CD4 T Lymphocytes Enumeration Techniques

Report of a Regional Workshop
Bangkok, Thailand, 24-27 November 2003

WHO Project: ICP BCT 001

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1. INTRODUCTION

The AIDS epidemic continues to spread in the South-East Asia Region (SEAR), which is the second most affected Region in the world after sub-Saharan Africa. To date, close to 40 million people throughout the world have been infected with human immunodeficiency virus (HIV), the virus that causes AIDS. Of these, almost 6 million are in the SEA Region. Estimated population prevalence rates per 100,000 population range from less than 1 in DPR Korea to over 1200 in Thailand. Over 99% of cases have been reported from three countries, Thailand, India and Myanmar.

The epidemic has affected persons in every country in the world. The majority of persons living with HIV infection and AIDS are found in the developing world where resources are poor and access to care and new treatments is limited. Despite the high prevalence rates, care for persons with HIV infection living in these countries was not given high priority, as a result of the high burden of disease due to other illnesses. In areas where the epidemic is most severe, the provision of care has been further compromised by the rapidly weakening economies and the costs of effective medications. Realizing the importance of anti-retroviral therapy, WHO has initiated various steps, the most notable being the commitment by the Director-General that within the next five years, at least 3 million HIV patients from developing countries shall be provided antiretroviral therapy (ART) by 2005 (“3 by 5” initiative).

The Global Fund for AIDS, Tuberculosis and Malaria has also identified ART therapy as one of the priority areas. The Regional Office is focusing attention on increasing access to treatment with antiretroviral drugs in preventing mother-to-child transmission and in the care of infected persons. With huge efforts being initiated in providing ART, a close monitoring of the patient who is on these drugs is also essential to ascertain the response. Enumeration of CD4 T lymphocytes is the most sensitive and specific indicator for this assessment. This indicator, together with the estimation of viral load constitutes two universally accepted monitoring tools. Since viral load has not been recommended by WHO, capacity building in CD4 T lymphocytes estimation is of great relevance.
The progressive depletion of CD4 T lymphocytes is the cardinal event in the pathogenesis of infection by the human immunodeficiency virus-1 (HIV). The number of these cells in the peripheral blood is the single most important parameter for monitoring the disease associated with HIV infection. Quantification of CD4 T lymphocytes is important in the staging and monitoring of patients infected with HIV. Throughout the course of the disease, the total T cell levels remain fairly constant despite a fall in CD4 T lymphocyte count, due to a concomitant rise in CD8 T lymphocytes. Therefore, the ratio of CD4 T lymphocytes to CD8 T lymphocytes is an additional important measure of disease progression. Measurement of lymphocyte subsets is done by flow cytometry which is the gold standard for enumeration of CD4 T lymphocytes. CD4 T lymphocytes enumeration is also utilized as a surrogate marker for HIV-induced damage; single CD4 T lymphocytes counts are used to assess the degree of immune deterioration; while repeated CD4 T lymphocytes tests define a declining slope of CD4 T lymphocytes counts, indicating the speed of progression towards AIDS. While on therapy, improvement in CD4 T lymphocytes counts is indicative of the success of therapy. In resource-poor countries, with the arrival of generic drugs for anti-retroviral therapy, the need for CD4 T lymphocytes counts has dramatically increased. CD4 T lymphocytes counts are the criteria for initiating ART as well as monitoring the therapeutic response in a patient.

Accordingly, a hands-on training course was organized at Siriraj Hospital, Bangkok, Thailand, from 24 to 27 November 2003 for enumeration of CD4 T lymphocytes through flow cytometry for those countries of the SEA Region where ART is expected to be taken up in a big way in the near future to facilitate effective utilization of scaled-up ART intervention. The detailed programme of the Workshop is placed as Annex 1. Fourteen participants from Bangladesh, India, Indonesia, Myanmar, Nepal, Sri Lanka and Thailand attended this Workshop. Five experts from India, Thailand and WHO facilitated the Workshop (see (1) List of Participants and (2) Programme at Annexes 1 and 2).

2. OBJECTIVES

The objectives of the workshop were as follows:

(1) To review the status of infrastructure available for enumeration of CD4 T lymphocytes in selected Member Countries;
(2) To provide hands-on training on flow cytometry including the interpretation of results;
(3) To discuss how to select and purchase the most appropriate technology;
(4) To provide guidance on quality assurance, maintenance and biosafety aspects; and
(5) To discuss the follow-up of strengthening national capacities for CD4 T lymphocytes estimation.

3. INAUGURAL SESSION

The workshop was inaugurated by Prof. Piyasakol Sakolsatayatorn, Dean of the Siriraj Hospital. Dr Jai Narain, Coordinator, HIV/AIDS & TB, WHO SEARO, gave a brief introduction to the WHO '3X5' strategy and stressed the need for building laboratory capacity in the Region for meeting the needs of the HIV/AIDS patients who are likely to receive Anti-Retroviral Therapy (ART). Dr Sudarshan Kumari, Regional Adviser, Blood Safety and Clinical Technology, WHO SEARO, welcomed all the participants and outlined the objectives of the workshop.

The technical session was chaired by Dr Suniti Solomon, YRG Care, Chennai, India. It began with a presentation by Dr Jai Narain, who detailed the WHO 3X5 strategy as well as the current scenario of HIV/AIDS problem in the Member Countries of the SEARO region. He also provided the current scenario of ART in the SEARO Region and highlighted the gaps that existed currently between those in need of ART vis a vis those who were actually taking ART. In order to bridge this gap, one of the requirements was to build laboratory capacity in terms of CD4 enumeration technology. Dr Gaby Vercauteren, BCT/HTP, WHO/HQ, Geneva, presented an overview of WHO-related CD4 activities. She outlined the various testing methods recommended by WHO for HIV testing, and CD4 enumeration. She also informed that WHO was in the process of developing uniform guidelines for CD testing across the world and stated that the draft guidelines would be posted on the Internet in January 2004 and requested all participants to view them and make suggestions, if any. She also stated that WHO was in the process of formulating an External Quality Assessment programme for CD4 estimation and making available reference reagents for this purpose to national reference laboratories in various Member Countries.
Country reports based on the WHO questionnaire were presented by Mrs Sirirat Likanonsakul from Thailand, Dr Ravi and Dr Balakrishnan from India and Dr Sondang Maryutka Sirait from Indonesia. It was observed that there was a need to evolve guidelines and/or Standard Operating procedures for CD4 testing in the Region, especially for specimen collection and transport.

Dr Vercautern, WHO/HQ, Geneva, made a presentation on the need for CD4 estimation in HIV/AIDS and the relevance of CD4 T cells in the pathogenesis of HIV/AIDS. This was followed by a presentation by Dr Kovit Pattanapanyasat of Siriraj Hospital, Bangkok, on the principles of flowcytometry, wherein he discussed the various physical principles involved in the technique of flowcytometry such as forward and side scattering of light; the basis of distinguishing the three cell populations viz. lymphocytes, monocytes and granulocytes; the principles of gating cells, the various types of flowcytometry such as single platform measurements using reference beads as controls; double and triple staining procedures, and their advantages and disadvantages. He also presented the various strategies for gating cells and the principles involved in selection of reagents and techniques for CD4 enumeration.

Dr. Sathien of Siriraj Hospital, Bangkok, made a presentation on haematology analyses: total leucocyte count, differentials and total lymphocyte counts. He explained the two principles of cell counting used in automated machines (electrical impedance and light scattering), the coefficient of variation between manual and automated methods, the principles of differential leucocyte enumeration (three part differential staining and the five part staining), and the factors that contribute to variation in differentials. Dr Sathien also explained the problems associated with automated differential counting such as lack of EQAS etc. Three part differential methods are considered adequate for use in the countries of the Region as the five part differential did not give any great advantage.

Dr Punneporn Wacinripee of Siriraj Hospital, made a presentation wherein she discussed the principles, procedures, limitations and quality control issues involved in the FACS count technology. Although FACS Count provides absolute CD8 values and CD4/CD8 ratios, these two parameters do not add great value to clinicians in the management of HIV/AIDS patients. It is the absolute CD4 values that are used by clinicians. On the contrary, CD8
measurements only add to more costs of the assay. Therefore, it was felt that manufacturers should either reduce the cost of reagents, or do away with the CD8 tube and reagents to bring down the cost.

Dr Kovit of Siriraj Hospital, Bangkok, presented the findings of comparative evaluations of FACS scan and FACS count in Thailand and stated that there was excellent correlation between the two systems. He also presented the comparative evaluation of FACS scan, FaCS count and the single platform cyflow method. The Single platform Cyflow method showed good correlation with FACS scan and FACS count systems.

Two brief presentation were made by Dr Vijay Lakshmi of Nizam Institute of Medical Sciences from Hyderabad and Dr Balkrishnan from the Voluntary Health Service Hospital, Chennai on their experience of using FACS Count method in India. Dr Balakrishnan also presented the comparative evaluation of Coulter cytosphere method, which is a non-flowcytometric assay and uses a simple microscope. This assay showed good correlation with FACS count results but within a limited range of CD4 counts especially in the <200 cell range. He also highlighted the advantages (low investment cost) and limitations of this technique.

The participants attended a laboratory demonstration of four methods – FACS Count staining method, FACS scan staining method, the Coulter cytosphere method and the single platform cyflow method. They were shown how these methods were used, results obtained and interpreted as well as required quality control procedures.

Dr Ilesh Jani, Mozambique, Africa, delivered two talks on calibration procedures for flow cytometry and the concept of panleucogating. He also summarized the various practical considerations required for panleucogating.

Two group assignments on haematology and flowcytometry were undertaken by participants. Both groups presented their answers to the questions provided in the exercise. Dr Jani pointed out that the majority of the answers were correct, except that both the groups missed out the importance of pipetting errors contributing to the variations in the results pointed out in the exercise. He made a presentation on Quality Control (QC) and Quality Assurance (QA) in CD4 testing and long and short term stabilization of blood in CD4+ T cell counting. In the QC/QA lecture, he outlined the definitions and concepts related to quality and discussed two
major aspects of quality control - related to the instrument as well as the procedures. In the presentation on the use of stabilized whole blood, he outlined the need for stabilization, the use of these cells in quality control of CD4 testing and also the advantages and limitations of the short term and long term stabilization procedures. This was followed by a presentation on gating strategies and their comparative evaluation of non flow cytometric methods such as Dyna dynabeads and Coulter cytospheres.

Group work was also undertaken on how to select and purchase the most appropriate technology for CD4 enumeration. The two groups presented the various criteria to be considered for choosing a particular technology. Dr Gaby, WHO, Geneva, who facilitated the group work, pointed out that one of the most important criteria was the end use needs assessment and the policymaker’s considerations in choosing an appropriate technology. Dr Kovit of Siriraj Hospital, Thailand, made two presentations on the need for EQAS in CD4 testing and the Thailand experience on running an EQAS programme for CD4 testing for the past three years. He pointed the various advantages and limitations of running an EQAS programme and valuable lessons learnt in carrying out this exercise. Dr Kovit also presented a summary of practical issues in the management of immunophenotyping methods such as laboratory design, equipment maintenance, staffing pattern and laboratory QC/QA issues. Mr Pavan Behl of Guava Technologies, New Delhi, India, presented a new technology for CD4 estimation called the micro capillary flowthrough cytometry. He highlighted on the low cost and the novelty of this new technology which was currently being validated at several centres.

Dr Jani briefed participants on the statistical methods used for comparison. He explained the difference between correlation and agreement between two technologies and explained the limitations of using correlation coefficients, r values and p values. He subsequently detailed the currently available appropriate methods of agreement analysis and discussed the advantages of this new approach. Dr Gaby stressed on the importance and need for safety in the laboratory, the six safety considerations in any laboratory. He also underlined the need for evolving a safety policy for every laboratory and also fixing responsibility for safety by appointing a safety officer in the lab. Dr Gaby and Dr Kovit subsequently spoke of the importance of equipment maintenance, especially the aspects pertaining to the preventive maintenance.
The participants were given a group assignment to identify the constraints and the needs in their respective nations which were currently impeding the process of implementing quality testing in CD4 estimations and formulated recommendations.

4. CONCLUDING SESSION

The workshop ended with a concluding ceremony where the participants thanked the WHO for providing a very useful learning experience at the workshop and Dr Kovit and his team from Siriraj Hospital for their excellent arrangements and hospitality.

5. RECOMMENDATIONS

For Member Countries

(1) SOPs should be developed and made available for the various CD4 technologies available currently.

(2) A national EQAS programme for CD4 testing centres should be set up.

(3) A concrete mechanism should be formulated for procurement of appropriate CD4 technologies based on their needs.

(4) A needs assessment should be carried out by laboratory experts to assess the existing capacity in the Region and identify laboratory infrastructure support required to meet the key elements of the WHO "3X5" strategy.

(5) With anti-retroviral therapy likely to be free of cost to HIV/AIDS patients, all Member Countries should work out a strategy with their respective national HIV/AIDS programme managers to ensure that CD4 testing also would be free of charge.

For WHO

(1) WHO should assess the need for laboratory capacity including for CD4 enumeration and quality assurance within the context of WHO-led "3 by 5" initiative in the Region.

(2) WHO should prepare a training plan on the basis of the need assessment in order to assist in rolling out ART in the SEA Region.
(3) WHO should ensure capacity at the regional level to provide technical support on laboratory aspects of “3 by 5” initiative by including in the resource mobilization plan a staff with laboratory background and the requirements for laboratory capacity building at country level as stated in # 2 above. (Action: HIV/AIDS & TB)

(4) WHO should develop uniform guidelines for the collection and transport of specimens for CD4 testing for use by all Member Countries in the SEA Region.
Annex 1

LIST OF PARTICIPANTS

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Dr Jai Narain
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WHO, SEAR, New Delhi
**Annex 2**

**PROGRAMME**

**Monday, 24 November 2003**

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<td>0830-0900 hrs</td>
<td>Registration</td>
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<td>0900-0930 hrs</td>
<td>Official opening ceremony</td>
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<td>1000-1015 hrs</td>
<td>Objectives</td>
<td>Dr Kumari</td>
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<td>1015-1030 hrs</td>
<td>Overview of 3 x 5 strategy on ARV Therapy</td>
<td>Dr Jai Narain</td>
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<td>1030-1045 hrs</td>
<td>Overview of the WHO CD4 related activities</td>
<td>Dr Vercauteren</td>
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<tr>
<td>1045-1130 hrs</td>
<td>Country reports:</td>
<td>All participants</td>
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<tr>
<td></td>
<td>• India</td>
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<td>1130-1145 hrs</td>
<td>Discussion</td>
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<td>1145-1200 hrs</td>
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<td>Participants</td>
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<td>1200-1245 hrs</td>
<td>The role of CD4 T cell counting in HIV/AIDS</td>
<td>Dr Vercauteren</td>
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<td>1245-1300 hrs</td>
<td>Discussion</td>
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<td>1400-1445 hrs</td>
<td>Overview of CD4 T cell enumeration technologies</td>
<td>Dr Jani</td>
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<td>1445-1530</td>
<td>Principles of flow cytometry</td>
<td>Dr Pattanapanyasat</td>
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<td>1530-1600 hrs</td>
<td>Discussion</td>
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<td>1615-1700 hrs</td>
<td>Gating strategies and selection of reagents related to lymphocyte subset counting</td>
<td>Dr Pattanapanyasat</td>
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<td>1700-1730 hrs</td>
<td>Discussion</td>
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1730-1745 hrs  Review of day 1

**Tuesday, 25 November 2003**

0900-0920 hrs  Flow cytometer calibration  Dr Jani
0920-0930 hrs  Discussion
0930-1015 hrs  Haematology analysers: total leucocyte, lymphocyte counts; differentials  Dr Sathien Sukpanichnant
1015-1030 hrs  Discussion
1045-1130 hrs  The concept of PanLeucogating  Dr Jani
1130-1300 hrs  Tour of the Institute

Practical exercises

1400-1545 hrs  Flow cytometry: group 1  Dr Pattanapanyasat
               Alternative technologies: group 2  Indian Expert
1600-1745 hrs  Alternative technologies: group 1  Indian Expert
               Flow cytometry: group 2

**Wednesday, 26 November 2003**

0830-0900 hrs  Review of practical experiments
0900-0945 hrs  Group work: paper exercises on haematology and flow cytometry  Dr Jani
0940-1015 hrs  Presentation of group work and  Dr Jani
1015-1030 hrs  Discussion
1045-1145 hrs  Group work: How to select and purchase the most appropriate technology  All facilitators
1145-1230 hrs  Presentation of group work discussion
1400-1500 hrs  Quality assurance: How to produce reliable results  Dr Jani
1500-1515 hrs  Discussion
1515-1545 hrs  Role of stabilizing reagents  Dr Jani
1600-1630 hrs  External quality assessment schemes  Dr Pattanapanyasat
1630-1700 hrs  Maintenance of equipment in the immunophenotyping laboratory  Dr Pattanapanyasat
1700-1715 hrs Discussion
1715-1730 hrs Review of day 3

Thursday, 27 November 2003

0900-0945 hrs Method comparison Dr Jani
0945-1000 hrs Discussion
1015-1045 hrs Safety in the immunophenotyping laboratory Dr Vercauteren
1045-1100 hrs Discussion
1100-1145 hrs Group work: identify the main problems/solutions in running a CD4 counting service
1145-1230 hrs Presentation group work and discussion
1230-1300 hrs Practical issues in the management of an immunophenotyping laboratory Dr Pattanapanyasat
1400-1415 hrs Post workshop test
1415-1530 hrs Recommendations
1530-1600 hrs Closure