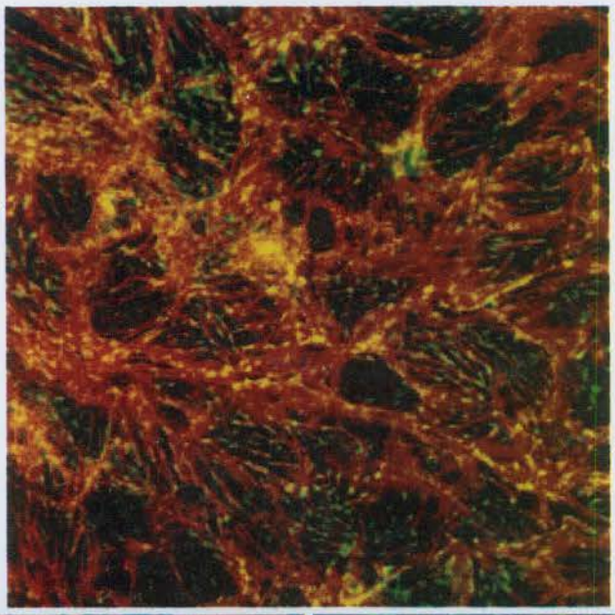
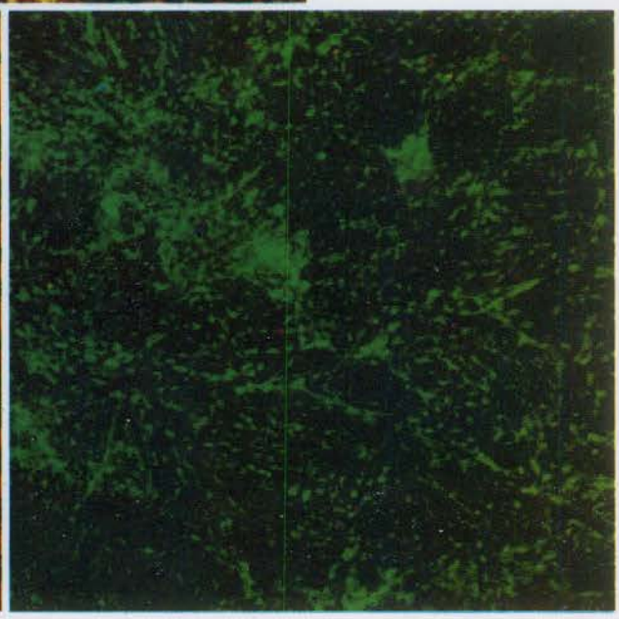
NATIONAL CENTRE FOR CELL SCIENCE

An autonomous institution of the Department of Biotechnology,
Government of India





ANNUAL REPORT
1998-99

N.C.C.S. Complex, Ganeshkhind, Pune 411 007

Dr. Murli Manohar Joshi,
Hon. Minister of Science and Technology, visited NCCS laboratories.

Participants in the special training course as a part of the technology transfer for cryopreservation of bone marrow cells.

Confocal laser scanning microscope.

CONTENTS

TERMS OF REFERENCE

- To receive, identify, maintain, store, grow and supply:
- Animal and human cells / cell cultures, cell lines of both existing (typed) and newly developed, hybrid cells including hybridomas;
- Tissues, organs, eggs (including fertilized) and embryos;
- Unicellular obligate pathogens, parasites and vectors, plasmids, genes and genomic libraries;

- Develop, prepare, quality control and supply culture media, other reagents and materials, and cell products independently and in collaborationwith industry and other organizations.

- Research and development in the above and cell cuclture related materials and products.

- To establish and conduct post graduate courses, workshops, seminars, symposia and training programmes in therelated fields

- To serve as National Reference Centre for tissue culture, tissue banking, cell products and data banketc. And to provide consultancy services tomedical, veterinary, pharmaceutical institutions, public health services and industries, etc. in the country.

- To provide and promote effective linkages on a continuous basis between various scientific and research agencies / laboratories and other otganizations including industries working in the country.

- To collaborate with foreign research institutions and laboratories and other international organizations in areas relevant to the objectives of the facility;

- To participate in such programmes as required in the country for the betterment of society and advancement of science and technology.

PREFACE

It is indeed my privilege, once again, to present this Annual Report of the National Centre for Cell Science (1998-99). I would like to present here an overview of the activities carried out during the last one year:

Cell Repository:

During 1998-99, a total of 388 cell cultures, comprising of 132 cell lines were supplied to 90 research institutes.

Research and Development:

A brief summary of the progress made under the various research projects is presented below:

On 5th June 1998, NCCS a special function was organized to formally transfer two technologies developed at NCCS, at the hands of Dr. Mrs. Manju Sharma, Secretary, Department of Biotechnology:

(I) *Technology for large scale expansion of human skin in culture for treatment of burns, vitiligo, and non-healing ulcers: In vitro* expansion of the human skin from small biopsies can be performed in tissue culture laboratories. In collaboration with health institutes such as K.E.M. Hospital, Pune and Mumbai, and Lokmanya Tilak Municipal Medical College and Hospital, Mumbai, such cultured epithelia sheets have been grafted to more than 85 patients successfully. It is now proposed to establish three units in different hospitals which could then undertake the work on continued basis. The expertise for this purpose will be provided by NCCS scientists. An MoU was signed by NCCS and KEM Hospital, Pune, LTMMC & H, Mumbai and Lok Nayak Jaiprakash Narayan Hospital, New Delhi.

(II) *Cryopreservation of cord blood and haematopoietic stem cells.* In order to overcome the limitations of temporary storage of aspirated human bone marrow, NCCS scientists have

standardized methods for its cryopreservation using a specially formulated medium. In collaboration with Cancer Research Institute, Mumbai, cryopreservation of umbilical cord blood has also been formulated. The cryopreserved bone marrow and cord blood form a ready source of the stem cells for transplantation purpose. National Institute of Virology, Pune, has offered to undertake testing of the samples received for cryopreservcation for detection of certain hazardous viruses like HIV, CMV, Hepatitis, etc. The cryopreservation technology has been transferred to the Armed Forces Medical College, Pune. A special training course was conducted for the AFMC staff, as a part of the technology transfer.

Experimental work on stem cell cryobiology, efficacy of trehalose as a cryoprotectant and infusion of fresh and frozen marrow in irradiated mice is being carried out further. In continuation with the previous studies on stromal cell biology, the recent experiments have underscored the deterministic role of stromal cells derived signaling in haematopoietic process. Using a depletion strategy for identification of the cellular target of erythropoietin, CD2 population completely abrogated TGF release, indicating a role of T lymphocytes in the process. Our recent data strongly suggests that one of the mechanisms in stimulation of a recipient's hematopoiesis could be mediated by the action of TGF beta 1 secreted by infused FLHCs and could provide rational framework on which Foetal Liver Infusion therapy can be further evaluated.

Varied aspects of experimental work on diabetes have been continued. Ability of the cytosolic extract from regenerating pancreas to reverse the status of STZ induced diabetes in mice has been evaluated. One of the fractions, FR4D, was most effective. This FR4D fraction was sequenced for 35 amino acids from the N-terminal and homology searches suggest this to be a novel protein. Further studies to identify the gene will be undertaken. Riboflavin as an additive to cryopreservation medium has been used for improved recovery of cryopreserved pancreatic islets. In a new line of investigation, studies have been undertaken to define

the conditions necessary for long term maintenance of differentiated exocrine gland acinar cells in vitro. Chitosan-gelatin appears to permit the maintenance of viability and functionality of acinar cells for more than 4 days. A protective role for glucose against the cytotoxic effects of the diabetogenic compound streptozotocin has been studied.

Using the *in vitro* wound healing models as reported earlier, we have further confirmed that inhibition of cell division by hydroxyurea does not inhibit cell migration. On the other hand, inhibition of protein synthesis by cycloheximide inhibits cell migration. Our efforts to identify and characterize the bone derived factor have indicated that the factor induces cellular migration of breast adenocarcinoma cells in vitro. An active component, a 55 kDa protein, was identified in one of the FPLC fractions of the crude extract.

Location of cyclin D 1 loci were identified in randomly growing population of SiHa cells by fluorescent in situ hybridization. This local localized near the nuclear membrane and / or the nuclioli in 73% an away from the nuclear membrane and / or nucleoli in 27% of the loci analysed. Further, two-colour FISH preparations were studied with the confocal laser scanning microscope and the relative positions of the cyclin D 1 loci in chromosome 11 territories were assessed in 3-dimensional reconstruction.

Studies on identification of oncogenes implicated in mouse melanomas using Cloudman menanoma Clone M3 Efforts were made to characterize the 1.2 kb gene using mRNA expression studies and FACS analysis of transfected cells. A partial sequence obtained mmanually shows no significant homology to the known genes involved in cell transformation.

Role of PKC in grown and differentiation in neuroblastoma cell lines Neuro 2A and its clone NB41A3 have been undertaken. Experimental data indicate that down regulation of PKC activity correlated with inhibition of proliferation.

We have undertaken molecular studies on the large number of bacteria which harbor in mosquito midguts. Thus far 32 types have been isolated, 8 of which were strongly haemolytic. Complete sequencing of PCR amplified 1.5 kb rRNA gene confirmed this to be Aeromonas jandei. This appears to be the first report of isolation of this organism from the mosquito midgut.

We have recently initiated work under new areas which include immunosuppression during experimental leishmanias: to establish role of non-T cell CD28 expression and function, the role of costimulatory molecules and the role of Leishmania receptors on macrophages; (ii) molecular characcterization of SMAR 1 protein: to identify DNA binding motifs, protein-protein interaction and construction of knock-out and transgenic mice of this protein. (iii) molecular and cellular basis of HIV pathogenesis: to identify differentially expressed molecules during HIV induced apoptosis and to elucidate the interaction of those molecules in the signalling cascade leading to cell death.

The Experimental Animal Facility has acquired BALB/cJ, C57BL/6J, DBA/2J and NOD/LtJ from Institute of Microbial Technology, Chandigarh. A single male mouse with spontaneous congenital cataract was detected in a production colony of BALB/c mice. This mutant colony is currently at F7 level of inbreeding. A true breeding mutant strain is being attempted.

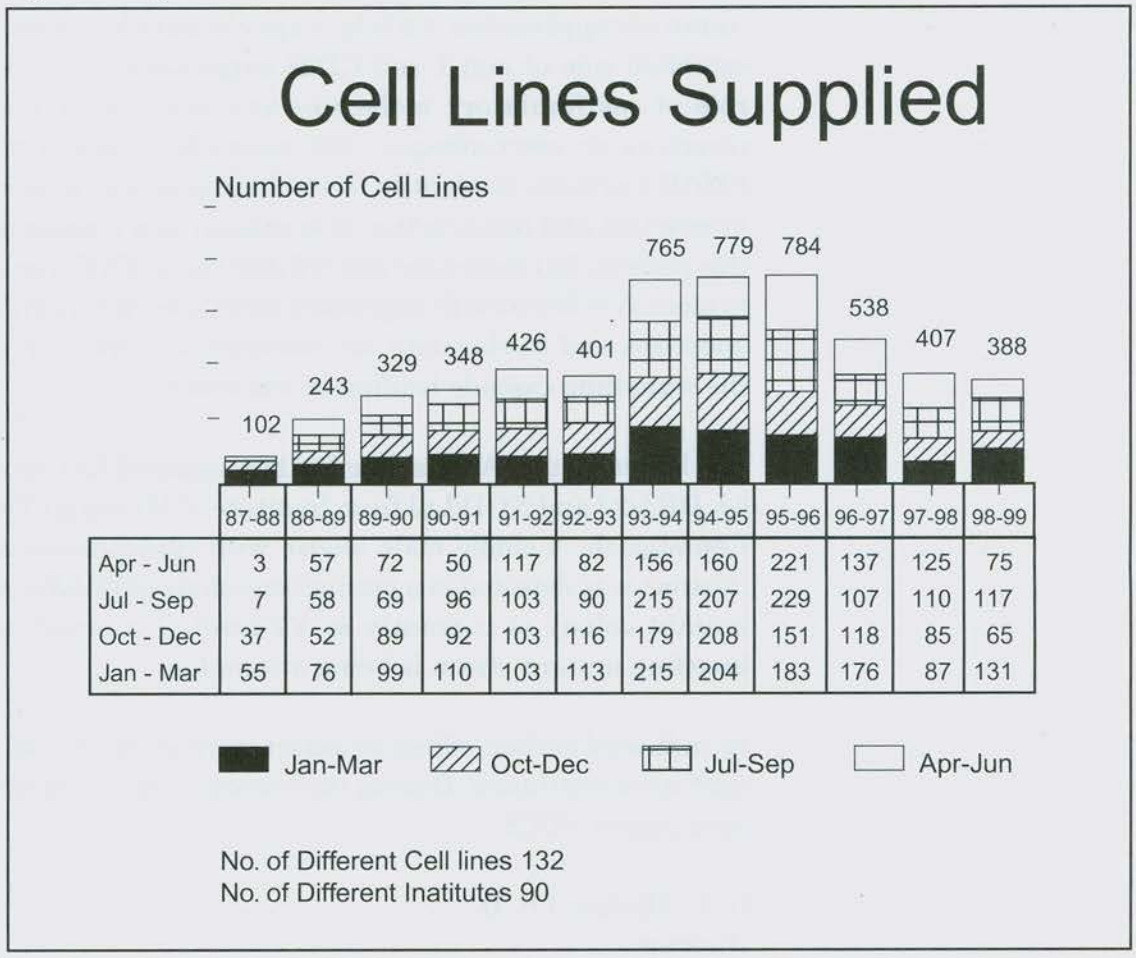
As indicated earlier, efforts to induct more scientific and technical staff were continued. During the current year, — new scientists have joined NCCS.

G. C. Mishra, Ph. D.
Director

CULTURE REPOSITORY

During 1998-99, a total of 388 cell cultures, comprising of 132 cell lines were supplied to 90 research institutes.

Figure 1. Cell lines supplied during 1998-99.



	87-88	88-89	89-90	90-91	91-92	92-93	93-94	94-95	95-96	96-97	97-98	98-99
Apr - Jun	3	57	72	50	117	82	156	160	221	137	125	75
Jul - Sep	7	58	69	96	103	90	215	207	229	107	110	117
Oct - Dec	37	52	89	92	103	116	179	208	151	118	85	65
Jan - Mar	55	76	99	110	103	113	215	204	183	176	87	131

Development of Th1 and Th2 specific costimulatory molecules

Participants:
G. C. Mishra
Chayya Iyengar
D. V. R. Prasad
Pinaki P. Banerjee
Vrajesh Parekh
Satyan Sharma
Sangeeta Shah

Collaborators:
Krishna Shastri

Date of initiation:
August 1997

Expected date of completion:
August 2002

Background:

T lymphocytes have an important role in most immunological responses. To exert their effector function, relatively quiescent T cells are activated during a complex interaction with antigen receptor (TcR) complex. Interaction of TcR with the major histocompatibility complex (MHC) or antibodies to TcR results in the initiation of signal transduction events and cellular activation. However stimulation of TcR alone is insufficient to activate most of the T cells. The current dogma is that at least two signals are required. The first signal is provided by the occupancy of TcR, which is MHC restricted and second non-MHC restricted signal(co-stimulatory signal) is delivered by certain molecules present on the surface of APCs. The co-recognition of the molecules on T cell and APC is, therefore, of crucial importance as the latter event results in the transduction to specific signals that eventually determines the outcome of an immune response.

Mouse T helper cells can be divided into two distinct types, Th1 and Th2, based on the patterns of cytokine secreted in response to antigen or mitogen stimulation. These different cytokine patterns result in different functions of two types of cells. Th1 cells mediate several reactions suitable for combating intercellular parasites, including delayed type hypersensitivity (DTH), macrophage activation and limited B cell help. Th2 cells provide strong help for B cells. Th1 and Th2 phenotypes may be important as effector cells in strong immune responses, and may account for the reciprocal nature of antibody and DTH responses, because Th1 and Th2 cells appear to be mutually inhibitory. Th1 cells produce IFN-g, which inhibits growth of Th2 cells whereas Th2 cells produce IL-4, IL-5, IL-10, etc. It has been postulated that these two T helper subsets are not only functionally different but also have qualitatively and quantitatively distinct requirements for co-stimulation. However, till date, no costimulatory molecule(s)

has been reported that preferentially activates either Th1 and Th2 like cells.

Recently, while analyzing the protein molecules from the surface of LPS-activated B cells, we found two phosphoglycoproteins of 38kd (B3) and 97-100kd (B2) which selectively activate Th cells to secrete IL-4 and IL-5. On the other hand, a protein molecule of 150kd (M150) present on the membrane of macrophages stimulates IL-2 and IFN-gproduction and proliferation of naïve and ovalbumin specific $CD4^+$ T cells.

Relevance:

The co-stimulatory molecules and the antibodies against them, their receptor on the target cell and antibodies against their receptor can be of immense importance in understanding the immunobiology of various pathogenic infections like malaria, TB, Leishmaniasis, leprosy as immunotherapeutic agent for selective regulation of immune system. Also, during organ transplantation antibody against Th1 costimulatory molecule can be of immense importance to increase the acceptability of the transplant. It may be mentioned there that development of Th1 and Th2 cells require specialized lymphokine melieu and it is very well established that both the subsets interfere with the development of each other. In pathogenic conditions where hosts require to eliminate pathogens by developing Th1 type of immunity, it has often been reported that the parasites dictate host to develop Th2 type of response which interferes in the development of required Th1 type immunity to eliminate the infection. Therefore , the present study proposes to understand the molecules in more details. The overall study will be carried out with the following aims:

Objectives:

1. To raise the antibodies and clone the genes of Th1 and Th2 specific co-stimulatory molecules identified by our lab.
2. The cloned genes will be transfected in non-professional APCs

and the transfected cells will be evaluated for their ability to help the Th cells, leading to their differentiation into Th1 and Th2 types.

3. To further investigate the role of Th1 specific costimulatory molecule,M150 in immunobiology of $CD4^+$ and $CD8^+$T cells.
4. Finally, attempts will be made to find the human homologue of these genes.

Work done:

1. Polyclonal antibodies against M150 have been raised in rabbit and syrian hamsters.
2. Hamsters were immunized intraperitoneally with 100mg of purified M150 followed by two booster injections of 50ug of M150 at 15 days interval. Three days after the last booster injection, antibody titer was checked on western blots and spleen cells were fused with myeloma cells (SP2/0). But we have failed to generate monoclonal antibodies against M150 because of heterofusions and lack of fusion partner in case of hamster and rabbit. So, Lewis rats have been immunized with M150 and now we do have polyclonal raised in them.
3. Fusions of immunized rat spleenic cells with Y3, SP2/0 and PAIOP3 are being carried out Screening of the mixed hybrids is in progress.
4. Polyclonal antibody has been successfully raised against one of the peptide stretches of M150 by linking the peptide with Key limpet hemocyanin(KLH) in rabbit,hamsters and rats. The activity of the antibodies have been checked in western blots, ELISAs and dot blots. The peptide polyclonal antibody has been purified using peptide affinitycolumn. This polyclonal is now being used to screen the cDNA library. Besides screening with polyclonal nti M150 is also in progress.
5. To further analyse the role of M150 in $CD4^+$ T cell activation We are attempting to look its role in Dendritic cells(DCs) mediated $CD4^+$T cell activation.. In this context successful attempts have been made to enrich spleenic DCs. Using these enriched spleenic DC preparations M150 blocking experiment has been carried out. Further confirmation of the results is

required and is underway. Studies on role of M150 in $CD8^+$T cell activation are also being carried out in an antigen specific system. SYNFEKL peptide of Ovalbumin(OVA) specific $CD8^+$T cell clones are being made.

6. We have identified A-20 cell line which expresses Th2 specific costimulatory molecules, B2 and B3, on their surface. Peptide sequencing from two stretches of B2 has shown 100% homology with GRP94 (gp96, Erp99, Endoplasmin) which belongs to the Heat Shock Protein 90 family. Keeping in mind the sequence homology , the increased expression of GRP94 and B2 upon LPS activation and heat shock(45', 45^0C) was studied on LPS activated B cell and A20. It is been found that expression of B2 increases at least two fold LPS activation and heat shock at 55hrs and 24 hrs of time point. It was also seen that after 24 hours of glucose starvation the expression of B2 in creases at least two fold and returning to normal levels by 29 hrs. The homology of B2 and endoplasmin has been further strengthened by western blot and FACS studies with anti-GRP94 antibody, besides immunoprecipitation using hsp90 monoclonal antibody AC-88. Further it has been found that A 20 gives a better costimulation to $CD4^+$T cells after giving a heat shock . The anti GRP 94 carboxy terminal antibody (Stressgen , Canada) was used to block the second signal to the $CD4^+$ T cell in assay system where anti CD3 antibody was used as the first signal. More than 90% blocking of T cell proliferation was observed when B cells were used as APC. Further experiments to firmly state the above results are being carried out along with lymphokine assays to confirm the Th2 type costimulatory activity of the molecule under study.
7. Polyclonal against B3 molecule has been made. Regarding the cloning of B3 efforts are being made. We have been able to complete the mRNA preparations f A20 cell line and the mRNA is currently being investigated for the presence of B3 molecule with the help of anti-B3 antibodies. However, the difficulty is that the anti-B3 antibodies are polyclonal but not affinity purified. Hence, we do see a mild background when the mRNA has been subjected to reticulocyte translation procedure.

Hence, we are going to trap the mRNAs and all homologous sequences present in the A20 cell line with the help of biotinylated oligos and magnetic attraction technology. This should help us in further analysis. Besides RT-PCRs will soon be carried out.

Outcome of the project:

Publications:

1. Javed N. Agrewala, Susmit Suvas, Aneesh Joshi, Archana Bhatnagar, Dass S. Vijay and Gyan C Mishra. M150 modulates the costimulatory signals delivered by B cells to T cells and enhances their ability to help B cells. *J. Interferon and Cytokine Res.(1998),18:297-304.*
2. Javed N. Agrewala, Susmit Suvas, Rakesh K. Verma and Gyan C. Mishra. Differential effect of anti-B7-1 and anti-M150 antibodies in restricting the delivery of costimulatory signals from B cells and macrophages. *J. Immunol.(1998), 160:1067-1077.*
3. Gobardhan Das, Harpreet Vohra, Bhaskar Saha, Javed N. Agrewala and Gyan C. Mishra. Leishmania donovani infection of a susceptible host results in apoptosis of Th-1 like cells: rescue of anti-leishmanial CMI by providing Th-1 specific bystander costimulation. Microbiology and Immunology, Vol. 142, no. 11. 1998.
4. Gobardhan Das, Harpreet Vohra, Bhaskar Saha, Javed N. Agrewala and Gyan C. Mishra. Apoptosis of Th1-like cells in experimental TB, Clinical Exp. Immunol. (In press).
5. Gabardhan Das, Harpreet Vohra, Kanury Rao, Bhaskar Saha and Gyan C. Mishra. Leishmania donovani infection of a susceptible host results in CD4+T cells apoptosis and decreased TH1 cytokine production, Scandinavian Journal of Immunol. (In press)
6. Manoj Raje and Gyan C. Mishra. Autoradiography. In Encyclopaedia of Immunology, 2nd edition, 1998, 296-300.

Patents: Nil
Products: Nil.

Culture of human skin keratinocytes, their 3-D epitheliation and grafting to burns, nevi, vitiligo and non-healing ulcer cases

Participants:
Manoj Mojamdar
Vaishali Chaubal
Sujit Nair
Ulka Ghate

Date of initiation:
May 1990

First phase completed:
May 1998

Expected date of completion of Second phase:
May 2000.

BACKGROUND:

There is a considerable loss of skin in burns cases. The growth of skin of these patients is slow and the raw wound becomes susceptible to severe infections. Autologous cultured epithelia offers the best alternative among the available wound covers.

Work done:

Phase 1: The culture of skin keratinocytes to 3-D epithelia has been standardised and the technology has been transferred to the user hospitals.

Phase 2: The scientific advisory committee member has suggested that we should concentrate our efforts on improving the growth rates of keratinocytes so as to cover the burn wound earlier than what was currently done.(15-21 days to 1-1.5 months) The two natural conditions wherein the keratinocytes grow faster than normal conditions are psoriasis and in dandruff. We have decided to study the dandruff causing yeast to see if they contain any wound healing and rapid epitheliation properties. The work is at the initiation phase.

Outcome of the project:

1.Technology transferred to user hospitals.

Publications:

G.Ulrich- Merzenich and Manoj Mojamdar: Skin Fibroblasts adjacent to Rheumatoid Arthritis joints produce large quantities of hyaluronan. **J. Invest Dermatol** (Communicated.)

Presentations:

Sujit Nair and Manoj Mojamdar. Culture of Human Skin Epithelia and its Clinical Applications. *Susamvad (Golden Jubilee Research Students Seminar),* University of Pune 4-5 December 1998.

Regulation of melanogenesis

Participants:
Manoj Mojamdar
Vaishali Chaubal
Sujit Nair
Ulka Ghate

Date of initiation:

Expected date of completion:

Background:

Skin colour identifies us, defines and categorizes us in the social and cultural hierarchy, makes us look healthy in the eyes of society. Pigmentary disorders are thus a social stigma and physiological trauma to the affected individual. Mammalian melanin are mainly of two types ; eu- (black) and pheo-melanin (red to yellow). Eumelanin protects the skin against UV radiation induced cancer. The regulatory switches between eu- and pheomelanin are poorly understood. ?-glutamyl transpeptidase(?-GTP), a transmembrane glycoprotein has been thought to be involved in the pheomelanogenic pathway.

Work done:

We have earlier shown that inactivation and subsequent recovery of this enzyme leads to hyper-melanization. In studies with irreversible inhibitors of ?-GTP there is a significant decrease in the tyrosinase activity (the main enzyme of the melanogenic pathway). These studies are being pursued at the gene and protein level to better understand the role of ?-GTP in melanogenesis. UV and more recently Nitric Oxide (NO) have been shown to regulate melanogenesis. The involvement of ?-GTP in these melanin synthesis regulation process is being investigated.

Outcome of the project:

Publications:

1. Anupma Sharma, Bhuvaneshwari Jagadisan, Virendar Sheorain, Vijaya Haldankar, and Manoj Mojamdar. (1998) Transient expression of high Molecular Weight, Heat Sensitive, Trypsin-Resistant Form of Tyrosinase in B16 Melanoma Cells. Pigment Cell Research 11:6 p375-79

Presentations:

Vaishali Chaubal and Manoj Mojamdar. Keratinocyte- Melanocyte

Publications:

G.Ulrich- Merzenich and Manoj Mojamdar: Skin Fibroblasts adjacent to Rheumatoid Arthritis joints produce large quantities of hyaluronan. **J. Invest Dermatol** (Communicated.)

Presentations:

Sujit Nair and Manoj Mojamdar. Culture of Human Skin Epithelia and its Clinical Applications. *Susamvad (Golden Jubilee Research Students Seminar),* University of Pune 4-5 December 1998.

Interactions for the Maintainance of Normal Skin Colour. *Susamvad (Golden Jubilee Research Students Seminar)* University of Pune 4-5 December 1998.

Bhuvaneshwari Jagadisan, Anupma Sharma, Virender Sheroin, and Manoj Mojamdar. Phenotypic Drift in B16 Melanoma Cells: Thus far and no Farther. Accepted for presentation in forthcoming Cell Biology Conference to be held in Feb 20-22 1999.

1. Manoj Mojamdar Remelanizaton in Amelanotic B16 Melanoma Cells : A Model for Investigating Commitment in Pigment Cells. Invited Talk for the forthcoming International Workshop on Pigmentation and Pigmentary Disorders to be held in March13-14th, 1999.

2. Vaishali Chaubal and Manoj Mojamdar Hyperpigmentation in B16 Melanoma Cells: Role of ?- Gluatmyl Transpeptidase. Accepted for presentation at the forthcoming International Workshop on Pigmentation and Pigmentary Disorders to be held in March13-14th, 1999.

3. Sujit Nair, Vaishali Chaubal, Yogesh Shouche and Manoj Mojamdar Hormone Induced Changes in the Expression of Melanogenic Enzymes. Accepted for presentation at the forthcoming International Workshop on Pigmentation and Pigmentary Disorders to be held in March13-14th, 1999.

Studies on cryopreservation and revival of haematopoietic cells

Participants:
Dr.L. S. Limaye, N.C.C.S., Pune.

Collaborators:
Dr. S.G. A. Rao. Head Stem Cell Biol., Div., C.R.I., Bombay.0
Dr. R. L. Marathe. Head Haematology Div., Jehangir Hospital, Pune.

Date of initiation:
Expected date of completion Dec.' 99

Background:

This project was initiated with two objectives :

- Ø Standardization of the known technology of bone marrow cryopreservation and transfer of the technology to hospitals that require it.
- Ø Research in the area of haematopoietic stem cell cryobiology.

Importance of the project:

The development of cryobiological methods for the safe preservation and storage of bone marrow provides a technological foundation for the wide spread clinical application of autologous HSC transplantation. Long term cryopreservation allows prophylactic storage for patients to be transplanted months to years later and for high risk population like people working in nuclear reactor plants and people with family history of leukemia.

Work done:

a) Technology Standardisation:

The technology was transfered to Armed Forces Medical College,Pune.A manual containing protocols for collection,processing and freezing and quality control for viral screening of stem cells from bone marrow and cord blood was jointly prepared by NCCS, CRI and NIV and was handed over to AFMC. Under the technology transfer programme a 6 member team of AFMC was trained at NCCS from 26OCT.to 10 NOV.98. The training comprised of two parts:

1 Basic Techniques in Tissue culture-Organised by Dr.R.R.Bhonde;

2 Cryopreservation and revival of human haematopoietic stem cells-Organised by Dr.L.S.Limaye

b) Research in the area of stem cell cryobiology

The experiments with Trehalose as an additive to the conventional freezing medium were continued further.Trehalose was added in combination with 10% DMSO in the conventional freezing medium and experiments were done using mouse and human haematopoietic cells.

1 Experiments on mouse bone marrow

CFU-S assays and engraftment assays were done by subjecting the mice to whole body irradiation and then infusing fresh and frozen marrow through their tail veins. The results are encouraging .In addition to the in vivo assays in vitro LTCIC assays were also set up to detect the efficacy of freezing (Table.1).

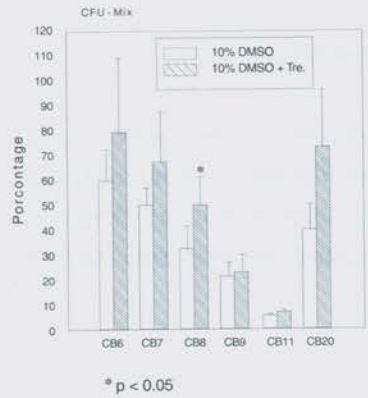
Set No.	*Type*	*Mean of CFU(mix) colonies formed from adh. + N.A. cells from 6 wells at the end of 4th Week.*
1.	*Irr. Stroma alone*	*nil*
2.	*Irr. stroma recharged with fresh marrow*	*499.60 ± 178*
3.	*Irr. stroma recharged with frozen marrow(10% D alone)*	*313.33 ± 61.9*
4.	*Irr. stroma recharged with frozen marrow(10% D + Tre.)*	*517.16 ± 98.8 ***

*** $p = 0.0108$*

2 Experiments on human haematopoietic cells

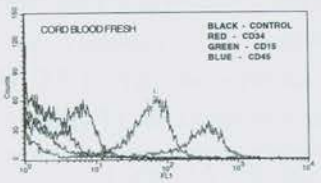
In order to test the efficacy of Trehalose in affording cryoprotection to human stem cells, a few samples of cord blood stem cells were

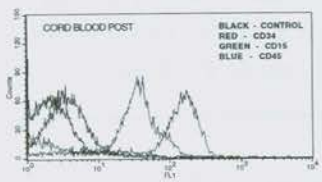
Fig. 2 : *Cryopreservation of cord blood samples with or without trehalose as an additive in the freezing mixture*



* $p < 0.05$

Fig. 3 : FACS analysis of surface markers.





frozen with or without Trehalose and subseqently revived. Viability, Nucleated cell recovery, CFU assays (Fig. 2) and FACS analysis of surface markers was done pre and post freezing. Results are encouraging (Fig. 3).

Proposed Work:

a) Technology Standardization:
Strengthening our previous data of small volumes in vials by freezing of large volumes of samples in cryobags and testing their revival efficacy.

b) Research:
A joint proposal by Dr. L.S.Limaye and Dr.V.P.Kale has been sbmitted to DBT for funding in DEC.98.The title of the proposal is "Bone marrow cryopreservation: Addition of membrane stabilisers and antioxidants in the conventional freezing mixture to conserve growth factor responsiveness and engraftment potential of hematopoietic stem cells"

Outcome of the Project:

Patents: Nil
Product: Nil
Publications: Nil

Stromal cell biology: Definition of the factors triggering cell-cell interactions in the haematopoietic cells

Participants:
Vaijayanti P. Kale
George Fernandes
Arundhati Khedkar

Collaborator:
L.C. PADHY

Date of initiation:
Dec. 1998

Probable date of completion:
Dec. 2001

Background:

Blood cell formation or haematopoiesis is a complex process where the multipotent stem cells present in few numbers, ordinarily resident in the bone marrow micro-environment orchestrate a complex sequence of events in cooperation with a large number of cytokines/ growth factors. The overall process involves the progress of primitive stem cells, through distinct stages, in an increasingly committed manner to reach a final destination of mature cells. Although the cell differentiation pathways originating from the pluripotent stem cell and culminating in fully differentiated end products are broadly understood, several aspects of this process still remain elusive.

Stem cells are found in intimate association with stromal cells in the haematopoietic micro- environment in vivo as well as in vitro. Several experiments have documented the importance of haematopoietic environment, which is defined by the stromal cells and the ECM components surrounding them. In long-term culture experiments, it has been clearly shown that bone marrow derived stromal cells play a crucial role in the stem cell proliferation and differentiation. Several experiments have underscored the need for cell-cell interactions that allow effective interactions or cross talk between the differentiating haematopoietic cells and the supportive stromal cells. In addition, several cytokines, interleukins, growth factors etc are required to convey specific signals to the differentiating progenitor cells at various stages of differentiating process. The role of various cytokines, interleukins etc. in this process is reasonably understood to allow designing of more precise experiments.

However, how stem cells decide whether to remain quiescent, to self renew or to differentiate remains to be elucidated. Similarly, it is not well understood what regulates lineage commitment. A line of our work is related to the still poorly understood problem: ' What determines or triggers the cellular cross talk in the haematopoietic compartment?' This undoubtedly is a central issue, because if the deterministic factors modulating specific cell to cell contact could be identified, it may pave the way to modulate haematopoiesis in a predetermined manner, at least in cell culture. Secondly, it may aid in the identification of defective steps in human disease process.

Work done:

We have reported earlier that TGFb released by bone marrow derived mononuclear cells (BM-MNC) in response to erythropoietin, confers an adhesive phenotype on the stromal cells leading to both homotypic as well as heterotypic interactions. The heterotypic interaction of stromal cells and the CD 34 + cells resulted in amplification of stem cells in a PKC and Ca+2 dependent pathway. We also demonstrated that the competency of stromal cells played a deterministic role in the stem cell behavior (AR 97-98).

Identification of cell type responsible for TGF release:

We carried out a 'depletion' experiment to identify the cellular target of erythrpoietin action. The BM-MNCs were depleted of

Fetal liver infusion: Its mode of action, efficacy of cryopreservation and its potential application in the management of cancer

Participants:
V. P. Kale
L. S. Limaye

Date of initiation:
March 1995

Date of completion:
September 1998.

(Supported by grants to VPK by the Department of Biotechnology, New Delhi)

Background:

Fetal Liver Infusion (FLI) therapy has been attempted in certain major disorders like aplastic anemia immunodeficiency syndrome, leukemia or metabolic disorders. Though the engraftment capacity of transplanted fetal liver cells has been successfully demonstrated in animal models, human subjects have extremely variable responses to the therapy, ranging from complete recovery on one hand to no response on the other. The responding patients, however, showed autologous recovery of haematopoiesis and no chimerism with donor fetal cells was evident. This observation indicated that the infused fetal liver cells secreted some stimulatory factors for the haematopoietic cells. The identity of the putative factors was not known at the time.

We undertook the project in order to identify the stimulatory factors secreted by fetal liver cells. We have reported previously that both Fetal LiverHaematopoietic cells (FLHCs) and the conditioned medium prepared from them (FLCM) stimulated colony formation of adult BM cells. We also demonstrated that the stimulatory activity of FLCM could be attributed to TGF b1 secreted by FLHCs(AR 98).

Work done:

Effect of neutralizing activity to TGF*b*1 on FLCM activity.

One way to rationalize the mode of action and to delineate the contribution of TGF b1 on the colony formation was to abrogate its biological activity in FLCM while it is being applied on colony

Determination of intracellular Ca+2 levels:

In order to determine the levels of intracellular Ca+2 levels in the stromal cells in response to TGF exposure, we treated the cells with various BAPTA derivatives having different kD values for Ca+2 followed by TGF treatment. The BM MNC were then exposed to these stromal cells and subsequently
plated for colony formation. From these experiments, it appears that the intracellular levels rise upto 1mM in response to TGF.

Role of integrin activation:

Since integrin activation is a downstream event of TGF signaling, we examined the effect stromal cells activated with various integrin specific peptides on the colony formation. We observed that the integrins a5b1 and a2bb3 enhanced the colony formation.

Outcome of the project:

The study has underscored the deterministic role of stromal cells derived signaling in haematopoietic process.

Publication: Nil
Patent: Nil
Product: Nil

Fig. 4 : A neutralizing antibody (AB) to TGF-b1 abrogates the stimulatory property of FLCM.

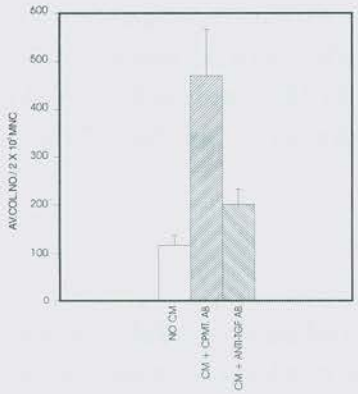


Fig. 5: FLCM-mediated stimulation of various types of progenitors derived from human BM MNC.

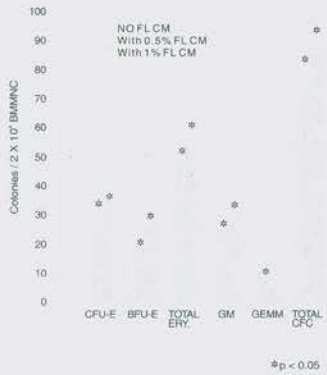
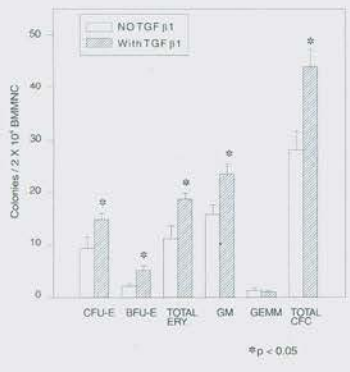


Fig. 6 : The stimulation induced by TGF-b1 is not lineage specific.



assays. We, therefore, chose a CM pretested for its stimulatory activity on colony formation when applied at I% level.The FLCM was then treated with a neutralizing antibody (Genzyme), and this preparation was evaluated in colony formation assays again. As seen in the figure 1, the CM treated with an antibody irrelevant to TGF b1 clearly showed the stimulation of colony formation nearly to the extent of fourfold. By contrast, when neutralizing antibody to TGF-b1 was used, the extent of stimulation seen in parallel experiments dropped to less than twofold. These results emphasize clearly that the TGF-b1 present in the FLCM had a positive stimulatory effect on colony formation.

Effect of FLCM and purified TGF-b1 on the various progenitors:

We have already reported that the stimulatory effect of FLCM could be mimicked by purified TGF-b1. We carried out separate experiments to examine whether the stimulation of colony formation by FLCM or TGF-b1 was of a general nature or was directed towards a specific lineage. For this purpose we carried out experiments under conditions such that discrete and morphologically distinguishable colonies formed for accurate enumeration of CFU-E, BFU-E, GM and GEMM type colonies. The low density of colonies formed per plate (<100) ensured that there was no physical mixing of the colonies. These colonies were identified by their morphology in five to six replicate experiments per set, and the data were subjected to statistical evaluation fot significance. In these experiments, we used IL-3(10 U/ml), GM-CSF (10 U/ml) along with Epo (1 U/ml).As shown in the figures 2 and 3, it is clear that the stimulatory effect seen was of a general type since both the erythroid and myeloid compartments were found to be stimulated nearly to the same extent.

Outcome of the project:

Our data strongly suggests that one of the mechanisms in stimulation of a recipient's hematopoiesis could be mediated by the action of TGF-b1 secreted by infused FLHCs and could provide rational framework on which FLI therapy can be further evaluated.

Publications:

Kale V. P. and Limaye L. S. (1999) Stimulation of adult human bone marrow by factors secreted by fetal liver hematopoietic cells: In vitro evaluation using semisolid clonal assay system. Stem Cells **17:** 107-116.

Patent: Nil
Product: Nil

Design and Development of Biocompatible Synthetic Matrices for Guided Tissue Regeneration, Differentiation Transplantation and Controlled Delivery applications

Participants:
R.R Bhonde
Makarand Risbud

Date of initiation:
August 1998

Expected date of completion:
July 2001.

Background:

Tissue engineering can be used to replace lost/failing tissues/ organs using cells and synthetic extracellular matrices and substitute largely to organ transplantation which is limited by the shortage of the donor tissue suitable for transplantation. Tissue engineering integrates the advantages of tissue/organ transplantation and purely synthetic prosthesis by combining cells with synthetic extracellular matrices that can be synthesised reproducibly on large scale.

Aim of the proposed work:

Design and Development of synthetic matrices for

1. Guided liver regeneration by culturing hepatocytes on synthetic macroporous matrices for replenishing functional and structural loss of liver in surgeries and diseases.
2. Pancreatic islet encapsulation and transplantation for its' possible application in treating insulin dependant diabetes.

Work carried out:

A novel hydrogel from Chitosan and polyvinyl pyrrolidone (PVP) is developed for its application in biomedical research. Hydrogel biocompatibility was tested using tissue culture methods. Semi-IPN's of PVP and chitosan were synthesised by cross-linking chitosan using glutaraldehyde. two compositions of hydrogels

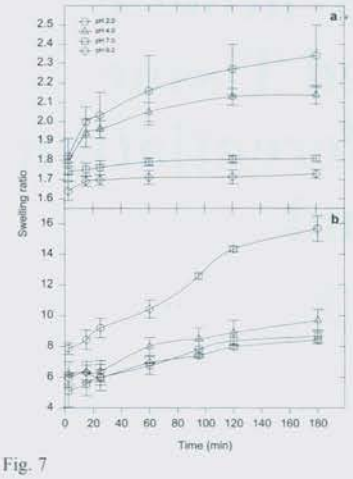


Fig. 7

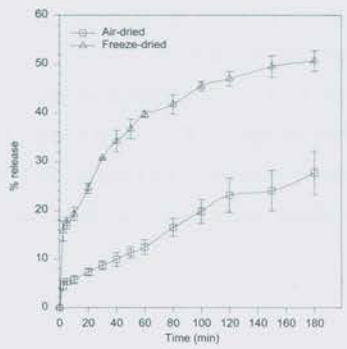


Fig. 8

HM1 and HM2 (1:1 and 2:1, *w/w* chitosan : PVP respectively) were prepared.. HM1 and HM2 were characterised by low protein adsorption which correlates with the their hydrophilic nature. Hydrogels allowed regulated transport of glucose and insulin across them. Hydrogel's molecular weight cut off were found to be around 149 kD.*In vitro* biocompatibility was found to be excellent with no cytotoxic effects on the cells (SiHa and HeLa). Islets cultured on the hydrogels retained their morphology and viability. Sophisticated image analysis system, used to study islet morphometry, confirmed the integrity of islets. Islets retained their functionality ascertained by insulin secretion. We propose chitosan/PVP membranes as potential candidates in islet immunoisolation. Hydrogel HM7 (blend 7:3) was dried either by air drying of freeze drying. Freeze dried hydrogel was characterised by scanning electron microscopy which revealed high surface porosity. Air-dried hydrogels were non porous and showed no open channel like structures. Both air and freeze-dried hydrogels showed pH sensitive swelling with pronounced swelling in pH 2. Acidic medium (pH 2) offered chain relaxation which was confirmed by Fourier transform infrared spectroscopy. Amoxicillin release from freeze dried hydrogel showed controlled release properties as compared to air dried hydrogels, making it an ideal candidate for the controlled delivery of antibiotics in the acidic environment of stomach.

Regeneration and Diabetes

Participants:
R. R. Bhonde
Anandwardhan Hardikar

Date of initiation:
1996

Expected date of completion:
2000

I. Pancreatic regeneration

Background :

We have already demonstrated that sub-total pancreatectomy in STZ diabetic BALB/c mice triggers neoislet formation and reversal of diabetic status. Further studies were focused to identify the nesidioblastotic factor(s) and test their potential on duct to islet cell transformation *in vitro* as well as assess their efficiency in the reversal of diabetic status *in vivo*.

Work carried out:

In vivo studies

A cytosolic extract was obtained from the regenerating pancreas at different days of regeneration. The potency of the extract was tested by its ability to reverse the diabetic status of STZ diabetic mice after intra-peritoneal injections of the extract for a period of 3 weeks. The animals were monitored throughout the study for their diabetic status and a glucose tolerance test (GTT) was also carried out at the

were then assessed for their potency to trigger neo-islet formation in STZ diabetic animals. The conditioned medium obtained from the *in vitro* islet cultures showed effects similar to that seen with the cytosolic extract form the regenerating pancreas. Animals retained a normoglycemic status by one month and remained normoglycemic with a normal GTT throughout the period of study. After HPLC fractionation, 8 fractions were obtained and are being tested for their potency to induce neo-islet cell formation. Out of these fractions 7 have been tested negative in one round of animal assay.

One fraction of the 8 HPLC separated fractions, was found positive in its ability to reverse diabetic status in STZ-diabetic BALB/c mice (n=10). Animals receiving the fraction (FR4D) remained normoglycemic as compared to sustained hyperglycemia and even death in the diabetic sham injected group. FR4D was sequenced for 35 amino acids from the N-terminal and homology searches reveals it to be a novel protein. Further studies are planned so as to reveal the gene coding for FR4D.

Regeneration of ß-cells:

Participants:
R.R Bhonde
Yogita Shewade

Date of initiation:

Expected date of initiation:

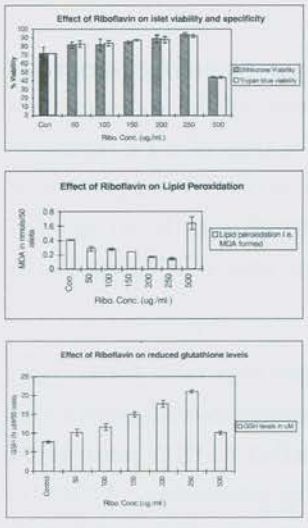


Fig. 9

II. Cryopreservation of Islets: Role of Riboflavin

Background:

Effective cryopreservation of pancreatic islets is valuable for the assessment of islet cell viability and functionality prior to transplantation. One proposed hypothesis of cryoinjury is the formation of oxygen free radicals formed during freezing and thawing. In a Attempt to find out antioxidant activity of various additives in a freezing mixture we tested effect of Riboflavin(Vitamin B2). Islets isolated from Balb/c mice were subjected to cryopreservation at -70^{0}C employing various concentrations of Riboflavin ranging from 50ug.-500ug./ml. in a freezing mixture consisting of RPMI-1640 supplemented with 20%(v/v) FCS, 2M Dimethyl Sulphoxide(Me_2So). Islets were revived after pre-determined storage period (30 days) and tested for their integrity, specificity and viability by Dithiozone and trypan blue staining respectively. The levels of reduced glutathione and lipid peroxidation in various batches of islets were measured. in cryopreserved islets basal and stimulated insulin release was also measured. It was found that viability of islets increased with the increased concentrations of riboflavin in the freezing mixture in a dose dependant manner upto 250 ug./ml. with concomitant decrease in MDA levels and increase in levels of reduced glutathione. the functionality of islets was also retained. However higher concentrations of riboflavin were found to be toxic as evidenced by high levels of lipid peroxidation and low levels of reduced glutathione. Riboflavin concentration of 250ug/ml. was found to be optimal for islet recovery (93 %). Perhaps this is the first report depicting the role and action of riboflavin leading to improved recovery of cryopreserved islets.

Regeneration O f Pancreas: III. Role of Acinar Cells: *Retention of functionality of Acinar cells on different Matrices.*

Participants:
R.R Bhonde
Savita Kurup

Date of initiation:

Expected date of completion:

Background:

The Acinar cells are highly differentiated with very little capacity of proliferation. The literature on maintenance of acinar cells *in vitro* indicates that these cells remain viable and functional for about 4 days only. To obtain secretory products of Acinar cells and to test their influence on the endocrine growth the long term maintenance is essential. Hence, studies were undertaken to define the conditions necessary for long term maintenance of differentiated exocrine gland Acinar cells *in vitro.*

Work carried out:

en for this study was Amylase Activity in terms of mM/min/mg protein and % viability on the 1st and 7th day. The various matrices taken for this purpose were 1% Gelatin, 0.5% ,1%& 2% Alginate,1% Chondroitin Sulfate,1% chitosan, 1:1 Chitosan:Gelatin, 1:2 Chitosan:Gelatin, 50mg/ml Laminin and 10mg/ml Collagen type IV. Of the various matrices used, 1:1Chitosan: Gelatin showed the maximum Amylase activity and Viability even after 7 days, therefore it was chosen for further studies. At the time of isolation, dispersed Acini in control or grown on Chitosan-Gelatin matrices responded to Carbamyl Cholin with 3.23 to 4.15 times increase in Amylase secretion in 60-mins period compared to non stimulated secretion in the same period(Table 1). After 7 days in culture, acinar cells still respond to Carbamyl Cholin with 2.6 times increase in Amylase secretion compared to non-stimulated secretion in Chitosan-Gelatin matrix, but only 1.69 times increase is seen in control. The % viability on 7th day in control is only 28% compared to 35% in Chitosan-

Gelatin matrix (Table 1). Indicating the fact that 1:1Chitosan:Gelatin permits the maintenance of acinar cells for more than 4 days as mentioned in the literature, in terms of functionality and viability.

S.NO	SAMPLE	AMYLASE ACTIVITY (uM/min/mg protein)	% VIABILITY
CONTROL (A)	FRESHLY ISOLATED		
i	Basal	0.257	100
ii	10^{-5}M Carbamyl Cholin	0.831	
(B)	CULTURE 7 DAYS		
i	Basal	0.036	28
ii	10^{-5} M Carbamyl Cholin	0.061	
1:1CHITOSAN: GELATIN (A)	FRESHLY ISOLATED		
i	Basal	0.202	100
ii	10^{-5} M Carbamyl Cholin	0.839	
(B)	CULTURE 7 DAYS		
i	Basal	0.319	35
ii	10^{-5} M Carbamyl Cholin	0.830	

Outcome of the project:

Publications

1. Shiras A, Parab P.B., **Bhonde R.R.**, New restrictions on animal experimentation where do we go from here, **Current Science** 75, 979-980, 1998.
2. **Bhonde R.R.**, Parab P.B., Muscle insulin resistance : A probable case of disuse of skeletal muscles, Lamarckism revisited. **Diabetoliga** 42 (2), 257-258 (1999).
3. Sitaswad S., **Bhonde R.R.**, Protective mechanism of glucose against streptozotocin induced free radical generation in vivo and in vitro, **Current Science** 76 (2) 231-233, 1999.
4. Hardikar A.A., Karandikar M.S., **Bhonde R.R.**, Effect of sub-total pancreatectomy on diabetic status in Balb/c mice. **Journal of Endocrinology** (IN PRESS).

Gelatin matrix (Table 1). Indicating the fact that 1:1Chitosan:Gelatin permits the maintenance of acinar cells for more than 4 days as mentioned in the literature, in terms of functionality and viability.

S.NO	SAMPLE	AMYLASE ACTIVITY (uM/min/mg protein)	% VIABILITY
CONTROL (A)	FRESHLY ISOLATED		
i	Basal	0.257	100
ii	10^{-5}M Carbamyl Cholin	0.831	
(B)	CULTURE 7 DAYS		
i	Basal	0.036	28
ii	10^{-5} M Carbamyl Cholin	0.061	
1:1CHITOSAN: GELATIN (A)	FRESHLY ISOLATED		
i	Basal	0.202	100
ii	10^{-5} M Carbamyl Cholin	0.839	
(B)	CULTURE 7 DAYS		
i	Basal	0.319	35
ii	10^{-5} M Carbamyl Cholin	0.830	

Outcome of the project:

Publications

1. Shiras A, Parab P.B., **Bhonde R.R.**, New restrictions on animal experimentation where do we go from here, **Current Science** 75, 979-980, 1998.
2. **Bhonde R.R.**, Parab P.B., Muscle insulin resistance : A probable case of disuse of skeletal muscles, Lamarckism revisited. **Diabetoliga** 42 (2), 257-258 (1999).
3. Sitaswad S., **Bhonde R.R.**, Protective mechanism of glucose against streptozotocin induced free radical generation in vivo and in vitro, **Current Science** 76 (2) 231-233, 1999.
4. Hardikar A.A., Karandikar M.S., **Bhonde R.R.**, Effect of sub-total pancreatectomy on diabetic status in Balb/c mice. **Journal of Endocrinology** (IN PRESS).

5. Shewade Y.M., Malati U.M., **Bhonde R.R.**, large-scale isolation of islets by tissue culture of audit mouse pancreas. **Transplantation proceedings** (IN PRESS)

6. **Bhonde R.R.**, Parab P.B., Sheorain V.S. An *in vitro* model for rapid screening of insulin secretogogue activity of oral hypoglycemics. ***In vitro* Cell Dev. Biol**. (IN PRESS)

7. Muragkar A, Unnikrishnan B, Padhy S, **Bhonde R.R**, Sinn E, Hormone anchored metal compounds : Synthesis, structure, spectroscopy and *in vitro* antitumor activity of 17 beta hydroxyandrost 4-en-one acetate thiosemicarbazone and its metal complexes, **Metal and Drug Bulletin Research** (IN PRESS)

8. Shewade Y., **Bhonde R.R.**, Riboflavin improves recovery of cryopreserved islets **Cryo-Letters.** (IN PRESS)

9. A.A.Hardikar, M.V. Risbud, **Bhonde R.R**, A simple microcapsule generator design for islet encapsulation. **J. Bioscience**. (IN PRESS)

Conferences Presentations

1. Parab PB, Katdare MR, Hardikar AA, Shah S and **Bhonde RR**, Immunomodulation of Experimentally induced IDDM by Shifting Gears from Th1 to Th2., ***Proc. of XXII All India Cell Biology Conference***, Trivendrum Feb. 1999

2. Makarand Risbud, Anandwardhan Hardikar, Sujata Bhat and **Bhonde RR** Chitosan-Polyvinyl Pyrrolidone Hydrogels As Candidates For Islet Immunoisolation An *In Vitro* Biocompatibility Evaluation, ***Proc. of XXII All India Cell Biology Conference,*** Trivendrum Feb. 1999

3. A.A. Hardikar and **Bhonde R.R,** Diabetes reversal ??? Reg Jerry @ nccs. pune. Awarded first prize for the best presentation at Pune University's Golden Jubilee Research Students Seminar.

Oxidative stress in diabetes mellitus and development of therapeutic strategies

Participants:
Sandhya Sitasawad

Date of initiation:
January1997

Expected date of completion:
2000

Figure 10: Effect of STZ on lipid peroxidation and viability in RIN cells. RIN cells were exposed to 1mM, 5mM and 10mM concentration of STZ. Lipid peroxidation was estimated and expressed in terms of nmole MDA/ mg protein. Viability was determined by Trypan blue dye exclusion test. Values represent an average of triplicates ± S.D.

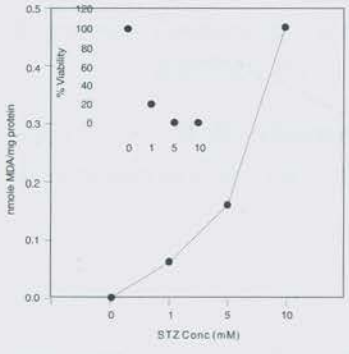
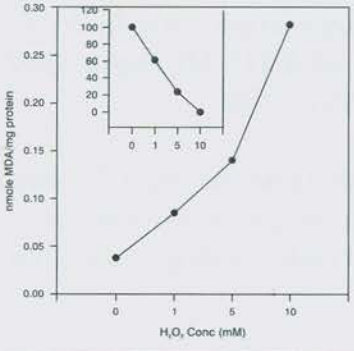


Fig. 11: Effect of H_2O_2 as a positive control on lipid peroxidation and viability in RIN cells. RIN cells were exposed to 1mM, 5mM and 10mM concentration of H_2O_2. Lipid peroxidation was estimated and expressed in terms of nmole MDA/mg protein. Viability was determined by Trypan blue dye exclusion test. Values represent an average of triplicates ± S.D



Background:

Diabetes mellitus a complex syndrome involving severe insulin dysfunction in conjunction with gross abnormalities in glucose homeostasis and lipid metabolism is progressive and is associated with high risk of atherosclerosis, kidney and nerve damage as well as blindness. Both types of diabetes are equally devastating with respect to their latter complications. Insulin-dependent diabetes mellitus (IDDM) is caused in the animals by the action of chemicals (streptozotocin and alloxan) or by the progressive autoimmune destruction of insulin-producing pancreatic beta cells in human. Although the pathogenesis of autoimmune IDDM has been extensively studied, the precise mechanisms involved in the initiation and progression of beta cell destruction remain unclear. A large number of evidences indicate that free radicals and oxidative stress may act as a common pathway to diabetes itself, as well as to its later complications.

The diabetogenic agent streptozotocin (STZ) is selectively toxic to insulin-secreting ? -cells of the pancreatic islets[1]. Recently it has been shown that STZ induced diabetes could be prevented by treatment with D-glucose and 5-thio-D-glucose[2]. Further it has been documented that alloxan induced cytotoxicity can also be reduced by glucose[3]. However, the mechanism of action of glucose in attenuating STZ or alloxan induced ?-cell injury has not been elucidated[2,3]. Present studies were therefore undertaken to ascertain the mode of action of STZ through free radical generation and its possible scavenging by glucose treatment employing *in vivo* and in vitro studies.

DM is one of the major sources of suffering and morbidity affecting millions of people throughout the world. In the past decade there has been three fold rise in the prevalence of DM. India is one of the worst affected countries and has around 20 million subjects with DM [4]. This number is likely to increase to 28 millions by the

Fig. 12 : Effect of D-Glucose on STZ induced lipid peroxidation in RIN cells and isolated islets. RIN cells and isolated islets were treated with 16mM concentration of D-Glucose prior to STZ treatment. Lipid peroxidation was estimated and expressed in terms of nmole MDA/mg protein. Values represent an average of triplicates ± S.D. ? Significantly different from control ($p< 0.05$), ? Significantly different from STZ treated cells ($p< 0.05$).

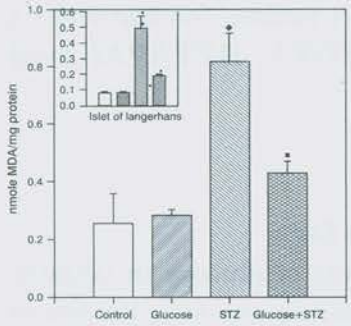
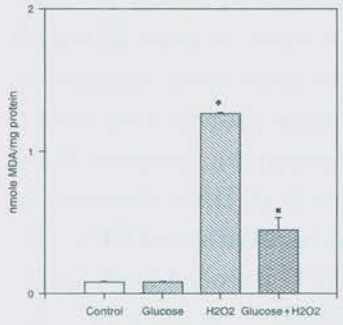


Fig. 13: Effect of D-Glucose on H_2O_2 induced lipid peroxidation in RIN cells. RIN cells were treated with 16mM concentration of D-Glucose prior to H_2O_2 treatment. Lipid peroxidation was estimated and expressed in terms of nmole MDA/mg protein. Values represent an average of triplicates ± S.D. ? Significantly different from control ($p< 0.05$), ? Significantly different from STZ treated cells ($p< 0.05$).



year 2000 and to 42 millions by the 2010. China, another highly populated country also has 20 million diabetics and their number is increasing each year by a 750, 000. DM adversely affects the ability to control blood glucose level[5].

Despite extensive research there is still a dearth of precise knowledge about the pathogenesis of diabetes. Several environmental factors like viruses, chemicals and diet are suggested to act as primary agents damaging pancreatic beta cells or as triggering agents of autoimmunity.

Free radicals are increasingly being implicated in the development and complications of DM. Relatively the low levels of free radicals scavenging enzymes and antioxidants in the beta cells make them most susceptible to free radical attack compared to other tissues[7-10]. Moreover, pancreatic beta cells contain a high levels of xanthine oxidase which is typical source of superoxide. Free radicals enhance an oxidative stress, non-enzymatic glycosylation of proteins and autoxidation of saccharides, polyol pathway and breakage of DNA and consequent increase in the activity of poly-ADP-ribose synthetase. Free radicals are also known to decrease the enzymatic and non-enzymatic antioxidant reserves[8,9] and inhibit the active calcium transport and calmodulin activated protein kinase activity[6]. Some of these aspects are required to be examined and understood experimentally inorder to develop therapeutic as well as preventive strategies.

Targets defined :

i) Determination of protective role of glucose against streptozotocin-induced lipid peroxidation, DNA and protein damage using the rat insulinoma cells (RINm5F) and mouse isolated islets.

ii) Determination of sodium nitroprusside (SNP) induced nitric oxide production in rat insulinoma cells (RINm5F).

iii) Role of the the metal compounds e.g. copper, zinc, iron and manganese in and the calcium/calmodulin antagonists which are non-specific inhibitors of nitric oxide on inhibition of nitric oxide production.

Fig.14: Effect of D-Glucose on STZ induced cell viability in RIN cells. RIN cells were treated with 16mM concentration of D-Glucose prior to STZ (1mM) treatment. Viability was determined by MTT assay and expressed as % Viability. Values represent an average of triplicates ± S.D. ? Significantly different from control ($p< 0.05$), ? Significantly different from STZ treated cells ($p< 0.05$).

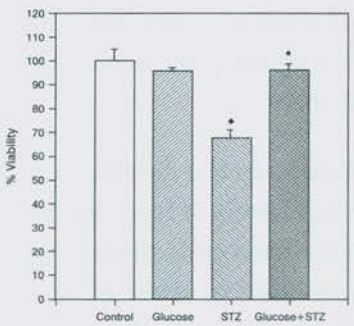
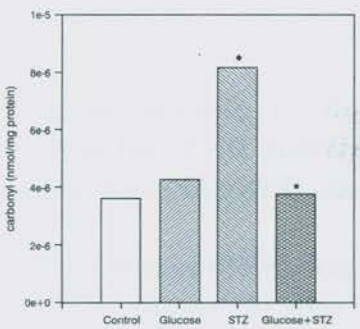


Fig.15: Effect of D-Glucose on STZ induced carbonyl content in RIN cells. RIN cells were treated with 16mM concentration of D-Glucose prior to STZ (1mM) treatment. Carbonyl groups formed were measured and expressed in terms of nmole carbonyl groups/mg protein. Values represent an average of triplicates + S.D. Significantly different from STZ treated cells ($p<0.05$).



iv) Their effect on the activity of antioxidant enzymes SOD and catalase, on lipid damage, protein damage, and DNA damage by apoptosis/cell cycle arrest and levels of insulin.

Work carried out during last year (1997-98)

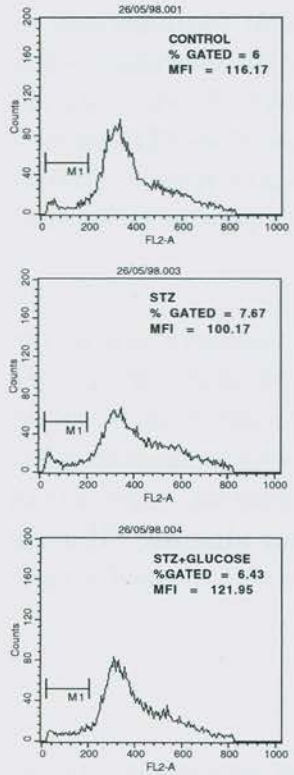
Experiments on the role of the diabetogenic agents STZ, alloxan and H2O2 in the dose and concentration dependent release of iron from the iron storage and transport protein ferritin and its inhibition by the iron chelators EDTA, DETAPAC and defrrioxamine.

Work carried out till date

Abstract of the work carried out so far :

There are many reports indicating role of glucose in cellular defences against environmental cytotoxic agents in a variety of cells, however the exact mechanism of action of glucose is not known. Present studies were therefore undertaken to investigate the role of glucose in its mechanism of action in protecting ?-cells *in vivo* and *in vitro* against cytotoxic effect of the diabetogenic compound streptozotocin (STZ). For *in vivo* studies, Balb/c male mice were treated with a single dose of STZ (200mg/kg body weight) with or without glucose treatment. It was found that none of the mice injected with glucose prior to STZ treatment became diabetic for 30 days whereas in only STZ treated group 7/8 mice became diabetic within 8-10 days. For in vitro studies RINm5F cells and isolated islets from Balb/c male mice were exposed to various concentrations of STZ with or without glucose treatment. Cytotoxic effect of STZ was studied by measuring levels of MDA as an index of lipid peroxidation and levels of DNA damage as measured by FACScan. It was found that incubation of RIN cells and isolated islets with STZ for 1 hr at 37°C induced high levels of MDA in a dose dependent manner. Similar results were also obtained when RIN cells and isolated islets were exposed to H_2O_2. The MDA levels were greatly reduced in RIN cells/islets by concomitant exposure to glucose and STZ. DNA damage was also reduced after pretreatment of RIN cells with glucose. In addition to this glucose also reduced carbonyl content of proteins in STZ

Fig. 16: Analysis of apoptosis in cells treated with STZ. Relative amount of apoptosis was measured. After 24 hr incubation without or with glucose, treated cells in suspension (optimally stained for surface immuno-fluorescence) were fixed with methanol and stained with propidium iodide. Since PI also intercalates RNA, RNAse was added incubated in the dark, at room temperature for 30 minutes and fluorescence intensity was measured



and glucose treated culture thus exerting its protective effect on proteins. Present data is thus suggestive of a protective role of glucose against pancreatic??-cell damage due to STZ adinistration or H_2O_2 exposure as evidenced by reduction in lipid peroxidation, DNA damage, protein damage as assessed by carbonyl content and higher viability of cells judged by trypan blue and MTT assay. This protective action could probably be attributed to its capacity to scavenge ·OH.

Outcome of the project

Patients: Nil
Products: Nil

Publications :

1. Effect of cryopreservation on lipid peroxidation in chick cornea. Sandhya L. Sitasawad & Milind S. Patole (1998) Indian Journal of Experimental Biology, 36 : 1025-1027
2. Membrane peroxidation and the neuropathology of Schizophrenia. Mahadik, S.P., Sitasawad,S., Mulchandani, M. (1998) In : "Phospholipid abnormalities in Spectrum Psychiatric Disorders" (In press)
3. Protective mechanism of glucose against streptozotocin-induced free radical generation in pancreatic islets in vivo and in vitro.
   Sandhya L Sitasawad and Ramesh Bhonde (1999) Current Science, 76 (2) 231-233.

Table 3: **Blood glucose status on day 15**

Group	Blood Glucose On 15th PI Day (mg/dL of Plasma)	Hyperglycemic
Normoglycemic		
Control	91 ± 11	0/8 8/8
Glucose treated	108 ± 17	0/8 7/7
Citrate Buffer	118 ± 15	0/7 7/7
STZ	360 ± 22	7/8 1/8
Glucose + STZ	110 ± 19	0/7 7/7

Immune reactions in low dose streptozocine induced autoimmune diabetes

Participants:
P. B. Parab
R. R. Bhonde

Date of initiation:
January 1998

Expected date of completion:
January 2000

Background:

Insulin dependent diabetes mellitus (IDDM) pathogenesis is invariably associated with T cell infiltration of the pancreatic islets (insulitis) and is characterised by a progressive T cell mediated destruction of insulin producing b cells. Low dose STZ induced autoimmune diabetes model in mice simulate type I (IDDM) diabetes with similar immuno pathologic profile to human disease.

In earlier studies we have observed b cell regeneration in the pancreas after intra peritoneal injections of islet cell culture supernatant (ICCS). In order to test the effect of ICCS on regeneration of b cells in IDDM animals, we have injected these animals either with ICCS or RPMI-1640 as control. Animals in both the groups were monitored for blood glucose, Glucose tolerance test, circulating islet cell antibodies at specific time intervals.

Work done:

Preliminary studies showed high levels of IgG2a and IgG1 levels in STZ treated animals as compared to the control animals. Group of RPMI treated STZ animals showed high levels of IgG2a (Th1) response which was decreased significantly (.0001) in ICCS treated STZ animals. Though IgG1 (Th2) levels in RPMI 10X treated STZ animals were initially low, significant increase in IgG1 levels was observed in ICCS treated animals.

None of the ICCS injected animals became normoglycemic at the end of the treatment however ICCS injected however, ICCS injected animal showed comparable GTT curve to control and

almost different GTT response as compared with RPMI treated animals. Attempts were made to study frequency of IFN-g and IL4 producing T lymphocytes in both experimental group. Methods are yet to be standardised.

Proposed work:

1. IFN-g, IL4, IL0-10, TNF-b cytokine profiles in ICCS treated and control animals.
2. Frequenty of cytokine producing T cells
3. Immunohistochemistry

Outcome of the project:

Publications:

1. R.R.Bhonde and P.B.Parab (1999) Muscle insuline Resistance : A probable case of disuse of skeletal muscle : Lamarkism revisited. *Diabetalogia,* **42**, 257-258.
2. A.S.Shiras, P.B.Parab and R.R.Bhonde (1998) New restriction on animal experimentation "Where do we go from here?" *Current Science,* **75** (10): 979-980.
3. Bhonde R.R., Parab P.B., Sheorin V.S. An in vitro model for rapid screening of insulin secretogogue activity of hypoglycemics. In vitro *Cell Dev. Biol.* (IN PRESS)

Patent:

1. A patent for "Improved nutrient composition useful for efffective maintenance of hybridoma cell line" (24/Bom/97). Accepted by Patent Office of India and will be advertised in official Gazette of India.

Workshop:

Organized a National Workshop on Hybridoma Technology and its' applications from 8th-23rd March, 1999 at National Centre For Cell Science at Pune. The workshop was funded by Department of Biotechnology.

Structure-function analysis of eukaryotic cells: (a) Epithelial-mesenchymal transition; (b) Interphase nucleus organization

Participants:
J. M. Chiplonkar
Madhura Vipra
Anurita Pais

Date of initiation:
July 1996

Expected date of initiation:
December 2000

(a) Epithelial-mesenchymal transition:

Background:

Epithelial cells form a tight continuous layer of cells covering a body surface or lining a body cavity. However, cohesiveness of epithelial cells gets modulated at times and they are induced to dissociate, disperse and migrate. Cell migration plays a central role in many physiological processes such as tissue formation and remodelling in embryogeneisi, angiogenesis and neovascularization, wound healing and tumour invasion and metastasis. Many factors are thought to be involved in the acquisition of cell motility. Growth factors, primarily known as regulators of cell proliferation can also stimulate cell motilityu in vitro. Growth factors are also essential for tissue repair and morphogenesis. They further play an importnt role in the expansion, invasion and metastasis of tumour cells. Some types of tumour cells are reported to produce growth factor receptors at various levels, thereby increasing their reactivities.

EMT provides one of the most striking examples of cellular transformations leading to cell motility. It is characterized by the loss of epithelial and acquisition of mesenchymal features. Cells lose their cohesive nature and are converted into individual motile fibroblatic cells. EMT is a complex process and can result from the interaction of more than one regulator. It has been reported that a scatter factor may promote EMT-like changes in vitro: e.g., HGFinfluences both cell growth and motility. Each

factor induces a particular transduction system and affect motility of cells but the net effect seen is a consequence of interaction of various transduction systems. It is important to understand the mechanism that triggers the specificity of the cellular response to the growth factor.

There is a growing body of evidence indicating that signal transduction generated by a given growth factor can follow multiple pathways. The choice of pathways that are activated my be dictated in part by the stimulus and in part by the cell itself. Using in vitro wound healing models, it has been shown that repopulation of the denuded areas occurred, by and large, by the cells migrating from the edges of the wound, rather than by cell division. This leads to the question ofhow and when the signalling pathways triggered by growth factors diverge in the cell cycle togenerate either mitogenic or motogenic response. Therefore it is important to understand the mechanisms that evoke the specificity of the cellular response to the growth factor.

Targets defined:

- Ø Characterization of epithelial cell line(s) for growth factor responsiveness, cell cycle time/ doubling time, duration of G1, S and G2 + M phases.
- Ø Analysis of 'early' and 'late' responses of the cell during wound healing.
- Ø Time kinetics of wound healing.
- Ø Wound healing response of G0 cells.
- Ø Synchronization of cells in different phases of the cell cycle
- Ø Response of synchronized cells to wound.
- Ø Effect of growth factors on wound healing.

Work done:

As reported previously, an in vitro wound healing model has been developed to study the process of epithelial-mesenchymal transition (EMT), induced by external stimuli such as growth

factors, etc. SiHa and MDBK cell lines were used for further experimental studies.

1. To assess the response of cells to pulse exposure of stimulants:

In order to determine whether continuous presence of the stimulants is required for the cells to respond, pulse exposures of 15, 30 and 60 minutes were used. Two stimulants were used, viz., foetal calf serum and epithelial growth factor (10 ng/ml), after making the wound in serum-starved monolayers. It was observed that these short exposures to the stimulants did not indice MDBK cells to migrate into the wounded areas. However, frequency of actively cycling cells appeared to be more (ass assessed by bromodeoxyuridine incorporation) than negative controls, but relatively less than the positive controls. It appeared from these results that short exposures could only induce the starved cells to proceed on cell divsion but could not induce these cells to migrate.

2. To assess cell migration in absence of cell division.

In order to confirm our earlier observation that the cells respond to the stimulus by preferring migration over cell division, cell division was arrested by mitomycin C and hydroxyurea treatment. Of the various concentrations tested, 10 ug/ml mitomycin C effectively blocked the cell division in MDBK. However, cell migration appeared unaffected and was to the same extent as in positive controls.

3. To assess the requirement for new protein synthesis for cell migration

We have previously shown that cels migrating into the wound showed up regulation of mesenchyme specific markers such as vimentin and fibroblast specific protein. In order to verify if the cell migration was dependent on new protein synthesis, we treated the cells with cycloheximide, which blocks synthesis of proteins.

Of the various concentrations tested, 5 ug/ml cycloheximide effectively blocked the cell migration into the wounds and was not toxic to the cells. Lower concentrations allowed the cells to migrate into the wound, similar to positive controls. These results indicated that new protein synthesis occurs in cells during migration.

4. To assess the effect of hepatocyte growth factor.

HGF is known to induce disruption and scattering of epithelial colonies, and EMT in some cells; it also stimulates mitogenic response. We evaluated the response of MDBK cells following treatment with HGF at various concentrations ranging from 2 to 40 ng/ml. At all these concentrations, HGF was increasingly mitogenic for these cells, proportional to the concentration of HGF, as assessed by bromodeoxyuriding incorporation. However, it did not induce any scattering of MDBK cells.

(b) Interphase nucleus organization:

Background:

In spite of rapid progress in understanding the architecture of the interphase nucleus, there is as yet little conception of how the various nuclear functions are organized in the volume of the nucleus. This is so partly because very little structural specialities of the nuclear contents have been visualized with the conventional technologies. Based on the data currently available, it appears more likely that genes will occupy domains or nuclear regions, rather than absolute points, and their location may change with respect to the cell cycle, cell physiology and / or their expression. It remains to be elucidated whether the specific location ofa gene in the interphase nucleus directly affects its expression. However, the possibility that a gene can affect its regulation by binding factors that determine its spatial localization in the cell nucleus is an important consideration that needs to be tested by experimentation. In this context, we have undertaken these studies to assess relative placement of a specific gene locus, cyclin D1, during its transcriptional activity and inactivity, which is

variable through the cell division cycle.

Targets defined:

Cell culture and related aspects.
Standardizaion of cytochemical techniques.
Standardization of immunocytochemical techniques.
Two-dimensional imaging and analysis.
Standardization of in situ hybridization.
Three-dimensional imaging and analysis.

Work done:

As reported previously, experimental protocols for simultaneous visualization of nuclear and extranuclear components using one- and two-colour immunofluorescence are being worked out. Similarly, protocols for fluorescent in situ hybridization are being standardized.

Simultaneous localization of cyclin D1 gene locus and chromosome 11 bearing the cyclin D1 gene was attempted in the interphase nuclei of randomly growing SiHa and MCF-7 cells. This was achieved by two colour fluorescent in situ hybridization using fluorescent label-tagged DNA probes. The protocols recommended by the manufacturer of the probes were followed. The preparations were studied using Kontron KS 400 Image Analysis System. Further, the preparations were also analysed with the confocal laser scanning microscope. In case of SiHa, majority of the cells showed 3 copies of chromosome 11 and 3 copies of cyclin D1 gene locus. In case of MCF-7, majority of the cells showed 5 to 7 copies of chromosome 11 territories as well as 5 to 7 copies of cyclin D1 gene locus.

One set of FISH preparations of SiHa cells was analysed in detail, using the image analysis system, for the relative location of the cyclin D1 gene loci, with reference to nuclioli and the nuclear membrane. Relative location, i.e., proximity or distality of the cyclin D1 loci were ascertained in relation to other observable structures such as nucleoli and / or nuclear membrane. From these

observations, it appeared that the cyclin D1 gene locus localized near the nuclear membrane and / or the nucleoli in 664 (=73%) and away from the nuclear membrane and / or nucleoli in 245 (=27%) of the 909 loci analysed from 307 nuclei.

Further, the two-colour FISH preparations from the same experiment were also studied with the confocal laser scanning microscope. The serial optical sections obtained from representative fields were used for 3-dimensional reconstructions. The relative positions of the cyclin D1 loci in chromosome 11 territories were assessed in these reconstructions.

In order to correlate the relative differences in the location of the cyclin D1 loci with their transcriptional status, further experimental studies using synchronized cell populations are in progress. Similarly, their characterization for cyclin D1 expressionis will also be carried out.

Outcome of the project:

Publications: Nil.
Patents: Nil
Products: Nil.

Conference:

1. Cytochemical analysis of epithelial mesenchymal transition. Jayant M. Chiplonkar and Madhura R. Vipra. XXII All India Cell Biology Conference, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, February 1999.

Identification and characterization of oncogenes implicated in melanoma

Participants:
Anjali Shiras
Varsha Shepal
Mandar Bhonde

Date of initiation:
August 1998

Expected date of completion:
August 2000

Background:

Melanoma, a cancer of the melanocytes, arises due to the uncontrolled perturbation in growth properties of melanocytes and disruption of the signal transduction cascade. Various oncogenes , cell adhesion molecules, and growth regulatory molecules of cdk family like p16 inactivation seem to be implicated in cell transformation , though an unequivocal association between oncogene expression and cell transformation have not been unrivalled for melanoma.

The project involves the identification of transforming genes in mouse melanomas using Cloudman Melanoma Clone M3 as an experimental cell system. With the automatic directional Cloning approach we have cloned a 1.2 Kb. cDNA from Clone M3 which on expression induces transformation in vivo and in vitro and also displays transfectability in culture across human, mouse and rat cell-lines. The project is aimed at characterization of this cDNA and understanding its role in the signal transduction cascade in reference to cell transformation.

Targets defined and work carried out:

The 1.2 Kb. cDNA on subcloning into vector CEV27 and transfecting across different recipient cell-lines displayed tumorigenicity in nude mice suggesting the significance of this gene in cell transformation. It was considered essential at this stage to sequence this cDNA and study its sequence homology to other known oncogenes and cell regulatory molecules.

The 1.2 Kb cDNA was sequenced by using primers from 5' and 3' ends on the automated DNA Sequenator. A major portion of the cDNA (about 700 base pairs) sequence is unambiguously

available and this sequence does not compare at the protein level with any known growth regulatory protein however the sequenced cDNA does show a very high level of homology to a Melanoma gene from Clone M2 on the EST DATA BANK. This information suggests that the cDNA is expressed at the mRNA level , however its translational product has not been identified.

Work is underway to obtain complete sequence of this cDNA and study the role of this molecule in the cell transformation process.

Outcome of the project:

Publications:

A.S. Shiras, P.B. Parab and R.R. Bhonde (1998) : New Restrictions on animal experimentation " Where do we go from here" Current Science 75(10) 979-80 : 1998.

N.Yardi, D.Divekar, H.Sant, Divate, P. , C.Apte, J.P.Singh, A.S.Shiras, S. Sitaswad and U.V. Wagh (1997) : Inter Individual Differences in Carbamazepine handling: Abstracted in Epilepsia : vol.38: Supplement 3

A.S. Shiras, V. S. Shepal, and Padma Shastry,(1999) : Identification of a novel clone from melanoma cDNA library expressing epithelial characters. In Proceedings of the " 18th. Annual Convention of IACR and National Symposium on Molecular Biology of Cancer."

A.S. Shiras, M.R. Bhonde (1999) : Cloning and characterization of a novel cDNA clone from a melanoma cDNA library. In Proceedings of the " XX11 All India Cell Biology Conference."

Patents: Nil.
Products: Nil.

Identification and characterization of a novel, bone derived factor that regulates breast cancer cell migration and metastasis.

Participants :
Gopal C. Kundu
Neeraj Tiwari
Subha Philip
Anuradha Pai

Collaborator:
Manoj Mojamdar

Date of initiation:
August, 1998.

Expected date of completion:
August, 2001.

Background:

Several types of cancers including the breast cancer are one of the major cause of death in India and around the World. Cellular migration and extracellular matrix (ECM) invasion are essential for embryonic development, inflammation, wound healing and most importantly for cancer metastasis. The invasion of cancer cell including the breast cancer is the first step in the complex multistep process that leads to the formation of metastasis. After local invasion of adjacent host tissue barriers, the cancer cell invades the vascular wall in order to disseminate. Cancer cells entering the circulation is able to evade host defenses, survive the mechanical trauma of the blood flow and arrest in the capillary bed of the target organs. Once arrested, the cancer cell again invades the vascular wall to enter the organ parenchyma. The extravasated cancer cell grows in a foreign environment different from the tissue of the origin and initiate a metastatic colony.

Breast cancer arises from epithelial cell populations that are profoundly responsive to steroid and polypeptide hormones. Hyperplasia and secretory differentiations of these cell populations occur regularly and the amplitude of these physiological processes is greatest during pregnancy, lactation and weaning respectively. The physiological processes signal discrete sets of cellular changes all controlled by a changing hormonal environment. Experimental evidence indicates that the role of hormones is central in the transformation of mammary epithelia towards neoplasia. In many instances, the endocrine dependency of breast cells such as MCF-7 or MDA-231 persist during their growth as established tumors, not only at the primary

locus of neoplasia in the breast, but in its metastases as well.

Bone is a source of various growth factors produced by osteoblasts. Osteoclast progenitors are derived from monocyte-macrophage lineage cells. Both osteoblasts and osteoclasts perform the task of bone remodeling as a tightly coordinated remodeling unit. Bone formation and resorption occur at discrete loci. The organization of skeletal tissue depends on selective interactions of cells with each other and with extracellular matrix during developmental and functional activity. The cell surface receptors, integrins have identified on the surface of osteoclast and osteoblast cells. Several ligands for integrins containing RGD cell adhesion sequences, have also discovered in both cell types. Bone is also one of the most common sites of metastasis in human breast, prostate and other cancers. Recent studies have shown that the inoculation of human breast cancer cells (MDA-231) to the nude mice developed osteolytic bone metastasis which is an unique step of osteoclast bone resorption. Although, there are more and more chemoattactant like factors are adding in the literature, the exact molecular mechanisms of migration and metastasis of human breast cancer cells to the bone is not clearly understood. Therefore, our work began with the hypothesis that the factor we are isolating from bone may have some role(s) for the control of cell motility and ECM-invasion of breast cancer cells and may regulate the osteolytic bone metastasis.

Targets defined:

1. To identify the factor from human bone extracts that induces the cellular migration and ECM-invasion of human breast adenocarcinoma cells (MDA-231) by cell migration and ECM-invasion assays.
2. To purify the factor by column chromatography including FPLC system and to check the purity by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue and silver staining.
3. To identify the N-terminal amino acid sequences of the factor by gas phase protein sequencer.
4. To produce the polyclonal antibody of the factor in rabbit and characterize it.

Fig. 17: Effect of partially purified human bone derived factor on human breast adenocarcinoma cell (MDA-MB-231) migration.

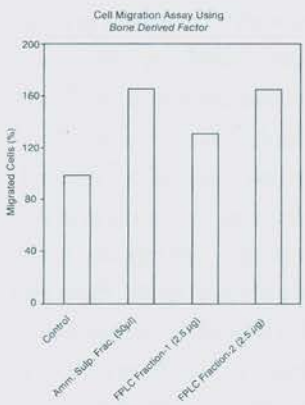
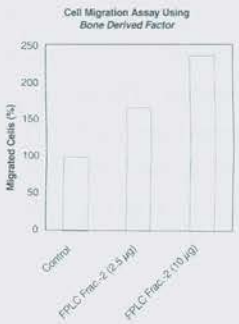


Fig.18: Effect of partially purified human bone derived factor on human breast adenocarcinoma cell (MDA-MB-231) migration in a dose dependent manner.



5. To clone the cDNA of the factor and sequence it. To demonstrate the role of the factor in bone metastasis using nude mice model.

Work carried out and proposed work:

Our data indicates that the factor isolated from the crude human bone extracts induces the cellular migration by human breast adenocarcinoma cells (MDA-231) in a dose dependent manner. The supernatants of the extracts are precipitated by ammonium sulfate and the ammonium sulfate fractions are separated by Sephadex G-50 chromatography. The active fraction containing partially purified factor is resolved by computer controlled FPLC system (Mono-Q column). All the fractions from FPLC are tested for cellular migration of MDA-231 cells by cell migration assay. The peak containing active fraction is analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue and silver staining. The active fraction showed one major and a minor protein bands. The samples are rechromatographed on FPLC (Mono-Q column) under different gradient condition and identified a sharp single peak. The activity and purity of this protein peak are analyzed by cell migration assay and SDS-PAGE respectively. The approximate molecular mass of this protein is 55 kDa in SDS-PAGE. Now we are in the process of purifying enough amount of protein for gas phase protein sequencing. As soon as the short amino acid sequences will be available, the sequences will be compared with published sequences by gene bank program. Attempts will be made to clone and characterize the cDNA of the factor if the sequences are not matched with any of the known sequences. In addition, the factor will be examined in ECM-invasion and cell adhesion assays. Furthermore, the polyclonal antibody of the factor will be generated and characterized. Finally, in order to delineate the role of the factor in bone metastasis, it will be tested in nude mice model.

Outcome of the project:

Publications: Nil
Patents: Nil
Products: Nil

Induction differentiation and apoptosis in neuroblastoma cells

Participants:
Padma Shastry
Nagarathna
DRK Mohan
Anmol Chandale
Jayashree Jagtap,

Date of initiation:
December 1993.

Expected date of completion:
December 1999

Background:

Neuroblastoma (NB) is the most common childhood tumour with the incidence having reported to have increased from 10% to 15 %. Surgery, irradiation, and chemotherapy have been employed as modalities of treatment and management of NB for many years. These lines of treatment have had some success, but provide only temporary relief, with a frequent recurrence of the disease. These factors have therefore led to a search for potential therapies in the management of NB. One of the striking features of NB is its ability to undergo spontaneous differentiation into a benign form of ganglioneuroma. Hence there is a growing interest in exploring approaches towards the induction of differentiation of NB cells as a treatment regime.

NB cell lines provide an excellent model for studying neuronal differentiation and the mechanisms therein. We have been working on the induction of differentiation using staurosporin (SSP), a broad range protein kinase inhibitor.

Protein kinase C (PKC) is a key element in signal transduction that controls cell growth and regulation and is believed to be associated with tumorigenesis.. The activity of this enzyme has been reported to be altered in different tumor types, and is linked to the initiation of growth in many cell lines. It has been reported that down regulation of PKC may be responsible for the induction of differentiation in neuroblastoma cells.

Objective: The present aspect of the study has been undertaken to investigate the role of PKC in growth and differentiation in neuroblastoma cell lines Neuro2A and its clone NB41A3.

Fig.19 : Proliferation assay of Neuro2a and NB41A3 cells was performed in the presence of different concentrations of SSP (1,10,25 and 50 nM) concentrations at different time periods (24 , 48, 72 hrs) as indicated.

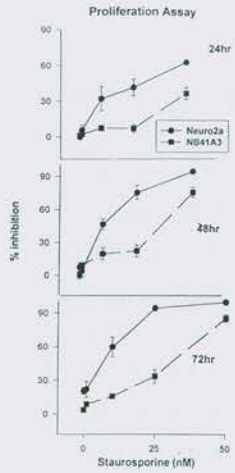
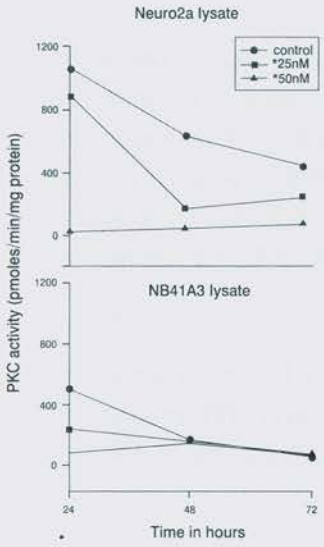


Fig. 20: PKC activity of whole cell lysate fraction in the presence of different concentrations of SSP (25 and 50 nM). Enzyme was isolated from Neuro2a and NB41A3 cells after treatment with SSP for different time periods (24, 48 and 72 hrs.).



Work carried out during the current year:

The two NB cell lines were treated with SSP for different time points The proliferation of the NB cells was assayed by 3H tdr studies. Cells were harvested at 24,48 and 72 h time periods with or without staurosporine treatment. There was dose and time dependent inhibition of proliferation upon treatment with SSP in both Neuro2a and NB41A3 cell lines. At 48Hrs and 72Hrs time points the difference was significant up to 25nM but not at 50nM concentration of SSP The pattern of inhibition is same as seen in proliferation assays in both the cell lines (Fig.19)

Phenotypic changes in NB cells were evaluated by examining the overall morphological changes indicated that treatment with SSP resulted in marked differentiation towards the neuronal phenotype. The cells became polar and showed long neurites with reduced cell density The NB41A3 cells remained clumped. SSP treatment induced cell spreading and loosening of clumps in these cells The results indicated that SSP at 25nM concentration induces morphological differentiation in Neuro2a and NB41A3.

PKC activity was assayed in total cell lysate, cytosolic and particulate fractions using a commercially available assay system The basal levels of PKC activity in total cell lysates was significantly higher in Neuro2a compared to NB41A3 at all the time points. The data with the cytosolic and particulate fraction in Neuro2a revealed higher enzyme activity at 48Hrs and 72Hrs where as in NB41A3 higher enzyme activity was observed at 24Hrs in the particulate fraction. The effect of SSP (25nM & 50nM) is depicted in Fig (20,21,22). At both the concentrations, the total cell lysate activity decreased significantly at 24Hrs,48Hrs & 72Hrs time points in Neuro2a and only at 24Hrs in NB41A3. The cytosolic fraction had lower activity at 48Hrs in both the cell lines with respect to the basal levels at the corresponding time point. SSP induced a dose dependent increase in the enzyme activity in the particulate fraction in Neuro2a at 48Hrs but not in NB41A3 cells. The results clearly demonstrate differential profiles in time course study in regard to total PKC activity in the two cell lines.

Fig. 21: PKC activity of cytosolic fraction in the presence of different concentrations of SSP (25 and 50 nM). Enzyme was isolated from Neuro2a and NB41A3 cells after treatment with SSP for different time periods (24,48 and 72 hrs.).

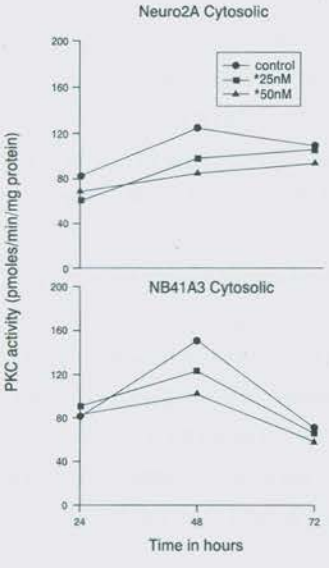
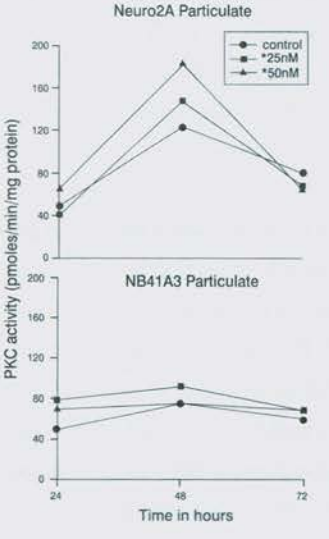


Fig. 22 : PKC activity of particulate fractiuon in the presence of different concentrations of SSP (25 nM and 50 nM). Enzyme was isolated from Neuro2a and NB41A3 cells after treatment with SSP for different time periods (24, 48 and 72 hrs.).



The data of the PKC activity and proliferation taken together in the two cell lines suggest that down regulation of PKC activity correlates with inhibition of proliferation. The data on proliferation indicated that Neuro2a cells were more sensitive to the SSP treatment with significant inhibition in DNA synthesis in a time course study. Our findings further suggest that the data on basal levels of PKC activity in tumors will be of significance in studies using PKC inhibitors as approach for therapeutic intervention.

Outcome of the project:

Mohan D.R.K, Nagarathnamma, M. Krishna, J. C. Jagtap, Padma Shastry. Differential responses of staurosporine on Protein Kinase C activity and proliferation in two murine neuroblastoma cell lines. *Cancer Letters (In press).*

Conferences:

Nagaratna R, Jayashree C.Jagtap, Padma Shastry. Staurosporine enhances binding of neuro 2a cells to type I collagen. Presented at 18th Annual Convention of Indian Association for cancer Research and National symposium on Molecular Biology of cancer, 19-21 February 1999. AIIMS, New Delhi.

Anmol Chandele, Debashis Mitra, Padma Shastry. Expression of Fas and Fas L in human neuroblastoma cell lines. Presented at XXII All India Cell Biology Conference 20-22 February 1999. Rajeev Gandhi Centre for Biotechnology, Thiruvananthapuram.

Molecular biological studies of mosquitoes

Participants:
M. S. Patole
Yogesh S. Shouche
Vyankatesh Pidiyar
P. Cyril Jaykumar
S. Prakash
Anagh Matapurlar

Date of Initiation:
April 1998

Expected date of completion:

Background:

Mosquitoes are responsible for transmission of several human diseases including malaria, filaria, Japanese encephalitis, dengue, yellow fever. Development of insecticide resistance strains has resulted in reappearance of these diseases in worrying proportions and genetic methods are being tried to control mosquito populations. This necessitates the need for complete understanding of molecular mechanisms and control circuits. With this goal in mind detailed molecular biological studies on mosquitoes are being undertaken, initially some housekeeping genes and there control elements are being investigated. Gene specific primer have been designed for the amplification of two such enzymes namely hexokinase and chitinase genes. Using these primers gene fragments are being cloned by PCR or RT-PCR amplification.

Work done:

For the studies of hexokinase gene, RNA was extracted from cultured Aedes aegypti cell line as well as larvae and used for RT-PCR. A 400 bp product obtained was cloned in pAT vector for use as a probe for screening expression cDNA library. Partial sequencing of these clones was done, but the sequence failed to show any homology with known hexokinase genes.

Chitinase was another candidate gene taken up for the studies as it shows developmental and tissue specific regulation. Using identical approach, primers designed from the conserved regions of known chitinase genes were used to PCR amplify the genomic DNAs of Aedes albopictus and Culex bitaeniorhynchus. Both showed the amplification of expected length (around 350 bp) fragment. The PCR product from Aedes albopictus was sequenced and showed homology to known chitinase sequences, thus confirming the identity. This was then cloned in the pBluescript

vector using single tube reaction procedure for the cloning of PCR products. Presence of chitinase PCR product in the while colonies was confirmed by sequencing.

Proposed work:

For the hexokinase gene, another set of primers has been designed from other conserved regions of the gene. This will be tried along with alternative approaches to pick the gene from the expression library. The cloned PCR product from chitinase gene will be used to pick up the genomic and cDNA clones for the gene and detailed studies on the organisation of the gene will be initiated. Generation of genomic library is a prerequisite for this, and it will be constructed.

Outcome of the project:

Publications:

1. Supriya G. Kshirsagar, Milind S. Patole and Yogesh S. Shouche (1998) Insect cell line authentication by DGGE. In Vitro Cellular & Developmental Biology – Animal 34: 665-667.
2. Yogesh S. Shouche, Milind S. Patole, Usha Pant, Shirish Paranjape and Kalyan Banerjee. Authentication of two cell lines developed from larval and pupal ovaries of Spodopteral litura by rRNA based method. Accepted for publication in In Vitro Cellular & Developmental Biology – Animal.

Studies on midgut flora of mosquitoes

Participanats:
Yogesh S. Shouche
M. S. Patole
Vyankatesh Pidiyar
P. Curo; Jaykumar
S. Prakash
Anagha Matapurkar

Date of Initiation:
April 1997

Expected date of completion:

Background:

Mosquitoes are responsible for transmission of several human diseases including malaria, filaria, Japanese encephalitis, dengue, yellow fever. Development of insecticide resistance strains has resulted in reappearance of these diseases in worrying proportions and genetic methods are being tried to control mosquito populations.

An alternative to generate genetically engineered mosquitoes is to manipulate their endosymbionts. Several species of mosquitoes are known to harbor endosymbionts belonging to Wolbachia sp. We screened the locla populations of mosquitoes using PCR primers that specifically amplify Wolbachia and they were found to be positive. We plan to carry out detailed analysis of these endosymbionts and their association with mosquitoes.

Mosquitoes harbor a large number of bacteria in their midgut. As this the first site any pathogen enter after entry in the mosquito body through blood meal, this becomes apromising site for the disease control. Members of gut flora can be genetically modified to express the factors that will block the development of pathogen in mosquito. We have undertaken complete study of this microflora. Male and female (before and after blood meal) mosquitoes were collected and dissected aseptically. The single guts were resuspended in sterile saline and plated on blood agar. Several types of bacteria were observed, some of which were strongly haemolytic. A quantitative increase and qualitative difference was observed in females after the blood meal. So far thirty two different types of bacteria have been isolated based on their colony morphologies and gram characters. Eight of these were found to be strongly haemolytic, which significant as they may have a role to play in nthe digestion of the blood meal.

All the attempts to culture organisms from the natural environments have underlined the limitations to culture the

bacteria from these. It is proposed that as little as 10% of the total flora is unable to grow under the controlled laboratory conditions. In order to ensure the survival of the genetically modified bacteria in the gut, it is necessary to have a complete picture of the qualitative and quantitative composition of the gut. For this culture independent methods are also being used. Bacterial small subunit ribosomal RNA (SSU rRNA 16 S rRNA) sequence analysis has neem popularly used for this.

Work done:

The haemolytic isolate was identified by complete biochemical analysis. It was identified as belonging to genus Aeromonas. This identification was independently confirmed by sending the isolate to Institute of Microbial Technology at Chandigarh, but the definitive identification up to species level could not be done. For this, complete sequencing of PCR amplified 1.5 kb rRNA gene was done and the isolate was found to be Aeromonas jandei. This was very interesting observation because this is the first report of isolation of this organism from the mosquito midgut. Normal habitat of this organism is water and it is occasionally associated with infant diorrhoea and opportunistic wound infections. This observation also emphasizes the importance of rRNA based identification memthods because biochemical characters of the isolate showed more resemblance to Aeromonal hydrophila. Sequencing of DNA of gyrase B gene showed the isolate to be very different from Aeromonas hydrophila.

Other isolates were analysed by RFLP of PCR amplified rRNA genes and those with identical RFLP were grouped together. A representative from each group was selected for identification by analysis of hypervariable region of rRNA gene. The results for this experiment are shown in Table 1.

Proposed work:

Significance of the presence of Aeromonas jandei in the mosquito

midgut will be investigated further. A representative library of PCR amplified rRNA genes from total midgut DNA will be prepared and studied for the understanding of complete microbial composition of the mosquito midgut.

Outcome of the project:

Publications:

1. Dighe, A., Yogesh S. Shouche and Ranade, D. R. (1998) Isolation of Selenomonas lipolyticum sp. Nov., an obligately anaerobic bacterium possessing lipolytic activity. Internatl. J. Systematic Bacteriol. 48: 783-791.

Table 4: Identification of bacterial isolates based on RFLP and 16 S rDNA sequencing

Isolate	Closest genera	S_ab value
A, B, C, L, M, N	Acinec\tobacter haemolyticus	0.685
6	A. Baumannii	0.585
D, E, J, 7a2, 7b, 8a, 10a, 10b	Proteus vulgaris	0.555
F	Staphylococcus epidermis	0.480
H*	Aeromonas jandai	0.920
O, G	Xanthomonas campestris	0.519
1b, 2a1, 2b1, 4a1, 4b, 9	Pseudomonas aeruginosa	0.606

· Complete gene has been sequenced.

Sequencing in progress: isolates I, K, P, Q, 1a, 2a2, 3, 4a2, 5b, 7c.

Characterization of immuno-suppression during experimental leishmaniasis in a susceptible and a resistant host.

Participants:
Bhaskar Saha
Nishant Singhal
Meenakshi B Pawa
K Venuprasad

Date of initiation:

Expected date of completion:
August 2001.

Background:

Leishmaniasis is caused by the protozoan parasite, *Leishmania sp.* The parasite resides and replicates obligatorily within the macrophages. Macrophages are one of the major antigen presenting cells which not only process and present the leishmanial antigen to T cells and thereby, provide the first signal but also express costimulatory molecules which, when engaged with appropriate ligand on T cells, deliver the second signal needed for T cells' optimal activation and effector functions. Therefore, from the stand-point of a parasite, it is logical to assume that Leishmania infection of macrophages may result in modulation of macrophage function aimed at the modulation of both the nature and the amplitude of T cell function to create a micro-milieu conducive for its survival. And all these strategies on behalf of the parasite is collectively termed as "Immune Evasion" strategy. On the other hand, the host also deploys a variety of attacking resorts which are aimed at containment of the parasite and elicitation of a protective and persistent anti-leishmanial immune response. As Leishmania gets into a macrophage, it is supposed to be destroyed by the oxidative killing mechanism of the macrophages. The resulting antigen may be presented to T cells to initiate an anti-leishmanial immune response. However, the T cells are not a population of single variety. They may be $CD4^+$ (T-helper or Th) or $CD8^+$ (T-cytotoxic or Tc). Both of them are able to secrete cytokines which may be grouped into two categories: Type 1 and Type 2. Type 1 cytokines include IL-2, IFN-g while Type 2 includes IL-4, IL-5 and IL-10. Therefore, based on cytokine profiles, there can be at least, four different types of T cells: Th1, Th2, Tc1 and Tc2. It has been proposed that while Th2 predominated response is exacerbative Th1 predominated response is protective. Mechanistically, Th1

cells produce IFN-g which activates macrophages to kill the intracellular parasite while Th2 cells produce IL-4 and IL-10 which not only inhibit the IFN-g induced macrophage activation but also inhibit Th1 cytokine secretion (so called CSIF). However, activation and secretion of cytokines of these subsets may mean that there has been antigen presentation and costimulation of T cells by the APC. On the other hand, *Leishmania* infection of macrophages causes down-regulation of B7-1 (CD80) and up-regulation of ICAM-1 (CD54) expression that eventuates in down-regulation of protective immune response. However, we do not know what happens to other costimulatory molecules like CD40, B7-2 (CD86) or M150 during Leishmania infection. In order to find out the basis of genetic resistance and susceptibility to *L. major* infection, we propose that the macrophages in BALB/c and C57BL/6 mice are different in terms of leishmania receptor expression: qualitative but not quantitative. The receptor may select out either a virulent parasite in BALB/c or an avirulent parasite as in C57BL/6 mice. The virulence of the parasite in BALB/c mice may depend on its ability to turn down anti-leishmanial immune response, e.g., by suppressing the costimulatory molecule expression, to down-regulate the activity of antigen processing, e.g., by inhibiting proton pump in the lysosome and thereby raising the pH in this organelle, and so on. Therefore, it needs be tested if there are differences in the receptor between these two mice strains.

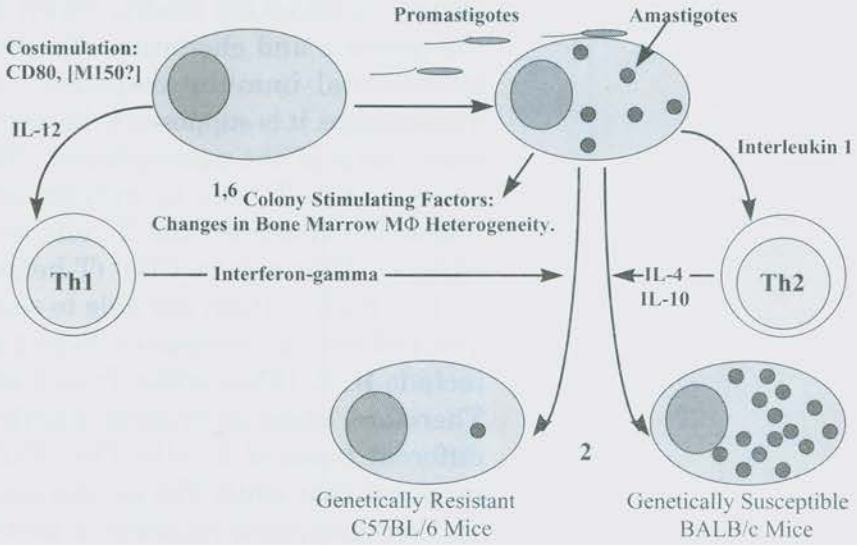


Fig. 23 : A summary of the interaction between macrophage, leishmania and T cells. Uninfected macrophages present leishmanial antigen to Th1 cells while leishmania-infected macrophages present the same to Th2 cells. Th1 cells secrete IFN-g to activate macrophages to kill the intracellular parasite while Th2 cells secrete IL-4 and IL-10 to inhibit the process and thereby promoting parasite growth.

Importance of the project.

The experiments are expected to reveal facts on the following aspects of leishmaniasis.

A. Role of non-T cell CD28 expression and function will be explored.
B. Role of different costimulatory molecules in Th subset polarization.
C. *Leishmania* receptors on macrophages as a possible basis of genetic resistance or susceptibility to Leishmania infection.

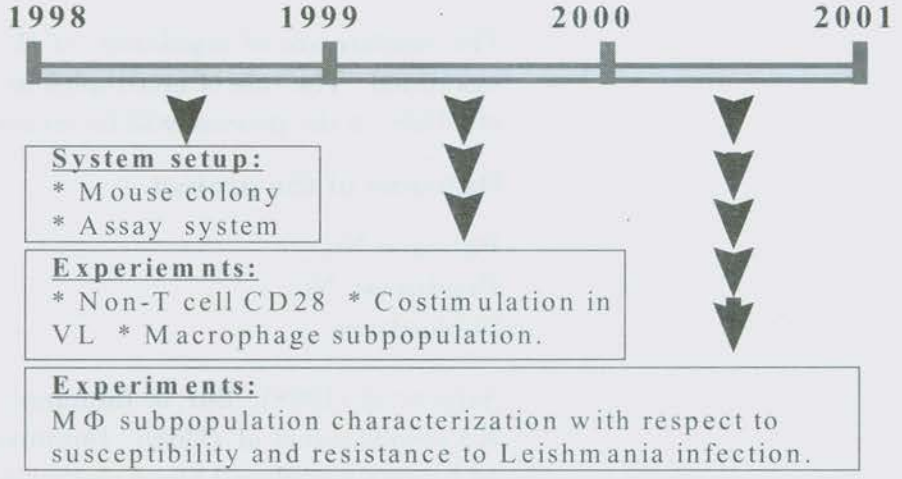
Targets defined and work carried out.:

A. 1.9.1998 - 31.8.1999. Set up of animal facility, Leishmania strains and Standardization of the assay systems.

B. 1.9.99 - 31.8.2000. Immunization of rats with macrophages from BALB/c and B6 mice. Generation of monoclonal antibodies which will be tested for differential blockade of Leishmania attachment to macrophages either from BALB/c or from C57BL/6.

C. 1.9.2000 - 31.8.2001. Identification of the molecule of difference for *Leishmania* attachment to the BALB/c and C57BL/6 macrophages.

: A Summary of Year-wise Program :



Work done till date:

CD28, described as a T cell costimulatory molecule so far, is found to be expressed on human peripheral blood neutrophils. CD28 expression on neutrophil reaches peak three hours after stimulation with PMA. Actinomycin D, an inhibitor of RNA transcription, down-regulates the PMA-induced CD28 expression suggesting that RNA synthesis is required for the regulation of CD28 expression. Staurosporin, an inhibitor of protein kinase C, inhibits the PMA-augmented CD28 expression suggesting that protein kinase C regulates the PMA-induced CD28 expression on neutrophils. Among the cytokines tested, IFN-g and IL-10 upregulate the CD28 expression on neutrophil within four hours of treatment with the respective cytokine. Immediately after CD28 cross-linking, IL-8 receptor A (IL-8RA) is found to be increased with concurrent increase in IL-8 induced chemotaxis. However, IL-8RA is found to be down-regulated three hours after CD28-cross-linking with concurrent decrease in IL-8 induced chemotactic migration. Similarly, treatment of neutrophils with PMA decreases IL-8RA down-regulation and decreased IL-8 induced chemotaxis. Thus, our results demonstrate for the first time that CD28 is expressed on human neutrophils which can be regulated and that it may play important role in neutrophil migration as required during inflammatory reactions.

Proposed work for the next year.

The mechanism of regulation of IL-8RA on neutrophil will be examined. The role of neutrophil in *Leishmaniasis* and the role of CD28 in the process will be investigated.

Outcome of the project.

Patents: Nil.
Products: Nil.
Publications:

Saha et al. (1998). Eur. J. Immunol. 28 : 4213-4220.
K Venuprasad et al. (1999). Immunobiology of CD28 expression on human peripheral blood neutrophils. (In Preparation).

SMAR1, a novel alternatively spliced gene product binds to scaffold / matrix associated retion at the T cell receptor locus

Participants:
Samit Chatopadhyay
Ruchika Kaul
Jatin Nagpal
Devraj Mogre

Collaborators:
Debashis Mitra,
Mitradas Panickar

Date of Initiation:
October 1998

Expected date of completion:
September 2001

Background:

Controls of V(D)J Recombination: Antigen receptor genes are assembled from component variable (V), diversity (D) and joining (J) gene segments through a process referred to as V(D)J recombination. The DNA sequence requirements for V(D)J recombination consists of highly conserved heptamer and nonamer DNA motifs separated by a spacer of 12 or 23 base pairs together known as 12 or 23 RSS (Recombinational Signal Sequences). In addition to these signal sequences, several lymphoid specific factors are known to be involved in V(D)J recombination. These include terminal deoxynucleotidyl transferase (Tdt) and most importantly recombination activating genes RAG1 and RAG2. Other proteins that are also required for V(D)J recombination include DNA-dependent protein kinase, XRCC4 and DNA ligase. In addition to all these factors there is another level of control through the *cis* regulatory elements. For example, the enhancer of TCR? locus is important for V(D)J recombination of the locus. Thus, the inclusion of enhancers such as E?, E?, E? and E? in recombination substrate activates D-J and V-(D)J recombination in transgenic mice. Furthermore, transcriptional activation in the germline gene segment often precedes their recombination implicating that enhancer may potentiate recombination through enhancer dependent germline promoters.

***Abnormal V(D)J recombination causes autoimmunity*:** In many cases it has been shown that autoimmune disease is associated with the clonal selection of T and B cells containing

specific receptor on the cell surface. Many of the exogenous and endogenous factors present or generated within the body become additive to the upregulation of autoimmune diseases both in human and mice. Recent reports suggests that in addition to many external and internal factors, the recombination of different receptor gene segments plays an important role in the formation of wrong joints in these segments. One of the hallmarks of SLE (Systemic Lupus Erythematosus) is the presence of autoantibodies against self antigens that are associated with the chromatin structure. Thus both the histones and DNA act as foreign antigens and severely affect normal immune responses. In this regard specific configuration of the receptor proteins get altered and misfolded such that they interact with the self instead of foreign antigens. In fact, anti-DNA and anti nucleosome antibodies show unusual V(D)J rearrangements that includes alternate D reading frames, inversion of the D segment and D-D fusions. In MRL mice that is prone to autoimmunity, atypical joining of V-D-J segments in B cells is more frequent compared to normal mice. This clearly indicates that proper joining of V(D)J segments is necessary to maintain the normal repertoire for immune responses. Thus, a clear understanding of the controls of V(D)J recombination is very important to solve the puzzle of development of autoimmune diseases.

Murine TCR? Locus: The murine TCR??locus spans 650kb, consisting of 20 V?s, two clusters of D? and J? gene segment and 2 C? exons. Genomic organization of TCR? locus is shown in Figure 1. To find out the chromatin changes associated with allelic exclusion and V(D)J recombination at the DP stage of thymocyte development and to identify *cis* regulatory elements involved, assays for DNase1 hypersensitive sites, DNA methylation and transcription in DN and DP thymocytes were perfomed. The assays were done within a 100kb region of TCR? locus starting 20kb upstream of D?1 to 50kb downstream of V?14 (Figure 1). Although, there were some differences in both transcription and methylation between DN and DP thymocytes, the results did not confirm the belief that the locus is inaccessible to the recombinase machinery at the later part of the T cell development. Upon DNaseI hypersensitivity assay, 11 sites were identified within the 100kb region (Chattopadhyay et al., J. Immunol 1998; 160,

1256) shown in Figure 1. Of these, HS9-11 present upstream of the D?1 segment, play an important role in the maintenance of chromatin structure. Deletion of these sites in mice, blocks recombination of D?1 to either J?s or V?s whereas recombination of D?2 to either of the J?s (J?1 and J?2) or V?s is not perturbed (Chattopadhyay et al.,1999, Immunity; 10, 1-20). Thus, local controls of V(D)J recombination exists within the locus. Apart from this site, two sites HS1 and 4 were found to be induced at the $CD4^+CD8^+$ double positive stage of thymocyte development. The induction of HS1 was most prominent among all. Most importantly, this site is present 400bp upstream of E? enhancer and is shown to work as silencer (Chattopadhyay et al., JBC 1998; 45, 29838).

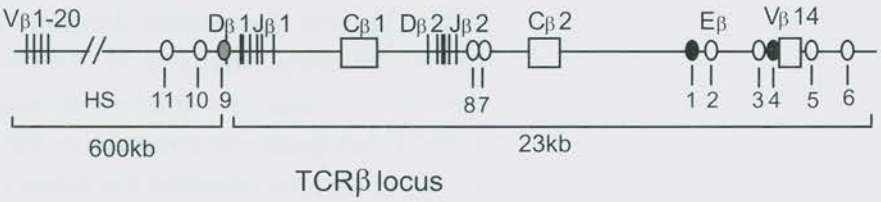


Fig. 24 : Vertical lines and boxes indicate Vß, Jß and Cß gene segments and horizontal arrows indicate transcriptional orientation of the respective gene segments.

Novel T cell specific protein SMAR1: By screening cDNA library of DP thymocyte, we identified a novel protein SMAR1 that binds to a putative MAR site within TCR? locus. SMAR1 was found to be over expressed in thymus during the transition from early to late stage development of T cells. The protein is expressed in many other tissues but is upregulated in thymus and B cells. By sequence homology, it has been shown that similar HS1 like sequence is also present next to the E? enhancer of human TCR? locus. Thus, HS1 is conserved in both human and mice. Our objective is to find out how SMAR1 controls V(D)J recombination and whether it plays any specific role in wrong joint formation during the recombination process. We predict that upon binding to HS1, SMAR1 might be affecting the normal function of E? enhancer by perturbing its interaction with promoters and other *cis* regulatory elements. This effect might be responsible for wrong joint formation during the recombination process. Thus, SMAR1 may play an important role in causing

autoimmune diseases by generating abnormal junctions of TCR genes. So far, abnormal joint formation in the receptor genes has been shown in murine models of SLE and very few information is available in case of human SLE patients. This project will thus give insight into the molecular mechanism of wrong joint formation both in T and B cell receptors in human SLE patients.

RELEVANCE

The proposal has arisen from one of the ongoing projects in the laboratory of the principal investigator. Our long term goal was to understand the role of the *cis* regulatory elements and their respective binding proteins in the control of V(D)J recombination of T cell receptor genes. PI has previously identified a *cis* regulatory element 400bp upstream of E? enhancer of T cell receptor beta (TCR?) gene, which has been characterized to be a MAR (Matrix Associated Region) site (Chattopadhyay et al., 1998a; Chattopadhyay et al., 1998b). By gel shift assays, it has been shown that two nuclear factors Cux and SATB1 bind to a 170bp DNA fragment comprising HS1 hypersensitive site (Figure 2). By transient transfection assays, it was shown that HS1 is a transcriptional silencer and may have a role in modulating E? enhancer function during V(D)J recombination. In addition to Cux and SATB1, a novel protein SMAR1 also binds to that site and alters chromatin structure in a stage and lineage dependent fashion. SMAR1 was sequenced and was found to exist in at least two forms, of which one is alternately spliced.

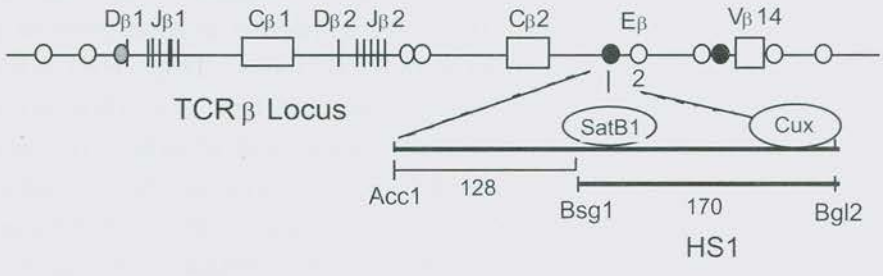


Fig. 25 : TCRß locus showing binding of proteins to HS1. The putative sites of different factors binding to HS1 are shown. Boxes indicating gene segments correspond to V? and C?. Dnase1 hypersensitive sites are shown by circles. Solid and semisolid circle represent strong and weakly induced HS sites. 128mer DNA sequence upstream of 170 HS1 fragment does not interact with any of the factors. SMAR1 binds to the 170mer DNA sequence where both Cux and SatB1 interact.

Since the identified protein is available with us, a thorough study on it can be done in this laboratory without much competition with other laboratories. By PCR analysis we found the expression of SMAR1 in human primary cells and cell lines. This stimulated PI to extend the study of this protein in human system as well as to characterize the function of SMAR1 in thymus during embryonic stage of mice. Upregulation of this protein only in the early embryonic development may indicate that the protein is important for V(D)J recombination at the early stage of T cell development. If this is correct, experiments will be performed to see blockage of the recombination process using antibodies to this protein. We will also get MRL mice which is prone to autoimmune disease and see the expression of different spliced forms of SMAR1 in thymus and quantitatively compare that to normal mice. These experiments will lead to understand the problems of abnormal junction formation during V(D)J recombination in SLE patients.

Objectives:

1. Identification of DNA binding motifs of SMAR1 by deleting 5' and 3' segments of HS1.

2. Deletion of different domains of the protein to further characterize DNA-binding and tetramerization domains.

3. Crosslinking experiments to show protein-protein interaction of SMAR1 with RAG proteins.

4. Introduction of SMAR1 protein into the thymus using retroviral mediated vectors and constructing knock-out and transgenic mice of SMAR1.

Work done:

We have identified a novel DNA binding protein SMAR1, the expression of which is upregulated in murine lymphoid cells, specifically in double positive T cells. SMAR1 binds to a *cis* regulatory element HS1, a MAR site, present next to the T cell

receptor beta (TCR?) gene enhancer E?. Homologous sequence is also present in human TCR? locus. Thus, HS1 sequence is conserved in human and mice. By PCR amplification for SMAR1, we show that one of the alternately spliced form of SMAR1 is abundant in human T cells but not in neutrophil or PBMC indicating that the spliced form of this protein is restricted to T cells. SMAR1 shows similarity with one of the B cell specific transactivator BRIGHT. Like BRIGHT, SMAR1 might play vital role in either transcription or in V(D)J recombination. By constructing transgenic and knock-out mice, its specific role in V(D)J recombination will be elucidated. In autoimmune prone MRL mice and human SLE patients, abnormal V(D)J recombination causes formation of wrong joints of TCR gene segments leading to recognition of self antigens. Involvement of SMAR1 in this aspect will be explored in detail.

Outcome of the project

Publications-

1. Whitehurst, C., Chattopadhyay, S. and Chen, J. (1999) Control of V(D)J recombinational accessibility of the Db1 gene segment at the TCR? locus by a germ line promoter. Immunity, 10, 1-20.

2. Chattopadhyay, S., Kaul, R. and Chen, J. (1999) SMAR1, a novel, alternatively spliced gene product, binds to scaffold/matrix associated region at the T cell receptor ? locus. Gene, (Under revision).

Patents- Nil
Products- Nil

Molecular & Cellular basis of HIV pathogenesis

Partcipants:
Debashis Mitra
Saurabh Gupta
Ajith Mathews
Sujata Bhade

Date of Initiation:
October, 1998.

Expected Date of Completion:
September, 2003.

Background:

Human immunodeficiency virus is the causative agent of acquired immunodeficiency syndrome (AIDS), which is defined by a reduction in the number of CD4+ T cells (less than 200 cells/ ?l) and the onset of opportunistic infections. The incidence of HIV infection has reached alarmingly high levels worldwide including India. Based on the studies done by UN AIDS program and scientists of ICMR, India, it is estimated that there are more than 3 million people infected with HIV in India and we will have the largest burden of HIV infection in the world by the turn of the century. The therapeutic strategies which are being used at present can reduce the viral load remarkably but is too expensive regimen to be used in Indian context. Thus it becomes our responsibility to put an all out effort to understand the pathogenesis of the virus and to develop new strategies to overcome the deadly disease. Keeping this in mind, I have initiated studies on some aspects of HIV-1 pathogenesis. The major objective is to gain more understanding of the virus and its interaction with he host cell, which will lead us to new anti-viral strategies. The specific aim of my plan is:

Molecular characterization of HIV induced apoptosis in T cells and elucidation of their interaction in the signalling cascade.

I would like to focus on one of the hallmark of HIV infection, i.e., CD4+ T cell depletion during the course of infection. Apoptosis has now been shown to be one of the major pathway for T cell depletion in AIDS, however, the molecular mechanism remains to be elucidated. Apoptosis appears to be greatly accelerated in HIV infection *in vitro*, and has a clinical correlate, T cells from HIV+ long term survivors undergo apoptosis in culture at a much slower rate than those from rapid progressors. Recent work has implicated two viral proteins, gp120 and Tat, in HIV induced apoptosis sensitization, however, how they promote cell death is

still unclear. The main objective of this study will be to identify differentialy expressed molecules during HIV induced apoptosis and to elucidate the interaction of those molecules in the signalling cascade leading to cell death. It will also involve identification, isolation and characterization of cysteine proteases in HIV induced apoptotic T cells. This study will also try to identify the role of two regulatory proteins of HIV, Tat and Nef in the apoptotic process. We will study the interaction of Tat and Nef with host cell proteins. Recently chemokine receptors has been implicated in HIV induced apoptosis but the mechanism remains unknown. We will try to elucidate the pathway involved in co-receptor mediated signal transduction in HIV induced cell death. Answers to these specific aims will permit not only a better understanding of the basic biologic process, but also the prospect of developing therapeutic agents to inhibit HIV-1 induced CD4+ T cell depletion.

Abstract of the work carried out:

As HIV infection experiments could not be initiated due to lack of facility, we have started staurosporine induced apoptosis in Jurkat T cell line as a model system to optimize the techniques. We have optimized propidium iodide staining of cells followed by FACS analysis and also DNA fragmentation assay. We have used RT-PCR to look for the expression of Fas and Bcl2 in control and apoptotic T cells. We have found that Fas is upregulated in staurosporine treated apoptotic T cells. We are trying right now to understand the pathway involved in staurosporine induced apoptosis in T cells.

We have also initiated transient transfection of T cell lines with plasmids encoding CAT reporter gene under the influence of HIV-1 LTR and plasmids encoding HIV-1 Tat for transactivation. The assay for CAT expression has also been optimized. We are trying to make a reporter construct expressing Green Fluorescent Protein (GFP) under the influence of HIV-1 LTR promoter. This construct will be useful in sorting out HIV infected cells from uninfected ones as described in the proposed work below.
In order to study the interaction of HIV-1 regulatory proteins

with host cell proteins, we have started cloning the genes for Tat and Nef in eukaryotic expression vectors and yeast shuttle vectors. We have obtained full length HIV-1 genome cloned in ??from NIH AIDS reference and reagent program. We are using that ??HXB2 clone to amplify different genes of the virus and clone them in eukaryotic expression vectors. We have been successful in cloning the complete Tat coding sequence in the vector pAS2-1, which will express Tat as a fusion protein with Gal4 DNA binding domain. The cloning of Nef is in progress. We are also trying to clone the cytoplasmic domain of the chemokine coreceptors CCR5 and CXCR4 to study their interaction in the signal transduction cascade in HIV infected cells.

Proposed work:

The proposed work for the next year involves making a stable T cell line expressing GFP protein under the influence of the HIV LTR promoter. Once we have the cell line, we will infect the cells with HIV. The infected cells will show increased expression of GFP due to transactivation by Tat. Then the infected cells will be sorted out from the uninfected cell using FACS. This pure population of infected cells will be then used to study the apoptotic pathway induced by HIV, using differential display of mRNA technique.

As mentioned before, we are also planning to study the interaction of two viral regulatory proteins, Tat and Nef, with host cell proteins. We are also interested in studying the signal transduction pathway of chemokine receptors in HIV induced apoptosis. For this purpose we will generate vectors expressing these proteins separately which will then be used in Yeast two hybrid system to study their interaction. The interactions will then be confirmed in Mammalian two hybrid assay.

Outcome of the Project:

Patents - Nil
Products - Nil
Publications - Nil

Studies on extracellular growth of erytrocytic stages of Plasmodium falciparum.

Participants:
P. R. Deshpande and et al.

Date of initiation:
August 1998.

Expected date of completion:
August 1999.

Background:

After successful establishment of continuous *in vitro* cultures of erythrocytic stages of human malaria parasite Plasmodium falciparum and other species, scientists were tried to culture P.falciparum without host RBCs. The parasite was able to survive for a couple of days only. However, while doing regular *in vitro* cultures of erythrocytic stages of P.falciparum by Trager and Jenson, some of the parasite cultures were started developing out side the RBCs spontaneously. The parasite stated growing extracellular continuously.

Work done:

The extracellular parasite were isolated from RBCs by using percoll density gradient and cultured in the malaria culture complete medium with and without RBCs extract. The parasites do not grow in any other media or conditions except the conditions which requied for erthrocytic stages cuslture. The parasite growth was studied by (3H) hypoxanthine incorporation and found that the parasites multiples by every 24 hours. The parasites like intracellular froms also take up (3H) ehnolamine butnot (3H) thymidine. However, the parasites were not effected by chloroquine drug which is leathal to erythrocytic forms. Whereas, iron chelators inhibits both extra and intracellular parasite growth and development is not affected by freezing (N2) in malaria cryo-medium and thawing. The parasite reactivity was studied with Mabs of P.falciparum by FACS. The Mabs : PON, POC and MSP 1 were positively reacted. PCR studies : The mutated parasite genomic DNA was used to amplifying P.falciparum specific primers by PCR. The preliminary data shows amplification of some of the primers.

Proposed Work:

SEM and TEM studies are in progress. FACS and immunoflurosence stage specific mabs, PCR and RT-PCR studies will be carried out.

Out come of the project:
Publications: Nil.
Patents: Nil.
Products: Nil.

Experimental Animal Facility

B. Ramanmoorthy
Kohale

The Experimental Animal Facility is an infrastructural service department of the Institute. It is a barrier-maintained facility for the breeding, maintenance and supply of high quality and standardized laboratory animals viz. inbred mice, rats, rabbits etc. for the ongoing research projects of the Institute. Defined barrier practices are followed scrupulously without any exception or allowance, with access to a select few personnel, to minimize the risk of microbial infection to the animals housed in the facility.

In order to initiate a scientific breeding program to propagate inbred lines, littermate breeder mice (pedigreed) of four different strains viz. BALB/cJ, C57BL/6J, DBA/2J and NOD/LtJ were procured from Institute of Microbial Technology, Chandigarh. The breeding of these mice is structured in a two-tier format, i.e. the Foundation colonies (FC) and the Production colonies (PC). The animals in the FC are propagated by strict full-sib pairing only. These colonies are the nuclear colonies for the long-term propagation of an inbred strain.

* A single male mouse with spontaneous congenital cataract was detected in a production colony of BALB/c strain of mice. Attempts are being made to establish the mode of inheritance. Also attempts are being made to separate and propagate the line as a true breeding mutant strain. The mutant colony is currently at F7 level of inbreeding.

Efforts are on to develop the necessary infrastructure for the maintenance and breeding of transgenic/knock-out mice in line with the emerging demand for these animals from the Scientists of the Institute.

The complete technical support and advice has been extended regularly to Scientists/ Research Scholars in the various aspects of animal experimentation namely, handling of laboratory animals, collection of blood and other samples, immunizations and the procurement of animals.

The breeding of laboratory animals has been planned to meet the needs of Scientists/Research Scholars for various animal

experiments. The details of the animals bred in the facility, procured from various sources, and supplied for various R & D activities are given below.

Sr. No.	Sps./Strain	Animals Procured	Animals Bred	Animals Supplied	Culled
1.	*Rat*				
	Wistar	———	357	192	105
	Lewis	12	32	4	
2.	Mice				
	BALB/c	10	5066	4642	1149
	C57BL/6	410	4154	1422	11 13
	SWISS	———	885	415	135
	NOD/LtJ	6	189	5	———
	DBA/2J	8	123	———	———
	Nude mice	32	———	32	2
	BALB/c*	———	502	———	58
3.	Mastomys	———	92	———	37
4.	*Hamster*	30	———	33	1
5.	*Rabbits*	5	7	5	1

* BALB/c with cataract mutation.

Library and documentation

N. V. Ramakrishnan
Panse

For sustained advancement of research and development programmes, easily accessible comprehensive information is an absolute pre-requisite. With this objective in view NCCS, Library and documentation facility over the years has developed necessary infrastructure and facilities to meet the information requirements of the centre. The Library and documentation facility of the centre added to its stocks 400 documents including books, reports, serial monographs and bound volumes of periodicals during the period 1998-1999

The electronic information sources available in the library which include number of CD-ROM databases such as Medline, Biotechnology abstract ,Journal citation report etc.

Facilities for direct access to international database hosts like STN is made available to users by way of renewing STN ONLINE password. The library continued to provide services like network based access to CD databases,electronic mail, internet access, current awareness services, SDI service on a regular basis on the latest R & D in any specific area chosen by the user.

The library is continued to be a member of Pune Library Network and is actively participating in the consortium.

Publications:

Growth of biotechnology literature: A bibliometric study
N.V.Ramakrishna and N.B.Pangannaya. Information Studies. Vol.5,No.1,1999,Page 25-37

Growth of Animal cell culture technology literature: A correlation between citation and publications based on growth curves

N.V.Ramakrishna and N.B.Pangannaya
Library Science with a Slant to Documentation and Information Studies (In Press) Published in March 1999 issue.

Bibliometrics of animal cell culture technology: A study based on Animal cell biotechnology
N.V.Ramakrishna and N.B.Pangannaya
Annals of Library Science and Documentation (In Press)

PUBLICATIONS AND CONFERENCES

Publications:

1. Javed N. Agrewala, Susmit Suvas, Aneesh Joshi, Archana Bhatnagar, Dass S. Vijay and Gyan C Mishra. M150 modulates the costimulatory signals delivered by B cells to T cells and enhances their ability to help B cells. *J. Interferon and Cytokine Res.(1998),18:297-304.*

2. Javed N. Agrewala, Susmit Suvas, Rakesh K. Verma and Gyan C. Mishra. Differential effect of anti-B7-1 and anti-M150 antibodies in restricting the delivery of costimulatory signals from B cells and macrophages. *J. Immunol.(1998), 160:1067-1077.*

3. Gobardhan Das, Harpreet Vohra, Bhaskar Saha, Javed N. Agrewala and Gyan C. Mishra. Leishmania donovani infection of a susceptible host results in apoptosis of Th-1 like cells: rescue of anti-leishmanial CMI by providing Th-1 specific bystander costimulation. Microbiology and Immunology, Vol. 142, no. 11. 1998.

4. Gobardhan Das, Harpreet Vohra, Bhaskar Saha, Javed N. Agrewala and Gyan C. Mishra. Apoptosis of Th1-like cells in experimental TB, Clinical Exp. Immunol. (In press).

5. Gabardhan Das, Harpreet Vohra, Kanury Rao, Bhaskar Saha and Gyan C. Mishra. Leishmania donovani infection of a susceptible host results in CD4+T cells apoptosis and decreased TH1 cytokine production, Scandinavian Journal of Immunol. (In press)

6. Manoj Raje and Gyan C. Mishra. Autoradiography. In Encyclopaedia of Immunology, 2nd edition, 1998, 296-300.

7. G.Ulrich- Merzenich and Manoj Mojamdar: Skin Fibroblasts adjacent to Rheumatoid Arthritis joints produce large quantities of hyaluronan. J. Invest Dermatol (Communicated.)

8. Anupma Sharma, Bhuvaneshwari Jagadisan, Virendar Sheorain, Vijaya Haldankar, and Manoj Mojamdar. (1998) Transient expression of high Molecular Weight, Heat Sensitive, Trypsin-Resistant Form of Tyrosinase in B16 Melanoma Cells. Pigment Cell Research 11:6 p375-79

9. Kale V. P. and Limaye L. S. (1999) Stimulation of adult human bone marrow by factors secreted by fetal liver hematopoietic cells: In vitro evaluation using semisolid clonal assay system. Stem Cells **17:** 107-116.

10. Shiras A, Parab P.B., Bhonde R.R., New restrictions on animal experimentation where do we go from here, **Current Science** 75, 979-980, 1998.

11. Bhonde R.R., Parab P.B., Muscle insulin resistance : A probable case of disuse of skeletal muscles, Lamarckism revisited. **Diabetoliga** 42 (2), 257-258 (1999).

12. Sitaswad S., Bhonde R.R., Protective mechanism of glucose against streptozotocin induced free radical generation in vivo and in vitro, **Current Science** 76 (2) 231-233, 1999.

13. Hardikar A.A., Karandikar M.S., Bhonde R.R., Effect of sub-total pancreatectomy on diabetic status in Balb/c mice. **Journal of Endocrinology** (IN PRESS).

14. Shewade Y.M., Malati U.M., Bhonde R.R., large-scale isolation of islets by tissue culture of audit mouse pancreas. **Transplantation proceedings** (IN PRESS)

15. Bhonde R.R., Parab P.B., Sheorain V.S. An *in vitro* model for rapid screening of insulin secretogogue activity of oral hypoglycemics. ***In vitro* Cell Dev. Biol.** (IN PRESS)

16. Muragkar A, Unnikrishnan B, Padhy S, Bhonde R.R, Sinn E, Hormone anchored metal compounds : Synthesis, structure, spectroscopy and *in vitro* antitumor activity of 17 beta hydroxyandrost 4-en-one acetate thiosemicarbazone and its metal complexes, **Metal and Drug Bulletin Research** (IN PRESS)

17. Shewade Y., Bhonde R.R., Riboflavin improves recovery of cryopreserved islets **Cryo-Letters.** (IN PRESS)

18. A.A.Hardikar, M.V. Risbud, Bhonde R.R, A simple microcapsule generator design for islet encapsulation. **J. Bioscience**. (IN PRESS)

19. Effect of cryopreservation on lipid peroxidation in chick cornea. Sandhya L. Sitasawad & Milind S. Patole (1998) Indian Journal of Experimental Biology, 36 : 1025-1027
20. Membrane peroxidation and the neuropathology of Schizophrenia. Mahadik, S.P., Sitasawad,S., Mulchandani, M. (1998) In : "Phospholipid abnormalities in Spectrum Psychiatric disorders (In press).
21. Protective mechanism of glucose against streptozotocin-induced free radical generation in pancreatic islets in vivo and in vitro. Sandhya L Sitasawad and Ramesh Bhonde (1999) Current Science, 76 (2) 231-233.

22. R.R.Bhonde and P.B.Parab (1999) Muscle insuline Resistance : A probable case of disuse of skeletal muscle : Lamarkism revisited. *Diabetalogia,* **42**, 257-258.

23. A.S.Shiras, P.B.Parab and R.R.Bhonde (1998) New restriction on animal experimentation "Where do we go from here?" *Current Science,* **75** (10): 979-980.

24. Bhonde R.R., Parab P.B., Sheorin V.S. An in vitro model for rapid screening of insulin secretogogue activity of hypoglycemics. In vitro *Cell Dev. Biol.* (IN PRESS)

25. Mohan D.R.K, Nagarathnamma, M. Krishna, J. C. Jagtap, Padma Shastry. Differential responses of staurosporine on Protein Kinase C activity and proliferation in two murine neuroblastoma cell lines. *Cancer Letters (In press).*

26. Supriya G. Kshirsagar, Milind S. Patole and Yogesh S. Shouche (1998) Insect cell line authentication by DGGE. In Vitro Cellular & Developmental Biology – Animal 34: 665-667.
27. Yogesh S. Shouche, Milind S. Patole, Usha Pant, Shirish Paranjape and Kalyan Banerjee. Authentication of two cell lines developed from larval and pupal ovaries of Spodopteral litura by rRNA based method. Accepted for publication in In Vitro Cellular & Developmental Biology – Animal.

28. Dighe, A., Yogesh S. Shouche and Ranade, D. R. (1998) Isolation of Selenomonas lipolyticum sp. Nov., an obligately anaerobic bacterium possessing lipolytic activity. Internatl. J. Systematic Bacteriol. 48: 783-791.

29. Saha et al. (1998). Eur. J. Immunol. 28 : 4213-4220.
30. K Venuprasad et al. (1999). Immunobiology of CD28 expression on human peripheral blood neutrophils. (In Preparation).

31. Whitehurst, C., Chattopadhyay, S. and Chen, J. (1999) Control of V(D)J recombinational accessibility of the Db1 gene segment at the TCR? locus by a germ line promoter. Immunity, 10, 1-20.

32. Chattopadhyay, S., Kaul, R. and Chen, J. (1999) SMAR1, a novel, alternatively spliced gene product, binds to scaffold/ matrix associated region at the T cell receptor ? locus. Gene, (Under revision).

33. Growth of biotechnology literature: A bibliometric study N.V.Ramakrishna and N.B.Pangannaya. Information Studies. Vol.5,No.1,1999,Page 25-37

34. Growth of Animal cell culture technology literature: A correlation between citation and publications based on growth curves N.V.Ramakrishna and N.B.Pangannaya Library Science with a Slant to Documentation and Information Studies (In Press) Published in March 1999 issue.

35. Bibliometrics of animal cell culture technology: A study based on Animal cell biotechnology N.V.Ramakrishna and N.B.Pangannaya Annals of Library Science and Documentation (In Press)

Conferences:

1. Sujit Nair and Manoj Mojamdar. Culture of Human Skin Epithelia and its Clinical Applications. *Susamvad (Golden Jubilee Research Students Seminar),* University of Pune 4-5 December 1998.

2. Vaishali Chaubal and Manoj Mojamdar. Keratinocyte-Melanocyte Interactions for the Maintainance of Normal Skin Colour. *Susamvad (Golden Jubilee Research Students Seminar)* University of Pune 4-5 December 1998.

3. Bhuvaneshwari Jagadisan, Anupma Sharma, Virender Sheroin, and Manoj Mojamdar. Phenotypic Drift in B16 Melanoma Cells: Thus far and no Farther. Accepted for presentation in forthcoming Cell Biology Conference to be held in Feb 20-22 1999.

4. Manoj Mojamdar Remelanizaton in Amelanotic B16 Melanoma Cells : A Model for Investigating Commitment in Pigment Cells. Invited Talk for the forthcoming International Workshop on Pigmentation and Pigmentary Disorders to be

held in March13-14th, 1999.

5. Vaishali Chaubal and Manoj Mojamdar Hyperpigmentation in B16 Melanoma Cells: Role of ?- Gluatmyl Transpeptidase. Accepted for presentation at the forthcoming International Workshop on Pigmentation and Pigmentary Disorders to be held in March13-14th, 1999.

6. Sujit Nair, Vaishali Chaubal, Yogesh Shouche and Manoj Mojamdar Hormone Induced Changes in the Expression of Melanogenic Enzymes. Accepted for presentation at the forthcoming International Workshop on Pigmentation and Pigmentary Disorders to be held in March13-14th, 1999.

7. Parab PB, Katdare MR, Hardikar AA, Shah S and Bhonde RR, Immunomodulation of Experimentally induced IDDM by Shifting Gears from Th1 to Th2., ***Proc. of XXII All India Cell Biology Conference***, Trivendrum Feb. 1999

8. Makarand Risbud, Anandwardhan Hardikar, Sujata Bhat and Bhonde RR Chitosan-Polyvinyl Pyrrolidone Hydrogels As Candidates For Islet Immunoisolation An *In Vitro* Biocompatibility Evaluation, ***Proc. of XXII All India Cell Biology Conference,*** Trivendrum Feb. 1999

9. A.A. Hardikar and Bhonde R.R**,** Diabetes reversal ??? Reg Jerry @ nccs. pune. Awarded first prize for the best presentation at Pune University's Golden Jubilee Research Students Seminar.

10. Jayant M. Chiplonkar and Madhura R. Vipra.Cytochemical analysis of epithelial mesenchymal transition. XXII All India Cell Biology Conference, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, February 1999.

11. Nagaratna R, Jayashree C.Jagtap, Padma Shastry. Staurosporine enhances binding of neuro 2a cells to type I collagen. Presented at 18th Annual Convention of Indian

Association for cancer Research and National symposium on Molecular Biology of cancer, 19-21 February 1999. AIIMS, New Delhi.

12. Anmol Chandele, Debashis Mitra, Padma Shastry. Expression of Fas and Fas L in human neuroblastoma cell lines. Presented at XXII All India Cell Biology Conference 20-22 February 1999. Rajeev Gandhi Centre for Biotechnology, Thiruvananthapuram.

Patent:

A patent for "Improved nutrient composition useful for efffective maintenance of hybridoma cell line" (24/Bom/97). Accepted by Patent Office of India and will be advertised in official Gazette of India.