RSA/23/2537
สำนักงานกองทุนสนับสนุนการวิจัย

รายงานฉบับสมบูรณ์

(1 ธันวาคม 2537 - 30 พฤศจิกายน 2540)

การผลิตโมโนโคลนอล แอนติบอดี ต่อ CD4 และ CD8 โปรตีน และการพัฒนาวิธีตรวจเพื่อช่วยพยากรณ์ความรุนแรงของโรคและใช้เป็นแนวทางการรักษาผู้ติดเชื้อเอดส์

Production of anti CD4 and CD8 monoclonal antibodies and development of techniques for prognosis of HIV infection and monitoring of therapy

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ภาควิชาภูมิคุ้มกันวิทยาคลินิก
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สารบัญ

บทคัดย่อ

โครงการวิจัยนี้มีวัตถุประสงค์เพื่อผลิตแอนติบอดี ต่อ CD4 และ CD8 โปรตีน แล้วนำแอนติบอดีที่ผลิตได้มาพัฒนาเป็นวิธีและน้ำยาเพื่อตรวจนับจำนวน CD4+ cells

จากการศึกษาใน 3 ปี (ธันวาคม 2537-พฤศจิกายน 2540) ผู้วิจัยได้ทำการศึกษาโดยมีรายละเอียดและผลการศึกษาดังนี้คือ

1. ผู้วิจัยสามารถแยก hybridomas ที่ผลิตโมโนโคลนอล แอนติบอดี ต่อ CD4 และ CD8 proteins ได้อย่างละ 1 โคลน ให้ชื่อว่า MT4 และ MT8 ตามลำดับ จากการศึกษาความจำเพาะของโมโนโคลนอล แอนติบอดี ที่ผลิตได้ พบว่า MT4 และ MT8 monoclonal antibody (mAb) สามารถทำปฏิกิริยาได้กับทั้ง native และ recombinant CD4 และ CD8 proteins ตามลำดับ MT4 และ MT8 mAb สามารถ inhibit ปฏิกิริยาของ standard CD4 (Leu3a) และ CD8 (Leu2) mAb ได้ และสามารถนำไปใช้ตรวจนับจำนวน CD4+ และ CD8+ cells ใน peripheral blood ได้เท่าๆ กับ การใช้ standard CD4 และ CD8 mAb ผลการศึกษายืนยันได้ว่า MT4 และ MT8 mAb ทำปฏิกิริยาจำเพาะกับ CD4 และ CD8 proteins ตามลำดับ

2. ผู้วิจัยได้นำวิธี DNA immunization มาประยุกต์ใช้ในการผลิตแอนติบอดีต่อ CD4 proteins ในหนูและในกระต่าย โดยพบว่าวิธี DNA immunization นี้สามารถกระตุ้นให้สัตว์ทดลองสร้างแอนติบอดีต่อ CD4 proteins ได้หลังการฉีด cDNA encoding CD4 protein (CD4-DNA) 1-3 ครั้ง จากการศึกษาในหนู พบว่า 4 ใน 5 ของหนูที่ฉีด CD4-DNA สร้าง anti-CD4 antibodies ขณะที่ 3 ใน 3 ของกระต่ายสร้าง anti-CD4 antibodies จากการศึกษาความจำเพาะของ CD4 polyclonal antibodies ที่ผลิตได้ พบว่าแอนติบอดีที่ผลิตได้นี้ทำปฏิกิริยาจำเพาะได้กับทั้ง native CD4 และ recombinant CD4 proteins

3. ผู้วิจัยได้พัฒนาวิธี Manual rosetting ขึ้นมาเพื่อตรวจนับจำนวน CD4+ cells และเมื่อนำวิธีที่พัฒนาขึ้นมานี้มาตรวจนับจำนวน CD4+ cells ในเลือดผู้ป่วยเอดส์และคนปกติจำนวน 40 คนเปรียบเทียบกับวิธีมาตรฐาน flow cytometry ผลการทดลองพบว่าวิธีทั้งสองให้ผลไม่แตกต่างกัน โดยมีค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.95 แต่เมื่อผู้วิจัยพัฒนาวิธี Manual rosetting ให้มีวิธีการทำที่ง่ายยิ่งขึ้น โดยการนำ MT4 monoclonal antibody ไปเคลือบบนเม็ดเลือดแดงแกะ และ tosyl-activated beads โดยตรง แล้วนำมาตรวจนับจำนวน CD4+ cells ในเลือดโดยวิธี Direct manual rosetting ผลการทดลองพบว่าจำนวน CD4+ cells ที่ได้โดยวิธี Direct manual rosetting นี้ให้ค่าต่ำกว่าวิธีมาตรฐาน flow cytometry อย่างมีนัยสำคัญ

4. ผู้วิจัยได้นำ MT4 mAb และแอนติบอดีต่อ CD4 โปรตีนที่ผลิตจากวิธี DNA immunization มาพัฒนาเป็นวิธี Sandwich ELISA เพื่อใช้ตรวจหา soluble CD4 proteins และเมื่อนำวิธีที่พัฒนาขึ้นมานี้มาหาปริมาณ soluble CD4 proteins ในเลือดผู้ติดเชื้อ HIV จำนวน 59 คน และคนปกติจำนวน 56 คน ผลการทดลองพบว่า ปริมาณ soluble CD4 proteins ในเลือดไม่มีความ

สัมพันธ์กับจำนวน absolute CD4+ cells โดยมีค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.187 และเมื่อเปรียบเทียบปริมาณ soluble CD4 proteins ในเลือดผู้ติดเชื้อ HIV กับคนปกติ พบว่าค่าที่ได้จากกลุ่มตัวอย่างทั้งสองไม่แตกต่างกัน

5. ผู้วิจัยได้นำ MT4 mAb มาผลิตเป็นน้ำยาตรวจนับจำนวน CD4+ cells เพื่อใช้กับเครื่อง flow cytometer ขึ้นมาใช้เอง โดยนำ purified MT4 mAb มาติดฉลากกับสาร fluorescein isothiocyanate (FITC) แล้วนำไปใช้ตรวจนับจำนวน CD4+ cells โดยวิธี flow cytometry และเมื่อนำ FITC labeled MT4 มาตรวจนับจำนวน CD4+ cells ในเลือดผู้ติดเชื้อเอดส์และคนปกติจำนวนอย่างละ 60 ราย เปรียบเทียบกับชุดน้ำยามาตรฐาน Simultest™ ของบริษัท Becton Dickinson ผลการศึกษาพบว่าเปอร์เซนต์และค่า absolute CD4+ cell counts ที่ได้จากน้ำยาทั้งสองชนิดใกล้เคียงกันมาก โดยมีค่าสัมประสิทธิ์สหสัมพันธ์ เท่ากับ 0.995 และ 0.996 สำหรับเปอร์เซนต์และค่า absolute CD4+ lymphocyte counts ตามลำดับ ผลการศึกษาในครั้งนี้ชี้ให้เห็นว่า น้ำยา FITC labeled MT4 ที่เตรียมขึ้นมาใช้เองนี้น่าจะเป็นน้ำยาอีกชนิดหนึ่งที่สามารถนำมาใช้ตรวจวัดระดับ CD4+ cells ในเลือดโดยวิธี flow cytometry ได้

ABSTRACT

The aims of this research are to generate antibodies against CD4 and CD8 proteins and use the generated antibodies to develop methods and reagents for CD4+ cell determination.

On completion of 3 years' work (December 1994-November 1997), the out puts from this study are as follows:

1. Two hybridomas that produced antibodies against CD4 and CD8 proteins, named MT4 and MT8 respectively, were generated. MT4 and MT8 monoclonal antibodies (mAb) were proved to be specific to CD4 and CD8 proteins as they reacted to native and recombinant CD4 and CD8, respectively. The MT4 and MT8 mAb could inhibit the reactivity of standard CD4 (Leu3a) and CD8 (Leu2) mAb. Furthermore, they could be used to enumerate CD4+ and CD8+ cells in blood samples as well as using standard CD4 and CD8 mAb. These results, therefore, demonstrate that generated MT4 and MT8 mAb are specific to CD4 and CD8 proteins, respectively.

2. DNA immunization was also used to produce polyclonal antibodies to CD4 proteins in mice and rabbits. By using this technique, anti-CD4 antibodies were induced in tested animals after 1-3 inoculations of cDNA encoding CD4 protein (CD4-DNA). In the mouse system, 4 out of 5 mice produced anti-CD4 antibodies after CD4-DNA immunization. In rabbits, 3 of 3 rabbits produced anti-CD4 antibodies after DNA immunization. These antibodies reacted with either native or recombinant CD4 molecules.

3. The manual rosetting method was developed for enumerating CD4+ cells. To evaluate this method, CD4+ cells in normal and HIV infected individuals were determined by using both manual rosetting and standard flow cytometry, simultaneously. The absolute CD4+ cell counts obtained from both methods were very similar with the correlation coefficient of 0.95. The manual rosetting technique was developed further for a simple procedure. MT4 mAb was directly coated on sheep red blood cells or tosyl-activated beads. These coated particles were then used to enumerated CD4+ cells in blood samples by direct manual rosetting technique. The CD4+ cell counts obtained from this direct manual rosetting, however, was significantly lower than those obtained by using standard flow cytometry.

4. Sandwich ELISA for quantifying soluble CD4 proteins was established by using generated MT4 mAb and rabbit anti-CD4 antibodies, which were obtained from DNA immunization. The sandwich ELISA was used to determine soluble CD4 proteins in blood

of 59 HIV infected and 56 healthy individuals. The study has shown that the levels of soluble CD4 proteins in tested blood were not correlated to the absolute number of CD4+ cells with the correlation coefficient of 0.187. When the levels of soluble CD4 proteins in HIV infected and healthy individuals were compared, no significant difference in soluble CD4 levels in both studied groups was observed.

5. To develop a home made reagent for CD4+ cell determination by flow cytometry, fluorescein isothiocyanate (FITC) was conjugated to affinity purified MT4 mAb. To evaluate this developed reagent, 30 HIV infected and 30 healthy individuals were determined for CD4+ cells by using both the commercial Simultest reagent kit (Becton Dickinson) and the home made FITC labeled MT4 mAb, simultaneously. The study has shown that both percentages and absolute CD4+ cell counts obtained from both reagents were equivalent. The correlation coefficient for regression analysis were 0.995 and 0.996 for percentages and absolute CD4+ cell counts, respectively. The results suggest that the home made FITC labeled MT4 mAb reagent is an acceptable alternative reagent for monitoring CD4+ cells in blood samples by flow cytometry.

บทนำ

เป็นที่ทราบกันดีอยู่แล้วว่าการแพร่กระจายของผู้ติดเชื้อไวรัสเอดส์ (human immunodeficiency virus; HIV) เป็นไปอย่างรวดเร็ว ปัจจุบันคาดว่าประเทศไทยมีผู้ติดเชื้อเอดส์แล้วเป็นจำนวนกว่าหนึ่งล้านคนและน่าจะยังมีผู้ติดเชื้อที่ยังไม่ได้รับการวินิจฉัยอยู่อีกเป็นจำนวนมาก มีการคาดการณ์ว่าในปี พ.ศ. 2543 จะมีคนไทยติดเชื้อเอดส์มากถึง 2-6 ล้านคน การแพร่ระบาดของโรคเอดส์อย่างรวดเร็วนี้ ทำให้เกิดผลกระทบอย่างรุนแรงต่อระบบสาธารณสุข สังคมและเศรษฐกิจของประเทศ

เมื่อเชื้อไวรัส HIV เข้าสู่ร่างกาย ผู้ติดเชื้ออาจไม่มีอาการหรือมีเพียงอาการของการติดเชื้อไวรัส (คล้ายไข้หวัด) เนื่องจากเชื้อ HIV เข้าสู่เซลล์ที่มี CD4 protein อยู่บนผิวเซลล์ (CD4+ cells) และทำให้เซลล์เม็ดเลือดขาวชนิด CD4+ cells ลดจำนวนลง การลดลงของเซลล์ดังกล่าวจะค่อยเป็นค่อยไป จนกระทั่งจำนวน CD4+ cells ลดลงน้อยกว่า 500 cells/mm^3 ผู้ป่วยจะเริ่มมีการติดเชื้อฉวยโอกาศ (opportunistic infection) การติดเชื้อจะรุนแรงขึ้นและผู้ป่วยอาจเป็นมะเร็งบางชนิดเมื่อจำนวน CD4+ cells ลดลงเหลือ 200 cells/mm^3 และผู้ป่วยส่วนใหญ่จะตายเมื่อจำนวน CD4+ cells ลดลงเหลือน้อยกว่า 50 cells/mm^3

ดังนั้นการตรวจนับจำนวน CD4+ cells ในผู้ติดเชื้อจึงสามารถนำมาใช้พยากรณ์ความรุนแรงของโรคและเป็นดัชนีบ่งชี้การให้การรักษาผู้ป่วยได้ National Institute of Health แห่งประเทศสหรัฐอเมริกาได้ให้ข้อแนะนำเกี่ยวกับการรักษาผู้ติดเชื้อ HIV ว่าถ้าจำนวน CD4+ cells ในผู้ป่วยมีมากกว่า 500 cells/mm^3 ยังไม่จำเป็นต้องให้ antiretrovirus treatment แต่ต้องตรวจนับจำนวน CD4+ cells อยู่อย่างสม่ำเสมอ และเมื่อจำนวน CD4+ cells ลดลงเหลือน้อยกว่า 500 cells/mm^3 จะต้องให้ยา antiretrovirus ในผู้ป่วยที่มี CD4+ cells น้อยกว่า 200 cells/mm^3 ควรได้รับยาป้องกันการติดเชื้อฉวยโอกาสเพิ่มเติมจากการให้ antiretroviral therapy. Public Health Service (PHS) ได้ให้ข้อแนะนำว่าผู้ติดเชื้อ HIV ทุกคนควรได้รับการตรวจนับจำนวน CD4+ cells ทุก 3-6 เดือน

การตรวจนับจำนวน CD4 cells ปัจจุบันนิยมใช้วิธี flow cytometry ซึ่งเป็นวิธีที่ให้ผลที่น่าเชื่อถือ แต่วิธีนี้ต้องการน้ำยาหรือ แอนติบอดีที่มีราคาสูงมาก ดังนั้นในการวิจัยนี้ ผู้วิจัยจึงมีความสนใจที่จะหาวิธีการใหม่หรือเตรียมน้ำยาสำหรับนำมาใช้ในการตรวจนับจำนวน CD4+ cells หรือ CD4 proteins ขึ้นมาใช้เอง โดยงานวิจัยนี้จะเริ่มตั้งแต่ การเตรียม recombinant CD4 และ CD8 proteins การผลิตแอนติบอดี ต่อ CD4 และ CD8 proteins แล้วนำ แอนติบอดีที่ได้ไปใช้ในการพัฒนาเป็นวิธีและน้ำยาตรวจนับจำนวน CD4+ cells

วัตถุประสงค์ของโครงการวิจัย

1. ผลิตแอนติบอดี ต่อ CD4 และ CD8 โปรตีนขึ้นมาใช้เอง
2. นำแอนติบอดีที่ผลิตได้ไปพัฒนาเป็นวิธีการและน้ำยาเพื่อตรวจนับจำนวน CD4+ cells
3. เปรียบเทียบวิธีที่พัฒนาขึ้นกับวิธี flow cytometry

การดำเนินงาน

1. การผลิตแอนติบอดีต่อ CD4 และ CD8 โปรตีน

เพื่อแอนติบอดีต่อ CD4 และ CD8 โปรตีน ในการศึกษานี้ได้นำวิธีการ 2 วิธีมาศึกษา คือ วิธี Hybridoma และวิธี DNA immunization โดยทำการศึกษาดังนี้

1.1 การผลิตโมโนโคลนอล แอนติบอดีต่อ CD4 และ CD8 โปรตีน มีวิธีการทำคือ

ทำการ immunize หนู Balb/C ด้วย CD4+ cell line หรือ CD8+ cell line จากนั้นนำ splenic cells ของ immunized mice มาเชื่อมกับ myeloma cells โดยมีวิธีการทำดังนี้ นำ 5 x 10^7 spleen cells มาผสมกับ myeloma cells (P3-X63-Ag8) 1 x 10^7 cells (ratio 5:1) ปั่นที่ 700 g นาน 5 นาที ดูด medium ทิ้งแล้วนำไปอุ่นที่ 37°C ประมาณ 10 นาที เติม 50% PEG solution 2 ml โดยค่อยๆ เติมในเวลา 30 วินาที เขย่าต่ออีก 30 วินาที จากนั้นเติม Iscove's modified Dulbecco's medium (IMDM) 5 ml ในเวลา 1 นาที แล้วเติม IMDM อีก 5 ml ปั่นที่ 700g 5 นาที แล้ว resuspend cells ใน 10%FCS-HAT selective medium 60 ml นำ 100 µl ของ cell suspension ไปเติมลงใน 96 well-plate ที่มี feeder cells อยู่ (เตรียม spleen feeder cells 100 µl/well ก่อน 1 คืน) นำ plate ไป incubate ที่ 37°C 5%CO_2 incubator ประมาณ 2-3 สัปดาห์ จากนั้นเก็บ culture supernatant มา screen หา clone ที่สร้างแอนติบอดีต่อแอนติเจนที่สนใจ โดยวิธี indirect immunofluorescent โดยใช้ CD4+ และ CD8+ cell line เป็นแอนติเจน จากนั้นตรวจยืนยัน supernatant ที่ให้ผลบวกอีกครั้งโดยใช้ CD4 และ CD8 transfected COS cells เป็นแอนติเจน

จากหลุมที่ให้ผลบวกจากscreening method นำ hybrid cells มาทำ single cell cloning โดยวิธี Limitting dilution จำนวน 3 รอบ จากนั้น expand hybrids ที่ได้ โดยส่วนหนึ่งนำไปเก็บใน liquid nitrogen อีกส่วนหนึ่งนำไปสร้างแอนติบอดีในหลอดทดลองหรือในช่องท้องหนู

1.2 การผลิตโพลีโคลนอล แอนติบอดีโดยวิธี DNA immunization มีวิธีการทำคือ

เริ่มต้นด้วยการเตรียม cDNA ที่กำหนดการสร้าง CD4 โปรตีนที่ insert อยู่ใน eukaryotic expression vector pπH3M โดยวิธี Cesium chloride-ethidium bromide gradient ultracentrifugation จากนั้นทำการฉีดหนู Balb/C หรือ กระต่ายด้วย plasmid DNA ที่เตรียมได้เข้าทาง intramuscular ทุกสัปดาห์ เจาะเลือดสัตว์ทดลองก่อนฉีดทุกครั้ง ตรวจหาระดับแอนติบอดีในซีรัมโดยนำไปทำปฏิกิริยากับ COS cells ที่ถูก transfect แล้วด้วย CD4-DNA โดยวิธี indirect immunofluorescent เมื่อแอนติบอดีมีระดับสูง ทำการเจาะเก็บซีรัมเพื่อนำไปศึกษาต่อไป

2. การศึกษา specificity ของแอนติบอดีที่เตรียมได้ โดยทำการศึกษาดังนี้

2.1 ศึกษาโดยให้ทำปฏิกิริยากับเซลล์ที่มีแอนติเจนที่สนใจอยู่บนผิวเซลล์

นำเซลล์ CD4+ cell lines, CD8+ cell lines และ CD4- cell line, CD8- cell lines มาทำปฏิกิริยากับแอนติบอดีที่เตรียมได้โดยวิธี indirect immunofluorescent แล้วตรวจสอบปฏิกิริยาด้วยกล้อง fluorescent หรือ flow cytometer

2.2 ศึกษาโดย immunoabsorption technique

นำแอนติบอดีที่ต้องการศึกษามา incubate กับ COS cells ที่ transfect แล้วด้วย CD4-DNA หรือ vector control ที่ 4°C นาน 30 นาที ปั่นเก็บ absorbed antibody นำ absorbed antibody และ pre-absorbed antibody มาทำปฏิกิริยากับเซลล์ที่มี CD4 protein บนผิวเซลล์ ตรวจหาปฏิกิริยาด้วยกล้อง fluorescent หรือ flow cytometer และเปรียบเทียบผลของปฏิกิริยาระหว่าง absorbed antibody และ pre-absorbed antibody

2.3 การศึกษาโดยการยับยั้งปฏิกิริยาของ specific antibody

นำ CD4+ cell line หรือ CD8+ cell line หรือ PBML มา incubate กับแอนติบอดีที่ต้องการทดสอบที่ 4°C 30 นาที ครบเวลา เติม standard CD4 mAb-PE หรือ standard CD8 mAb-PE (ซื้อจากบริษัท Becton Dickinson) แล้ว incubate ที่ 4°C 30 นาทีล้างเซลล์ 3 ครั้งด้วย 1%BSA-PBS 0.02% azide แล้วตรวจวิเคราะห์ด้วย flow cytometer คำนวนหา % inhibition ของ mean fluorescent intensity (MFI)

2.4 การศึกษาโดยการตรวจนับจำนวน CD4+ cells หรือ CD8+ cells ในเลือดโดยใช้แอนติบอดีที่เตรียมได้เปรียบเทียบกับ standard CD4 mAb หรือ standard CD8 mAb

เก็บเลือดใน heparin แล้วแยก peripheral blood mononuclear cells (PBMC) โดยวิธี Ficoll-Hypaque centrifugation นำ PBMC มาทำปฏิกิริยากับแอนติบอดีที่เตรียมได้หรือ standard monoclonal antibodies โดยวิธี indirect immunofluorescent ตรวจวิเคราะห์ด้วย flow cytomter และเปรียบ %CD4+ cells และ %CD8+ cells ที่ได้ระหว่างแอนติบอดีที่เตรียมได้กับ standard monoclonal antibodies

3. การพัฒนาวิธีและน้ำยาสำหรับตรวจนับจำนวน CD4+ cells

3.1 การตรวจนับ CD4+ cells โดยวิธี Manual Rosetting

เก็บเลือดโดยใช้ K_3-EDTA เป็นสารกันเลือดแข็ง นำ 1 ml ของเลือดผสมกับน้ำยาแตกเม็ดเลือดแดง FACS lysing solution (Becton Dickinson) 5 ml incubate ที่อุณหภูมิห้อง 10 นาที ล้างเซลล์ 2 ครั้งด้วย 1%BSA-PBS Azide แล้ว resuspend cells ด้วย 1%BSA-PBS

Azide 250 μl แล้วเติม AB serum ลงไป 25 μl แล้วนำไป incubate ในน้ำแข็ง 30 นาที ดูดเซลล์ 25 μl ใส่ใน microcentrifuge เติม 10 μl anti-CD4 monoclonal antibody แล้ว incubate ในน้ำแข็ง 30 นาที ครบเวลา ปั่นล้าง 2 ครั้งด้วย 1%BSA-PBS Azide แล้ว resuspend ด้วย 1%BSA-PBS Azide 40 μl เติม 10 μl ของ anti-mouse immunoglobulins coated beads (Dynal) แล้วนำไป rotate 1 ชั่วโมง ครบเวลานำเซลล์ 10 μl ไปผสมกับน้ำยา Turk's solution 10 μl แล้วนับ % rosettes ใน hemacytometer ด้วยกล้องจุลทรรศน์ธรรมดา โดยนับ rosettes ในเม็ดเลือดขาวอย่างน้อย 200 เซลล์

3.2 การตรวจหาปริมาณ soluble CD4 protein ในเลือดโดยวิธี Sandwich ELISA

ทำการเคลือบ anti-CD4 monoclonal antibody ใน ELISA 96 well plate เติม serum หรือ plasma แล้ว incubate ที่ 37°C 1 ชั่วโมง ล้างเพลต 3 ครั้ง แล้วเติม rabbit anti-CD4 antibody (ที่ได้โดยวิธี DNA immunization) incubate ที่ 37°C 1 ชั่วโมง ล้างเพลต 3 ครั้ง แล้วเติม anti-rabbit immunoglobulin-peroxidase แล้ว incubate ที่ 37°C 1 ชั่วโมง ล้างเพลต 3 ครั้ง เติม TMB substrate แล้ววัดค่า OD ที่ความยาวคลื่น 450 nm.

3.3 การเตรียมน้ำยา FITC labeled anti-CD4 mAb เพื่อตรวจนับจำนวน CD4+ cells

การเตรียมน้ำยานี้เริ่มที่การเตรียม anti-CD4 monoclonal antibody (MT4) ในรูป ascites จากนั้นเตรียมแอนติบอดีให้บริสุทธิ์โดยวิธี Affinity chromatography นำ purified anti-CD4 mAb ที่ได้มาติดฉลากด้วย fluorescein isothiocyanate (FITC) โดยวิธี Alkaline reaction ทำการแยก unbound FITC ออกจาก FITC labeled anti-CD4 mAb โดยวิธี Ultrafiltration (Amicon)

ทำการพิสูจน์ประสิทธิภาพของน้ำยา FITC labeled MT4 mAb ที่ได้โดยการนำไปตรวจนับจำนวน CD4+ cells ในเลือดคนปกติและผู้ป่วยเอดส์โดยวิธี Direct immunofluorescent แล้วตรวจหาปริมาณ CD4+ cells ด้วย flow cytometer เปรียบเทียบผลที่ได้กับวิธีมาตรฐาน flow cytometry โดยใช้ชุดน้ำยา Simultest (Becton Dickinson)

ผลที่ได้รับจากการดำเนินงาน

1. การผลิตแอนติบอดีต่อ CD4 และ CD8 โปรตีน

1.1 การผลิตโมโนโคลนอล แอนติบอดีโดยวิธี Hybridoma

จากการศึกษาโดยนำ CD4+ และ CD8+ cell line มาเป็นแอนติเจนในการฉีดหนูเพื่อผลิตโมโนโคลนอล แอนติบอดี หลังการ fusion ทำการ screen hybrids ที่ได้โดยนำ supernatant มาทำปฏิกิริยากับ CD4+ และ CD8+ cells (โดยวิธี indirect immunofluorescent) จากนั้นจึงนำ supernatants ที่ให้ผลบวกมาทดสอบกับ CD4-DNA transfected COS cells และ CD8-DNA transfected COS cells จากการศึกษาครั้งนี้ผู้วิจัยสามารถแยก hybridomas ที่ผลิตโมโนโคลนอล แอนติบอดี ต่อ CD4 และ CD8 protein ได้อย่างละ 1 โคลน ให้ชื่อว่า MT4 และ MT8 ตามลำดับ ทั้ง MT4 และ MT8 มี isotype เป็นชนิด IgM

จากการศึกษาความจำเพาะของแอนติบอดีทั้งสองพบว่า

1. MT4 monoclonal antibody (mAb) ทำปฏิกิริยาได้กับ CD4-DNA transfected COS cells แต่ไม่ทำปฏิกิริยา CD8-DNA transfected COS cells ทางตรงข้าม MT8 mAb ทำปฏิกิริยาได้กับ CD8-DNA transfected COS cells แต่ไม่ทำปฏิกิริยา CD4-DNA transfected COS cells
2. MT4 และ MT8 mAb ทำปฏิกิริยาจำเพาะกับ cell line expresing CD4 และ CD8 protein ตามลำดับ แต่ไม่ทำปฏิกิริยากับ CD4- หรือ CD8- cell lines
3. MT4 และ MT8 mAb สามารถ inhibit standard anti-CD4 mAb (Leu3) และ standard anti-CD8 mAb (Leu2) ในการจับกับแอนติเจนจำเพาะ
4. เมื่อนำ MT4 และ MT8 mAb มาใช้ตรวจนับจำนวน CD4+ และ CD8+ cells ในเลือดพบว่าให้ค่าใกล้เคียงกับการใช้ standard anti-CD4 mAb และ standard anti-CD8 mAb

ดังนั้นจึงยืนยันได้ว่า MT4 และ MT8 เป็นโมโนโคลนอล แอนติบอดี ต่อ CD4 และ CD8 โปรตีนตามลำดับ

ดูรายละเอียดของผลการทดลองใน manuscript: Kasinrerk W, Tokrasinwit N, Naveewongpanit P. Production of CD4 monoclonal antibody and development of home made reagent for CD4+ lymphocyte determination. J Med Ass Thai (submitted, 1997) ซึ่งได้แนบมาด้วยแล้ว

1.2 การผลิตโพลีโคลนอล แอนติบอดีโดยวิธี DNA immunization

ในช่วง 5-6 ปีที่ผ่านมา ได้มีการศึกษาการกระตุ้นระบบภูมิคุ้มกันต่อไวรัสและโปรตีนหลายชนิดโดยการฉีด DNA ที่กำหนดการสร้างโปรตีนนั้นเข้าไปในสัตว์ทดลองแทนการฉีดด้วยโปรตีนแอนติเจน เรียกวิธีดังกล่าวว่า DNA immunization หรือ DNA vaccination ในการศึกษานี้ผู้วิจัยได้นำเอาวิธี DNA immunization มาใช้ในการผลิตแอนติบอดีต่อ CD4

โปรตีน การศึกษานี้เริ่มต้นด้วยการฉีด DNA encoding CD4 protein (CD4-DNA) เข้าทาง intraquadriceps muscle ของขาหลังของหนู Balb/C 100 μg/dose ทุกสัปดาห์ เก็บเลือดหนูทาง retro-orbital plexus ก่อนการฉีดทุกครั้ง แล้วนำซีรัมมาทดสอบหา anti-CD4 antibody activity โดยวิธี indirect immunofluorescent โดยใช้ CD4-DNA transfeted COS cells และ CD8-DNA transfected COS cells เป็นแอนติเจน ผลการทดลองพบว่า 4 ใน 5 ของหนูที่ฉีดด้วย CD4-DNA สร้างแอนติบอดีต่อ CD4 โปรตีน โดยมี antibody titer 1:40 ถึง 1:160

นอกจากนี้ ยังได้ศึกษาการสร้างแอนติบอดีต่อ CD4 โปรตีนโดยวิธี DNA immunization ในกระต่าย โดยในการศึกษานี้ผู้วิจัยได้ศึกษาในกระต่าย 3 ตัว คือ ตัวที่ 1 ฉีด sucrose ก่อน 20 นาที แล้วฉีด CD4-DNA ตัวที่ 2 ฉีด sucrose ก่อน 20 นาที แล้วฉีด CD4-DNA ผสมกับ chloroquine ตัวที่ 3 ฉีดยา bupivacaine ก่อน 24 ชั่วโมง แล้วฉีด CD4-DNA จากนั้นเก็บซีรัมทุกสัปดาห์แล้วนำซีรัมมาทดสอบหา anti-CD4 antibody activity โดยวิธี indirect immunofluorescent โดยใช้ CD4-DNA transfeted COS cells และ CD8-DNA transfected COS cells เป็นแอนติเจน ผลการทดลองพบว่า กระต่ายทั้ง 3 ตัวสามารถสร้างแอนติบอดีต่อ CD4 proteins โดยที่กระต่ายตัวที่ 2 ซึ่งฉีดด้วย sucrose และ CD4-DNA + chloroquine ให้ผลดีที่สุด โดยสามารถกระตุ้นให้กระต่ายสร้างแอนติบอดีหลังการฉีด CD4-DNA เพียง 7 วัน (1 dose) ระดับของแอนติบอดีของกระต่ายทั้ง 3 ตัวเพิ่มขึ้นเมื่อฉีด CD4-DNA ครั้งต่อๆ ไป และมี antibody titer ถึง 1:500 หลังการฉีด CD4-DNA 6 ครั้ง

จากการศึกษาความจำเพาะของแอนติบอดีที่ผลิตได้ทั้งในหนูและกระต่าย พบว่า

1. แอนติบอดีที่ผลิตได้สามารถทำปฏิกิริยาได้กับ CD4-DNA transfected COS cells แต่ไม่ทำปฏิกิริยากับ mock transfected COS cells
2. แอนติบอดีที่ผลิตได้ทำปฏิกิริยาจำเพาะกับ CD4+ cell lines แต่ไม่ทำปฏิกิริยากับ CD4- cell lines
3. แอนติบอดีที่ผลิตได้สามารถ inhibit standard anti-CD4 mAb (Leu3a) ในการจับกับ CD4 proteins บน CD4+ cells
4. แอนติบอดีที่ผลิตได้สามารถนำมาใช้ตรวจนับจำนวน CD4+ cells ในเลือดคนปกติและผู้ป่วยเอดส์ โดยให้ค่าใกล้เคียงกับการใช้ standard anti-CD4 mAb (Leu3a)

ผลการศึกษานี้แสดงให้เห็นว่า วิธี DNA immunization สามารถนำมาใช้ผลิตแอนติบอดีต่อ leukocyte surface protein ได้

ดูรายละเอียดของผลการทดลองใน reprints 2 เรื่อง: Kasinrerk W, Tokrasinwit N, Piluk Y. Production of mouse anti-CD4 antibodies by DNA-based immunization. Asian Pac J Aller Immunol 1996 และ Kasinrerk W, Tokrasinwit N, Changtamroung K. Production of anti-CD4 antibodies in rabbits by DNA immunization. Asia Pac J Mol Biol Biotech. 1997 ซึ่งได้แนบมาด้วยแล้ว

3. การพัฒนาวิธีและน้ำยาสำหรับตรวจนับจำนวน CD4+ cells

3.1 **การตรวจนับ** CD4+ cells โดยวิธี Manual Rosetting

วัตถุประสงค์หนึ่งของงานวิจัยนี้คือการพัฒนาวิธีการตรวจนับจำนวน CD4+ cells ในเลือดขึ้นมาใช้แทนวิธี flow cytometry โดยได้พัฒนาวิธี Manual rosetting ขึ้นมาโดยอาศัยหลักการคือ นำ whole blood มาแตกเม็ดเลือดแดงออกก่อนด้วย red blood cell lysing buffer (Becton Dickinson) จากนั้นนำเซลล์ที่ได้ (lysed whole blood) มาทำปฏิกิริยากับ anti-CD4 mAb และ anti-mouse immunoglobulin coated beads ตามลำดับ แล้วตรวจนับจำนวน CD4 rosette ด้วยกล้องจุลทรรศน์

จากการศึกษาในคนปกติจำนวน 16 รายและผู้ป่วยเอดส์จำนวน 24 ราย โดยนำ lysed whole blood มาตรวจหา %CD4+ cells โดยวิธี Manual rosetting และคำนวนเป็นจำนวน absolute CD4+ cells/µl. (โดยอาศัยค่า white blood cell count) เปรียบเทียบกับวิธีมาตรฐาน Simulset (flow cytometric analysis) ผลการทดลองพบว่า %CD4 positive cells ในเม็ดเลือดขาวที่ได้จากทั้ง 2 วิธีไม่แตกต่างกัน โดยมีค่าสัมประสิทธิ์สหสัมพันธ์ (correlation coefficient) เท่ากับ 0.93 ค่าเฉลี่ยของ % CD4+ cells โดยวิธี Manual rosetting และ flow cytometry เท่ากับ 6.35 และ 6.32 ตามลำดับ ค่าที่ได้จากทั้ง 2 วิธีไม่มีความแตกต่างอย่างมีนัยสำคัญ (paired t test; $p>0.05$) เมื่อเปลี่ยน %CD4+ cells เป็น absolute number (cells/µl) พบว่าค่าที่ได้จากทั้ง 2 วิธีไม่แตกต่างกัน โดยมีค่าสัมประสิทธิ์สหสัมพันธ์ (correlation coefficient) เท่ากับ 0.95 ค่าเฉลี่ย absolute CD4+ cell count ของวิธี Manual rosetting และ flow cytometry เท่ากับ 529.04 และ 515.75 cells/µl ตามลำดับ โดยไม่มีความแตกต่างอย่างมีนัยสำคัญ (paired t test; $p>0.05$) วิธี Manual rosetting นี้ไม่ให้ผลบวกกับ monocytes และ granulocytes ในเลือดเลย

ดูรายละเอียดผลการทดลองใน manuscript: Kasinrerk W, Tokrasinwit N, Intharasut S, Naveewongpanit P. Manual rosetting method for enumerating CD4 positive cell. J Med Lab Sci (in press, 1997) ซึ่งได้แนบมาด้วยแล้ว

จากนั้นผู้วิจัยได้ศึกษาถึงการพัฒนาวิธี Manaul rosetting ให้มีวิธีการทำที่ง่ายยิ่งขึ้น โดยการเคลือบ MT4 mAb กับเม็ดเลือดแดงแกะ และ tosyl-activated beads แล้วนำมาตรวจนับจำนวน CD4+ cells โดยตรง โดยมีผลการศึกษาดังนี้คือ

1. นำ MT4 mAb ascites ไปเกาะบนเม็ดเลือดแดงแกะ โดยใช้ glutaraldehyde แล้วนำ MT4 coated SRBC ไปตรวจนับจำนวน CD4+ cells โดยการศึกษานี้ได้นำ CD4+ cell line Sup

T1 มาใช้ในการศึกษา ผลการทดลองพบว่า Sup T1 form rosette กับ MT4 coated SRBC เพียง 5% ทั้งๆที่มากว่า 90% ของ Sup T1 express CD4 proteins บนผิวเซลล์ ผลการทดลองนี้แสดงให้เห็นว่า การใช้ MT4 ascites ไปเคลือบ SRBC มีความไวไม่ดีพอ

2. นำ MT4 mAb ไปเกาะกับ tosyl-activated magnetic beads (Dynal) โดยการศึกษานี้เริ่มต้นด้วยการ purified MT4 mAb จาก ascites โดยวิธี affinity chromatography แล้วนำ purifed MT4 mAb ที่ได้ไป coat กับ tosyl-activated magnetic beads จากนั้นจึงนำ MT4 coated beads ไปตรวจนับจำนวน CD4+ cells โดยการศึกษานี้เริ่มต้นด้วยการนำ CD4+ cell line Sup T1 และ Molt4 มาใช้ในการศึกษา ผลการทดลองพบว่าทั้ง Sup T1 และ Molt4 สามารถ form rosette กับ MT4 coated beads ได้มากกว่า 90% ซึ่งแสดงให้เห็นว่า MT4 coated beads สามารถ form rosette กับ CD4+ cells ได้ จากนั้นจึงนำ MT4 coated beads ไปตรวจนับจำนวน CD4+ cells ในเลือดคนปกติจำนวน 5 รายเปรียบกับวิธี flow cytometer ผลการทดลองพบว่า ค่าที่ได้โดยวิธี rosetting ให้ผลต่ำกว่าวิธีมาตรฐาน flow cytomter (ตารางที่ 1) ซึ่งแสดงให้เห็นว่าวิธีนี้มีความไวไม่พอที่จะตรวจนับ human CD4+ cells ซึ่งมีโมเลกุลของ CD4 บนผิวเซลล์น้อยกว่า CD4+ cell lines

3.2 การตรวจหาปริมาณ soluble CD4 protein ในเลือดโดยวิธี Sandwich ELISA

เพื่อนำการตรวจหาปริมาณ soluble CD4 ในเลือดมาเป็นดัชนีบ่งชี้จำนวน CD4+ cells จึงทำการ set up วิธี sandwich ELISA เพื่อตรวจหาปริมาณ soluble CD4 ในเลือด โดยอาศัยหลักการคือใช้ MT4 mAb เคลือบเพลต เติมตัวอย่างตรวจ แล้วเติม rabbit anti-CD4 antibody (polyclonal antibody จากวิธี DNA immunization) และ anti-rabbit immunoglobulin-HRP และ TMB substrate โดยการศึกษานี้เริ่มที่การไตเตรทหาปริมาณที่เหมาะสมของแอนติบอดีและ conjugate ที่ใช้ โดยใช้ recombinant CD4 protein ที่ได้จาก CD4-DNA transfectd COS cells เป็น positive control และ recombinant CD1a protein ที่ได้จาก CD1-DNA transfectd COS cells เป็น negative control ในการ set up sandwich ELISA เมื่อได้ conditions ที่เหมาะสมแล้วจึงนำมาตรวจหาปริมาณ soluble CD4 ในเลือดผู้ติดเชื้อ HIV จำนวน 59 คน และคนปกติจำนวน 56 คน จากการเปรียบเทียบค่า OD ที่ได้กับจำนวน absolute CD4+ cell count ที่ทำควบคู่ไปด้วย ผลการทดลองพบว่า ปริมาณ soluble CD4 protein ที่ได้ไม่มีความสัมพันธ์กับ absolute CD4+ cell count เลย (ตารางที่ 2 และรูปที่ 1) โดยมีค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.187

เมื่อเปรียบเทียบปริมาณ soluble CD4 proteins ในเลือดผู้ติดเชื้อ HIV กับคนปกติ พบว่าค่าที่ได้จากกลุ่มตัวอย่างทั้งสองไม่แตกต่างกัน (ตารางที่ 3 และรูปที่ 2)

ตารางที่ 1 แสดง %CD4+ cells และ absolute CD4+ cells ในเลือด โดยวิธี direct manual rosetting และ วิธี flow cytometry

Sample No.	**%CD4+ cells**		**Absolute CD4 count***	
	Rosette**	**Flow cyto.*****	**Rosette**	**Flow cyto.**
1	**8.4**	**12.5**	**730**	**1086**
2	**9.1**	**15.8**	**646**	**1122**
3	**8.3**	**16.0**	**672**	**1296**
4	**8.5**	**16.4**	**650**	**1255**
5	**13.7**	**22.0**	**863**	**1386**

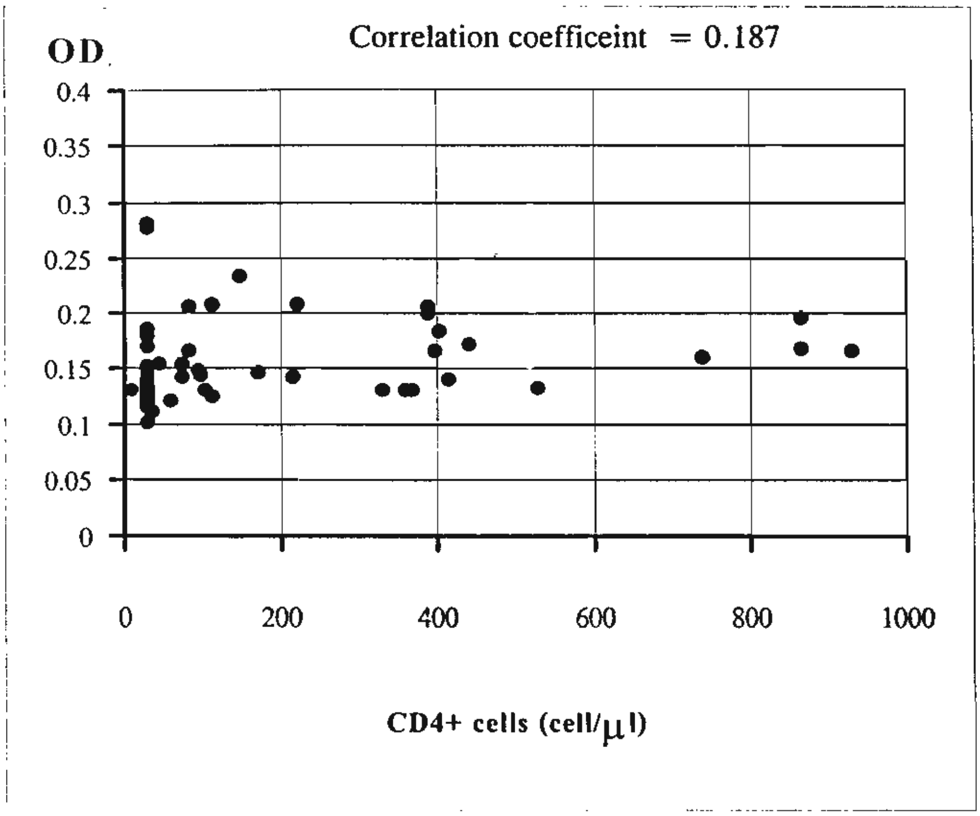
* cell/µl

** Direct manual rosetting technique

*** Flow cytometry

ตารางที่ 2 แสดงค่า CD4+ cells และปริมาณ soluble CD4 proteins ในผู้ติดเชื้อ HIV

Sample No.	CD4+ cell (cells/μl)	OD	Sample No.	CD4+ cell (cells/μl)	OD
1	10	0.132	31	30	0.123
2	220	0.208	32	30	0.179
3	30	0.128	33	864	0.169
4	30	0.281	34	440	0.172
5	83	0.206	35	30	0.119
6	30	0.148	36	388	0.199
7	30	0.152	37	30	0.180
8	30	0.101	38	359	0.131
9	30	0.134	39	30	0.278
10	30	0.115	40	30	0.131
11	81	0.166	41	169	0.147
12	30	0.127	42	30	0.141
13	528	0.133	43	30	0:000
14	213	0.143	44	414	0.141
15	367	0.131	45	104	0.131
16	59	0.120	46	97	0.144
17	403	0.185	47	110	0.207
18	147	0.234	48	30	0.129
19	395	0.167	49	740	0.161
20	865	0.195	50	329	0.131
21	36	0.112	51	30	0.117
22	30	0.127	52	44	0.154
23	110	0.124	53	388	0.205
24	931	0.166	54	30	0.132
25	30	0.171	55	30	0.132
26	73	0.143	56	30	0.181
27	30	0.130	57	30	0.123
28	30	0.150	58	95	0.149
29	73	0.153	59	30	0.186
30	30	0.124			

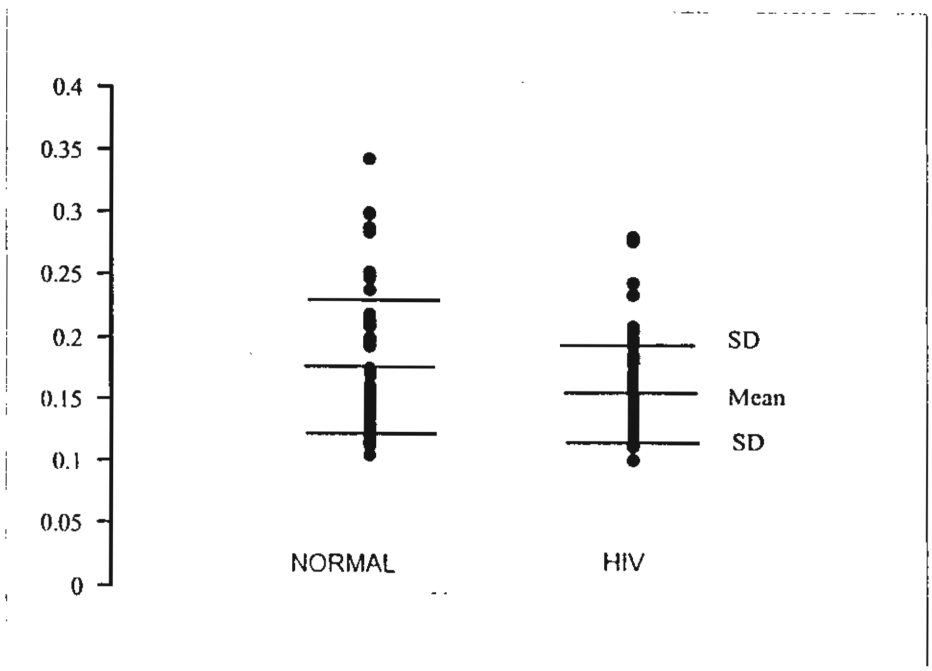


รูปที่ 1 แสดงความสัมพันธ์ระหว่างจำนวน CD4+ cells และ ปริมาณ soluble CD4 protein ใน ผู้ติดเชื้อ HIV จำนวน 59 ราย

ตารางที่ 3 แสดงปริมาณ soluble CD4 proteins ในเลือดคนปกติและผู้ติดเชื้อ HIV

Sample No.	Normal	HIV
1	0.342	0.243
2	0.219	0.132
3	0.128	0.208
4	0.161	0.128
5	0.122	0.281
6	0.238	0.206
7	0.149	0.148
8	0.135	0.152
9	0.115	0.101
10	0.122	0.134
11	0.152	0.115
12	0.113	0.166
13	0.125	0.127
14	0.248	0.133
15	0.135	0.143
16	0.158	0.131
17	0.158	0.120
18	0.172	0.185
19	0.129	0.234
20	0.116	0.167
21	0.213	0.195
22	0.141	0.112
23	0.168	0.127
24	0.175	0.124
25	0.142	0.166
26	0.211	0.171
27	0.147	0.143
28	0.208	0.130
29	0.287	0.150
30	0.299	0.153

Sample No.	Normal	HIV
31	0.152	0.124
32	0.148	0.123
33	0.261	0.179
34	0.157	0.169
35	0.199	0.172
36	0.127	0.119
37	0.139	0.199
38	0.199	0.180
39	0.160	0.131
40	0.193	0.278
41	0.155	0.131
42	0.159	0.147
43	0.144	0.141
44	0.195	0.141
45	0.140	0.131
46	0.214	0.144
47	0.115	0.207
48	0.161	0.129
49	0.252	0.161
50	0.199	0.131
51	0.120	0.117
52	0.104	0.154
53	0.247	0.205
54	0.249	0.132
55	0.193	0.132
56	0.285	0.181
57		0.123
58		0.149
59		0.186



รูปที่ 2 แสดงปริมาณ soluble CD4 proteins ในคนปกติและผู้ติดเชื้อ HIV

3.3 การเตรียมน้ำยา FITC labeled MT4 mAb เพื่อตรวจนับจำนวน CD4+ cells

เนื่องจากปัจจุบันวิธีมาตรฐานที่ใช้ในการตรวจนับจำนวน CD4+ cells ในเลือดคือวิธี flow cytometry แต่พบว่าน้ำยา (แอนติบอดี) ที่ใช้ในวิธีดังกล่าวนี้มีราคาแพงมาก ทำให้วิธีนี้ไม่เหมาะกับประเทศไทยที่มีงบประมาณน้อยแต่มีการระบาดของโรคเอดส์สูง ไม่เหมาะกับการนำมาใช้ตรวจ absolute CD4 count ในผู้ติดเชื้อที่ยังไม่แสดงอาการ และเนื่องจากการศึกษาที่ผ่านมา ผู้วิจัยสามารถผลิตโมโนโคลนอล แอนติบอดีต่อ CD4 โปรตีน (MT4) ได้ จึงได้นำ MT4 mAb มาผลิตเป็นน้ำยาตรวจนับ CD4+ cells สำหรับใช้กับเครื่อง flow cytometer ขึ้นมาใช้เอง โดยการศึกษาเริ่มต้นด้วยการ purify MT4 mAb ด้วยวิธี affinity chromatography แล้วนำ purified MT4 มาติดฉลากกับสาร fluorescein isothiocyanate (FITC) โดยวิธี alkaline reaction แล้วนำไปใช้ตรวจนับจำนวน CD4+ cells ด้วยเครื่อง flow cytometer เพื่อประเมินประสิทธิภาพของน้ำยาที่เตรียมขึ้นมา จึงได้ทำการตรวจนับจำนวน CD4+ lymphocytes ในเลือดผู้ติดเชื้อเอดส์และคนปกติจำนวน 60 ราย โดยทำการเปรียบเทียบระหว่างน้ำยา FITC labeled MT4 ที่เตรียมขึ้นกับชุดน้ำยามาตรฐาน Simultest™ ของบริษัท Becton Dickinson ผลการศึกษาพบว่าเปอร์เซนต์และค่า absolute CD4+ cell counts ที่ได้จากน้ำยาทั้งสองชนิดใกล้เคียงกันมาก โดยมีค่าสัมประสิทธิ์ สหสัมพันธ์ เท่ากับ 0.995 และ 0.996 สำหรับเปอร์เซนต์และค่า absolute CD4+ lymphocyte counts ตามลำดับ ผลการศึกษาในครั้งนี้ชี้ให้เห็นว่า น้ำยา FITC labeled MT4 ที่เตรียมขึ้นมาใช้เองนี้น่าจะเป็นน้ำยาอีกชนิดหนึ่งที่สามารถนำมาใช้ตรวจวัดระดับ CD4+ cells ในเลือดโดยวิธี flow cytometry ได้

ดูรายละเอียดผลการทดลองใน manuscript เรื่อง Kasinrerk W, Tokrasinwit N, Naveewongpanit P. Production of CD4 monoclonal antibody and development of home made reagent for CD4+ lymphocyte determination. J Med Ass Thai (submitted, 1997) ซึ่งได้แนบมาด้วยแล้ว

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1. ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

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4. การเชื่อมโยงทางวิชาการกับนักวิชาการอื่นๆ ทั้งในและต่างประเทศ

1. มีการติดต่อและแลกเปลี่ยนความรู้และผลงานวิจัยกับ Dr. Hannes Stockinger จาก Institute of Immunology, University of Vienna ประเทศออสเตรีย อยู่เสมอ

2. มีการติดต่อและแลกเปลี่ยนความรู้กับ นายแพทย์ปรีดา มาลาสิทธิ์ ศูนย์อณูชีววิทยาทางการแพทย์ คณะแพทยศาสตร์ ศิริราชพยาบาล มหาวิทยาลัยมหิดล อยู่เสมอ

5. การเชื่อมโยงทางวิชาการกับนักวิชาการในสถาบันเดียวกัน

มีการติดต่อ แลกเปลี่ยนความคิด และสารเคมีเสมอกับ ผศ. ดร. ปรัชญา คงทวีเลิศ และ รศ. นพ. นพพร สิทธิสมบัติ และคณาจารย์ในภาควิชา อยู่เสมอ

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Production of Mouse Anti-CD4 Antibodies by DNA-Based Immunization

Watchara Kasinrerk, Niramon Tokrasinwit and Yaiyarat Piluk

SUMMARY The intramuscular injection of plasmid DNA encoding an antigenic protein has been developed recently as a tool for immunization. DNA-based immunization was shown to generate immune responses against the encoded antigen in diverse animal species. In this report, we present the use of DNA-based immunization for the production of antibodies to CD4, a human leukocyte surface molecule. Mice were injected intramuscularly with eukaryotic expression vector containing cDNA encoding CD4 protein, termed CD4-DNA, and were subsequently assayed for anti-CD4 antibody production by indirect immunofluorescence. Sera collected from 2 of 3 inoculated mice reacted with CD4 expressing transfected COS cells and Sup-T1 cells. Anti-CD4 antibody activity was abolished by adsorption with CD4 molecule exppressing cells. $CD4^+$ cell depleted lymphocytes were also used to confirm the specificity of the anti CD4 antibodies present in immune serum. CD4-DNA immune serum reacted with approximately 1/3 of freshly isolated lymphocytes but to very few cells in the $CD4^+$cells-depleted preparation. CD4-DNA immunized sera was used to enumerate $CD4^+$cells in the peripheral blood of 8 healthy donors and 2 AIDS patients. The number of $CD4^+$ cells estimated by DNA immunized sera was very similar to estimates using standard anti-CD4 monoclonal antibody Leu3a. DNA-based immunization is therefore capable of raising antibodies to human leukocyte surface antigens. This technology may be useful for producing antibodies to other cell surface antigens in mice or other animals.

The feasibility of directly injecting purified closed-circular plasmid DNA that encodes antigenic protein for producing both antibodies and cell mediated immune responses has recently been demonstrated.[1,2] This strategy can be defined as the physical delivery of an antigen encoding expression vector *in vivo* for the induction of antigen expression and the elicitation of specific immune responses. The DNA-based immunization technique has several advantages over conventional protein antigen immunization. DNA is simple to produce and purify than protein antigens and is highly stable.

Leukocyte surface antigens are molecules which are of critical importance for the function of human leukocytes. Antibodies raised against surface molecules have become a major tool in immunophenotyping and characterizing the structure of these surface molecules.[3] Highly pure antigen is required to produce polyclonal antibodies against a leukocyte surface molecule. DNA-based immunization therefore has the potential to be a novel alternative to standard antigen immunization. We report here an investigation into the production of antibodies to leukocyte surface antigens by DNA-based immunization. We found that injec-

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tion of plasmid DNA encoding human CD4 protein into mouse quadriceps muscle is capable of eliciting anti-CD4 antibodies. These antibodies reacted with either recombinant CD4 or native CD4 molecules. The DNA immunization technique is simple and rapid and produces antibodies capable of immunophenotyping leukocyte surface molecules.

MATERIALS AND METHODS

Cells and cell lines

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy donors or AIDS patients by Ficoll-Hypaque density centrifugation.[4] Sup T-1 cells, a human T cell line expressing high levels of surface CD4 protein,[5] were cultured in RPMI 1640 supplemented with 15% fetal calf serum (FCS; Gibco, Grant Island, NY), 40 μg/ml gentamycin and 2.5 µg/ml amphotericin B in a fully humidified atmosphere of 5% CO_2 at 37°C. COs cells were grown in Minimum Essential Medium (MEM; Gibco) containing 5% FCS and antibiotics at 37°C in a 5% CO_2 atmosphere.

Plasmid DNA for immunization

cDNA encoding CD4 protein was inserted into an eukaryotic expression vector πH3M (designated CD4-DNA), which was a kind gift from Dr. H Stockinger, University of Vienna, Vienna, Austria. πH3M is a high-efficiency eukaryotic expression vector containing a simian virus 40 (SV40) origin of replication and synthetic transcription units. The transcription units consist of a chimeric promotor composed of human cytomegalovirus AD169 immediate early enhancer sequences fused to the human immunodeficiency virus (HIV) long terminal repeat (LTR) sequences. A polylinker containing two *Bst*XI sites is inserted immediately downstream from the LTR sequence. The SV40 small tumor(t) antigen splice and early region polyadenylylation signals, which derived from pSV2, are placed downstream from the polylinker.[6]

Plasmid DNA preparations

Plasmid DNA was transformed into competent *E. coli* MC1061/p3 and the transformed bacteria were grown with vigorous shaking in 250 ml Terrific Broth per 1 liter flask. After overnight cultivation, cells were harvested and lysed by an alkaline lysis procedure.[7] DNA was then purified by cesium chloride-ethidium bromide density gradient ultracentrifugation.[7] The resulting DNA was phenol/chloroform-extracted, ethanol precipitated and resuspended in TE (10 mM Tris, 1 mM EDTA) pH 8.0 for transfection or immunization into mice. The concentration and purity of each DNA preparation was determined by OD260/280 reading.[7]

DNA immunization

Isolated plasmid DNA was injected weekly into the quadriceps muscle of both hind legs (100 μg per leg) of 4-8 week-old Balb/C female mice. Blood samples were collected from the retro-orbital venous plexus of ether-induced anesthetized mice prior to each DNA inoculation. Serum samples were then separated and stored at -20 °C.

DEAE-Dextran transfection of COS cells

Plasmid DNA encoding CD4 or CD8 proteins were transfected into COS cells using the DEAE-Dextran transfection method.[8] Briefly, 1×10^6 COS cells were transfered to 6 cm tissue culture dishes (NUNC, Ros kilde, 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. Cells were cultured in MEM containing 5% FCS overnight, washed once, and recultured with the same medium for another 2 days to allow expression of CD4 or CD8 proteins.

Immunofluorescence analysis

The specificity of anti-CD4 protein antibodies was assessed by indirect immunofluorescence using FITC-conjugated sheep anti-mouse immunoglobulin antibodies (Dakopatts, Glostrup, Denmark). To block non-specific Fc receptor mediated antibody binding, tranfected COS cells, Sup T-1 cells or PBMC were incubated for 30 minutes at 4 °C with 10% human AB serum before staining. Blocked cells were then incubated for 30 minutes at 4 °C with various dilutions of tested antisera or anti-CD4 monoclonal antibody (MAb) (Leu 3a; Becton Dickinson, Sunnyvale, Ca) or anti-CD8 MAb (Leu 2a;

Becton Dickinson). After washing, cells were incubated with the FITC-conjugate for another 30 minutes. Membrane fluorescence was analyzed under a fluorescence microscope or flow cytometer (FACSCAN, Becton Dickinson). For flow cytometric analysis, individual populations of blood cells were gated according to their forward and side scatter characteristics.

Detection of anti-CD4 antibody activity and immunoadsorption

Serum samples from immunized mice were tested for anti-CD4 antibody activity by indirect immunofluorescence using either transfected COS cells or the Sup-T1 cell line. Stained cells were analyzed by fluorescent microscopy or FACSCAN. Immunoadsorption of anti-CD4 antibody from immunized serum was performed to confirm the specificity of anti-CD4 antibodies. Briefly, CD4-DNA immunized serum was incubated with 1×10^7 Sup-T1 cells or CD4 or CD8 expressing transfected COS cells for 60 minutes at 4°C. After incubation, the cells were removed by centrifugation. Anti-CD4 antibody activity was then re-analyzed by using CD4 or CD8 expressing transfected COS cells or Sup-T1 cells by indirect immunofluorescence.

CD4⁺ cell depletion

PBMC were treated with anti-CD4 MAb Leu3a (Becton Dickinson) at 4°C for 30 minutes. The treated cells were then mixed with sheep anti-mouse IgG-coated Dynabeads (M450; Dynal, Oslo, Norway). Cells that bound the iron conjugate were separated with a magnet in accordance with the recommended protocol (Dynal).

Enumeration of the percentage of $CD4^+$ cells in clinical specimens

PBMC were isolated from heparinized blood of healthy donors or AIDS patients by the standard Ficoll-Hypaque density centrifugation methed.[4] Isolated PBMC were stained with CD4-DNA immunized serum, non-immunized serum or anti-CD4 MAb Leu3a by indirect immunofluorescence. Membrane fluorescence was analyzed on a FACSCAN.

RESULTS

Large-scale preparation of CD4-DNA and expression of recombinant CD4 protein in COS cells

CD4-DNA was amplified in *E. coli* MC 1061/p3, and the plasmid DNA were isolated from transformed bacteria using the alkaline lysis procedure, and then purified by CsCl-EtBr gradients. The DNA yields were determined by OD 260/280 reading after completion of all the purification steps. In 3 lots of DNA preparations, the OD 260/280 ratios were between 1.8-2.0 The yields of purified plasmid DNA were approximately 3 mg/litre of bacteria.

The isolated plasmid DNA were then transfected into COS cells and analyzed for CD4 protein expression by indirect immunofluorescence. The CD4-DNA transfected COS cells showed strong positive reaction with anti-CD4 MAb Leu3a, but did not react with anti-CD8 MAb leu2a. These results indicated that the isolated CD4-DNA encodes CD4 protein and the encoded protein can be expressed on eukaryotic COS cells.

Production of anti-CD4 antibodies by CD4-DNA immunization

Three Balb/C mice were immunized with CD4-DNA at one-week intervals. For negative controls, mice were immunized with M6-DNA, which encodes a human leukocyte surface molecule,[8] or with πH3M vector lacking any expressible gene insert. The anti-CD4 antibody activity in sera was evaluated by indirect immunofluorescence using CD4-DNA and CD8-DNA transfected COS cells as antigens. Serum anti-CD4 antibody activity was detected in 2 of the 3 immunized mice injected with CD4-DNA (Table 1). Neither preimmune sera from the same mice nor control sera was reactive (Table 1.)

The reactivity of anti-CD4 antibodies was also investigated using Sup-T1 cell line. This cell line was positive with CD4-DNA immunized sera, but negative with preimmune sera (Fig.1). Anti-CD4 titers were 1:160 and 1:40.

Anti-CD4 antibody specificity

CD4-DNA immunized serum was adsorbed with either Sup-T1 cells, CD4-DNA transfected COS cells or CD8 -DNA transfected COS cells. The anti-CD4 antibody activity of adsorbed sera was then re-analyzed with CD4-DNA transfected COS cells, CD8-DNA transfected COS cells and Sup-T1 cells. As shown in Table 2, the activity of anti-CD4 antibodies was eliminated after adsorption with Sup-T1 or CD4-DNA transfected

Table 1. Anti-CD4 antibody activity in sera before and after DNA immunization

Mouse No.	Immunization	Serum	Immunofluorescent reactivity CD4-COS[a]	CD8-COS[b]
1	CD4-DNA	Pre-immunization	-	-
		Dose 1	-	-
		Dose 2	-	-
		Dose 3	+	-
		Dose 4	+	-
		Dose 5	+	-
		Dose 6	+	-
		Dose 7	+	-
		Dose 8	+	-
2	CD4-DNA	Pre-immunization	-	-
		Dose 6	+	-
		Dose 7	+	-
		Dose 8	+	-
3	CD4-DNA	Pre-immunization	-	-
		Dose 1	-	-
		Dose 2	-	-
		Dose 3	-	-
		Dose 4	-	-
		Dose 5	-	-
		Dose 6	-	-
		Dose 7	-	-
		Dose 8	-	-
4	Vector-DNA[c]	Pre-immunization	-	-
		Dose 6	-	-
		Dose 7	-	-
		Dose 8	-	-
5	M6-DNA[d]	Pre-immunization	-	-
		Dose 6	-	-
		Dose 7	-	-
		Dose 8	-	-

[a] COS cells transfected with CD4-DNA were strongly positive with anti-CD4 MAb Leu3a.
[b] COS cells transfected with CD8-DNA were strongly positive with anti-CD8 MAb Leu2a.
[c] Vector lacking any expressible gene insert.
[d] Plasmid DNA encoding M6 protein.[8]

COS cells. In contrast, anti-CD4 activity was still detected in serum after adsorption with CD8-DNA transfected COS cells.

Peripheral lymphocytes before and after $CD4^+$ cell depletion were used to confirm the specificity of anti-CD4 antibodies. As shown in Table 3, CD4-DNA immunized serum stained approximately 30% of cells in freshly isolated lymphocytes, but only about 4% in a $CD4^+$ cell depleted population. As a control, anti-CD4 MAb Leu3a was used, and a similar reaction pattern was found (Table 3.) Preimmune serum did not react with either the depleted or non-depleted population.

Enumeration of the percentage of $CD4^+$ cells in peripheral blood samples using CD4-DNA immunized serum

To further characterize the anti-CD4 antibody activity in CD4-DNA immunized serum, we used this serum for the enumeration of $CD4^+$ cells in peripheral blood. PBMC from 6 healthy subjects and 2 AIDS patients were stained with CD4-DNA immunized serum and anti-CD4 MAb Leu3a side by side. The stained cells were analyzed by flow cytometry. Both methods gave similar results (Table 4.) Non-immunized normal mouse serum was included in all experiments and gave no reaction (Table 4.).

DISCUSSION

Several investigators have demonstrated that the immunization of mice with a plasmid DNA vector

Table 2. Immunoadsorption of anti-CD4 antibody activity generated by CD4-DNA immunization.

Serum dilution	Adsorbed cells	Immunofluorescent reactivity[a]		
		CD4-COS[b]	CD8-COS[c]	Sup-T1
1:40	None	+	-	+
	Sup-T1	-	-	-
	CD4-COS[b]	-	-	-
	CD8-COS[c]	+	-	+
1:80	None	+	-	+
	Sup-T1	-	-	-
	CD4-COS[b]	-	-	-
	CD8-COS[c]	+	-	+

[a] Immunofluorescent reactivity was analyzed by fluorescent microscopy

[b] CD4-DNA transfected COS cells.

[c] CD8-DNA transfected COS cells.

Table 3. Percentage of $CD4^+$ cells in peripheral blood lymphocytes before and after $CD4^+$ cell depletion determined by staining with CD4-DNA immunized serum and anti-CD4 MAb Leu3a

Donor	Anti-CD4 MAb Leu3a		DNA immunized serum[a]		Pre-immune serum	
No.	Before[b]	After[c]	Before	After	Before	After
1	33[d]	4	31	4	1	1
2	25	2	28	2	0.7	0.3

[a] Serum obtained from CD4-DNA immunized mouse.

[b] Before $CD4^+$ cell depletion

[c] After $CD4^+$ cells depletion.

[d] Percentage of $CD4^+$ cells was determined by flow cytometric analysis.

containing genes results in the induction of immune responses to the encoded proteins.[1,2] The use of DNA-based vectors as an alternative to antigen immunization is a novel strategy, now under development and evaluation. Plasmid vectors containing several genetic elements are required to drive the intracellular expression of the foreign gene insert. These genetic elements include (i) a transcriptional promotor, (ii) an optional enhancer element to augment gene expression, (iii) the foreign gene encoding an antigenic gene product, and (iv) RNA-processing elements, primarily a polyadenylation signal and an optional intron elements.[2] In addition, the plasmids should contain two bacterium-specific genetic sequences to allow large scale production of DNA, *i.e.* an antibiotic selectable marker to permit the identification and isolation of bacterial cells successfully transduced with the gene of interest, and a bacterial origin of replication to facilitate large scale amplification of the plasmid DNA within this host cell. Once the DNA enter the mammalian cells, the encoded proteins are expressed through normal cellular transcription and translation mechanisms. Immunization with DNA-based plasmids has been attempted successfully in various species including mice, chicken, ferrets, cattle, and non-human primates by various routes of administrations.[9-15] Most experiments, however, have been conducted with DNA.

cDNA encoding CD4 protein constructed in the eukaryotic expression vector πH3M was used to produce antibodies to leukocyte

Table 4. Determination of the percentage of CD4$^+$ lymphocytes using anti - CD4 MAb Leu3a and CD4-DNA immunized serum

Subject No.	% Positive cells in lymphocytes		
	Anti-CD4 MAb Leu3a	DNA immunized serum[a]	Non-immunized serum
1	39.1	39.7	0.9
2	33.5	33.1	1.0
3	29.2	30.4	2.0
4	31.5	31.4	0.9
5	26.6	32.5	1.9
6	37.5	39.1	1.2
7[b]	5.0	5.4	0.5
8[b]	0.5	0.5	1.1

[a] Serum obtained from CD4-DNA immunized mouse.
[b] Subject Nos. 7 and 8 were AIDS pateints and by standard Simutest flow cytometry the percentage of CD4$^+$ lymphocytes were 7 and 2, respectively.

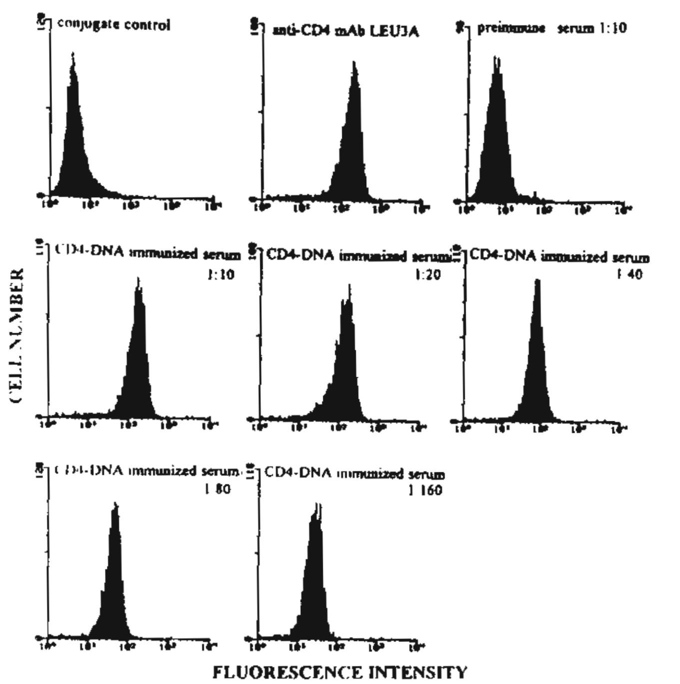

Fig. 1. Immunofluorescent analysis of the reactivity of anti-CD_4 antibodies with the Sup-T1 cell line. Sup-T 1 cells were stained with either anti-CD4 MAb Leu3a, pre-immune serum or CD4-DNA immunized serum. The intensity of fluorescence was measured by FACSCAN with logarithmic amplification.

surface antigens. The transcription units presented in the πH3M vector meet the criteria for plasmid vectors used in DNA-based immunization. This study demonstrates that mice inoculated with πH3M containing encoding CD4 protein induces anti-CD4 antibody; 2 of 3 inoculated mice generated anti-CD4 antibodies detectable by immunofluorescence. Anti-CD4 antibodies bound either recombinant CD4 proteins which were expressed on transfected COS cells, or native CD4 proteins on lymphocytes and Sup-T1 cells. Direct intramuscular injection of plasmid DNA has been widely used to induce antibody production, due to its simplicity and effectiveness. However, a large quantity of DNA is required (approximately 100-300 μg/inoculation).[12,13,16-18] Alternatively, delivery of DNA-coated gold beads into epidermis by a biolistic device has been shown to generate immune responses.[10,19,20] This biolistic transfection required less DNA than intramuscular inoculations.

CD4-DNA immunized serum was used to count CD4$^+$ cells in peripheral blood and gave comparable results to standard methods in 6 healthy PBMC and 2 AIDS patients. A reduction in circulating CD4$^+$cells is characteristic of AIDS patients,[21] and was found in our 2 patiens using both CD4-DNA immunized serum and anti-CD4 MAb Leu3a.

The direct introduction of genes into mouse muscle and its expression *in vivo* may allow the processing of gene products and lead to the presentation of an effective target antigen. This DNA-based immunization strategy is simple and rapid. To our knowledge, production of antibody to the human leukocyte surface antigen using DNA immunization technology has not been previously reported. Anti-CD4 antibodies, as we demonstrated here, can be used to determine cell surface molecules, and have many other potential applications.

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Production Of Anti-CD4 Antibodies In Rabbits By DNA Immunization

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Abstract. DNA immunization is a new methodology, where antigen encoding DNA plasmids are injected directly into muscle or skin with the purpose of eliciting an immune response to the gene product. In this report, DNA encoding human CD4 protein were immunized into rabbits for the production of anti-CD4 antibodies. Rabbits were immunized intramuscularly with CD4-DNA in three different procedures. Anti-CD4 antibodies were induced in all immunized rabbits. The rabbit that was pre-treated with sucrose solution, followed by injection of CD4-DNA containing chloroquine, produced anti-CD4 antibodies after only one DNA inoculation. This was faster than those receiving the same pre-treatment, but followed by injection of DNA without chloroquine or pre-treatment with Bupivacain. The generated antibodies bound either recombinant or native CD4 proteins. The CD4-DNA immunized serum could inhibit the binding of standard CD4 mAb Leu3a to CD4 proteins. This result, therefore, confirmed the specificity of rabbit anti-CD4 antibodies generated by DNA immunization. Rabbit anti-CD4 antiserum was used to enumerate $CD4^+$ cells in the peripheral blood of 5 donors. The percentage of $CD4^+$ cells obtained by DNA immunized serum was very similar to those obtained using the standard CD4 monoclonal antibody, Leu3a. These studies, thus, demonstrate that DNA immunization is an effective method for the production of hyperimmune globulin products in rabbits.

INTRODUCTION

DNA immunization refers to the induction of an immune response to a protein expressed *in vivo*, subsequent to the introduction of its encoding DNA. In contrast to classical protein immunization, where antigens are administered, DNA immunization involves the administering of genetic material encoding the antigen. The antigen is, therefore, produced within the cells of the immunized individual and induces the immune responses. The possibility of using direct transfer of plasmid DNA to induce an immune response to the expressed protein was first indicated by Acsadi *et al.*[1] Subsequently, several investigators have demonstrated the feasibility of using direct injection of plasmid DNA for the induction of protective immunity against various pathogens[2-9] and the production of hyperimmune globulin products.[10,11]

Immunization with DNA-based plasmid has been successfully attempted in several tissues by various routes of administering.[12] The intramuscular injection has been demonstrated as the most efficient route to transfer an aqueous solution of plasmid DNA.[12] This method, however, still results in a low efficiency of gene transfer and considerable variability in gene expression.[13,14] Davis *et al.* have demonstrated that the variability of gene transfer by intramuscular injection was reduced by pre-treatment of a hypertonic sucrose solution.[14] Some investigators demonstrated that the efficiency of gene transfer by using pure DNA in regenerating muscle was better than in normal muscle.[15-17] The administration of toxic agents tending to cause muscle necrosis and repair, either prior to or concurrently with injection of DNA, has been used to increase the expression of encoded protein and enhance the immune responses.[2-4,18]

In the present study, the production of anti-CD4 antibodies by immunizing cDNA encoding CD4 protein, CD4-DNA, into rabbits was studied. It was found that the immunization of CD4-DNA could induce anti-CD4 antibody production. Pre-

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treatment of rabbit, with hypertonic sucrose solution and followed by injection of plasmid CD4-DNA containing chloroquine, was able to induce anti-CD4 antibody production after only one DNA inoculation .

MATERIALS AND METHODS

Plasmid DNA and antibodies. cDNA encoding CD4 protein was inserted into an eukaryotic expression vector πH3M (designated CD4-DNA)[11], which was kindly donated by Dr. H. Stockinger, University of Vienna, Vienna, Austria. πH3M is a high - efficiency eukaryotic expression vector contining a simian virus 40 (SV40) origin of replication and synthetic transcription units. The transcription units consisting of a chimeric promotor composed of human cytomegalovirus AD169 immediate early enhancer sequences fused to the human immunodeficiency virus (HIV) long terminal repeat (LTR) sequences. The SV40 small tumor (t) antigen splice and early region polyadenylation signals, which derived from pSV2, are placed downstrem from the polylinker.[19] CD4 monoclonal antibody (mAb) Leu 3a, PE-labeled Leu3a and CD8 mAb Leu 2a were purchased from Becton Dickinson (Sunnyvale, CA). FITC-conjugated sheep F(ab')2 anti-mouse immunoglobulins antibodies were purchased from Dakopatts (Glostrup, Denmark).

Cells and cell lines. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy donors by Ficoll-Hypaque density centrifugation. Sup T-1 and Molt4, human T cell lines which expressed high levels of surface CD4 proteins constitutively, were cultured in RPMI 1640 supplemented with 15% fetal calf serum (FCS; Gibco, Grant Island, NY), 40 µg/ml gentamycin and 2.5 µg/ml amphotericin B in a fully humidified atmosphere of 5% CO_2 at 37°C. K562, a human erythro-myeloid cell line lacking in CD4 expression, were cultured in the same medium. COS cells were grown in Minimum Essential Medium (MEM; Gibco) containing 5% FCS and antibiotics at 37°C in a 5% CO_2 atmosphere.

DNA preparations. For large scale preparation, the plasmid DNA were transformed into competent *E. coli* MC1061/p3 and the resulting bacteria were grown with vigorous shaking in 250-ml Terrific Broth per 1-liter flask. After overnight cultivation, cells were harvested and lysed by the alkaline lysis procedure.[19] DNA were then purified by cesium chloride-ethidium bromide density gradient ultracentrifugation.[19] It was subsequently resuspended in phosphate buffer saline (PBS) pH 7.2. The concentration and purity of DNA preparation was determined by OD260/280 reading.[19]

DNA immunization. Three rabbits were injected intramuscularly with isolated plasmid DNA in three different ways. Rabbit number 1 was injected with 250 µl of hypertonic 25% sucrose solution 20 minutes prior to the administering of 500 µg of isolated CD4-DNA. In rabbit number 2, a pre-injection of 25% sucrose solution was also performed prior to the administering of plasmid DNA. However, 500 µg of CD4-DNA containing 200 µM chloroquine diphosphate was used as an immunizing agent. In rabbit number 3, 100 µl of 0.5% Bupivacain hydrochloride solution (Marcaine; Astra, Sodertalje, Sweden) was administered 24 hours before injecting with 500 µg CD4-DNA. The same procedure of DNA immunization was performed in each rabbit at one-week intervals. Blood samples were collected from immunized rabbits prior to each DNA inoculation. Serum samples were then separated and stored at -20°C.

DEAE-Dextran transfection of COS cells. Plasmid DNA encoding CD4 or CD8 proteins were transfected into COS cells using the DEAE-Dextran transfection method.[20] Briefly, 1×10^6 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. Cells were then cultured overnight in MEM containing 5% FCS, washed once, and re-cultured with the same medium for another 2 days to allow expression of encoded proteins.

Immunofluorescence analysis. The production of antibodies against CD4 proteins was assessed by indirect immunofluorescence using FITC-conjugated sheep anti-mouse immunoglobulins antibodies. 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. Cells were then incubated for 30 minutes at 4°C with various dilutions of tested antisera, CD4 or CD8 mAb. After washing, cells were incubated with the FITC-conjugate for another 30 minutes. Membrane fluorescence was analyzed under a fluorescent microscope or flow cytometer (FACSCalibur, Becton Dickinson). For flow cytometric analysis, individual populations of leukocytes were gated according to their forward and side scatter characteristics.

Inhibition of standard CD4 mAb binding by rabbit anti - CD4 antibodies. $CD4^+$ cells were pre-incubated with CD4-DNA immunized serum or pre-immune resum for 30 minutes on ice. The PE-labelled CD4 mAb Leu3a (Becton Dickinson) was then added to the pre-stained cells, which were incubated for another 30 minutes. Membrane fluorescence was analyzed by a flow cytometer. The percentage of inhibition of fluorescence intensity was calculated from the mean fluorescence intensity of the sample in the presence and absence of rabbit serum.

Enumeration of the percentage of CD4⁺ cells in blood samples. PBMC were isolated from heparinized blood by the standard Ficoll-Hypaque density centrifugation method. Isolated PBMC were stained with rabbit CD4-DNA immunized serum or CD4 mAb Leu3a by indirect immunofluorescence. Membrane fluorescence was analyzed on a flow cytometer.

RESULTS

Preparation of plasmid DNA encoding CD4 protein. Plasmid DNA encoding CD4 protein, CD4-DNA, were transformed into *E. coli* MC 1061/p3. The plasmid DNA were, then, isolated from the transformed bacteria by CsCl-EtBr gradients 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 were between 1.8-2.0. The yields of isolated CD4-DNA were approximately 3 mg/liter of bacteria.

To verify whether the isolated plasmid DNA can be expressed to the encoded protein in eukaryotic cells, the isolated DNA were transfected into COS cells and analyzed for CD4 protein expression by indirect immunofluorescence. COS cells, which were transfected with all isolated CD4-DNA, showed strong positive reaction with CD4 mAb Leu3a, but negative with CD8 mAb Leu2a.

Production of rabbit anti-CD4 antibodies by CD4-DNA immunization. Three rabbits were immunized with CD4-DNA at one-week intervals as described in materials and methods. The sera were collected 7 days after each DNA immunization. The anti-CD4 antibody activity in sera was evaluated by indirect immunofluorescence using CD4-DNA and CD8-DNA transfected COS cells as antigens. The anti-CD4 antibodies was induced in all immunized rabbits (Table 1). The antibodies were detected after four, one and two DNA immunizations for rabbits number 1, 2 and 3, respectively. The anti-CD4 antibody titer of 1:500 was present in all immunized rabbits after six DNA immunizations.

The specificity of generated anti-CD4 antibodies was confirmed by using $CD4^+$ cell lines, Sup-T1 and Molt4, and $CD4^-$ cell line K562 as antigens. Sup T1 and Molt4 were positive with CD4-DNA immunized sera, but negative with preimmune sera (Fig. 1). K562, in contrast, was negative with both immune and preimmune sera (Fig. 1).

Inhibition of standard CD4 mAb binding by CD4-DNA immunized serum. The specificity of anti-CD4 antibody presence in the CD4-DNA immunized serum was analyzed by the inhibition test. CD4-DNA immunized and preimmune serum were used to inhibit the binding of standard CD4 mAb Leu3a to CD4 proteins on Molt4 cells. As shown in Fig. 2, 81% of mean fluorescence intensity (MFI) obtained from the binding of PE-labelled Leu3a was inhibited by CD4-DNA immunized serum, whereas, only 3% inhibition was observed by preimmune serum.

Enumeration of the percentage of CD4⁺ cells in peripheral blood lymphocytes using CD4-DNA immunized serum. To further characterize the anti-CD4 antibody activity in CD4-DNA immunized serum, this serum was used for the enumeration of $CD4^+$ cells in peripheral blood lymphocytes. PBMC from 5 subjects were stained with CD4-DNA immunized serum and CD4 mAb Leu3a, side by side. The percentage of $CD4^+$ cells in the lymphocyte population was analyzed by a flow cytometer and a comparison made between using CD4-DNA immunized serum and standard CD4 mAb. As shown in Table 2, the percentage of $CD4^+$ cells obtained by using both antibodies was very similar.

DISCUSSION

Over the past five years, the principle of DNA immunization has been demonstrated in several different animal models. The expression vectors used have encoded antigens from several viruses, bacteria and parasite antigens as well as leukocyte surface proteins.[2-11] Currently, it is clear that the induction of both humoral and cellular immunity is possible with the DNA immunization strategy. Several studies showed that the DNA immunization technique has several advantages over classical protein immunization. Large scale production is easier and less expensive to produce and maintain quality control for DNA than for protein antigens. DNA, in addition, is highly stable material. DNA immunization, therefore, promises to be an attractive alternative to the classical vaccines and an immunizing agent for the preparation of hyperimmune serum.

Several DNA delivery methods have been used to introduce plasmid DNA into animals.[12] The most efficient route to transfer pure plasmid DNA is intramuscularly, when it is injected as an aqueous

Table 1. Anti-CD4 antibody activity in sera before and after DNA immunization

Rabbit No.	Serum[c]	Immunofluorescent reactivity			
		CD4-COS[a]		CD8-COS[b]	
1	Pre-immunization	-	-	-	-
	Dose 1	-	-	-	-
	Dose 2	-	-	-	-
	Dose 3	-	-	-	-
	Dose 4	1+[e]	w+	-	-
	Dose 5	3+	2+	-	-
	Dose 6	3+	3+	-	-
2	Pre-immunization	-	-	-	-
	Dose 1	1+	w+	-	-
	Dose 2	2+	1+	-	-
	Dose 3	3+	3+	-	-
	Dose 4	4+	4+	-	-
	Dose 5	4+	4+	-	-
	Dose 6	4+	4+	-	-
3	Pre-immunization	-	-	-	-
	Dose 1	-	-	-	-
	Dose 2	1+	w+	-	-
	Dose 3	3+	2+	-	-
	Dose 4	4+	4+	-	-
	Dose 5	4+	4+	-	-
	Dose 6	4+	4+	-	-

[a] COS cells transfected with CD4-DNA were strongly positive with CD4 mAb Leu3a.

[b] COS cells transfected with CD8-DNA were strongly positive with CD8 mAb Leu2a.

[c] Sera were collected seven days after the indicated dose of immunization and tested for the presence of anti-CD4 antibodies by indirect immunofluorescence.

[d] Serum dilutions.

[e] Positive reactivity: w+; weakly positive, 1+ to 4+; degree of fluorescent intensity.

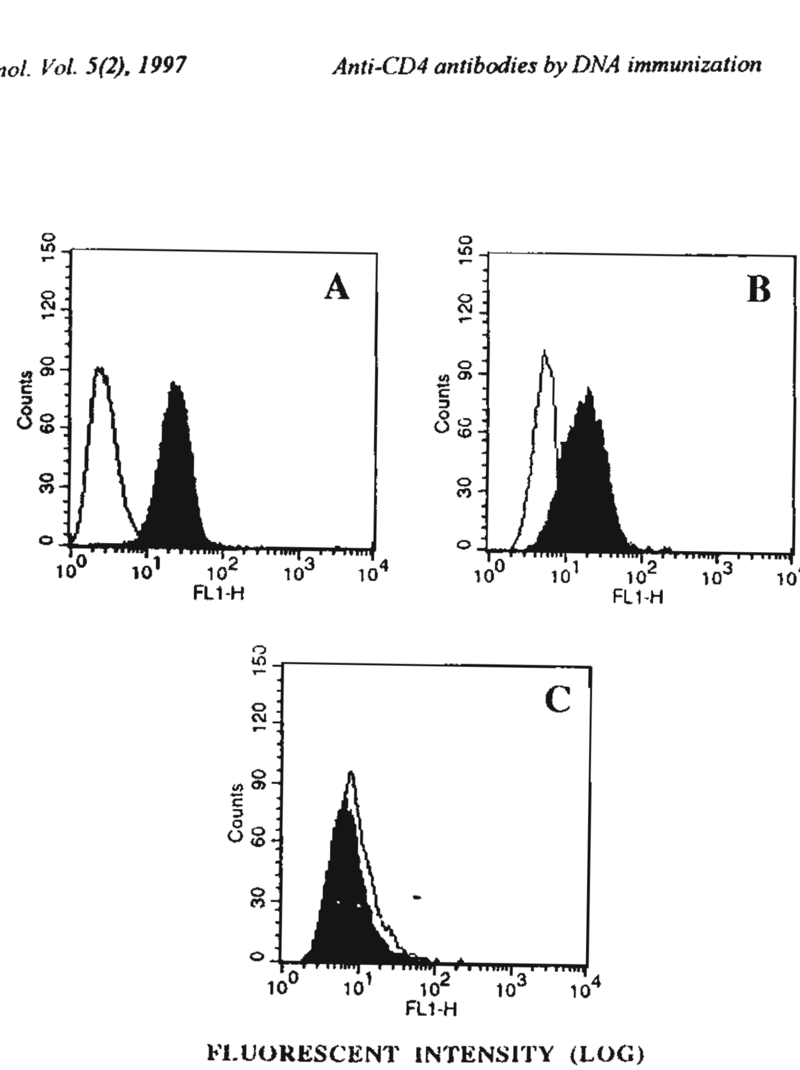

Figure 1. Immunofluorescence analysis of the reactivity of anti-CD4 antibodies with Sup-T1, Molt4 and K562 cell lines. Sup-T1 (A), Molt4 (B) and K562 (C) were stained with either preimmune serum or CD4-DNA immunized serum. Shaded peaks and unshaded peaks represent the immunofluorescence profiles of cells stained with CD4-DNA immunized and preimmune sera, respectively.

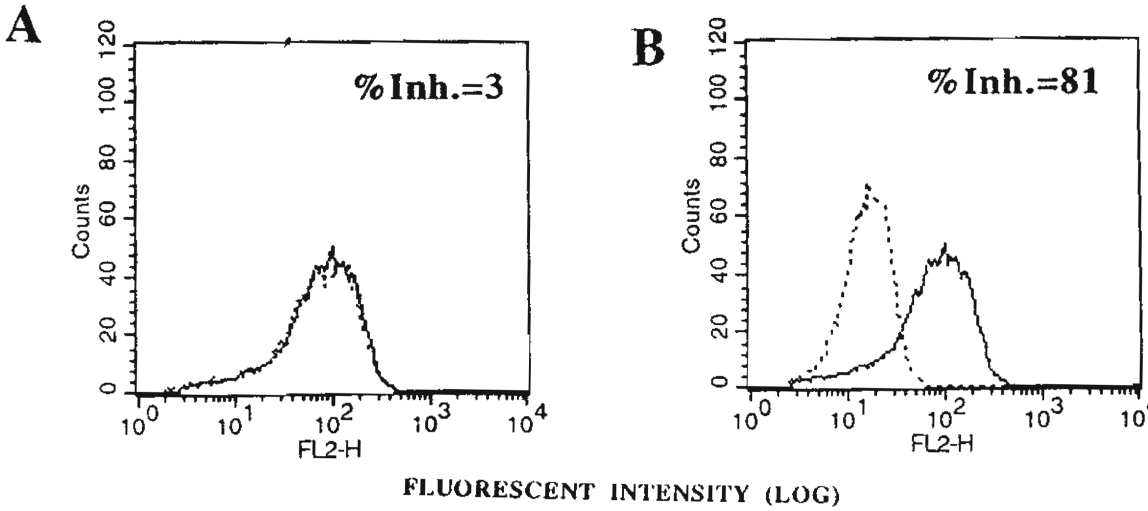

Figure 2. Inhibition of standard CD4 mAb binding by CD4-DNA immunized serum. Preimmune serum (A) or CD4-DNA immunized serum (B) was used to inhibit the binding of PE-labelled Leu3a. Solid lines represent the immunofluorescence profiles of cells stained with PE-labelled Leu3a. Dashed lines represent the immunofluorescence profiles of cells pre-incubated with preimmune sera or CD4-DNA immunized. The percentage of mean fluorescence intensity inhibition was indicated in each figure.

Table 2. Determination of the percentage of CD4$^+$ lymphocytes using CD4 mAb Leu3a and CD4-DNA immunized serum

Subject No.	% Positive cells in lymphocytes	
	CD4 mAb Leu3a	CD4-DNA immunized serum
1	24	25
2	36	36
3	27	30
4	46	48
5	31	33

solution (12). This route, however, still results in a low efficiency of gene transfer and a considerable variability of gene expression. It is likely that this situation is due largely to the limitation of diffusion by physical factors such as the organization of the connective tissue, and extent of the extracellular matrix (13,14). The diffusion barrier can be overcome by pretreating mature muscle with a hypertonic solution of sucrose, which presumably forces the myofiber apart, thus, opening channels in the connective tissue. Intramuscular injection of hypertonic sucrose solution 20 minutes prior to the administering of DNA into mice, gave an increase in reporter gene expression (14).

The efficiency of gene transfer, using pure DNA in skeletal muscle, is at least ten times more effective in regenerating muscle than in normal mature muscle.[15-17] Administration of toxic agents tended to cause muscle necrosis and repair. Therefore, several toxic agents have been used to pre-treat muscle for induction of regenerating muscle (2-4,18). Injection of the local anesthetic, Bupivacain, 24 hours prior to injection of DNA has been used to increase the expression of HIV gp 160 constructs and enhance immune responses (3,4).

In an attempt to produce a large amount of antibodies to the human CD4 protein, this study carried out the introduction of DNA encoding CD4 protein into rabbits. DNA encoding CD4 protein were injected into 3 rabbits in different ways. The first rabbit was pre-treated with sucrose solution, followed by CD4-DNA injection. The second rabbit was also pre-treated with sucrose solution, but this was followed by injection of mixed CD4-DNA and chloroquine. The third rabbit was pre-treated with local anesthetic, Bupivacain. Inoculation with CD4-DNA induced anti-CD4 antibody production in all tested rabbits. The rabbit that was pre-treated with sucrose solution, followed by injection of CD4-DNA containing chloroquine, however, produced anti-CD4 antibodies faster than the others. By this method, the antibodies could be detected only 7 days after the first DNA inoculation. This observation is probably due to the effect of sucrose pre-treatment and chloroquine, which was added into the DNA solution. Chloroquine is a weak base that can diffuse readily across phospholipid membrane, thus, raising the pH in the lysosome or endosome and reducing the activity of lysosomal enzymes (21). In the presence of chloroquine, the degradation of injected DNA within host cells was, therefore, decreased. Finally, the expression of encoded protein was increased and enhanced the induction of the immune responses.

In the previous study in mice, where CD4-DNA were directly immunized in the absence of any reagent, the anti CD4-antibodies were induced in 2 of 3 immunized mice (11). The antibodies were detected in immunized mice after more than 3 weeks of DNA administrations (11). In the present study, in contrast, all immunized rabbits produced anti-CD4 antibodies and the rapid induction of anti CD4 antibodies production was observed. The high efficiency and rapid induction of antibody production was observed. The high efficiency and rapid induction of antibody production may due to the effect of pre-treatment of rabbits and the reagent added in the CD4-DNA solution. However, to indeed verify the effect of these reagents for enhancing the immune response by DNA immunization, more experiments are required.

The titer of anti-CD4 antibodies generated by the DNA immunization was high. Generated anti-CD4 antibodies bound either recombinant CD4 proteins which were expressed on transfected COS cells, or native CD4 proteins on human lymphocytes and T cell lines. These rabbit anti-CD4 antibodies could inhibit the binding of standard CD4 mAb Leu3a to CD4 proteins. These results, therefore, confirm the specificity of rabbit anti-CD4 antibodies generated by DNA immunization. Rabbit anti-CD4 antiserum was used for determining CD4$^+$

cells in peripheral blood. The percentage of CD4$^+$ cells in 5 healthy lymphocytes gave comparable results to standard CD4 mAb Leu3a.

DNA immunization has been demonstrated to induce antibody response against several encoded antigens in various animals. The present report conveys the experiment performed in rabbits, which demonstrated that CD4-DNA injection induces anti-CD4 antibody production. The pre-treatment of rabbit, with hypertonic sucrose solution and injection of plasmid DNA in the presence of chloroquine, induced the antibody production after only one DNA inoculation.

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Manual rosetting method for enumerating CD4 positive cells

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Short title : Manual rosetting method for CD4 count

ABSTRACT

The measurement of CD4 positive cell levels is clinically useful in monitoring immune status and is used to guide the therapy of human immunodeficiency virus infection. The standard method for determination of the number of CD4 positive cells is carried out by flow cytometric analysis. This technique, however, requires expensive, complex instrumentation as well as trained technologists. The rapid growth of HIV infection in developing countries has increased the need for a simple and cost-effective method for CD4 positive cell monitoring. Therefore, the purpose of this study was to establish a non flow cytometric method, named the manual rosetting method, for quantifying CD4 positive cell numbers. In this investigation, 24 HIV infected and 16 healthy individauls were enumerated for CD4 positive cells by using both standard flow cytometry and the manual rosetting method simultaneously. The mean percentage of CD4 positive cells in the total of white blood cells from samples performed by the manual rosetting method was 6.31%, while the standard method yielded a mean of 6.48%. The correlation coefficient for regression analysis was 0.93. The mean of absolute CD4 positive cells obtained from both methods were 529.04 and 515.75 cells/μl, respectively. The correlation coefficient for regression analysis was 0.95. The interference of granulocytes and monocytes with the manual rosetting method was evaluated. Very few CD4 rosetting cells were detected in granulocyte and monocyte populations. It was concluded that the manual rosetting method appears to have the potential to quantify CD4 positve cells in the limited laboratory facilities of developing countries and have a strong correlation with standard flow cytometric technology.

Keywords: CD4 count, CD4 percentage, flow cytometry, manual rosetting method

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CD4 positive cell enumeration is an important immunodianostic measurement used in evaluating patients infected with human immunodeficiency virus (HIV). The progressive depletion of CD4 positive cells is associated with the likelihood of clinical complications and the development of AIDS [1,2]. The United States Centers for Disease Control and Prevention proposed that a CD4 positive cell count less than 200 cells/μl be used as one of the criteria for AIDS in the case definition[3]. CD4 positive cell counts are also used for monitoring HIV seropositive individuals into various treatment protocols [4-7].

In most laboratories, the standard method for CD4 positive cell enumeration involves the use of a flow cytometric method [8]. By this method, CD4 positive cells in whole blood specimens are identified by immunophenotyping and analyzing the results by using a flow cytometer. The results obtained in this manner are a proportion of lymphocytes that are CD4 positive cells or a percentage of CD4 positive cells. These results must be combined with a hematology determination, which provides the absolute number of circulating lymphocytes and calculated to the absolute CD4 positive cell count. This standard flow cytometric method requires expensive instruments and reagents as well as trained technologists, thus limiting the availability of CD4 positive cells quantified in developing countries.

Recently, several methods that do not require flow cytometer were developed for enumerating CD4 cells [9-13]. However, the reagents used are still expensive. Some methods still require special and expensive equipment [9,11-13]. In this study, another non-flow cytometric method, named the manual rosetting method, was developed for enumerating CD4 positive cells. By this method, lysed whole blood samples were stained with CD4 mAb and the stained cells were then incubated with sheep anti-mouse immunoglobulin coated beads. The rosettes, which were CD4 positive cells, were then counted by a light microscope. This technique is simpler and less expensive than the flow cytometric method. The clinical utility of this assay is very attractive in developing countries where HIV prevalence is high and funds for flow cytometry and service are limited.

MATERIALS AND METHODS

Subjects

EDTA anticoagulated whole blood was obtained from HIV infected and normal individuals for evaluation of CD4 positive cell counts by flow cytometry and the manual rosetting method. HIV infection diagnosis was made on the basis of positive testing for the presence of anti-HIV antibodies by both GPA (Gel particle agglutination; Serodia HIV, Fujirebio, Japan) and ELISA (3rd generation Abbott anti-HIV1/HIV2, Abbott laboratory, USA).

Enumeration of CD4 positive cells by flow cytometry

100 μl of blood was incubated at room temperature with 20 μl of each Simultest reagent panel (Becton Dickinson, Moutain View, CA) in separate tubes. The Simultest reagent panel was composed of two-color reagent pairs of leukoGATE (CD45-FITC/CD14-PE), control IgG1-FITC/IgG2-PE, CD3-FITC/CD4-PE, and CD3-FITC/CD8-PE. After 15-30 min incubation, red cells were lysed by the addition of FACS lysing buffer (Becton Dickinson) to the tubes. Samples were subsequently fixed with 1.0% paraformadehyde and analyzed using a FACScan flow cytometer with Simulset software (Becton Dickinson). The results obtained in this manner were the percentages of CD4 positive cells in the lymphocyte population. The absolute CD4 positive cell counts were then calculated as the product of the total white blood cell count, percentage of lymphocytes, and the percentage of CD4 positive cells.

Enumeration of CD4 positive cells by the manaul rosetting technique

For the manual rosetting technique, 1 ml of whole blood was incubated with 5 ml of FACS lysing buffer (Becton Dickinson) at room temperature for 10 min. The resulting lysed whole blood (LWB) was then washed twice with phosphate buffered saline containing 1% bovine serum albumin and 0.02% sodium azide (1% BSA-PBS-Azide), and resuspended with 250 μl of the same solution. To block the Fc receptors, the cell suspension was added with 25 μl of human AB serum and incubated for at least 30 min on ice. Aliquots (50 μl) of LWB were added into a 1.5 ml microtube followed by 10 μl of anti-CD4 mAb (Leu3a, Becton Dickinson), and placed on ice for

30 min. As a control, 1%BSA-PBS-Azide was added instead of antibody. Stained cells were then washed twice with 1%BSA-PBS-Azide and resuspended with 50 μl of the same solution. Ten microlitres of sheep anti-mouse IgG coated beads (Dynal, Oslo, Norway) were added. The beads were washed once and resuspended in 1%BSA-PBS-Azide with 10%AB human serum prior to use. The blood/bead mixture was gently rotated on a platform shaker at room temperature for 1 h. After incubation, the mixture was gently resuspended and 10 μl of it was added to 10 μl of Turk's solution (2% acetic acid and 0.01% gentain violet). This was then loaded into the chamber of a hemacytometer for counting. Cells that had formed rosettes with three or more beads were counted as CD4 positive cells (Fig. 1). The percentage of CD4 positive cells was obtained by counting at least 200 white blood cells. The absolute CD4 positive cell counts were then calculated from the total number of white blood cells and the obtained percentage of CD4 rosetting cells.

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. The PBMC were washed twice with 1% BSA-PBS-Azide and adjusted to a final concentration of 1 $x10^7$ cells/ml with the same solution.

Preparation of granulocytes

Granulocytes were isolated from the pellet of the Ficoll-Hypaque density gradient isolation of PBMC by the dextran sedimentation method. Cells were then washed twice and the contaminated red blood cells were removed by using hypotonic lysis with ammonium chloride buffered. To block non-specific Fc receptor-mediated binding of antibody, human AB serum was added to a final concentration of 10%.

Preparation of E^+ and E^- populations

To isolate E^+ and E^- populations, 2-aminoethylisothiouronium-hydrobromide treated sheep red blood cells (AET-SRBC)[14] were added to the PBMC, incubated at 37°C for 10 min, and then centrifuged at 600g for 2 min. Cells were kept at 4°C overnight. The rosette forming cells in the pellet were resuspended and isolated further by Ficoll-Hypaque gradient centrifugation. E^+ and E^- fractions were collected and the sheep red blood cells were then lysed with ammonium chloride buffered. E^+ and E^- cells were then resuspended in 1%BSA-PBS-Azide with 10% human AB serum.

Statistical analysis

The CD4 positive cell counts obtained from flow cytometry and the manual rosetting method were compared by using the student paired t-test. The correlation coefficient (r) of both methods was established for all samples by linear regression analysis. Sensitivity was defined as the number of individuals with CD4 positive cell counts < 200 cells/μl by both methods divided by the number of individuals with CD4 cell counts < 200 cells/μl by flow cytometry. Specificity was defined as the number of individuals with CD4 positive cell counts > 200 cells/μl by both methods divided by the number of individuals with CD4 cell counts > 200 cells/μl by flow cytometry.

RESULTS

Comparison of the manual rosetting method and flow cytometric method

The manual rosetting method was developed for the enumeration of CD4 positive cells in countries where funds for flow cytometry are limited. Blood specimens from 24 HIV-seropositive and 16 HIV-seronegative donors were enumerated for CD4 positive cells by standard flow cytometry (Simulset) and the manual rosetting method. In the case of flow cytometry, the percentage of CD4 positive cells in white blood cells was calculated from the percentage of CD4 positive cells in the lymphocyte population (obtained from flow cytometric analysis), and the white blood cell count and leukocyte differential (obtained using standard laboratory procedures). A correlation plot comparing the percentage of CD4 positive cells in white blood cells obtained from both methods is shown in figure 2. Linear regression analysis resulted in a slope of 0.76 and an intercept of 1.67 % when data from the two methods were compared. The correlation coefficient (r) of this regression analysis was 0.93. The mean value of the percentage of CD4 positive cells for samples tested by standard flow cytometry and the manual rosetting method were 6.31 % and 6.48%, repectively. No significant difference in these values was seen ($p > 0.05$).

The absolute number of CD4 positive cells were then calculated from the percentage of CD4 positive cells and the number of white blood cells per µl. A correlation plot which compares between the absolute CD4 positive cell count obtained from the reference flow cytometric method and the manual rosetting method is shown in figure 3. The mean value for samples tested by standard flow cytometry and the manual rosetting method were 515.75 and 529.04 cells/µl, respectively. Linear regression analysis of both methods resulted in a slope of 0.80 and an intercept of 114.53 cell/µl. The correlation coefficient for this regression analysis was 0.95. Again, no significant differences or trends were seen in these values ($p>0.05$).

Since the CD4 positive cell count of less than 200 cells/µl was used as the critiria for AIDS in the case definition [3], the data obtained from both methods were also evaluated, based on the CD4 positive counts of more or less than 200 cells/µl. This analysis demonstrated that the manual rosetting method was 89% sensitivity and 96% specificity (Table I).

Effect of granulocytes and monocytes on the manual rosetting method

Potential sources of interference with the rosetting method could be due to cell types other than CD4 positive T lymphocytes. To address this question, granulocytes, which do not express CD4 protein [15], were isolated from 4 normal donors and used as subjects for the manual rosetting method. By immunofluorescent analysis, the isolated granulocyte populations contained 0.6-5 % CD3 positive T cells, 0.3-1.4 % CD4 positive cells and 0-0.4% CD14 positive monocytes (Table II). Very few rosette forming cells, range 0.3-0.7 %, were observed in these granulocyte populations (Table II). These results indicated that granulocytes did not interfere with the manual rosetting method.

The interference of monocytes was also investigated. PBMC were separated into E^+ and E^- populations by sheep red blood cell rosetting [14]. The E^- population isolated from 4 normal donors consisted of more than 57% CD14 positive monocytes (range, 57-78%) and less than 3% of CD3 positive lymphocytes (range, 0.6-3%) (Table III). The rosette forming cells obtained from the manual rosetting method were barely detectable in these E^- populations (range, 1-4%). The results indicated that monocytes were not detected by the manual rosetting method. In contrast, in the E^+ population which contained a majority of CD3 positive cells and CD4 positive lymphocytes (Table IV), a high number of rosette forming cells were observed (Table IV).

DISCUSSION

The absolute number of CD4 positive cells is an important marker for the prognosis and classification of the state of the disease, and monitoring for the therapy of HIV infection[1-7]. The accepted standard method for the enumeration of CD4 positive cells is flow cytometry. However, problems facing the clinical laboratory are that flow cytometry generally does not lend itself to routine high-volume testing, and requires expensive equipment and reagents. In addition, specially trained personnel are required to operate flow cytometry instrumentation. This technology, is therefore, costly for adaptation as a routine method in laboratories in developing countries. Alternative methodologies for CD4 positive cell determination that should be simple, inexpensive and reliable are urgently needed for use in laboratories, especially in developing countries. To support this requirement, we introduce a non-flow cytometric method called the manual rosetting method for determination of CD4 positive cells.

The principle of the manual rosetting method is that lysed whole blood samples are pre-stained with CD4 mAb and the stained cells are then incubated with anti-mouse immunoglobulin coated beads. The CD4 rosette forming cells are then enumerated by a light microscope. The obtained percentage of CD4 positive cells are then calculated to the absolute number by multiplying with the white blood cell count. The manual rosetting method described here is simple, needs no any special instruments and is a cost-effective method for quantifying CD4 positive cells. A high degree of correlation between standard flow cytometry and the manual rosetting method has been found for both percentage and absolute CD4 positive cells (correlation coefficient, 0.93 and 0.95, recpectively). Furthermore, the manual rosetting method has high sensitivity and specificity for identifying individuals with less than 200 cells/μl of CD4 positive cells.

It is known that peripheral blood monocytes express CD4 molecules on their surface[16] and that these cells can interfere with and affect the accuracy of the flow cytometric measurement of CD4 positive lymphocytes[8,17]. Therefore, the standard flow cytometric method generally employs a T-cell specific antibody, CD3 antibody, to discriminate between target cells and contaminated cells[18,19]. In this study, experiments were performed to confirm that the manual rosetting method was able to discriminate between the CD4 positive lymphocytes and monocytes. Four normal blood samples were separated into E^+ and E^- populations using a conventional AET-sheep red blood cell rosetting technique[14]. The E^- population, which contained 60-80% CD14 positive monocytes and less than 3% CD3 positive T cells was used to examine the issue of potential interference from monocytes. It was found that in the E^- population, by using the manual rosetting method, a very low number of rosette formimg cells (range 0.6-3%) were determined. In contrast, in the E^+ population, which contained more than 50% CD3 positive T cells, a high number of rosette formimg cells (range 44-60%) were determined. The resultant near-zero rosette formation in the E^- population indicated that there was no interfering effect from monocytes in the enumeration of CD4 positive cells by the manual rosetting method. This may be due to the low density CD4 expression on the monocyte surface[16]. Monocytes, therefore, do not form rosettes with anti-mouse immunoglobulin coated beads. Granulocytes were also tested for interference and their affect on the accuracy of the measurement of CD4 positive cells. As predicted, by the manual rosetting method, approximately 1% (range 0.6-1%) of CD4 rosetting cells were detected in these populations. These results indicated that granulocytes do not affect the accuracy of this manual rosetting method.

In summary, a non-flow cytometric method, named the manual rosetting method, was introduced for the enumeration of CD4 positive cells. The manual rosetting method is simple, inexpensive and meets the growing demand for CD4 counts, especially in developing countries where HIV prevalence is high.

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Table I. Sensitivity and specificity of the manual rosetting method compared with flow cytometry.

	Manual rosetting *		
	< 200 cells/μl	> 200 cells/μl	Total
Flow cytometry			
< 200 cells/μl	16	2	18
> 200 cells/μl	1	21	22
Total	17	23	40

*89% sensitivity and 96% specificity.

Table II. Interference of granulocytes with the manual rosetting method.

Sample No.	Immunofluorescence			Manual rosetting
	%CD3 pos.	%CD4 pos.	%CD14 pos.	%CD4 rosette
1	0.6*	0.3	0	0.8
2	0.6	0.3	0	0.4
3	4.6	1.4	0.4	1.1
4	1.7	0.8	0.2	0.6

Granulocytes were isolated from 4 healthy donors and were determined for percentages of CD3, CD4 and CD14 positive cells by indirect immunofluorescent technique. An aliquot of cells was enumerated for CD4 positive cells by the manual rosetting method.

* % positive cells.

Table III. Interference of monocytes with the manual rosetting method.

Sample	Immunofluorescence		Manual rosetting
No.	%CD3 pos.	%CD14 pos.	%CD4 rosette
1	1.0*	78	4.0
2	0.6	57	1.0
3	1.6	77	2.8
4	3.0	73	2

The E^- population was isolated from 4 healthy donors by sheep red blood cell rosetting and determined for the percentages of CD3 and CD14 positive cells by indirect immunofluorescent technique. An aliquot of cells was enumerated for CD4 positive cells by the manual rosetting method.

* % positive cells.

Table IV. Enumeration of CD4 positive cells in the E^+ population by the manual rosetting method.

Sample	Immunofluorescence			Manual rosetting
No.	%CD3 pos.	%CD4 pos.	%CD14 pos.	%CD4 rosette
1	95*	58	2	60
2	77	48	0.8	53
3	66	42	1.8	44
4	75	48	3.0	58

The E^+ population was isolated from 4 healthy donors by sheep red blood cell rosetting and determined for percentages of CD3, CD4 and CD14 positive cells by indirect immunofluorescent technique. An aliquot of cells was enumerated for CD4 positive cells by the manual rosetting method.

* % positive cells.

FIGURE LEGENDS:

FIG. 1. Representative picture in a hemacytometer chamber of CD4 positive cell binding beads.

FIG. 2. Correlation plot comparing the % CD4 positive cells measured with the manual rosetting method and the standard flow cytometric method. The linear regression is expressed by the equation Y=1.67 + 0.76x (r=0.93).

FIG. 3. Correlation plot comparing absolute CD4 positive cell count measured with the manual rosetting method and the standard flow cytometric method. The linear regression is expressed by the equation Y=114.53 + 0.80x (r=0.95).

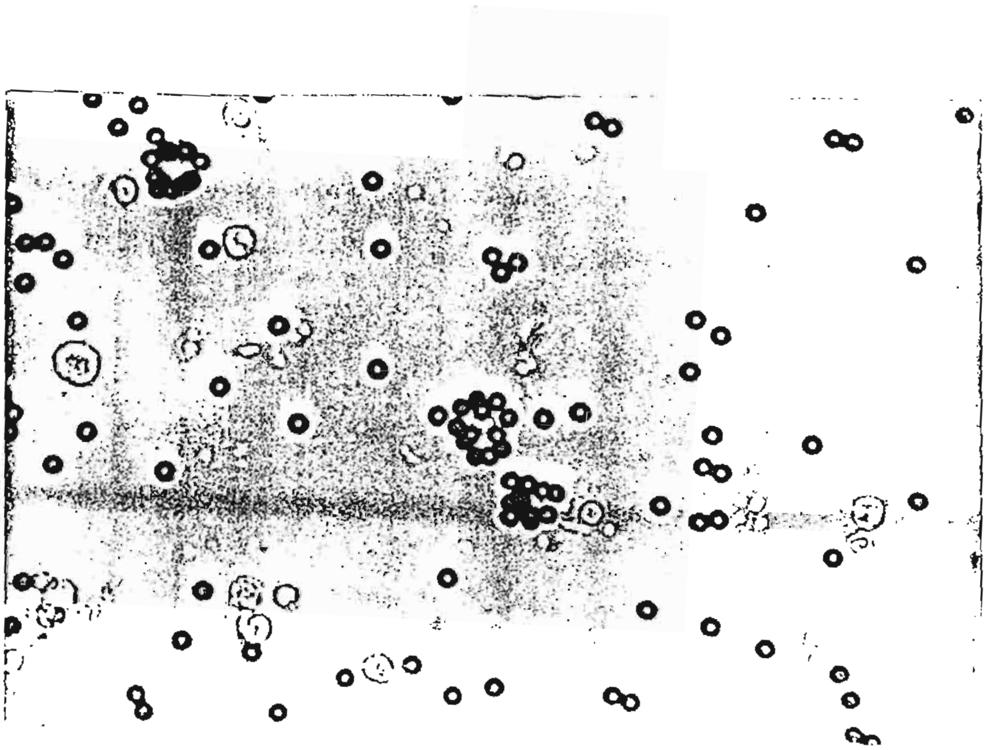

Fig.1

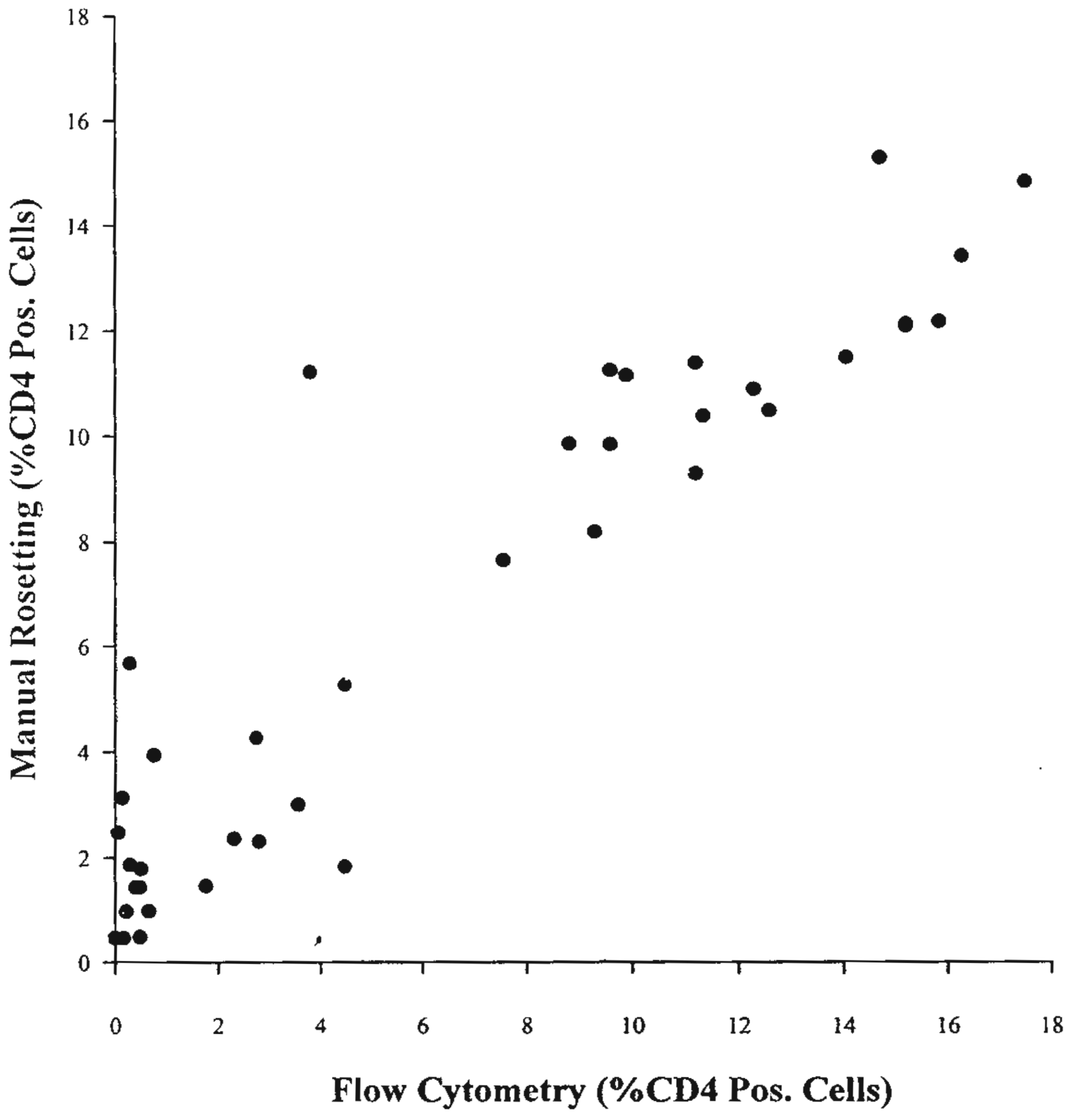
Manual Rosetting (%CD4 Pos. Cells)
Flow Cytometry (%CD4 Pos. Cells)
0
2
4
6
8
10
12
14
16
18

Fig. 2

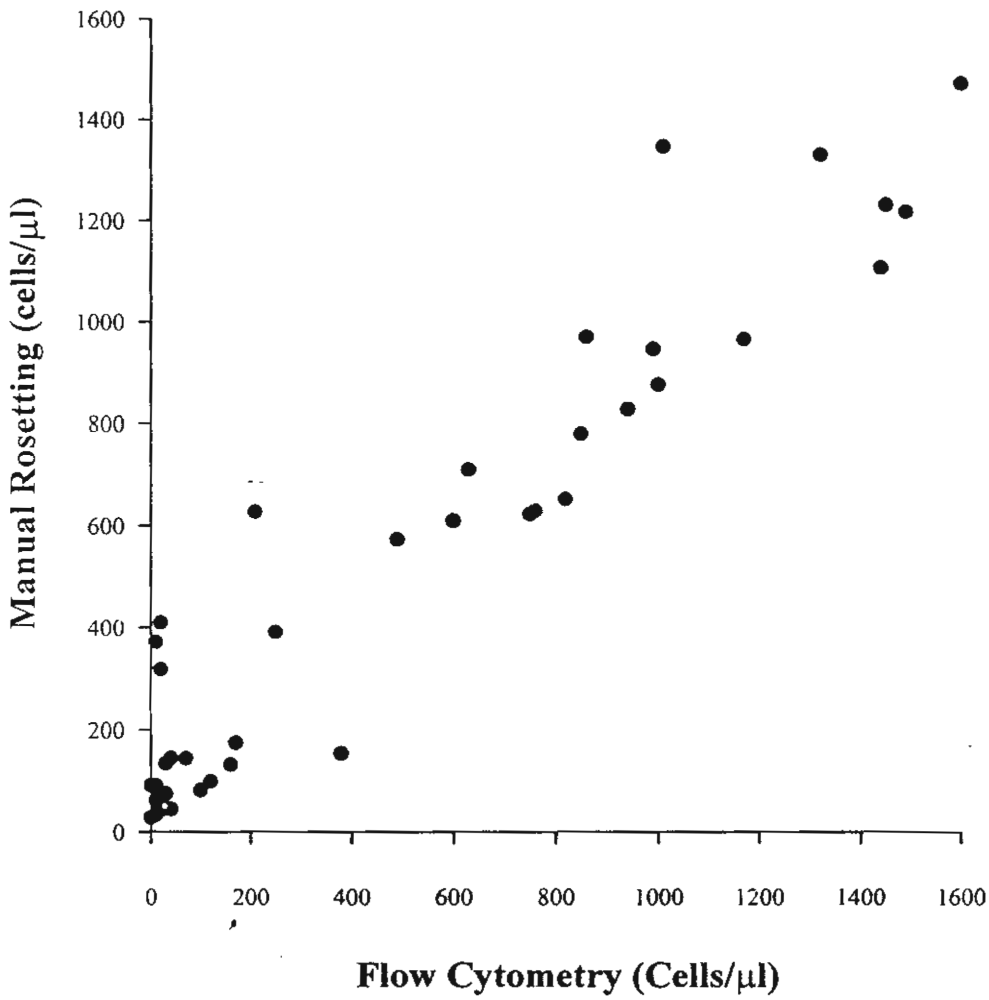
1600
1400
1200
1000
800
600
400
200
0
Manual Rosetting (cells/µl)
0
200
400
600
800
1000
1200
1400
1600
Flow Cytometry (Cells/µl)

Fig. 3

Production of CD4 monoclonal antibody and development of home made reagent for CD4+ lymphocyte determination

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Abstract

A hybridoma secreting monoclonal antibody (mAb) specific to CD4 protein was generated. This monoclonal antibody, named MT4, was proved to be specific to CD4 protein as it reacted with CD4-DNA transfected COS cells, CD4+ cell lines and CD4+ lymphocytes. Furthermore, MT4 mAb inhibited the binding of standard CD4 monoclonal antibodies to CD4 proteins on CD4+ cells. To develop a home made reagent for CD4+ lymphocyte determination by flow cytometry, fluorescein isothiocyanate (FITC) was conjugated to MT4 mAb. To evaluate the developed reagent, 30 HIV infected and 30 healthy individuals were determined for CD4+ lymphocytes by using both commercial Simultest™ reagent kit and home made FITC labeled MT4 mAb simultaneously. The study has shown that both percentages and absolute CD4+ lymphocyte counts obtained from both reagents were equivalent. The correlation coefficient for regression analysis was 0.995 and 0.996 for percentages and absolute CD4+ lymphocyte counts, respectively. The results suggest that home made FITC labeled MT4 reagent is an acceptable alternative reagent for monitoring CD4+ lymphocytes in blood samples by flow cytometry.

การผลิตโมโนโคลนอล แอนติบอดี ต่อ CD4 โปรตีน และพัฒนาเป็นน้ำยาเพื่อตรวจวัดระดับ CD4+ Lymphocytes

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ในการศึกษานี้สามารถเตรียมไฮบริโดมาที่ผลิตโมโนโคลนอล แอนติบอดีที่จำเพาะต่อ CD4 โปรตีนได้จำนวน 1 โคลน โมโนโคลนอล แอนติบอดีนี้ได้ให้ชื่อว่า MT4 และได้พิสูจน์ยืนยันความจำเพาะต่อ CD4 โปรตีนแล้วพบว่าแอนติบอดีนี้สามารถทำปฏิกิริยาได้กับ CD4-DNA transfected COS cells, CD4+ cell lines และ CD4+ lymphocytes นอกจากนี้แอนติบอดีนี้ยังสามารถยับยั้งการจับกันของ CD4 โมโนโคลนอล แอนติบอดีมาตรฐานกับ CD4 โปรตีนบนผิวของ CD4+ cells ได้ เพื่อผลิตน้ำยาตรวจนับ CD4+ lymphocytes ขึ้นมาใช้เอง จึงนำสาร fluorescein isothiocyanate (FITC) มาติดฉลากกับ MT4 แล้วนำไปใช้ตรวจนับจำนวน CD4+ lymphocytes โดยวิธี flow cytometry เพื่อประเมินประสิทธิภาพของน้ำยาที่เตรียมขึ้นมา ได้ทำการตรวจนับจำนวน CD4+ lymphocytes ในเลือดผู้ติดเชื้อเอดส์และคนปกติอย่างละ 30 ราย โดยทำการเปรียบเทียบระหว่างน้ำยา-FITC ติดฉลาก MT4 กับชุดน้ำยามาตรฐาน Simultest™ ผลการศึกษาพบว่าเปอร์เซนต์และค่า absolute CD4+ lymphocyte counts ที่ได้จากน้ำยาทั้งสองชนิดไม่แตกต่างกัน โดยมีค่าสัมประสิทธิ์ สหสัมพันธ์ เท่ากับ 0.995 และ 0.996 สำหรับเปอร์เซนต์และค่า absolute CD4+ lymphocyte counts ตามลำดับ ผลการศึกษาในครั้งนี้ชี้ให้เห็นว่า น้ำยา FITC ติดฉลาก MT4 ที่เตรียมขึ้นมาใช้เองนี้น่าจะเป็นน้ำยาอีกชนิดหนึ่งที่สามารถนำมาใช้ตรวจวัดระดับ CD4+ lymphocytes ในเลือดโดยวิธี flow cytometry ได้

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Accurate and reliable measures of CD4+ lymphocytes are essential for the assessment of the immune system of human immunodeficiency virus (HIV)-infected persons[1-3]. The pathogenesis of acquired immunodeficiency syndrome (AIDS) is largely attributable to the decrease in CD4+ lymphocytes[4-8]. Progressive depletion of CD4+ lymphocytes is associated with an increased likelihood of clinical complications [9,10]. Consequently, the Public Health Service (PHS) has recommended that CD4+ lymphocyte levels be monitored every 3-6 months in all HIV-infected persons[11]. The measurement of CD4+ T lymphocyte levels has been used to establish decision point for initiating prophylaxis[12], anti-viral therapy[13] and monitoring the efficacy of treatment[14-16]. It is also used for prognostic indicators in patients who have HIV disease[17,18]. Moreover, CD4+ lymphocyte levels are a criterion for categorizing HIV-related clinical conditions by CDC's classification system for HIV infection and surveillance case definition for AIDS among adults and adolescents[19].

The standard method for CD4+ lymphocyte enumeration involves the use of flow cytometric method[20,21]. This method, CD4+ lymphocytes in whole blood specimens are identified by immunophenotyping and analyzing the results using a flow cytometer. The results obtained in this manner are a percentage of CD4+ lymphocytes. These results must be combined with a hematology determination which provides the total white blood cell count and the percentage of lymphocytes (differen-tial) and calculated to the absolute CD4+ lymphocyte count. The standard flow cytometric method, however, requires very expensive reagents, i.e., fluorescent dye labeled monoclonal antibodies specific to leukocyte surface molecules. This technique, thus, limits the availability of CD4 + lymphocyte enumeration in developing countries.

In this report, a monoclonal antibody (mAb) specific to CD4 protein was generated. The generated CD4 mAb was, then, developed to be a home made reagent for CD4+ lymphocyte determination by flow cytometer. This home made reagent could be used cheaply to enumerate CD4+ lymphocytes in blood samples as well as commercial products. The clinical utility of the reagent produced is very attractive in developing countries where HIV prevalence is high and funds for flow cytometry and services are limited.

MATERIALS AND METHODS

Production of monoclonal antibody to CD4 protein

Human T cell line, Sup T1, 1 x 10^7 cells were injected intraperitoneally into a BALA/c mouse. A booster immunization was followed a week later with intravenous injection of 1 x 10^6 Sup T1 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 as previously described[22]. After that, cells were distributed into 672 wells of 96 well-plates. Two weeks later, hybridomas were identified by an inverted microscope . Cell culture supernatants from hydridoma containing wells were screened for antibody against CD4 protein. The positive clone was re-cloned three rounds by limiting dilution. To produce high concentration of CD4 mAb, the cloned hybridomas were injected intraperitoneally into BALC/c mice that were pre-treated with 2, 6, 10, 14-tetramethyl-pentadecan (Pristane). Ascitic fluid containing CD4 mAb was harvested, usually, 10-20 days after hybridoma inoculation.

Screening for CD4 specific monoclonal antibody

Hybridoma cell culture supernatants were firstly analyzed by indirect immunofluorescence using Sup T1 cells as antigens. The positive supernatants were screened further for antibody specific to CD4 protein by the same technique but using cDNA encoding CD4 protein transfected COS cells as antigens. In all experiments, COS cells transfected with cDNA encoding unrelated protein were used as negative control.

DEAE-Dextran transfection of COS cells

To prepare cDNA encoding protein of interests, the cDNAs, which had been constructed into an eukaryotic expression vector πH3M, were transformed into competent *E. coli* MC1061/p3. After that, plasmid DNAs were purified by cesium chloride-ethidium bromide density gradient ultracentrifugation[23]. The resulting DNAs were phenol/chloroform-extracted and ethanol precipitated, then resuspended in TE (10mM Tris, 1mM EDTA) pH 8.0. The Plasmid DNAs were transfected into COS cells using the DEAE-Dextran transfection method[24]. Briefly, 1x10^6 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 min at room temperature. COS cells were then cultured overnight in MEM containing 5% FCS, washed once, and re-cultured with the same medium for another 2 days to allow expression of the encoded proteins.

Indirect immunofluorescence analysis

The specificity of antibody against CD4 protein was assessed by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulin antibodies (Immunotech, Coultrer Corporation, Miami, FL). 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. Blocked cells were then incubated for 30 minutes at 4°C with culture supernatants or mAb. After washing, cells were incubated with the FITC-conjugate for another 30 minutes. Membrane fluorescence was analyzed by flow cytometer (FACSCalibur, Becton Dickinson, San Jose, Ca).

Determination of isotype of monoclonal antibody

The isotype of mAb was determined by capture ELISA (Sigma, St. Louis, MO) 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 used as conjugate. The reactivity was visualized by using 3',3',5',5'-tetramethylbenzidine (TMB) as substrate.

Inhibition of standard CD4 monoclonal antibody binding by MT4 monoclonal antibody

CD4+ cells (peripheral blood lymphocytes or human T cell lines) were pre-incubated with MT4 mAb or irrelevant mAb for 30 minutes on ice. Phycoerythrin (PE)-labeled CD4 mAb Leu3a (Becton Dickinson) or FITC-labeled CD3 mAb Leu4 (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 of the sample in the presence and absence of first un-labeled mAb.

Fluorescent labeling of MT4 monoclonal antibody

MT4 mAb was purified from MT4 hybridoma induced ascitic fluid by using UltraLink™ Immunobilized Mannan Binding Protein column (Priece, Oud-Bejerland, The Netherlands) according to the recommended protocol (Priece). The concentration of purified MT4 was measured by reading the absorbance at 280 nm and adjusted to 2 mg/ml in PBS containing 0.1 M $NaHCO_3$. Fluorescein isothiocyanate (FITC; Sigma) ,dissolved in DMSO at a concentration of 10 mg/ml, 25 µl was slowly added to 1 ml of antibody (2mg/ml). The mixture was rotated at room temperature for 90 min. The free fluorescein dye was, then, removed by ultrafiltration using Centricon concentrator (MW cut-off 10,000; Amicon, Beverly, MA) and equilibrated with PBS. The ratio of fluorescein to protein was estimated by measuring the absorbance at 495 nm and 280 nm. The concentration of the FITC labeled antibody was measured by reading the absorbance at 280 nm.

Enumeration of CD4+ lymphocytes by FITC labeled MT4

One hundred microliters of K_3EDTA-whole blood was incubated at room temperature with 50 µl of FITC labeled MT4 (40 µg/ml). After 30 min room temperature incubation, 2 ml of lysing buffer (Becton Dickinson) was added and let stand at room temperature in the dark for 10 min for lysis of red blood cells. Cells were then washed once with 2 ml of PBS containing 0.1% sodium azide. Samples were subsequently fixed with 1% paraformadehyde and analyzed by using a FACSCalibur flow cytometer with CELLQuest software (Becton Dickinson). The lymphocyte population was gated according to their size and granularity using light scattering, i.e, forward scatter (FSC) and side scatter (SSC) with linear scale. The percentage of CD4+ lymphocytes in the gated population was determined by using FITC fluorescene-1 (FL1) and FSC. The absolute CD4+ lymphocyte count (cells/µl) was then calculated as the product of the total white blood cell count, percentage of lymphocytes, and the percentage of CD4+ lymphocytes.

In some cases, contamination of red blood cells occurred within the gated cells. The contaminated red blood cells were gated out from the lymphocyte population by making another gate using FL1 and FSC. The percentage of CD4+ cells in the lymphocyte population was then re-calculated from the number of lymphocytes in second gated population.

Enumeration of CD4+ lymphocytes by commercial Simutest^{TM} reagent

One hundred microliters of K_3EDTA-whole blood was incubated at room temperature with 20 μl of each Simultest^{TM} reagent panel (Becton Dickinson) in separate tubes. The Simultest^{TM} reagent panel was composed of two-color reagent pairs of leukoGATE (CD45-FITC/CD14-PE), control IgG1-FITC/IgG2-PE, CD3-FITC/CD4-PE, and CD3-FITC/CD8-PE. After 15-30 min room temperature incubation, 2 ml of lysing buffer (Becton Dickinson) was added and let stand at room temperature in the dark for 10 min for lysis of red blood cells. Cells were then washed once with 2 ml of PBS containing 0.1% sodium azide. Samples were subsequently fixed with 1 % paraformadehyde and analyzed using a flow cytometer with Simultest IMK-lymphocyte software (Becton Dickinson). The absolute CD4+ lymphocyte count was then computed from the total white blood cell count and the percentage of lymphocyte.

RESULTS

Production of CD4 monoclonal antibody

After fusion, cell culture supernatants from hybridoma containing wells were tested for reactivity with surface antigens of Sup T1 cells by indirect immuno-fluorescence technique. Forty-three culture supernatants showed clearly positive. To screen further for hybridomas that produced CD4 specific antibody, all positive supernatants were tested again by the same technique, but CD4 transfected COS cells were used as the antigen. One of these culture supernatants reacted with CD4 transfected COS cells, but not with mock transfections. The hybridomas in this culture well were then re-cloned three times by limiting dilution. A final clone (3E8) that gave the same reaction pattern with transfected COS cells was propagated and re-named MT4. By using capture ELISA for isotype characterization, MT4 mAb was proved to be of IgM isotype.

Characterization of MT4 monoclonal antibody specificity

To confirm the specificity of generated mAb, MT4 mAb was used to stained CD4+ T cell lines, Sup T1[(25)] and Molt4[(26)], and CD4- cell lines, K562[(27)] and U937[(28)] by indirect immunofluorescence and analyzed by flow cytometry. As predicted, MT4 mAb reacted to both CD4+ cell lines, but not to CD4- cell lines. Then, MT4 mAb was used to stain peripheral blood lymphocytes by the same technique. The results were compared to those obtained by using standard CD4 mAb Leu3a. As shown in Table 1, percentages of positive cells in 5 donors obtained by using both antibodies were very similar.

To characterize the specificity of MT4 mAb further, MT4 was used to inhibit the binding of standard CD4 mAb to CD4 proteins on both peripheral blood lympho-cytes and CD4+ cell lines. Peripheral blood mononuclear cells from 3 normal donors were, firstly, incubated with MT4 or control antibodies. Then, PE labeled CD4 mAb Leu3a or FITC labeled CD3 mAb Leu4 was added and the fluorescence intensity was determined by a flow cytometer. It was found that MT4 mAb inhibited the binding of standard CD4 mAb Leu3a in all 3 donors tested with the percent inhibition of 97, 98 and 97, respectively. The irrelevant mAb control, M6, had no inhibitory effect with the percent inhibition of 4, 8 and 3, respectively. In contrast, MT4 mAb did not inhibit the binding of standard CD3 mAb Leu 4 (% inhibition of 1, 1 and 0, respectively). The FACS profiles were similar for each donor and one of which is shown in Fig. 1. CD4+ cell lines, Sup T1 and Molt4, were also used to confirm these results by the same technique and it was found that MT4 strongly inhibited the binding of standard CD4 mAb (PE labeled Leu3a) to both cell lines (Fig. 2). Whereas, irrelevant antibodies had no effect (Fig. 2).

Enumeration of CD4+ lymphocytes by FITC labeled MT4 monoclonal antibody

In order to develop a home made reagent for enumerating CD4+ lymphocytes in blood samples by using the generated CD4 mAb, fluorescent dye (FITC) was conjugated to the MT4 mAb. The FITC labeled MT4 was then used to determine CD4+ lymphocytes in blood samples. For performing CD4+ lymphocyte determination using the home made reagent, Blood sample was incubated with FITC labeled MT4, after that, red blood cells were lysed and the stained cells were analyzed by a flow cytometer with CELLQuest software. By flow cytometric analysis, the lymphocyte population was firstly gated using FSC and SSC (Fig. 3A and 3C). Fluorescent labeled cells in the gated lymphocytes were then determined according to their fluorescence intensity by using FL1 and FSC (Fig. 3B and 3D). The results obtained in this manner were the percentages of CD4+ cells in the lymphocyte population. In some cases, a number of red blood cells were contaminated in the lymphogate (Fig. 3E). Since red blood cells are smaller in size and less fluorescent than lymphocytes (Fig.3E), those contaminated could be gated out by making another gate using FL1 and FSC (Fig. 3F; R2). Then, the percentage of CD4+ cells in the lymphocyte population was re-calculated from the number of cells in the second gated population (Fig. 3F).

By the method mention above, the lymphocyte populations were gated out from other cells according to their size and granularity. It was possible that some non-lymphocytes could have been contaminated in the lymphogate and affect the accuracy of the flow cytometric measurement of CD4+ lymphocytes. To address this question, 40 blood samples (20 healthy and 20 HIV infected persons) were stained with PE labeled CD14 /FITC labeled CD45 mAb and analyzed for non-lymphocytes in the lymphocyte population that had been gated by using FSC and SSC. As shown in Table

2, very few monocytes and granulocytes were detected in the gated lymphocytes. The FACS profile from one donor is shown in Figure 4.

To evaluate the accuracy of the home made reagent, CD4+ lymphocytes from 30 healthy and 30 HIV infected persons were determined by using home made FITC labeled MT4 and standard Simultest™ reagent kit. As shown in Table 3, both percentages and absolute CD4+ lymphocyte counts obtained by both reagents were very similar with no statistics significantly difference. A correlation plot comparing the percentages and absolute number of CD4+ lymphocytes obtained from both methods are shown in Figure 5 and 6, respectively. Linear regression analysis resulted in a slope of 0.971 and an intercept of 0.933 when the percentage of CD4+ lymphocytes from the two methods were compared (Fig.5). The correlation coefficient of the percentage CD4+ lymphocytes obtained from both methods was 0.995 (Fig 5). When the absolute CD4+ lymphocyte counts were compared, linear regression analysis resulted in a slope of 0.996, an intercept of 8.914 and the correlation coefficient obtained from both methods was 0.996 (Fig. 6). These results indicated that home made FITC labeled MT4 reagent can be used to enumerate CD4+ lymphocytes in blood samples in equivalent to those given by the commercial reagent.

DISCUSSION

The absolute number of CD4+ lymphocytes is an important marker for the prognosis and classification of the state of the disease, and monitoring for the therapy of HIV infection[12-19]. CD4+ lymphocyte counts must be monitored every 3-6 months in all HIV-infected persons[11]. The accepted standard method for the enumeration of CD4+ lymphocytes is flow cytometry[20,21]. By this technique, the CD4+ lymphocyte number is the product of three laboratory techniques: the white blood cell count, the percentage of lymphocytes and the percentage of CD4+ lymphocytes. Measuring the percentage of CD4+ lymphocytes is carried out by immunophenotyping and analyzed by a flow cytometer. However, a problem facing the clinical laboratory is that flow cytometry requires very expensive reagents. This technology is, therefore, costly for adaptation as a routine method in laboratories in developing countries. In this part of the world, inexpensive and reliable reagent is urgently needed. To support this requirement, anti-CD4 monoclonal antibody was generated in our laboratory. The generated monoclonal antibody was then conjugated to fluorescein isothiocyanate (FITC) and used as a homa made reagent for enumerating CD4+ lymphocytes.

By using conventional hybridoma technique[22], in this study, a hybridoma producing CD4 specific monoclonal antibody, named MT4, was obtained. The specificity of MT4 mAb was confirmed as it reacted with CD4-DNA transfected COS cells, CD4+ cell lines and CD4+ lymphocytes. Furthermore, MT4 mAb inhibited the binding of standard CD4 monoclonal antibodies to CD4 proteins on CD4+ cells.

The MT4 mAb was then conjugated to FITC by alkaline reaction. The ratio of fluorescein to protein was estimated by measuring the absorbance at 495 nm and 280 nm. In this study, the ratio of 0.6 was obtained and this ratio was in the recommended ratio for the optimal conjugation of FITC to antibody[29].

The home made FITC labeled MT4 was then used to enumerate CD4+ lymphocytes by flow cytometer using CELLQuest software. By flow cytometric analysis, the lymphocyte population was firstly gated according to their size and granularity using FSC and SSC. The percentages of CD4+ lymphocytes in the gated lymphocytes were then determined by using FL1 and FSC parameters. It is known that monocytes also express CD4 molecules on their surface[30]. Therefore, if monocytes were contaminated in the lymphogate, these cells can affect the accuracy of the flow cytometric measurement of CD4+ lymphocytes. The commercial Simultest™ reagent kit, thus, generally employs PE labeled CD14/FITC labeled CD45 (leukoGate) for setting gate around the lymphocyte cluster. It also employs a T lymphocyte specific antibody, CD3 mAb, to discriminate between CD4+ lymphocytes and contaminated monocytes. By using the home made FITC labeled MT4 mAb, however, lymphocytes were gated according only to their size and granularity. To clarify whether non lymphocytes had been contaminated in the lymphocyte population that had been gated by FCS and SSC, 40 blood samples were stained with PE labeled CD14/FITC labeled CD45 and analyzed for monocytes and granulocytes in the lymphogate. It was found that very few monocytes and granulocytes were in the gated lymphocytes. The results indicated that parameters FSC and SSC can be used to gate the lymphocyte population.

In some samples, contamination of red blood cells within the lymphogated was occurred. This contamination could also affect the accuracy of the flow cytometric measurement of CD4+ lymphocytes. To correct this affect, the contaminated red blood cells had to be gated out. According to their size and auto fluorescent, contaminated red blood cells could be easily gated out by making an additional gate using FL1 and FSC. The percentage of CD4+ cells in the lymphocyte population was then re-analyzed from the second gated population.

In this study, CD4+ lymphocytes from a total of 60 blood samples were evaluated by both the home made FITC labeled MT4 reagent and the standard Simultest™ reagent kit. It was concluded that the home made reagent provides results which are equivalent to those given by the commercial Simultest™ reagent kit. A very high degree of correlation between both reagents has been found in both percentage and absolute CD4+ lymphocytes. The results suggest that home made FITC labeled MT4 reagent is an acceptable alternative reagent for monitoring CD4+ lymphocytes in blood samples.

In summary, a home made reagent for determining CD4+ lymphocytes in blood samples by flow cytometry has been developed. This reagent is more cost effective than available commercial reagents. Therefore, it is appropriate for use in measuring CD4+ lymphocytes in either asymptomatic HIV infected persons or AIDS patients. We believe that this home made FITC-labeled MT4 meets the growing demand for CD4 counts, especially in developing countries where HIV prevalence is high.

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Table 1. Percentage of positive cells in peripheral blood lymphocytes determined by staining with MT4 mAb and standard CD4 mAb Leu3a.

Donor	Monoclonal antibody		
No.	MT4	Leu3a	Myeloma control[a]
1	32[b]	32	0
2	37	39	0
3	30	32	0
4	33	33	0
5	24	25	0

[a] Myeloma induced ascitic fluid which was used as negative control.

[b] Percentage of positive cells was determined by flow cytometric analysis.

Table 2. Determination of monocytes, granulocytes and lymphocytes in the lymphogate using FSC and SSC.

Donor[a] no.	% cells in lymphogate Mono.	Gran.	Lymph.	Donor no.	% cells in lymphogate Mono.	Gran.	Lymph.
1	0.3	4.7	95.0	21	0.3	6.5	93.2
2	0.0	6.7	93.3	22	0.9	3.8	95.3
3	0.2	5.8	94.0	23	1.1	3.0	95.9
4	0.1	4.4	95.5	24	0.8	4.1	95.1
5	0.2	3.6	96.2	25	1.1	2.5	96.4
6	0.2	4.7	95.1	26	0.8	4.4	94.8
7	0.2	5.9	93.8	27	0.8	3.1	96.1
8	0.1	7.4	92.5	28	1.0	5.0	94.0
9	0.1	6.7	93.1	29	0.5	4.7	94.8
10	2.0	4.2	93.8	30	0.4	7.1	92.5
11	0.3	5.9	93.7	31	1.0	2.4	96.5
12	0.5	4.0	95.5	32	0.2	3.3	96.5
13	0.6	7.3	92.1	33	1.0	6.0	93.0
14	0.9	4.9	94.2	34	0.4	2.2	97.4
15	1.2	3.4	95.3	35	1.0	5.2	93.8
16	0.8	4.9	94.2	36	0.5	5.5	94.0
17	0.2	5.3	94.5	37	0.6	3.4	96.0
18	1.1	5.2	93.6	38	0.2	6.4	93.4
19	0.1	2.9	97.0	39	1.2	3.8	95.0
20	0.5	5.1	94.4	40	0.9	4.8	94.3

[a] Donor no. 1-20 were healthy donors. ; Donor no. 21-40 were HIV infected donors.

Table 3. Percentages and absolute CD4+ lymphocyte counts determined by home made FITC labeled MT4 and Simultest^{TM} reagent kit.

Donor	%CD4+ lymph[c]		Abs. CD4+ lymph[d]		Donor	%CD4+ lymph		Abs. CD4+ lymph	
no.	Simul.[b]	MT4[b]	Simul.	MT4	no.	Simul.	MT4	Simul.	MT4
H1[a]	23	23	441	441	N1[a]	33	32	1433	1390
H2	24	26	507	549	N2	35	34	947	920
H3	36	35	661	643	N3	35	35	1062	1062
H4	24	27	513	578	N4	32	31	594	575
H5	16	17	256	272	N5	28	28	902	902
H6	13	15	346	400	N6	34	32	993	934
H7	17	17	371	371	N7	32	32	737	737
H8	15	15	322	322	N8	32	34	609	647
H9	19	22	512	593	N9	39	39	1024	1024
H10	3	4	18	23	N10	24	24	504	504
H11	22	22	779	779	N11	40	37	963	891
H12	25	27	378	408	N12	42	40	816	777
H13	7	7	101	101	N13	39	36	888	820
H14	12	13	277	277	N14	29	29	1107	1107
H15	1	1	5	5	N15	35	35	735	735
H16	23	24	883	921	N16	28	26	908	843
H17	11	12	721	786	N17	45	46	1035	1058
H18	1	1	17	17	N18	47	47	954	934
H19	22	23	408	427	N19	32	32	730	730
H20	12	11	297	272	N20	36	37	647	665
H21	4	5	73	91	N21	32	35	860	941
H22	3	2	25	27	N22	38	39	1098	1127
H23	1	1	6	6	N23	33	35	1200	1273
H24	1	1	8	8	N24	35	35	635	635
H25	22	23	409	428	N25	44	41	687	640
H26	38	37	717	699	N26	36	36	1109	1109
H27	15	15	151	151	N27	24	24	714	714
H28	11	13	257	304	N28	30	32	744	793
H29	10	11	192	212	N29	42	41	1005	982
H30	0	0	0	0	N30	28	30	1540	1650
Mean	14.37	15.00	321.70	337.03	Mean	34.63	34.47	906.00	903.97
SD	10.30	10.48	258.66	268.27	SD	5.85	5.54	239.41	251.21

[a] Donor H1-30 were HIV infected donors. ; Donor N1-30 were healthy donors.

[b] Simul.; Simultest^{TM} reagent kit / MT4; home made FITC labeled MT4

[c] %CD4+ lymphocytes

[d] Absolute CD4+ lymphocyte count (cell/cu.mm.)

Figure legends:

Fig. 1. Inhibition of standard CD4 mAb binding to lymphocytes by MT4 mAb. Peripheral blood lymphocytes were pre-incubated with MT4 mAb (B and D) or without mAb (A and C). PE-labeled CD4 mAb Leu3a (A and B) or FITC labeled CD3 mAb Leu4 was added to the pre-stained cells. The membrane fluorescence intensity was analyzed by flow cytometry.

Fig. 2. Inhibition of standard CD4 mAb binding to CD4+ cell lines by MT4 mAb. Molt4 (A-C) or Sup T1 (D-F) were pre-incubated with MT4 mAb (B and E), M6 mAb (C and F) or without mAb (A and D). PE-labeled CD4 mAb Leu3a was added to the pre-stained cells and the membrane fluorescence intensity was analyzed by flow cytometry.

Fig. 3. Flow cytometric analysis of CD4+ lymphocytes using FITC labeled MT4 mAb. Lymphocytes in the blood samples were gated according to FSC and SSC (A and C; gate R1). The percentages of CD4+ lymphocytes were determined by FL1 and FSC (B and D). In the case of red blood cell contamination (E), the contaminated red blood cells were gated out according to FL1 and FSC (F; gate R2). Samples in A, B and C, D were taken from healthy and AIDS patient, respectively. The percentage of CD4+ lymphocytes detected in each sample was indicated.

Fig. 4. Determination of monocytes and granulocytes in the lymphocytes gated by using FSC and SSC. Lymphocytes in the blood samples were gated according to FSC and SSC (A). The gated cells were analyzed for bright CD45+ lymphocytes, weak CD45+ granulocytes and CD14+ monocytes according to their fluorescent reactivity (B). The percentage of cells in each quadrant was indicated.

Fig. 5. Scattergram of percentage of CD4+ lymphocytes from 60 blood samples as determined by home made FITC labeled MT4 and commercial Simultest™ reagent.

Fig. 6. Scattergram of absolute CD4+ lymphocyte counts from 60 blood samples as determined by home made FITC labeled MT4 and commercial Simultest™ reagent.

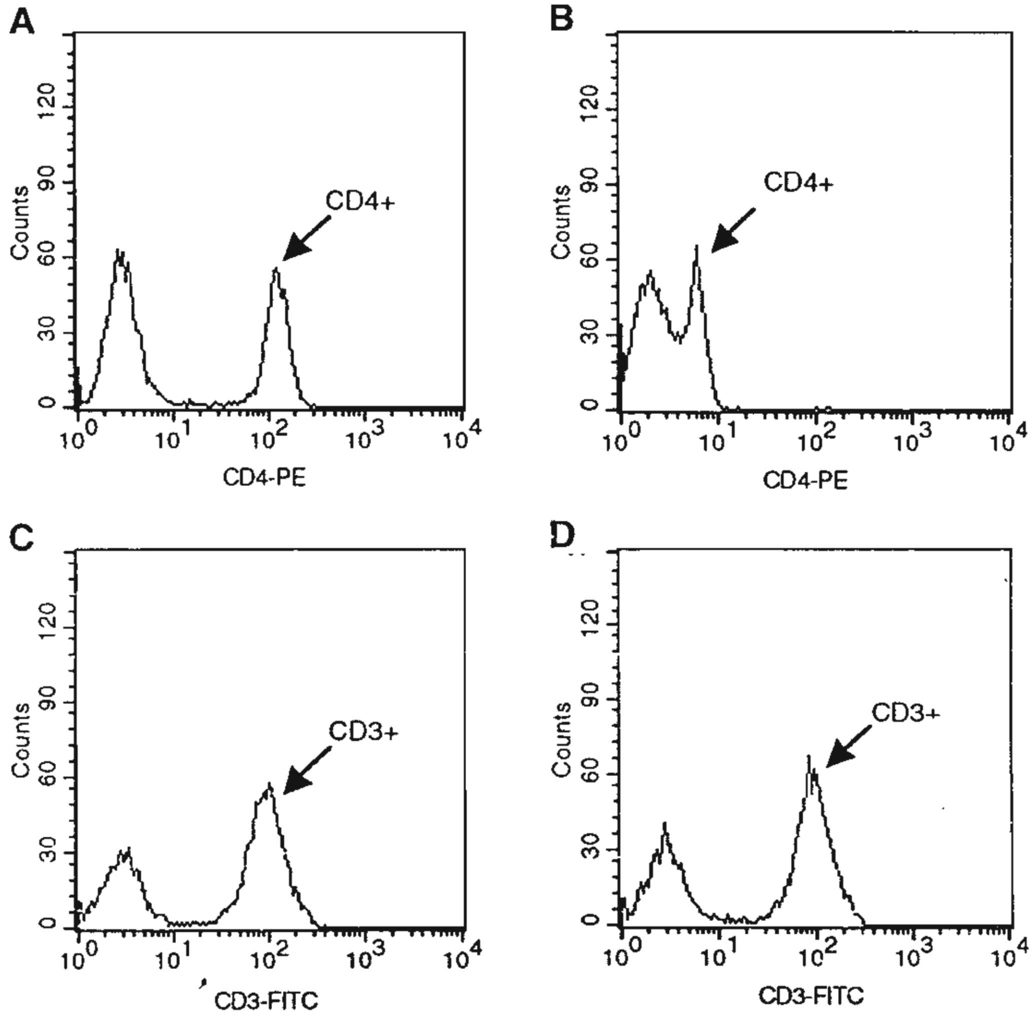
A
B
C
D
Counts
CD4+
CD3+
CD4-PE
CD3-FITC
0
30
60
90
120
10^0
10^1
10^2
10^3
10^4

Fig 1

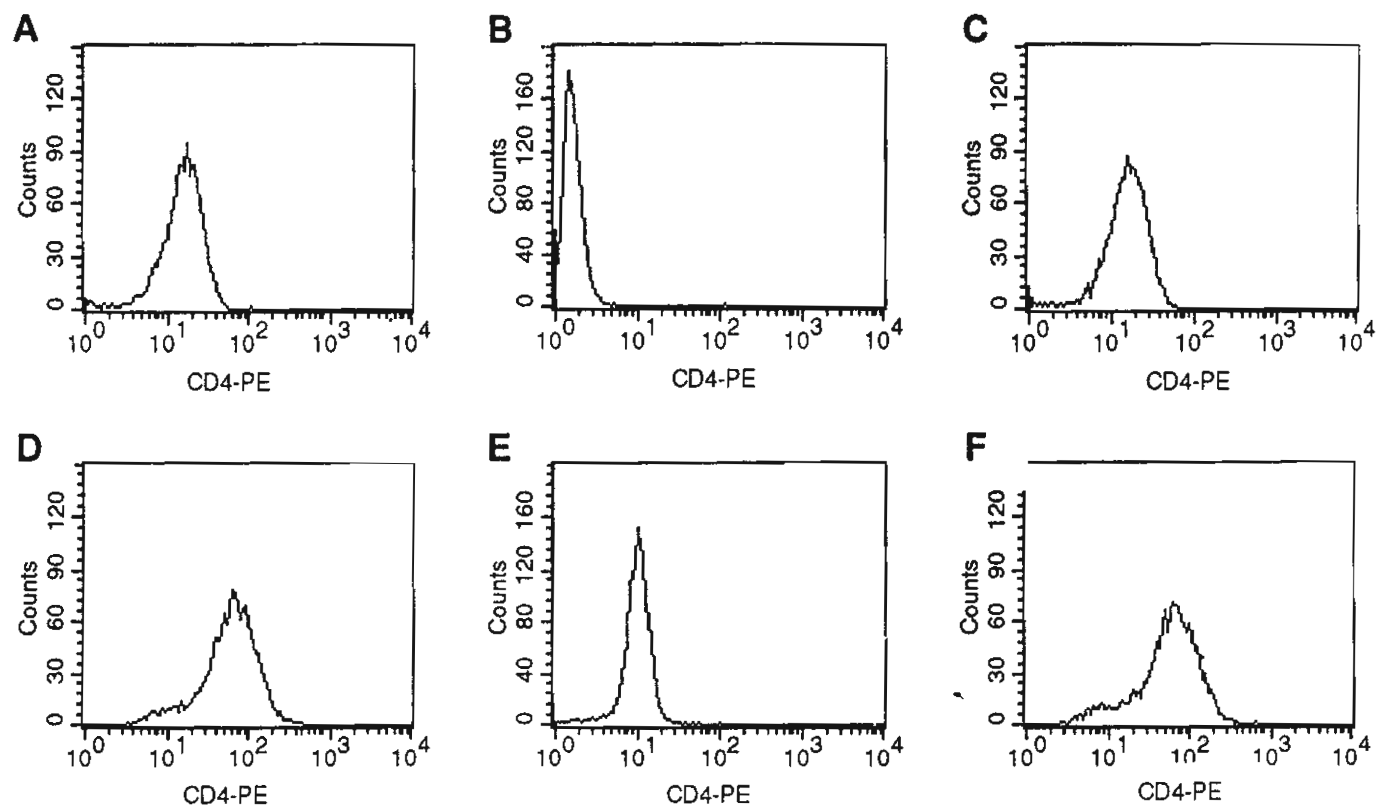

Fig. 2

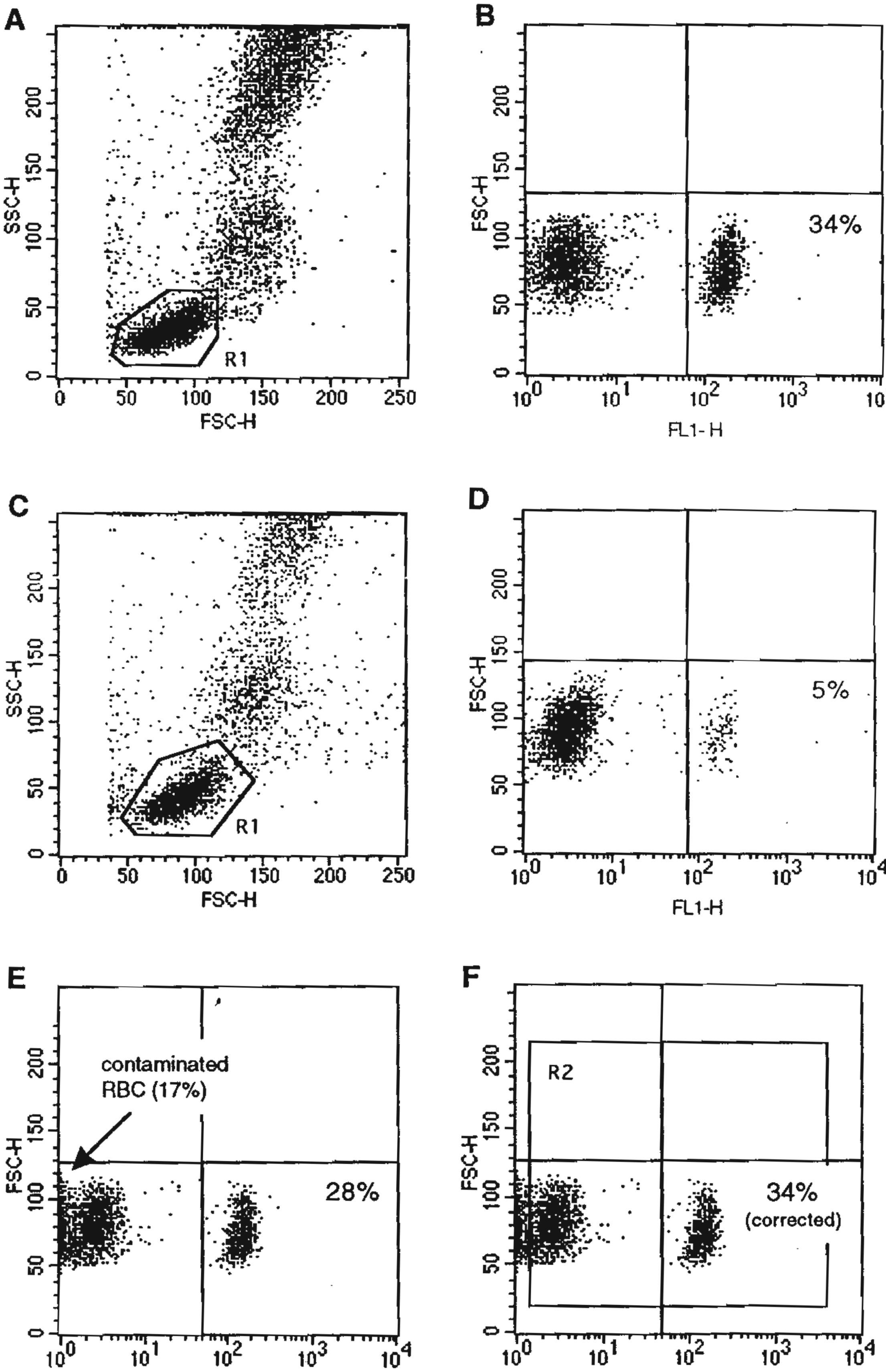
A
B
C
D
E
F
SSC-H
FSC-H
FL1-H
R1
R2
34%
5%
28%
contaminated
RBC (17%)
34%
(corrected)

Fig. 3

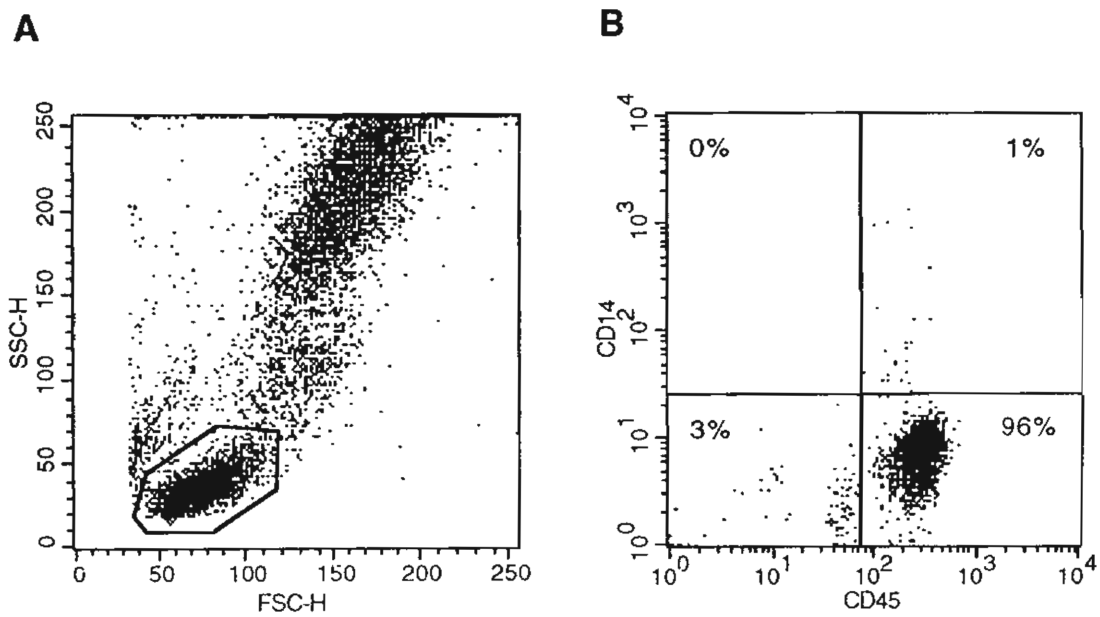

Fig. 4

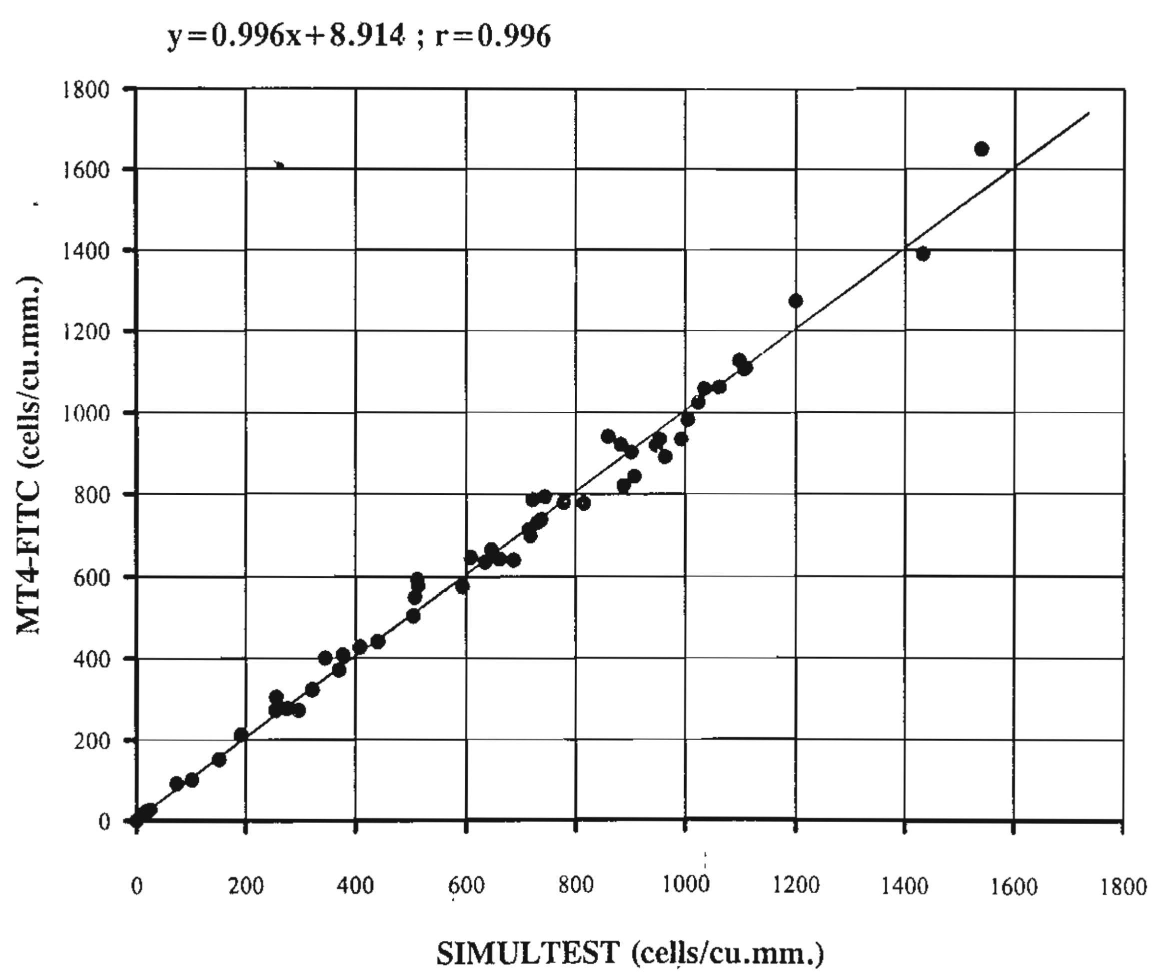
y=0.996x+8.914 ; r=0.996
MT4-FITC (cells/cu.mm.)
1800
1600
1400
1200
1000
800
600
400
200
0
0
200
400
600
800
1000
1200
1400
1600
1800
SIMULTEST (cells/cu.mm.)

Fig. 6