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Technical Report 21

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THAILAND

APPLICATION ON MONOCLONAL ANTIBODIES FOR PROGESTERONE MEASUREMENT

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APPLICATION ON MONOCLONAL ANTIBODIES FOR PROGESTERONE MEASUREMENT

Report prepared for
the Government of the Republic of Thailand

by

the International Atomic Energy Agency
acting as Executing Agency for
the United Nations Development Programme

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Report Title: **APPLICATION OF MONOCLONAL ANTIBODIES FOR PROGESTERONE MEASUREMENT**

Ref: **IAEA 544 THA/5/031-053**
Task No. BL 11-05/053

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Counterpart: **Professor M. Kamonpatana**
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Date of visit: **20/07/91 - 09/08/91**

Duties

1. Provide instructions on the maintenance of hybridoma cell lines, their culture and harvesting of monoclonal antibodies.

2. Assist the counterparts to develop work plans for using monoclonal antibodies in RIA for measuring progesterone.

3. Assess the need for and feasibility of establishing a laboratory for producing monoclonal antibodies against progesterone.

ACTIVITIES AND REMARKS RELATING TO DUTIES OF MISSION

My visit to Chulalongkorn University took place after the completion of the UNDP/IAEA Project 'Improving Food and Animal Production' (THA/5/031), and at a time when plans towards a new project submission were being formulated by the original participants and other relevant parties. Under such circumstances, and after the thorough review of the project by consultant Dr J. Vercoe, it would not be relevant to me to record any comments constituting an "assessment of the status of project implementation".

Appendix 1 contains a schedule of my activities during my visit.

DUTY 1

I gave two two-hour lectures to the staff of the Biochemistry Unit and other laboratories in the Faculty of Veterinary Medicine. the first of these concerned the structure of antibody molecules, the basis of antibody specificity in terms of the molecular structure of the antibody-binding site (this paid particular attention to the recently solved structure of the binding site of DB3, a monoclonal antibody specific for progesterone); and the theory and practice of immunisation. The second lecture dealt with the details of the derivation of antibody-secreting hybridoma cell lines in tissue culture and a survey of current biotechnological methods for the manipulation and exploitation of both the antigen-binding (Fab) and the constant (Fc) regions of antibody molecules.

During my stay I also spent time with Ms. Kitiya Srisakvattana maintaining and assessing the growth of two plasmacytoma cell lines (one mouse line, one rat line) in the conditions of the facilities of the Biochemistry Unit. This served as an assessment of both the equipment and the prevalence of potential contaminating microbes in those laboratories. We also used the equipment available to freeze these cells for storage in liquid nitrogen, and later on we thawed them again to check the success of the procedures. With Ms. Srisakvattana I also undertook a detailed examination of the availability of equipment and facilities appropriate to different aspects of monoclonal antibody derivation and production (see DUTY 3, below).

DUTY 2

During her stay in the laboratory in Cambridge (under IAEA and British Council funding) Ms. Srisakvattana had derived a mouse monoclonal antibody (mAb) against progesterone (called AFRC MAC 296 or KS2/239.308.11) of good specificity relative to related steroids, and of reasonable sensitivity. In Bangkok Ms. Sirima Sravasi has been assessing the performance of this mAb for measurement by ELISA of progesterone in milk and

plasma samples from cattle and buffalo. Although this antibody has performed well on milk samples from cattle, there have been difficulties in the other cases. The failures with buffalo are not surprising since these animals have substantially lower progesterone levels than cattle; the results with cattle plasma are encouraging but the sample variances are too high. We discussed potential improvements to the assay of bovine samples, including alternative procedures for "blocking" to improve background values. We also discussed other strategies to improve assay sensitivity. The present ELISA method demands that progesterone in the sample should compete with progesterone conjugated to bovine serum albumin (BSA) coated to the plastic wells of a microtitre plate. I suggested that the bivalency of an IgG antibody such as MAC 296 might pose a limitation to assay sensitivity, since both binding arms need to be competed-off for displacement to be measured. Assay sensitivity would theoretically be improved, either by the use of monovalent Fab fragments of MAC 296, or (more radically) coating the wells with purified MAC 296 and measuring competition between progesterone and a progesterone-enzyme conjugate. It was agreed that these modifications could be investigated, though I suspect that the improvements would not bring the assay with this mAb into the range required for buffalo samples.

Professor Kamonpatana and I discussed the general problem of assay sensitivity using mAbs compared with polyclonal antisera. It is evident that at present the most sensitive reagents available for the assay of steroid hormones are polyclonal antisera. These do derive, however, from carefully selected bleeds, usually from groups of rabbits of which only one or two respond appropriately to the immunogen. In my experience (and I think this is likely to be the general case) monoclonal antibodies are usually derived from mice which have been immunised but not subjected to the same criteria of selection as is imposed on the bleeds from rabbits. In my opinion mAbs as sensitive as polyclonal reagents, with the attendant advantages of permanence of supply, reproducibility etc., will be generated once a strict selection protocol is established at the immunisation stage. Rats, from which blood samples are more easily obtained than mice, and which are a good species for mAb production, might represent the best animals for such an approach.

DUTY 3

(a) The need for a monoclonal laboratory. In my opinion the establishment of basic monoclonal antibody technology in the Faculty of Veterinary Medicine is desirable. Professor Kamonpatana's previous successes in establishing new methodologies (see Vercoe report) indicate that the Biochemistry Unit is an appropriate place for such an undertaking. The initial need is for local production of the antibody MAC 296 (described above) for use in the progesterone ELISA. If this was the sole requirement then it is clear that

contract production elsewhere would be in order. There are, however, grounds on which to argue that an extension of the anti-steroid mAb work would be highly beneficial. An example is the need for more sensitive progesterone assays in relation to the buffalo as discussed above (DUTY 2). The establishment of a permanently available monoclonal reagent would be of great practical value, in the face of long-term uncertainty of supply. Similarly there is a perceived need to establish mAb based ELISA assays for further steroids such as testosterone and oestrone sulphate. It is my view that once a diversity of mAb requirements develops it becomes of advantage to produce antibody locally, especially in fields of study where purchase prices are high and protective attitudes are common.

There are further aspects to 'need'. I gained the impression that beyond the Biochemistry Unit there were laboratories in the Faculty of Veterinary Medicine which would benefit from the presence of monoclonal antibody technology for the development of assays, eg. in studies of various parasitic diseases (see also below).

The feasibility of a monoclonal antibody laboratory

I believe that the establishment of a monoclonal antibody laboratory is feasible. To my mind there are three elements required for success: (i) the provision of adequate laboratory space and equipment; (ii) the achievement of technical proficiency in the various aspects of the technology; (iii) the presence of a "fund of knowledge" to problem-solve and guide specific work projects.

In relation to (i). The laboratory space in which Ms. Srisakwattana and I carried out our trial culture experiments was certainly adequate and our trials of maintenance and freezing/thawing of cell lines were successful. Equipment requirements are dealt with in Appendix 2. One particular issue, animal supply and accommodation, I will return to below.

In relation to (ii) and (iii). There are several aspects of mAb technology: immunisation; tissue culture; immunochemistry. In her training in the UK Ms. Srisakwattana has achieved considerable technical proficiency in tissue culture and has begun to get to grips with immunochemistry. Opportunities for training of this sort, eg. in Europe, North America, Japan, Australia, New Zealand, are certainly the best approach as long as there are only few trained workers in Thailand. The so called "fund of knowledge" I referred to above is a more knotty problem. In my own experience with monoclonal antibody technology the free exchange of technical information and ideas with colleagues in other institutes or facilities has been crucial to the successful establishment of procedures. I understand that this process has, in the past, been somewhat alien to attitudes in Bangkok. Perhaps this problem can be overcome, especially by scientists who have

been trained abroad. I do feel that there is an opportunity for successful and fruitful establishment of monoclonal antibody technology if workers in different establishments (eg. Faculty of Veterinary Science; Departments of the Medical School, and the forthcoming Institute of Biotechnology) can manage to collaborate.

The above opinions on feasibility relate to the aspects of mAb technology involving tissue culture work and immunochemistry/protein biochemistry. The stage of the work involving immunisation of mice or rats prior to the generation of novel hybridomas is a separate problem. The animal holding facilities I saw were inadequate. I understand from discussion with Professor Songkram Luangtongkum (Dean of the Faculty of Veterinary Science) that plans for improved small animal facilities are being developed. I must stress the importance of this aspect: mycoplasma derived from poorly maintained rats and mice can cause great or even total loss of efficiency in hybridoma work. I recommend that the Faculty of Veterinary Science strive for the highest possible standards of small animal care in line with practices in the 'developed' world. Indeed my affirmative opinion on the 'feasibility' of the full establishment of monoclonal antibody technology, ie. including de novo generation of original hybridomas, is contingent upon the establishment of adequate small animal facilities.

Continuing on the issue of animals, in order to reduce small animal use to a practical minimum, I would counsel against the use of animals for the medium-to-large-scale production of mAbs in the form of ascitic fluid, and for the pursuit of in vitro production methods[*]. I would also prefer the use of an in vitro cell line as a "feeder" cell rather then peritoneal cells from mice, if a suitable cell line can be identified. If these suggestions are followed then small animal holding could be reduced to just those animals used for immunisation and the selection of a good immune spleen donor. It would be ideal if these animals were purchased as high grade stock from a quality supplier. In this way local breeding would be unnecessary and gradual accumulation of rodent pathogens avoided.

CONCLUSIONS

The trial experiments conducted in Professor Kamonpatana's Unit went well and it is clear that the conditions for tissue culture practice are adequate. Similarly I believe that the immunochemistry aspects of mAb technology could also be set up. Both of these aspects, of course, require a financial outlay on equipment. The conditions for animal maintenance

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[*] see Pannel , R. and Milstein, C. Journal of Immunological Methods (1991) (in press), for a convenient in vitro method suitable for use with a roller apparatus.

are not yet suitable, but are expected to be upgraded. I would argue for a minimum, but high quality, provisions of small animals for mAb work.

In these simple practical terms, the establishment of a monoclonal antibody laboratory is feasible. I believe, however, in order for such an undertaking to be worth the investment, that there must be a genuine and realistic medium-term ambition to use such a laboratory for basic research and development. This means the production of useful new mAbs and/or the production and purification of existing mAbs for use in novel assay systems. Investment in a monoclonal antibody laboratory would not be justified just for the sole endpoint of producing previously-developed mAbs in tissue culture supernatant form. It also means, (i) a serious commitment to train and educate sufficient staff (leaders and assistants) in both the scientific and technical aspects of the appropriate areas of immunology, and (ii) a willingness to collaborate with other local groups working in this field of study.

RECOMMENDATIONS

1. To counterpart Institution

If and when a decision is made to initiate monoclonal antibody-related vork in the Biochemistry Unit of the Faculty, however modest, careful planning vith respect to equipment, upgrading of small animal holding facilities and staff training should be initiated. The degree of commitment in all of these aspects vill depend upon the extent to which the different aspects of mAb technology are to be undertaken.

2. To Royal Thai Government (RTG)

The use of monoclonal antibodies now covers fields of investigation which range widely in their concerns from fundamental research to industrial/agricultural applications. It is both realistic and of potential strategic value to consider the establishment of mAb technology in laboratories in Chulalongkorn University. I believe it could provide a useful component of an independent research base, especially if the RTG took measures to encourage inter-faculty and inter-institutional cooperation in this field of research. The RTG should also consider the means to facilitate overseas training at an academic level (M.Sc. & Ph.D.) in the field of immunology to underpin this technology, since UNDP only supports project-related training.

3. To UNDP/IAEA

I strongly encourage support for the development of monoclonal antibody technology at Chulalongkorn University. The group under Professor Kamonpatana has previously proven its ability to establish new technologies so that the Biochemistry Unit, Faculty of Veterinary Sciences would be an appropriate location for the effort, especially in relation to their programmes of steroid hormone assays and immunointervention. The IAEA should also consider provision of further opportunities for technical training of staff overseas. Coordination with RTG in this training and education aspect, with respect to immunology, would be of benefit.

APPENDIX 1: SCHEDULE

22 July 1991	- Arrange programme - Discussion on project activities - Seeing facilities concerned
23 July 1991	- Discussion on milk progesterone EIA
24 July 1991	- 9:30-11:30 am. Lecture "Structure of Antibodies and Mechanism of Immune Responses" - Recommendations for setting up facilities for monoclonal antibody production
25 July 1991	- 9:30-11:30 am. Lecture "Derivation of monoclonal antibodies. New antibody technologies". - Tissue culture set up
26-28 July 1991	- Free
29 July 1991	- Tissue culture set up (continued)
30 July 1991	- Cryopreservation of cell
31 July 1991	- Animal availability, holding etc. (in this department or another department?)
1 August 1991	- Animal immunisation etc.; protein biochemistry
2 August 1991	- Thawing of cryopreserved cells
3-4 August 1991	- Free
5 August 1991	- Discussion of frozen-cell storage practice
6 August 1991	- Discussion of specific project proposal with Professor Maneewan Kamonpatana
7 August 1991	- 9:30-11:30 am. Lecture "New Major Histocompatibility Complex (MHC) Genes involved in the Assembly of Antigenic Peptides into MHC Class I Molecules" - Medical School Final assessment of equipment needs
8 August 1991	- Visit to UNDP HQ. Meeting with Mr Sunil Saigal, Mr Johann Stuyt and Mrs Pattum Sunitwong-na Ayuttayah; Report preparation
9 August 1991	- Leaving Bangkok

APPENDIX 2: MONOCLONAL ANTIBODY PRODUCTION CHECK LIST

Immunization of animals

LOU/c *Rats (but can use any other) i.p., i.m. or s.c.
BALB/c Mice (" ")

Materials : needles
syringes
adjuvants - complete Freund's adjuvant
- incomplete " "
- Superfos (Al $(OH)_3$ -based)
- combinations

Bleeding rats/mice

mice : using scalpel on stretched tail (apparatus) (or from paraorbital plexus) — with or without anaesthesia

rats: from tail by needle
from tail by cutting and cauterisation by cardiac puncture — with anaesthesia

Before fusion (final injection) : rats i.v. >> i.p. (>> = much better than)
mouse i.v. >> i.p.

Fusion : remove spleen
- ether pot, ether dispenser for anaesthesia and killing rats
- instruments (sterile)
- medium (see 'medium' section)
- plasticware (see 'plastic' section)

centrifuge for sedimenting cells
" myeloma cells
- grow in flasks
- NSO should be OK
- Y3Ag1.2.3 may require trypsin-EDTA and recovery at 37° C for 1 hr (with inversion)

Polyethylene glycol
Hypoxanthine - Azaserine

Freezing mixture : dimethylsulphoxide + FCS
-70° C freezer
liquid nitrogen storage container for 1 ml vials

Medium & FCS

Purchase powder + bicarbonate (make up in Milli Q water)

filtration
- membranes
- bottle
- filter system - large
  - bench top
- fetal calf serum - discussed
- antibiotics penicillin, streptomycin, gentamycin, ciprofloxacin, fungizone

cloning
- mice for feeders
- or feeder cell lines (preferred)
- 96 well microtitre plate (flat, with lid)

Antibody preparation
(medium scale)

(i) ascites : pristane/IFA
(mice or rats genetically compatible with spleen + myeloma)

(ii) culture : large flasks
: spinner, roller systems
(Centrifuge, Concentration system (Amicon))

N.B. culture method recommended over ascites method

Purification

(a) by affinity - Anti-mouse Ig or anti-rat Ig
- staphyloccal protein A

(b) by ion-exchange chromatography

(c) salt precipitation (ammonium sulphate)

chromatography : columns, pumps, fraction collector, spectrophotometer or colorimetric (Bradford) assay
: dialysis membrane
: SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Biochemistry

- test for size (M.W.) of Immunoglobulin chains
- test for purity of purified preparation
- will tell you if mAb is IgG or IgM

Equipment - SDS-PAGE Apparatus, power pack and chemicals

Isotyping of monoclonal antibodies using specific anti-isotype reagents

- Ouchterlony gel precipitation test
- Kits by ELISA
  or by haemagglutination
- Small scale concentration : vacuum dialysis

plasticware

Syringes

Petri dishes

50 ml centrifuge tubes - recyclable

flasks (i) small
(ii) medium
(iii) large

Plates 24 w. flat
96 w. flat

Freezing tubes

10 ml plastic tubes

Vacuum pump and suction line

Pipette cans

Gilsons & tips

Sterilizing - autoclave
- oven

Lab hygiene - 74 OP alcohol for surfaces
- Virkon, cheap disinfectant
- Hibiscrub for hands

Washing (glassware/pipettes) - distilled water washes

"Note

- Permission for use of cell lines

NSO - Dr C. Milstein, MRC Cambridge

Y3Ag1.2.3 - Dr C. Milstein, MRC Cambridge

IR983F - Dr H. Bazin, University of Louvain, Belgium

- Mycoplasma testing

Stain	Hoechst 33258 (Sigma)
HBSS	without phenol red
Fix	(Carnoy) methanol glacial acetic acid
Mounting medium	Citric acid monohydrate Disodium phosphate glycerol

[1]need for care with health of rats. Avoid contact with feral rats

- diseases dangerous to man
- Weill's disease (leptospirosis)
- Hantan virus (Korean haemorrhagic fever)

APPENDIX 3: Equipment Needs

Major

Laminar flow hood

-70oC freezer

Liquid nitrogen storage vessel

Amicon Concentrator & hollow fibre cartridges

SDS-PAGE GEL apparatus + power pack

peristaltic pump

fraction collector

chart recorder

medium filtration system (pressure vessel + filter stand)
roller apparatus (in 37 C room)

Minor

mouse bleeding stand

ether pot and dispenser

vacuum pump (for suction line)

pipette cans and rack

Gilsons

Other

Several other small and 'consumable' items mentioned in Appendix 2