 of 612 wells contained antibodies which reacted to monocytes, but did not, or reacted weakly to lymphocytes. To screen further for hybridomas that produced CD14 specific antibody, all 12 positive supernatants were tested again by the same technique, but CD14-DNA transfected COS cells were used as the antigen. Five out of 12 culture supernatants reacted with CD14 expressing COS cells, but not with mock CD147 transfectants. The hybridomas in these positive wells were then re-cloned two rounds by limiting dilution. The final clones which gave positive reactivity with CD14 expressing transfectants but negative to mock transfectants were propagated and re-named MT14/1, MT14/2, MT14/3, MT14/4 and MT14/5, respectively. MT14/1, MT14/2, MT14/3, MT14/4 and MT14/5 MAbs were IgG1, IgG2b, IgG1, IgG1 and IgM isotypes, respectively.

After single cell cloning, all generated hybridoma clones were grown at a high density and the culture supernatants were collected for further studies. However, a hybridoma clone, MT14/5, stopped producing antibody. Therefore, this MT14/5 clone was discarded.

Characterization of the specificity of generated CD14 MAbs

To confirm the specificity of generated MAbs, all MT14 MAbs were used to stained monocytes, granulocytes and lymphocytes isolated from 5 healthy donors by indirect immunofluorescence, and analyzed by flow cytometry. As predicted, all generated MT14 MAbs strongly reacted to monocytes, weakly reacted to granulocytes and were negative with lymphocytes. The reaction patterns were absolutely identical to those obtained by using reference CD14 MAb, MEM-18. The typical FASC profiles of MT14 and MEM-18

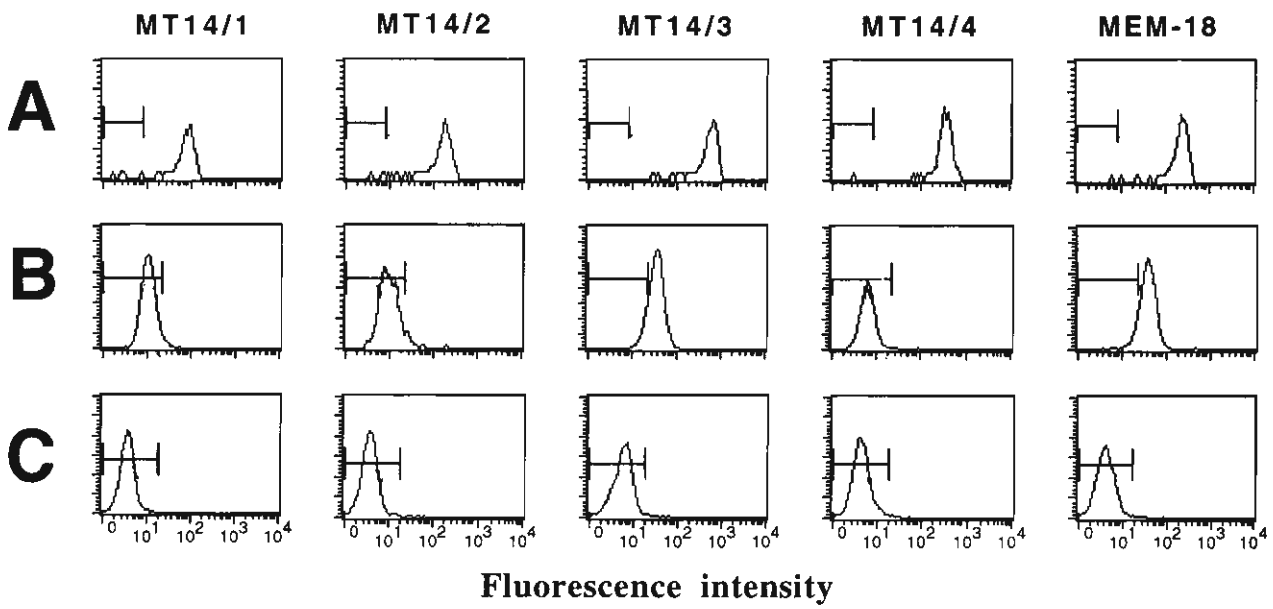


Fig. 1 Immunofluorescence analysis of the reactivity of MT14/1, MT14/2, MT14/3, MT14/4 and referent MEM-18 MAbs with monocytes (A), neutrophils (B) and lymphocytes (C). The marker represents background fluorescence of negative control MAb.

CD14 transfectants were then used to immunize mouse and spleen cells were then fused with myeloma by conventional hybridoma technique. After fusion, the hybridoma culture supernatants were screened using CD14 transfected COS cells and mock transfected cells as antigens. By this screening procedure, supernatants from 5 hybridoma clones showed positive reactivity with CD14 transfectants but not to mock transfectants. With peripheral blood cells, these five antibodies strongly reacted to monocytes, weakly reacted to neutrophils, but did not react to lymphocytes. The staining patterns, together with the transfectant experiments, indicated that the generated MAbs are specific to CD14 protein. Obtaining 5 hybridoma clones produced antibodies to a surface molecule within one fusion is unusual. Our experiment, therefore, demonstrated that the high efficiency COS cell expression system can be used to produce very good antigen for production of monoclonal antibody. In addition to CD14 MAbs, using the same strategy, 3 clones of anti-CD99 MAbs were also produced in our laboratory (data not shown).

After 2 rounds of single cell cloning, however, a hybridoma clone termed MT14/5 stopped producing antibody. The cessation of antibody producing by hybridomas due to their having an unstable assortment of chromosomes has been described.[30] The epitope recognized by the generated CD14 MAbs was analyzed using the inhibition test. One of the generated MAbs, named MT14/3, completely inhibited the binding of referent CD14 MAb Leu-M3.[22,23] This result indicated that MT14/3 MAb may react to CD14 molecule at the same or at a very close epitope of that recognized by referent CD14, Leu-M3, MAb. Leu-M3 was an anti-CD14 MAb derived from the hybridization of mouse Sp2/0 myeloma cells with spleen cells of BALB/c mice immunized with peripheral blood monocytes from a patient with rheumatoid arthritis.[22] This MAb was now used as a LeukoGate reagent in SIMULTEST reagent kit developed by Becton Dickinson. In contrast to MT14/3, MT14/4 MAb enhanced the binding of Leu-M3 MAb with an unknown mechanism. This enhancement effect was also observed in the CD147 system.[31]

A series of studies was performed to analyze the function of the CD14 molecule and indicated that CD14 functioned as a receptor for LPS.[13,14,15] LPS was shown to bind to CD14, a process mediated by the plasma protein LBP, and blockade of the CD14 molecule with some anti-CD14 MAbs prevented LPS induced TNF release by monocytes.[13,15] We therefore raised the question whether our generated CD14 MAb bind to the epitope which is involved in LPS binding. To address this question, monocytes were incubated with autologous plasma, which used as a source of LBP, in the presence of CD14 MAb and FITC-labeled LPS. It was found that the generated CD14 MAb, MT14/3, inhibited LPS binding. In the same experiment, the isotype matched control showed no effect on LPS binding. These results indicated that MT14/3 MAb recognizes an epitope on CD14 which is involved in LPS binding.

In conclusion, in the present study, we introduce an alternative method for preparation of antigen for immunizing mice in the production of monoclonal antibody. We used the high-efficiency COS cell expression system to induce the over expression of the protein of interest on COS cell surface. This type of antigen was shown to be a very effective antigen for the production of monoclonal antibody against cell surface molecules. Using this type of antigen, we could produce 5 MAbs directed against CD14 protein within one fusion. This strategy is, therefore, very efficient in production of monoclonal antibody against any cell surface protein when cDNA clone is available.

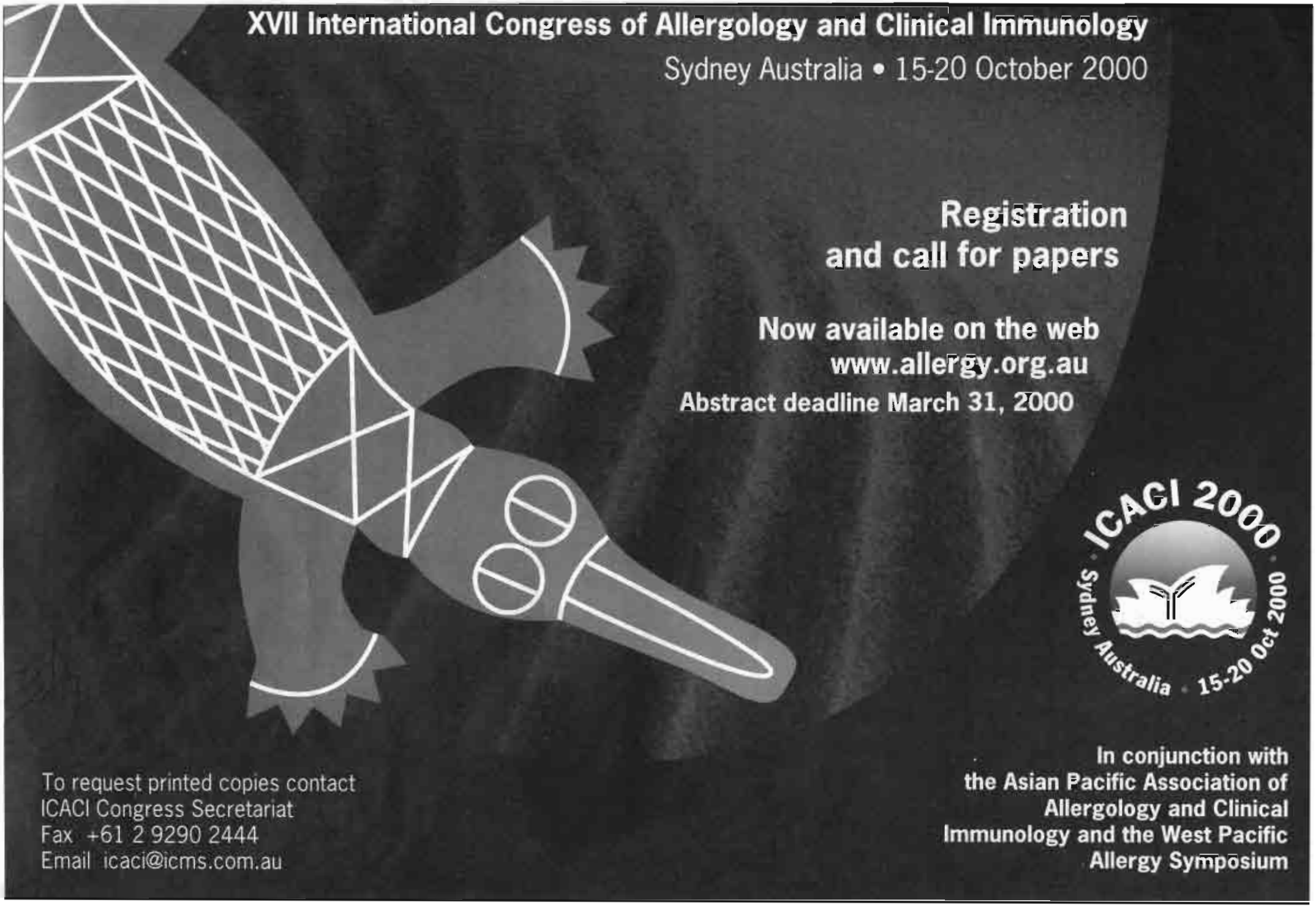
ACKNOWLEDGEMENTS

This work was financially supported by the Thailand Research Fund, the National Science and Technology Development Agency of Thailand and by the Ministry of University Affairs. S. Moonsom is a research assistant supported by The National Science and Technology Development Agency of Thailand (NSTDA). The authors would like to thank Dr. H. Stockinger for providing the cDNA encoding CD14 protein.

REFERENCES

1. Lasser A. The mononuclear phagocytic system. Human Pathol 1983; 14: 108-26.
2. van Furth R. Mononuclear phagocytes in immunity, infection and pathology. Oxford: Blackwell Scientific, 1975.
3. Johnston RB. Current concepts: immunology. Monocytes and macrophages. N Engl J Med 1988; 318: 747-52.
4. Zembala M, Asherson GL. Human monocytes. London: Academic Press Limited, 1989.

5. Tizard IR. Immunology. 4th ed. New York: Saunder College Publishing, 1995.
6. Stachura J. Cytochemistry of monocytes and macrophage. In: Zembala M, Asherson GL, eds, Human monocytes. London: Academic Press Limited, 1989; p. 27-36.
7. Koski IR, Poplack DG, Blaese RM. A non-specific esterase stain for the identification of monocytes and macrophages. In: Bloom BR, David JR, eds, *In vitro* methods in cell-mediated and tumor immunity. New York: Academic Press, 1976; p. 359-62.
8. Knapp W, Dorken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGK. Leukocyte typing IV. Oxford: Oxford University Press, 1989.
9. Schlossman SF, Boumsell L, Gilks W, Harlan JM, Kishimoto T, Morimoto C, Ritz J, Shaw S, Silverstein RL, Springer TA, Tedder TF, Todd RF. Leukocyte typing V. New York: Oxford University Press, 1994.
10. Montaner LJ, Collin M, Herbein G. Human monocytes: Isolation, cultivation, and applications. In: Herzenberg LA, Weir DM, Herzenberg LA, Blackwell C, eds, Weir's handbook of experimental immunology. Volume IV: The integrated immune system. 5th ed. Oxford: Blackwell Science, 1996; 155: 1-11.
11. Gadd S. Cluster report: CD14. In: Knapp W, Dorken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGK, eds, Leukocyte typing IV. Oxford: Oxford University Press, 1989; p. 787-9.
12. Kasinrerk W, Baumruker T, Majdic O, Knapp W, Stockinger H. CD1 molecule expression on human monocytes induced by granulocyte-macrophage colony-stimulating factor. J Immunol 1993; 150: 579-84.
13. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990; 249: 1431-3.
14. Hailman E, Lichensrein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, Busse LA, Zukowski MM, Wright SD. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J Exp Med 1994; 179: 269-77.
15. Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ. Structure and function of lipopolysaccharide binding protein. Science 1990; 249: 1429-31.
16. Goyert SM, Haziot A, Jiao D, Katz IR, Kruger C, Ross J, Schtt C. CD14 cluster workshop report. In: Schlossman SF, Boumsell L, Gilks W, Harlan JM, Kishimoto T, Morimoto C, Ritz J, Shaw S, Silverstein RL, Springer TA, Tedder TF, Todd RF, eds, Leukocyte typing V. New York: Oxford University Press, 1994, p. 778-90.
17. Baggiolini M, Dewald B, Walz A. Interleukin-8 and related cytokines. In: Gallin JI, Goldstein IM, Synderman R, eds, Inflammation: basic principles and clinical correlates. 2nd ed. New York: Raven Press, 1992, p. 247-63.
18. Hirono T, Akira S, Taga T, Kishimoto T. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. Immunol Today 1990; 11: 443-9.
19. Koj A. Initiation of acute phase response and synthesis of cytokines. Biophys Acta 1996; 1317: 84-94.
20. Pohlman TH, Harlan JM. Endotoxin-endothelial cell intreraction. In: Morrison DC, Ryan JR, eds, Bacterial endotoxic lipopolysaccharide. Vol 1: Molecular biochemistry and cellular biology. Boca Raton: CRC Press, 1992, p. 347-71.
21. Goyert S, Tesio L, Ashman L, Ball E, Basil V, Garrido F, Hogg N, Horejsi V, Howell A, Jayaram Y, Look AT, Ruiz-Cabllo F, Schmitt D, Schneider M, van der Schoot CE. Report on CD14 cluster Workshop. In: Knapp W, Dorken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGK, eds, Leukocyte typing IV. Oxford: Oxford University Press, 1989, p. 789-94.
22. Dimitriu-Bona A, Burmester G, Waters S, Winchester R. Human mononuclear phagocyte differentiation antigens. I. Patterns of antigenic expression on the surface of human monocytes and macrophages defined by monoclonal antibodies. J Immunol 1983; 130:145-52.
23. Herrmann F, Komischke B, Odenwald E, Ludwid WD. Use of monoclonal antibodies as a diagnostic tool in human leukemia. I. Acute myeloid leukemia and acute phase of chronic myeloid leukemia. Blut 1983; 47: 157-63.
24. Aruffo A, Seed B. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc Natl Acd Sci USA 1987; 84: 8573-7.
25. Kasinrerk W, Fiebinger E, Stefanova I, Baumruker T, Knapp W, Stockinger H. Human leukocyte activation antigen M6, a member of the immunoglobulin superfamily, is the species homologue of rat OX-47, mouse basigin and chicken HT7 molecule. J Immunol 1992; 149:847-54.
26. Sambrook J, Fritsch EF, Maiatis T. Molecular cloning: A laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.
27. Kasinrerk W, Tokrasinwit N, Phunpae P. CD147 monoclonal antibodies induce homotypic cell aggregation of monocytic cell line U937 via LFA-1/ICAM-1 pathway. Immunol 1999; 96: 184-92.
28. Seed B. An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. Nature 1987; 329: 840-42.
29. Kasinrerk W. Transfection of gene into eukaryotic cells by DEAE-Dextran transfection. J Med Tech Assoc Thailand 1990; 18: 105-8.
30. Harlow E, Lane D. Monoclonal antibodies. In: Harlow E, Lane D, eds, Antibodies: A laboratory manual. New York: Cold Spring Harbor Laboratory, 1988, p. 139-242.
31. Stockinger H, Ebel T, Hansmann C, Koch C, Majdic O, Prager E, Patel DD, Fox DA, Horejsi V, Sagawa K, Shen DC. CD147 (neurothelin/basigin) workshop panel report. In: Kishimoto T, Kikutani H, Borne AEGK, Goyert SM, Mason D, Miyasaka M, Moretta L, Okumura K, Shaw S, Springer T, Sugamura K, Zola H, eds, Leukocyte typing VI. New York: Garland Publishing, 1997, p. 76-9.


XVII International Congress of Allergology and Clinical Immunology
Sydney Australia • 15-20 October 2000
Registration
and call for papers
Now available on the web
www.allergy.org.au
Abstract deadline March 31, 2000
ICACI 2000
Sydney Australia
15-20 Oct 2000
To request printed copies contact
ICACI Congress Secretariat
Fax +61 2 9290 2444
Email icaci@icms.com.au
In conjunction with
the Asian Pacific Association of
Allergology and Clinical
Immunology and the West Pacific
Allergy Symposium

ELSEVIER

Immunology Letters 71 (2000) 33–41

Immunology Letters

www.elsevier.com/locate

CD99 monoclonal antibody induce homotypic adhesion of Jurkat cells through protein tyrosine kinase and protein kinase C-dependent pathway

Watchara Kasinrerk [a,*], Niramon Tokrasinwit [a,1], Seangdeun Moonsom[1] [a], Hannes Stockinger [b]

[a] *Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand*
[b] *Institute of Immunology, Vienna International Research Cooperation Center at NFI, University of Vienna, Brunner Strasse 59, 1235 Vienna, Austria*

Received 21 September 1999; accepted 26 October 1999

Abstract

CD99 is a 32 kDa cell surface glycoprotein which is involved in cell adhesion. Engagement of the CD99 molecule by CD99 monoclonal antibodies has been shown to induce homotypic aggregation of various cell types. By using a newly established CD99 monoclonal antibody, MT99/3, we show here that LFA-1/ICAM-1 independent cell adhesion pathways are activated via CD99. Engagement of the CD99 molecule by MT99/3 induced homotypic cell aggregation of Jurkat T-cells within 30 min reaching its maximal level within 4 h. The Jurkat cell aggregation was not blocked by addition of CD11a (LFA-1) and CD54 (ICAM-1) mAbs. Furthermore, MT99/3 treatment did not alter the expression of LFA-1 and ICAM-1 molecules. Induction of Jurkat homotypic aggregation by MT99/3 was, however blocked by the protein kinase C inhibitor, sphingosine, the protein tyrosine kinase inhibitor, genistein, and by actin filament polymerization blocking agent, cytochalasin B. Thus, these observations suggest that CD99 can mediate β2-integrin independent cell adhesion that depends on activation of protein kineses and reorganization of the cytoskeleton. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: CD99; Cell adhesion; Protein kinase C; Protein tyrosine kinase

1. Introduction

Lymphocyte adhesion is one of the critical steps in the regulation of immune processes involving cellular localization, effector recognition, and activation phenomena. Engagement of various cell surface molecules with natural ligands or specific antibodies, such as CD147, CD99, CD43, CD14, CD9 or CD4, has been demonstrated to induce leukocyte homotypic aggregation [1–7]. Some of these adhesion events seem to be mediated by the LFA-1/ICAM-1 pathway [1,3–5,7] as the LFA-1 or ICAM-1 monoclonal antibodies (mAbs) block homotypic cell aggregation triggered via these molecules. The current hypothesis is that the engagement of these molecules would induce an 'inside-out' signal that increases the affinity and/or level of leukocyte integrins for their ligand(s) [8].

The CD99 (E2 or MIC2) molecule is a leukocyte surface protein with a molecular weight of 32 kDa encoded by the MIC2 gene [9]. Sequence analysis of the cDNA indicates that CD99 is a cell membrane glycoprotein that does not belong to any known family of proteins [10–12]. The extracellular domain is glycosylated with *O*-linked sugars followed by a putative transmembrane domain and a short cytoplasmic domain [12]. Although the function of the CD99 molecule is still unclear, it has been implicated in two processes: cell adhesion and cell death. In T-cell adhesion events, CD99 mAbs blocked spontaneous T-cell rosette forma-

* Corresponding author. Tel.: +66-53-945080; fax: +66-53-221890.
E-mail address: watchar@chiangmai.ac.th (W. Kasinrerk)

[1] N. Tokrasinwit and S. Moonsom are researchers supported by The Thailand Research Fund (TRF) and The National Science and Technology Development Agency of Thailand (NSTDA), respectively.

0165-2478/00/$ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0165-2478(99)00165-0

tion [13,14]. Furthermore, CD99 mAbs were shown to induce homotypic aggregation of double positive (CD4 + CD8 +) thymocytes [2], T-cell lines [2] and B-cell line [3]. Recently, a truncated form of the CD99 molecule, named CD99 type II, was described [3]. Overexpression of the truncated form of CD99 inhibited cell adhesion. Apoptosis induced via the CD99 molecule has also been demonstrated [15]. This apoptosis induction is not followed by detectable DNA fragmentation, and does not belong to the Fas/CD95 pathway. Because of these cellular events induced via CD99, the molecule was proposed to be important for developmental processes such as hematopoietic cell differentiation [15,16].

In the present study, we analyzed the molecular mechanism underlying CD99 mediated cell adhesion. A newly established unique CD99 mAb, MT99/3, induced homotypic cell aggregation of Jurkat cells via a LFA-1/ICAM-1 independent pathway. The Jurkat cell aggregation induced by the CD99 mAb was inhibited by the protein kinase C (PKC) inhibitor, sphingosine, the protein tyrosine kinase (PTK) inhibitor, genistein, and the actin filament polymerization blocking agent cytochalasin B.

2. Materials and methods

2.1. Cells and cell lines

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood from healthy donors by density gradient centrifugation over Ficoll–Hypaque solution (Sigma, St. Louis, MO). Neutrophils were isolated by centrifugation through Ficoll Hypaque density gradients followed by dextran sedimentation. Cells were washed three times with PBS and used for immunophenotyping. Human T-cell lines (Jurkat, Molt4 and Sup T1), a monocytic cell line (U937), and an erythroid–myeloid cell line (K562) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 40 μg/ml gentamicin and 2.5 μg/ml amphotericin B in a humidified atmosphere of 5%CO_2 at 37°C. COS7 cells were cultured in MEM (Gibco) containing 10% FBS and antibiotics.

2.2. Reagents and antibodies

Cytochalasin B, genistein and sphingosine were purchased from Sigma. All reagents were dissolved in DMSO (Sigma). CD4 mAb MT4 (IgM isotype) [17] as well as isotype matched mAb P-3E10 (IgG2a) recognizing an un-defined cell surface molecule were generated in our Department. CD11a mAb MEM-25 (IgG1) and CD54 mAb MEM-111 (IgG2a) were kindly provided by Dr Vaclav Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic). CD11a mAb HI111 (IgG1) and CD54 mAb HA58 (IgG1) are phycoerythrin (PE) conjugates kindly provided by Becton Dickinson (Thailand). PE conjugated X40, an anti-Keyhole limpet hemocyanin (KLH) mAb, from Becton Dickinson, was used as isotype matched negative staining control.

For purification of the mAbs, affinity chromatography was used. IgM isotype mAbs were purified from ascites by using an anti-mouse IgM coated sepharose column (Zymed Laboratory, San Francisco, CA) according to the method described elsewhere [18]. IgG isotype mAbs were purified by using a protein A coated sepharose column (Pharmacia, Uppsala, Sweden).

2.3. Isolation of the cDNA coding for the CD99 molecule

The cDNA encoding the CD99 molecule was isolated essentially by using 'Brian Seed's eukaryotic expression cloning system' [19] and a one round panning technique described previously [20]. Briefly, thirty dishes of COS cells were transfected with a cDNA expression library prepared from the human T-cell line HPB-ALL by the DEAE-dextran transfection method. Three days after transfection, cells were panned with CD99 mAbs on dishes coated with sheep F(ab')2 anti-mouse Ig antibodies. Episomal DNA was collected from the adherent cells by the Hirt procedure and transformed into *E. coli*. Seventy bacteria colonies were separately grown in a 5-ml liquid culture overnight. Then 1.5 ml of ten bacterial suspensions were pooled and one-third of the isolated plasmid DNA was transfected into COS cells. After 2 days, COS cells were stained with CD99 mAb by the indirect immunofluorescence technique and analyzed by microscopy. Plasmid DNA of the individual colonies of the CD99 positive pools were again transfected into COS cells and re-analyzed.

For nucleotide sequencing, the cDNA insert was subcloned into pBS(+ / −) phagemid (Stratagene, La Jolla, CA). Double-stranded DNA was sequenced according to the dideoxynucleotide chain-termination method, using T7, T3 and internal oligonucleotide primers.

2.4. Hybridomaproduction

A female BALB/c mouse was intraperitoneally immunized three times at a 2-week interval with 1×10^7 CD99 transfected COS cells. After three immunizations, mice were intravenously boostered with 1×10^6 transfectants. Splenocytes were collected and fused with P3-X63Ag8.653 myeloma cells by a standard hybridoma technique using 50% polyethylene glycol. After HAT medium selection, culture supernatants were

firstly analyzed for antibody reactivity against U937 cells using indirect immunofluorescence assay. The positive supernatants were then differentially screened for CD99 specificity using CD99 transfected COS cells. By this procedure, CD99 mAb MT99/3, which is of the IgG2a isotype, was obtained.

2.5. Immunofluorescence analysis

Cells were analyzed by indirect immunofluorescence using FITC-conjugated sheep F(ab')2 anti-mouse immunoglobulin antibodies (Immunotech/Coulter Corporation, Miami, FL). To block nonspecific Fc-receptor-mediated binding of mAbs, cells were pre-incubated for 30 min at 4°C with 10% human AB serum before staining. Membrane fluorescence was analyzed on a FACSCalibur (Becton Dickinson, Sunnyvale, CA) flow cytometer. Individual populations of blood cells were gated according to their forward and side scatter characteristics.

2.6. Labeling of cell surface proteins and immunoprecipitation

For surface labeling, cells were biotinylated by using Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) (5 mM) for 1 h at 4°C. The reaction was quenched by washing the cells once with 1 mM glycine in PBS and then twice with PBS. Cells (5×10^7) were solubilized in 1 ml lysis buffer (1% NP-40, 50 mM Tris–HCl pH 8.2, 100 mM NaCl, 2 mM EDTA, 5 mM iodoactamide, 1 mM PMFS, and 10 μg/ml aprotinin). Cell lysates were precleared with protein A-sepharose beads coated with mouse immunoglobulins. Precleared lysates were then mixed with purified mAb coated protein A-sepharose beads for 24 h at 4°C. After immunoprecipitation and SDS-PAGE, biotinylated proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS for 1 h at room temperature. The blocked membrane was incubated for 1 h at room temperature with avidin-peroxidase (Dako, Glostrup, Denmark), and the biotinylated proteins were visualized by the chemiluminescence detection system (Pierce).

2.7. Homotypic cell aggregation assay

Jurkat cells were washed three times with RPMI-1640 medium and resuspended to a concentration of 2.5×10^6 cells/ml with RPMI-1640 supplemented with 10% FBS and antibiotics. One hundred microliters of cell suspension was then distributed into 96 well flat-bottomed microplates (NUNC). mAbs were added to each well to a final concentration of 2.5 μg/ml to obtain the total volume of 200 μl. Plates were incubated in a CO_2 incubator and examined for homotypic cell aggregation under a phase-contrast inverted microscope (Olympus, Tokyo, Japan) at various time points. The degrees of cell aggregation were scored as follows: 0, no aggregation (>90% of cells were unaggregated); 1+, the majority of cells were un-aggregated, but a few small clusters of <20 cells were observed (this level of adhesion is typical of the spontaneous adhesion exhibited by many lymphoblastoid cell lines including Jurkat); 2+, 50% of cells were in medium-sized aggregates (20–50 cells), with the remainder as single cells; 3+, nearly all cells were in medium-sized to large aggregates (>50 cells) with only a few (<20%) unaggregated cells; and 4+, >90% of the cells were in large aggregates. Photographs were taken with an Olympus camera under an inverted microscope.

In order to study the effect of LFA-1 and ICAM-1 antibodies on CD99 mediated cell aggregation, purified mAbs to CD11a (MEM-25) and CD54 (MEM-111) were added into wells containing Jurkat and CD99 mAb MT99/3. Cell aggregation was then determined and scored as described above.

To study the effect of cytochalasin B or genistein on CD99 mAb induced cell aggregation, Jurkat cells were cultured with purified MT99/3 (2.5 μg/ml) in the presence or absence of cytochalasin B or genistein for 4 h. To study the effect of sphingosine, Jurkat cells were pre-incubated with sphingosine at 37°C in a 5% CO_2 incubator for 15 min. Cells were washed twice and re-cultured with purified MT99/3 mAb.

2.8. LFA-1 and ICAM-1 expression on CD99 mAb treated Jurkat

Jurkat cells were incubated with or without 2.5 μg/ml of CD99 mAb MT99/3 for 30 min 1, 2 and 4 h in a CO_2 incubator at 37°C. Cells were then washed once and stained with PE-labeled mAbs HI111(CD11a), HA58 (CD54) and X40 (isotype matched control). Stained cells were analyzed by a flow cytometer.

3. Results

3.1. Isolation of a CD99 cDNA clone and production of a CD99 mAb

To isolate the cDNA encoding the CD99 protein, a high-efficiency COS cell expression system was employed [19]. By using this technique, a cDNA clone, termed H11, was isolated from a HPB-ALL library. The nucleotide sequence of the insert was partially determined and compared with the EMBL nucleotide database. This comparison indicated that the H11 cDNA insert was identical to the cDNA of CD99.

To generate new CD99 mAbs, a mouse was immunized with H11 plasmid transfected COS cells and

spleen cells from the immunized mouse were fused with myeloma cells using the standard hybridoma technique. The specificity of the antibodies secreted by the hydridomas was determined by indirect immunofluorescence using CD99 transfected COS cells. By this screening, a CD99 mAb, designated MT99/3 (IgG2a isotype), which strongly bound to CD99 transfectants but not bound to mock or any other transfectants was obtained. To confirm that MT99/3 is a CD99 mAb, the reactivity of this mAb to various cell lines and peripheral blood cells was studied and compared with reported reactivity of established CD99 mAbs. All cell lines tested including the human monocytic cell line U937, the erythroid/myeloid cell line K562 and the T-cell lines Jurkat, Sup T1 and Molt4 were positive with MT99/3 (Fig. 1). With peripheral blood mononuclear cells, the MT99/3 mAb bound to lymphocytes and monocytes but not to neutrophils ($n = 7$) (Fig. 2A–C). Red blood cells of seven healthy donors were also studied for staining by MT99/3. Red blood cells from five donors were positive (Fig. 2D), and these from two donors were negative (Fig. 2E). All of these staining patterns were similar to those found in previous studies using CD99 mAbs [12–14,21–23]. Together with the transfectant experiments, these results indicate that MT99/3 is a CD99 mAb.

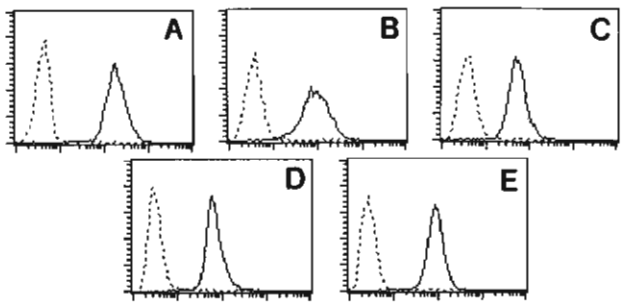


Fig. 1. Immunofluorescence analysis of the reactivity of mAb MT99/3 with U937 (A), K562 (B), Jurkat (C), SupT1 (D) and Molt4 (E) cells. Solid lines represent the immunofluorescence profiles of cells stained with MT99/3 mAb and dashed lines represent background fluorescence of the negative control mAb.

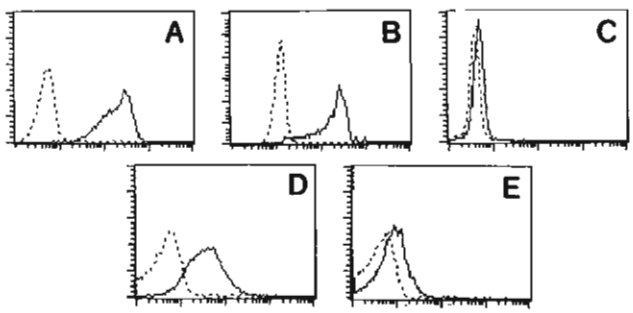


Fig. 2. Immunofluorescence analysis of the reactivity of mAb MT99/3 with peripheral blood lymphocytes (A), monocytes (B) neutrophils (C), red blood cells which show positive (D) and negative (E) reactivity, respectively. Solid lines represent the immunofluorescence profiles of cells stained with MT99/3 mAb and dashed lines represent background fluorescence of the negative control mAb.

These data were confirmed by biochemical characterization of the cell surface antigen recognized by MT99/3. mAb MT99/3 precipitated a broad protein band of ≈ 27–34 kDa from lysates of surface-biotinylated Jurkat cells (Fig. 3). This broad zone contained a major protein band of 32 kDa and a minor protein band of 28 kDa. A similar precipitation pattern was described for other CD99 mAbs [3,12,14,21–24]. P-3E10, an IgG2a isotype matched control mAb, which recognizes an un-clustered leukocyte surface antigen, precipitated a protein band of 55 kDa, and MT4, an irrelevant mAb, did not precipitate any protein band (Fig. 3).

3.2. MT99/3 induces homotypic cell aggregation of Jurkat cells via a LFA-1/ICAM-1 independent pathway

As CD99 mAbs were reported to induce homotypic aggregation of several lymphoid cells [2,3], we investigated this phenomenon by using our new generated CD99 mAb. Purified mAb MT99/3 induced homotypic cell aggregation in Jurkat cells (Table 1 and Fig. 4) but not in U937 and KS62 cells (data not shown). mAb MT99/3 induced Jurkat cell aggregation was dose-dependent. Some smaller cell aggregates were already observed with 1 μg/ml of purified MT99/3 and maximal aggregation was seen when 2.5 μg/ml or higher concentrations of the mAb were applied (data not shown). We also studied the kinetic of MT99/3 induced cell aggregation; after 30 min small aggregates were visible which gradually increased and reached their maximal size (4+) at 4 h (Table 1).

Homotypic aggregation induced by CD99 mAbs has been shown to be both LFA1 dependent [3] and LFA-1 independent [2]. We, therefore, examined the effect of LFA1 and ICAM-1 mAbs on MT99/3 mediated Jurkat cell aggregation. In our studies, Jurkat cells were cultured in the presence of MT99/3, MT99/3 + MEM-25 (a CD11 a mAb), or MEM-25 only. We found that MEM-25 (50 μg/ml) by itself slightly induced Jurkat aggregation after 1 and 2 h incubation, however, in the presence of MT99/3 or MT99/3 + MEM-25 the degree of aggregation was increased (Table 2). These results indicated that mAb MEM-25 had no effect on MT99/3 mediated cell aggregation. Also MEM-111, an ICAM-1 mAb, did not block MT99/3 induced cell aggregation (Table 2). Furthermore, we studied the level of LFA-1 and ICAM-1 expression on Jurkat cells after engagement of CD99 by MT99/3. As determined by PE-labeled HI111 (CDT 1a) and HA58 (CD54) mAbs the expression of LFA-1 and ICAM-1 molecules did not change upon MT99/3 treatment (Table 3). These results indicate that homotypic aggregation of Jurkat cells induced by CD99 mAb is LFA-1/ICAM-1 independent.

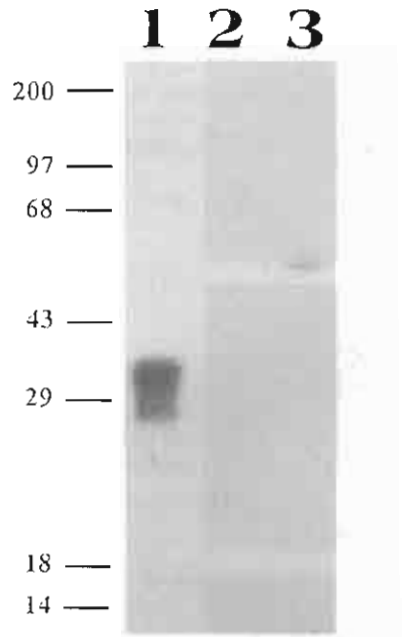


Fig. 3. Biochemical characterization of the cell surface antigen recognized by mAb MT99/3. SDS-PAGE analysis of immunoprecipitates obtained from lysates of biotin labeled Jurkat cells using MT99/3 (lane 1), MT4 control mAb (lane 2) and P-3E10 control mAb (lane 3). Electrophoresis was performed under reducing conditions. The positions of molecular weight markers are indicated on the left in kDa.

3.3. Sphingosine, genistein and cytochalasin B block cell aggregation induced by mAb MT99/3

To analyze whether intracellular signaling is required for mAb MT99/3 induced Jurkat cell aggregation, we analyzed the effect of various inhibitors of protein kineses and cytoskeleton reorganization. Table 4 and Fig. 4 show that the PKC inhibitor sphingosine and the PTK inhibitor genistein blocked mAb MT99/3 induced Jurkat cell aggregation in a dose-dependent manner. Cytochalasin B, an actin filament polymerization blocking agent, also blocked completely mAb MT99/3 induced cell aggregation, which indicates a requirement for an intact cytoskeleton (Table 4 and Fig. 4). In all experiments, DMSO diluent controls had no effect on cell aggregation (data not shown). Cell viability for all inhibitors at all concentrations used was analyzed and found to be greater than 95%.

4. Discussion

In this report, we have isolate a cDNA encoding the CD99 molecule and used this cDNA to generate CD99 transfectants for immunization to produce new CD99 mAbs. One CD99 mAb, named MT99/3, was established and found to induce homotypic cell aggregation of Jurkat cells. This CD99 mAb mediated cellular effect involves PKC and PTK-dependent intracellular signaling pathways but is independent of the integrin LFA-1 and its counterreceptor ICAM-1.

The CD99 molecule is a 32 kDa transmembrane glycoprotein which was assigned at the Fifth International Workshops on Human Leukocyte Differentiation Antigens [25]. The CD99 molecule has been demonstrated previously to be involved in cell adhesion [2,3,13–15,26] and apoptosis [15]. To further investigate the role of CD99 in cellular adhesion, we established new CD99 mAbs. For this purpose, we isolate the CD99 cDNA and used it to transfect COS cells for immunization and mAb screening. One mAb specific for the CD99 molecule, named MT99/3, was obtained. MT99/3 mAb precipitated a major protein band of $\approx$ 32 kDa which is the same as that described for the CD99 molecule [12,14,22,24,25]. A 28 kDa minor protein band was also precipitated by MT99/3. This 28 kDa polypeptide presumably represents the unsialylated, fully glycosylated CD99 molecule as suggested by previous biochemical studies [12,14] or a truncated form of the CD99 molecule which was recently described [3]. The epitope recognized by MT99/3 was broadly expressed on hematopoietic cell lines, human lymphocytes and monocytes, but not on neutrophils. This staining pattern is in agreement with that described for other CD99 mAbs [12,13,21–23]. On red blood cells, we found that the epitope recognized by mAb MT99/3 is heterogeneously expressed as erythrocytes from five out of seven donors reacted with mAb MT99/3. This is probably due to the quantitative polymorphism of CD99 expression on human erythrocytes, involving the blood group Xg phenotype [12,21,23]. Together, the biochemical data and the staining pattern confirmed that mAb MT99/3 is directed to the CD99 molecule.

Table 1
Homotypic cell aggregation of Jurkat cells induced by mAb MT99/3[a]

MAb	Homotypic cell aggregation			
	Incubation time (h)			
	0.5	1	2	4
MT99/3	1[b]	2	3	4
P-3E10	0	1	1	1
MT4	0	1	1	1
–	0	1	1	1

[a] Jurkat cells were cultured for 30 min, 1, 2 and 4 h in the presence or absence of mAb MT99/3 (2.5 μg/ml). Homotypic cell aggregation was determined under an inverted microscope.

[b] Degree of cell aggregation was graded as described in Section 2. Results are representative of ten separate experiments.

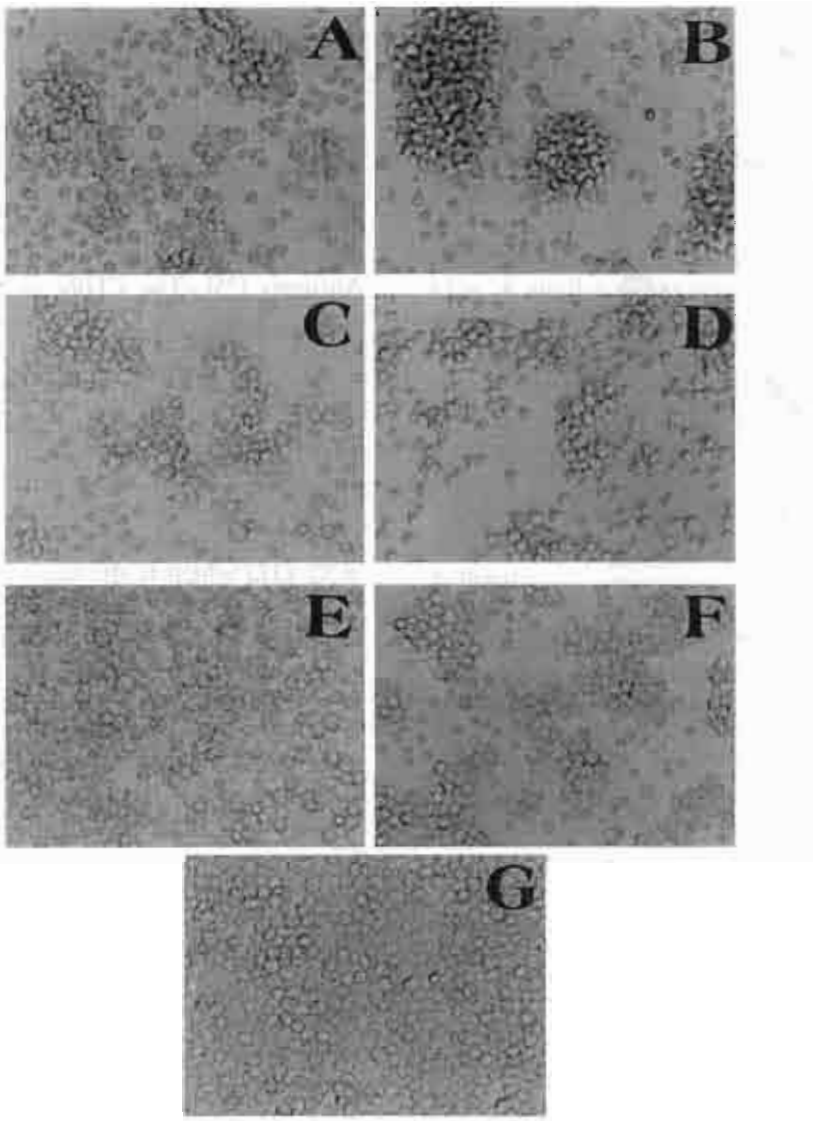


Fig. 4. Photomicrographs (× 100) of homotypic cell aggregation induced by mAb MT99/3. Jurkat cells were incubated for 4 h with medium (A), 2.5 μg/ml MT99/3 mAb (B). MT4 control mAb (C) and P-3E10 control mAb (D). Jurkat cells were pretreated with sphigosine (20 μM) and subsequently cultured in the presence of MT99/3 (E). Jurkat cells were culture with mAb MT99/3 in the presence of genistein (25 μg/ml) (F) or cytohalasin B (50 μM) (G).

Table 2
Effect of LFA-1 and ICAM-1 mAbs on Jurkat cell aggregation induced by mAb MT99/3[a]

Conditions	Homotypic cell aggregation				
	Incubation time (h)				
	0.5	1	2	3	4
Jurkat alone	0[b]	1+	1+	1+	1+
Jurkat + MT99/3	1+	2+	3+	4+	4+
Jurkat + MT99/3 + MEM-25 (50 μg/ml)	1+	2+	3+	4+	4+
Jurkat + MT99/3 + MEM-111 (50 μg/ml)	1+	2+	3+	4+	4+
Jurkat + MT99/3 + MEM-111 (100 μg/ml)	1+	2+	3+	4+	4+
Jurkat + MEM-25 (50 μg/ml)	0	1+	2+	4+	4+
Jurkat + MEM-111 (50 μg/ml)	0	1+	1+	1+	1+
Jurkat + MEM-111 (100 μg/ml)	0	1+	1+	1+	1+

[a] Jurkat cells were cultured for 30 min, 1, 2, 3 and 4 h with or without mAb MT99/3 in the presence or absence of purified MEM-25 (CD11a mAb) or MEM-111 (CD54 mAb). Homotypic cell aggregation was determined under an inverted microscope.

[b] Degree of cell aggregation was graded as described in Section 2.

Table 3
Expression of LFA-1 and ICAM-1 molecules on mAb MT99 3 treated Jurkat cells[a]

Treatment time	Mean fluorescence intensity					
	X40-PE[b]		HI111-PE[b]		HA58-PE[b]	
	Untreated	MT99 3-treated	Untreated	MT99 3-treated	Untreated	MT99 3-treated
30 min	3.4	3.5	27.8	36.7	23.4	31.7
1 h	3.5	3.6	34.8	36.2	29.2	33.9
2 h	3.5	3.5	34.3	37.7	28.9	32.3
4 h	3.8	3.4	44.6	41.4	35.1	32.1

[a] Jurkat cells were incubate for 30 min, 1, 2, and 4 h at 37°C with or without mAb MT99 3. Cells were washed and stained with indicated mAbs and analyzed by a flow cytometer.

[b] X40-PE, negative control antibody; HI111-PE, anti-CD11a mAb; HA58-PE, anti-CD54 (ICAM-1) mAb. Results are the mean of the mean fluorescence intensity of two separate experiments.

Table 4
Effect of various inhibitors on Jurkat cell aggregation induced by mAb MT99.3[a]

Culture conditions	Concentration of inhibitors	Target of inhibitors	Degree of aggregation
Jurkat alone	-	-	1
Jurkat + MT99 3	-	-	4
Jurkat + MT99 3 + sphingosine	20 μM	PKC	1
Jurkat + MT99 3 + sphingosine	10 μM	PKC	3
Jurkat + MT99 3 + sphingosine	5 μM	PKC	4
Jurkat + MT99.3 + genistein	25 μg/ml	PTK	1
Jurkat + MT99 3 + genistein	12.5 μg/ml	PTK	3
Jurkat + MT99.3 + genistein	6.5 μg/ml	PTK	4
Jurkat + MT99 3 + Cytochalasin B	50 μM	Actin filament	1
Jurkat + MT99 3 + Cytochalasin B	25 μM	Actin filament	1
Jurkat + MT99 3 + Cytochalasin B	12.5 μM	Actin filament	1

[a] Jurkat cells were incubated for 4 h with medium or 2.5 μg/ml MT99 3 mAb, or pretreated with sphigosine followed by culturing in the presence of mAb MT99/3. For genistein or cytochalasin B, Jurkat cells were culture with mAb MT99/3 in the presence of genistein or cytohalasin B. Results are representative of two separate experiments.

Intercellular adhesive events are one of the critical steps in the regulation of immune processes. Engagement of various cell surface molecules with specific ligands or mAbs generate proadhesive signals that induce cell adhesion [1–7,27]. In the present study we demonstrate that the CD99-specific mAb MT99/3 can induce homotypic aggregation of Jurkat cells. However, U937 and K562, which also express CD99 molecules, did not show cell aggregation in response to MT99/3. Thus, our results indicate that MT99/3 specifically induce homotypic aggregation of a T-cell line but not of myeloid cell lines.

Interaction of LFA-1 with ICAM-1 is important in a variety of adhesion-dependent interactions of immune cells [8,28,29], and engagement of various cell surface molecules with specific ligands or mAbs activates LFA-1 at the cell surface [1,3,27,30]. In this report, we found that the Jurkat cell aggregation induced by MT99/3 was not inhibited by LFA-1 and ICAM-1 mAbs, even when high concentrations (100 μg/ml) of the blocking antibodies were used. The blocking capacity of these LFA-1 (MEM-25) and ICAM-1 (MEM-111) mAbs was demonstrated previously: they could inhibit cell aggregation of U937 cells induced by mAb M6-1D4, a CD147 mAb [1]. Therefore, these results indicate that engagement of the CD99 molecule by CD99 mAb MT99/3 induces aggregation of Jurkat cells which is independent of LFA-1/ICAM-1 pathways. This finding is in agreement with that shown in thymocytes by Bernard et al. [2]. However, it is in contrast to the CD99-induced aggregation of IM-9 cells reported to be LFA-1 dependent [3]. At the moment, we do not have any explanation for this discrepancy. One possibility could be that CD99 is involved in regulation of different cell adhesion events in T-cells (Jurkat) versus B-cells (IM-9). Alternatively, the difference between CD99 mediated induction of LFA-1 dependent and independent cell adhesion mechanisms could be a result of the methods used: cross-linking of the CD99 mAb with anti-mouse IgG was performed to induce IM-9 aggregation [3] while we and Bernard et al. [2] could induce cell aggregation with soluble CD99 mAbs.

Up-regulation of LFA-1 expression on the surface of IM-9 and Jurkat cells by CD99 mAb has been described [3]. Our results, however, demonstrate that engagement of CD99 by our newly established mAb MT99/3 did not increase expression of CD11a (LFA-1 α_L chain) and CD54 (ICAM-1) molecules on Jurkat cells. This observation together with the finding that LFA-1 and ICAM-1 mAbs cannot block MT99/3 induced cell aggregation indicate the independence of the CD99 adhesion pathway described here from LFA-1/ICAM-1. As aggregation of thymocytes induced by CD99 mAbs could also not be blocked by mAbs to β1, β2 and β3 integrins, Bernard et al. [2] postulated that an unidentified integrin or a peculiar epitope of a known integrin is involved in CD99-mediated cell aggregation.

Signal transduction, the process of transmission of an extracellular message to the interior of the cell, requires the activation of specific cell membrane receptors and downstream intracellular signaling cascades. Several specific ligands or antibodies against cell surface receptors have been reported to induce intracellular signal transduction resulting in a physiological response of the cell. Homotypic aggregations of monocytic and lymphoid cells are mediated by PKC and/or PTK-dependent intracellular signaling pathway after engagement of cell surface molecules [5,30–33]. We, therefore, asked whether PKC and/or PTK-dependent intracellular signaling pathways are involved in CD99 mAb mediated cell aggregation. Our results show that the PKC inhibitor sphingosine and the PTK inhibitor genistein significantly blocked cell aggregation induced by MT99/3. This finding indicates that CD99 mediated cell adhesion of Jurkat cells is an active process involving both PKC and PTK dependent intracellular signaling pathways. While this manuscript was in preparation, Wingett et al. [34] reported that ligation of CD99 resulted in pronounced tyrosine phosphorylation of an $\approx$29 kDa protein and suggested that a specific CD99 linked signal transduction pathway may exist.

Cell adhesion mediated by several adhesion molecules requires an intact cytoskeleton for establishment and maintenance of stable cell conjugates. In this report, paralysis of the cytoskeleton by cytochalsin B completely abrogated adhesion induced by mAb MT99/3, implying that Jurkat aggregation induced via CD99 mAb is strongly associated with cytoskeletal reorganization.

In conclusion, we provide evidence here that engagement of the CD99 molecule by a specific CD99 mAb newly generated by us activates intracellular signaling pathways in Jurkat cells resulting in LFA-1/ICAM-1 independent cell adhesion. We suggest that our CD99 mAb mimics a natural ligand of CD99, the identification and characterization of which will uncover the function of the CD99 system and its involvement in cell adhesion events of the immune system.

Acknowledgements

The authors wish to thank Dr Vaclav Horejsi for providing mAbs to LFA-1 and ICAM-1. This work was supported by the National Science and Technology Development Agency of Thailand, The Thailand Research Fund, and by SFB005 of the Austrian Science Fund.

References

[1] W. Kasinrerk, N. Tokrasinwit, P. Phunpae, Immunology 96 (1999) 184–192.
[2] G. Bernard, D. Zoccola, M. Deckert, J.P. Breittmayer, C. Aussel, A. Bernard, J. Immunol. 154 (1995) 26–32.
[3] J.H. Hahn, M.K. Kim, E.Y. Choi, et al., J. Immunol. 159 (1997) 2250–2258.
[4] Y.H. Nong, E. Remold-O'Donnell, T.W. LeBien, J. Exp. Med. 170 (1989) 259–267.
[5] R.P. Lauener, R.S. Geha, D. Vercelli, J. Immunol. 145 (1990) 1390–1394.
[6] A. Masellis-Smith, G.S. Jensen, J.G. Seehafer, J.R. Slupsky, A.R.E. Shaw, J. Immunol. 144 (1990) 1607–1613.
[7] G.S. Kansas, J.C. Cambier, T.F. Tedder, Eur. J. Immunol. 22 (1992) 147–1527.
[8] R.O. Hynes, Cell 69 (1992) 11–25.
[9] R. Levy, J. Dilley, R.I. Fox, R. Warnk, Proc. Natl. Acad. Sci. USA 76 (1979) 6552–6556.
[10] S.M. Darling, P.J. Goodfellow, B. Pym, G.S. Banting, C. Princhared, P.N. Goodfellow, Cold Spring Harbor Symp. Quant. Biol. 51 (1986) 205–212.
[11] G.S. Banting, B. Pym, S.M. Darling, P.N. Goodfellow, Mol. Immunol. 26 (1989) 181–188.
[12] C. Gelin, F. Aubrit, A. Phalipon, B. Raynal, S. Cole, M. Kaczorek, A. Bernard, EMBO J. 8 (1989) 3253–3259.
[13] A. Bernard, F. Aubrit, B. Raynal, D. Pham, L. Boumsell, J. Immunol. 140 (1988) 1802–1827.
[14] F. Aubrit, C. Gelin, D. Pham, B. Raynal, A. Bernard, Eur. J. Immunol. 19 (1989) 1431–1436.
[15] G. Bernard, J.P. Breittmayer, M. de Matteis, P. Trampont, P. Hofman, A. Bernard, J. Immunol. 158 (1997) 2543–2550.
[16] E.Y. Choi, W.S. Park, K.C. Jung, S.H. Kim, Y.Y. Kim, W.J. Lee, S.H. Park, J. Immunol. 161 (1998) 749–754.
[17] W. Kasinrerk, N. Tokrasinwit, P. Naveewongpanit, J. Med. Assoc. Thai. 81 (1998) 879–892.
[18] E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1988.
[19] B Seed, A. Aruffo, Proc. Natl. Acad. Sci. USA 84 (1987) 3365–3369.
[20] W. Kasinrerk, E. Fiebiger, I. Stefanova, T. Brumruker, W. Knapp, H. Stockinger, J. Immunol. 149 (1992) 847–854.
[21] P.N. Goodfellow, P. Tippett, Nature 289 (1981) 404–405.
[22] A. Bernard, V. Gay-Bellile, M. Amiot, B. Caillou, P. Charbord, L. Boumsell, J. Immunol. 132 (1984) 2338–2344.
[23] F. Latron, D. Blanchard, J.P. Cartron, Biochem. J. 247 (1987) 757–764.
[24] A.C. Petty, P. Tippett, Vox Sang 69 (1995) 231–235.
[25] S. Schlossman, L. Boumsell, W. Gilks, et al., Leukocyte Typing V, Oxford University Press, Oxford, 1994.
[26] C. Aussel, G. Bernard, J.P. Breittmayer, C. Pelassy, D. Zoccola, A. Bernard, Biochemistry 32 (1993) 10096–100101.
[27] R. Pardi, L. Inverardi, J.R. Bender, Immonol. Today 13 (1992) 224–230.

[28] T.A. Springer, Nature 346 (1990) 425–434.
[29] E.C. Butcher, L.J. Picker, Science 272 (1996) 60–66.
[30] G.S. Kansas, T.F. Tedder, J. Immunol. 147 (1991) 4094–4102.
[31] W. Mourad, R.S. Geha, T. Chatila, J. Exp. Med. 172 (1990) 1513–1516.
[32] N. Odum, H. Yoshizumi, Y. Okamoto, N. Kamikawaji, A. Kimura, Y. Nishimuru, T. Sasazuki, Human Immunol. 35 (1992) 71–84.
[33] H.M. Lorenz, T. Harrer, A.S. Lagoo, A. Baur, G. Eger, J.R. Kalden, Cell Immunol. 147 (1993) 110–128.
[34] D. Wingett, K. Forcier, C.P. Nielson, Cell Immunol. 193 (1999) 7–23.