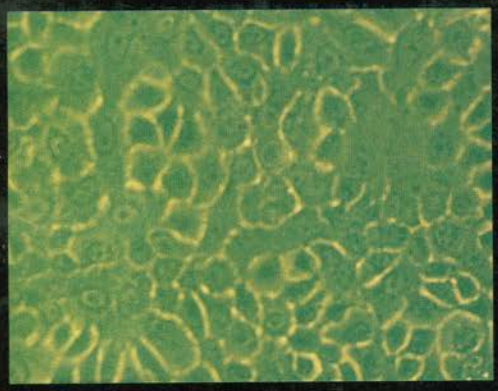
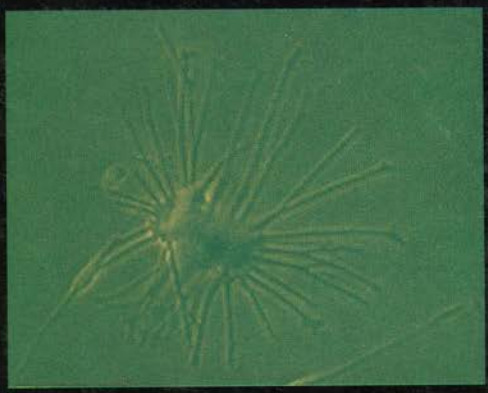
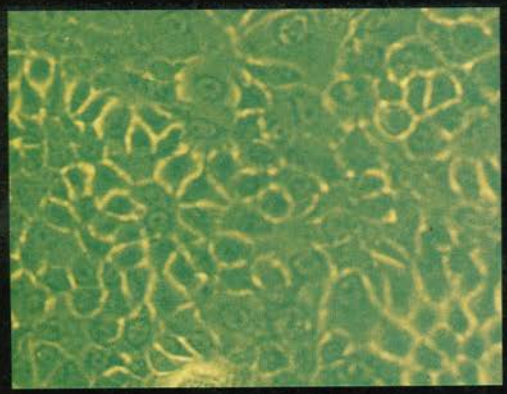
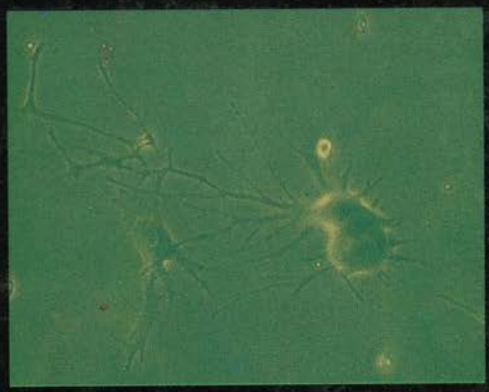
NATIONAL CENTRE FOR CELL SCIENCE









Annual Report 1997-98

NATIONAL CENTRE FOR CELL SCIENCE

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

ANNUAL REPORT
1997-98

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

Cover photo

Keratinocytes (top left panel) and melanocytes (top right panel) were plated in two separate areas of the same petri dish. The melanocyte dendrites are randomly oriented. The keratinocytes were selectively irradiated with 10 mJ UV-C (254 nm) (bottom left panel). The melanocyte dendrites tend to reorient towards the irradiated keratinocytes (bottom right panel). Phase contrast photomicrographs of live cultures (x 750). See page 11 for details.

National Centre for Cell Science
Ganeshkhind, Pune 411 007.
Annual Report 1997-98.

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Contents

Terms of reference	**1**
Preface	**2**
Culture Repository	**6**
Research projects	**7**
Development of Th1 and Th2 specific costimulatory molecules	**8**
Culture of human skin keratinocytes, their 3-D epitheliation and grafting to Burns, nevi, vitiligo and non-healing ulcer cases	**12**
Regulation of melanin skin pigmentation	**13**
Studies on cryopreservation and revival of haematopoietic cells	**16**
Stromal cell biology: definition of the factors triggering cell-cell interactions in the haematopoietic cells	**19**
Fetal liver infusion: its mode of action, efficacy of cryopreservation and its potential application in the management of cancer	**23**
Regeneration of pancreatic beta cells	**31**
Oxidative stress in diabetes mellitus and development of therapeutic strategies	**46**
Identification and characterization of oncogenes implicated in melanoma	**54**
Inducing differentiation and apoptosis in neuroblastoma cells	**58**
Structure function analysis of eukaryotic cells: (I) Epithelial mesenchymal transition; (II) Interphase nucleus organization	**63**

Molecular cross-talk between bacteria / bacterial antigens with human mucosal epithelial and submucosal mesenchymnal cell systems using cell culture as a model 68

Molecular biological studies on mosquitoes 71

Identification and characterization of protective antigens in lymphatic filariasis 75

Immune reactions in streptozotocin (STZ) induced autoimmune diabetes 76

Role of hemozoin in immune response 77

Supporting units 79

Library and Documentation
Animal House
Media

Publications and Conferences 82

Audit Report 85

Plates

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 collaboration with industry and other organizations.
- Research and development in the above and cell culture related materials and products
- To establish and conduct post graduate courses, workshops, seminars, symposia and training programmes in the related fields;
- To serve as National Reference Centre for tissue culture, tissue banking, cell products and data bank etc. and to provide consultancy services to medical, 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 organizations 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.

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Preface

It is my privilege, once again, to present this Annual Report of the National Centre for Cell Science, (1997-98). I present here an overview of the activities carried out during the last one year:

Cell Repository:

During 1997-98, a total of 407 cell cultures, comprising of 131 cell lines were supplied to 90 research institutes. Four hundred and thirty eight users have registered for availing the cell supply facility. A total of Rs. 1,88,400/- was received from the users as handling charges during the last year.

Research and development:

A short summary of the progress made in the various research projects is presented below.

The co-stimulatory molecules and the antibodies against them, their receptor on the target cell and antibodies against the receptors 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. These molecules are therefore being studied in detail. We have analyzed the role of M 150 in CD4+ T cell activation. B7-1 and M150 are potent costimulatory molecules expressed on B cells and macrophages. We have examined the capacity of Abs against B7-1 and M150 in differentially inhibiting the costimulatory signals delivered by macrophages and B cells to OVA specific CD4+ T cells. M150 has also been shown to act as a co-stimulatory molecule in the activation of CD8+ T cells.

The culture of skin keratinocytes for clinical use was continued and 9 more cases of vitiligo have been treated with cultured epithelia grafting. Follow up data from 7 cases revealed that 6 of the cases have retained their pigmentation for more than three months; two cases did not show pigmentation.

Regulation of melanin skin pigmentation is being evaluated from two aspects: dendritogenesis and phosphorylation in melanogenesis. B16 melanoma model is being used for assessing the role of melanogenic enzymes. During remelanization,. These cells expressed a high molecular weight form of tyrosinase.

Studies on the identification of melanoma oncogene(s) were continued using Cloudman Melanoma Clone M3 as an experimental cell system. Efforts were done this year to characterize the 1.2 kb gene in detail using mRNA expression studies and FACS analysis of the transfected cells. Attempts are also under way to sequence this gene to understand its role in the signal transduction cascade.

Neuroblastoma cell lines provide an excellent model for studying neuronal differentiation and the mechanisms therein. We have been studying the induction of differentiation using staurosporin, a broad range protein kinase inhibitor. The results indicate that cycloheximide, a protein synthesis inhibitor, enhanced staurosporine-induced differentiation in NB cells.

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. As reported earlier, the technology for cryopreservation of bone marrow has been standardized and is now ready for transfer to the hospitals to enable setting up of stem cell banks.

We have previously reported that bone marrow mononuclear cells released TGF beta 1 in response to erytropoietin. TGF beta 1 in turn was found to confer an adhesive phenotype on the stromal cells leading to both homotypic as well as heterotypic interactions. The heterotypic interaction of CD34+ stem cells with the stromal cells was found to result in amplification of stem cells. The signal transduction pathways involved in the stem cell amplification as well as the amplified population of the stem cells are being evaluated. These studies have revealed that the stromal regulation of stem cell proliferation appears to be dependent on the activation of PKC and rise in intracellular Ca+2 mediated vial PC-PLC pathway.

Fetal liver infusion (FLI) therapy has been attempted in certain major disorders like immunodeficiency syndrome, aplastic anemia, leukemia and genetic or metabolic disorders. Human subjects have shown extremely variable responses

to transplantation, even with a single clinical categories. Studies have been initiated with a view to understand the mechanism of autologous recovery of adult hematopoiesis in response to FLI. These studies will pave the way to establish quality control criterion for the "infusion material" and to improve the success rate of FLI therapy. The results obtained so far indicate that the fetal liver cells bring about stimulation of haematopoiesis by secretion of TGF beta 1.

A variety of physiological aspects of pancreatic beta cells are being studied using in vivo and in vitro model systems to understand the pathogensis of diabetes. These include regeneration of the beta cells, role of multigenerational malnutrition, intra-uterine protein malnutrition, oxidative stress in diabetes, and immune reactions in streptozotocine induced autoimmune diabetes.

The events occurring during migration of cells using an *in vitro* wound healing model have been extended further to record the alterations in a variety of markers such as cytokeratins, desmosomal proteins, epithelial specific antigen, fibroblast surface protein, vimentin, extracellular matrix components, DNA synthesis, etc. These studies indicate that there is a striking down regulation in epithelial markers and a concomittent up regulation of fibroblast markers. Progression of the cell division cycle appears to be temporarily and reversibly arrested in the cells, which have been induced to migrate.

Studies on the lamina propria fibroblasts have shown that production of proinflammatory cytokines and adhesion molecules by these cells, which do not classically belong to the immune system, can influence the local inflammatory reaction at the intestinal mucosal site during the LPS interaction.

Development of insecticide resistant strains of mosquitoes has resulted in reappearance of the diseases like malaria, filaria, Japanese encephalitis, dengue, etc. This necessitates the need for complete understanding of molecular mechanisms and control circuits. We have therefore undertaken detailed molecular biological studies on the mosquitoes. Local mosquito populations have been screened using PCR primers that specifically amplify the endosymbiont Wolbachia and they were found to be positive. We plan to carry out detailed analysis of these endosymbionts and their association with mosquitoes.

We have initiated studies on hemozoin pigment that is released into blood along with the merozoits as schizont infected erythrocyte bursts. Hemozoin has been found to inhibit proliferation of PHA stimulated peripheral blood mononuclear cells.

The facility for small laboratory animals has been upgraded and has started functioning in the new environmentally controlled experimental animal facility. The library and documentation area has also been strengthened by the addition of 600 documents. The library is now equipped with a dedicated LAN with 3 terminals and maintains a number of databases. The electronic mail and Internet access facilities are made available to the users.

As indicated previously, our efforts to induct more scientific and technical staff have been continued. During the current year, 3 new scientists have joined NCCS.

G. C. Mishra, Ph. D.
Director

Culture repository

During 1997-98, a total of 407 cell cultures, comprising of 131 cell lines were supplied to 90 research institute. Four hundred thirty eight users have registered for availing the cell supply facility. A total of Rs. 1,88,400/- was received from the users as handling charges during the last year.

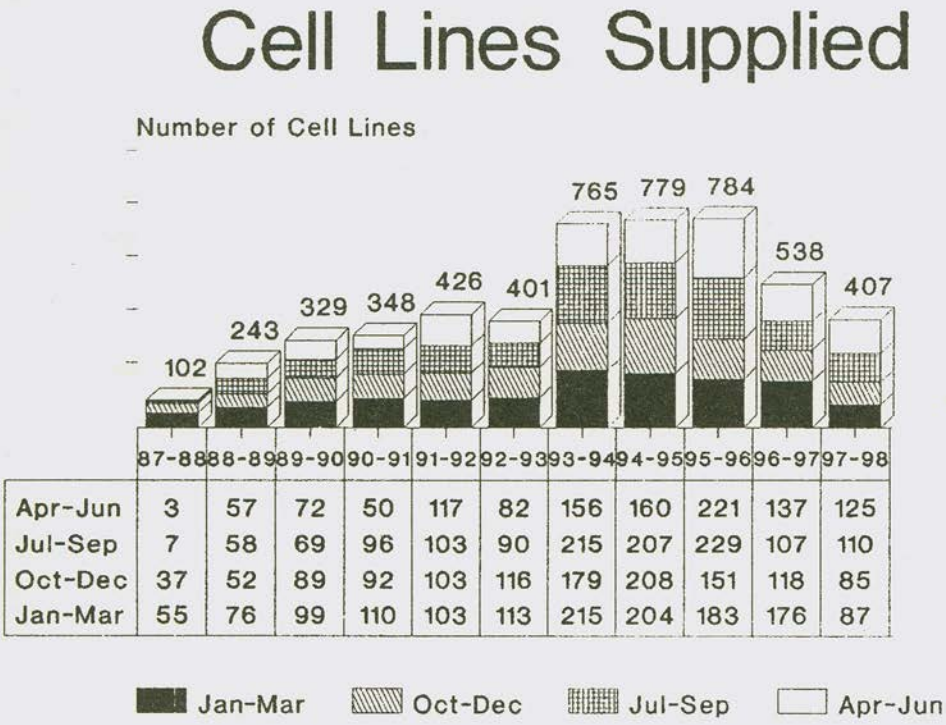


Figure 1. Cell lines supplied during 1997-98.

Research projects

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: Bhaskar Saha
P. B. Parab
Yogesh Shouche

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-γ, 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 or 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-γproduction 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. We have further analysed the role of M150 in $CD4^+$ T cell activation. B7-1 and M150 are potent costimulatory molecules expressed on B cells and macrophages. We have examined the capacity of Abs against B7-1 and M150 in differentially inhibiting the costimulatory signals delivered by macrophages and B cells to OVA specific $CD4^+$ T cells. The antiB7-1 Ab significantly blocked the proliferation of Th cells, MLR,T cell help to B cells, and secretion of IFN-γ when B cells were used to provide costimulation, but not when macrophages were used. In contrast Anti M150 antibody significantly decreased the proliferation of Th cells, MLR and production of IFN γ when macrophages were utilized to provide costimulatory signals, but not when B cells were used as APC. However, when macrophages activated with IFN-γ were used as a source of costimulation, like anti M150 Ab, Ab to B7-1 also down regulated the activation of Th cells. The significance of this finding is that M150 is a potent first costimulatory signal for initiating proliferation and secretion of IFN-γ and providing cognate help for B cells by Th cells when the macrophage is used as an accessory cell. M150 induced IFN-γ production induces the expression of B7-1 on the surface of the macrophages, which then delivers a second cosignal for Th cells. B7-1 works efficiently when B cell provides cosignal. Both of the molecules promote Th1 activity, as

evidenced by the inhibition of the secretion of IFN-γ but not IL-4 by Th cells with anti M150 and B7-1 antibodies.

2. Our lab has also found that M150 acts as a co-stimulatory molecule in activation of $CD8^+$ T cells. Our finding indicate that, on being co-stimulated by M150, $CD8^+$ T cells proliferate and acquire functional molecules like granazymes and perforin which can help in lysis of target cells.
3. Polyclonal antibodies against M150 have been raised in rabbit and Syrian hamsters. Rabbit was immunized intraperitoneally with 200μg of purified M150 followed by three booster injections of 100μg of M150 at 15 days interval. The sera were tested on both ELISA and western blot working at 1: 50,000 dilution (Plate I, Fig. 3).
4. Hamsters were immunized intraperitoneally with 100μg 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) at a ratio of 5:1. Screening of the mixed hybrids is in progress.
5. We have identified A-20 cell line, which expresses Th2 specific costimulatory molecules, B2 and B3, on their surface. In order to raise polyclonal antibody, 10^7 activated A-20 cells were immunized intraperitoneally in rats and C57BL/6. After giving two boosts with 10^6 activated A-20 cells at 7days interval, the sera was tested for antibodies against B2 molecule.
6. Polyclonal antibody has been raised against B3 molecule and gene has been partially cloned.

Outcome of the project:

Publications:

1. 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. 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.*

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

Date of Initiation:
Expected date of completion:

Background:

The cultures of skin keratinocytes for clinical uses were continued and 9 more cases of vitiligo have been treated with cultured epithelia grafting. Follow up data revealed that 5 of the cases have retained their pigmentation for more than three months. Two cases did not show pigmentation and 2 cases have yet to come for the first follow up.

The cultured epithelia are fragile, difficult to transport and hence bioartificial membranes for the growth of human fibroblasts and keratinocytes are needed. So far we have prepared Collagen-chitosan membranes. While cells grew on collagen matrix, they did not grow on chitosan alone or on collagen-chitosan matrix. Collagen-hyaluron matrix preparation is being standardized.

Outcome of the project:

Publications: Nil
Patents: Nil
Products: Nil

Regulation of melanin skin pigmentation

Participants: Manoj Mojamdar

Date of Initiation:
Expected date of completion:

Background:

Work carried out:

1. Oxidative melanogenesis:

Treatment of intact B16F10 cells with sodium periodate inactivates the ectoenzyme portion of γ-GTP -glutamyl transpeptidase. The levels of γ-GTP in these cells recovered over a period of 24-72 hrs. Parallel to this recovery the cells became hypermelanotic. Tyrosinase activities of sodium periodate treated B16 cells also increased over the same time period peaking at 48 hrs. Retreatment of these periodate treated cells with borohydride abrogated the recovery of γ-GTP and abolished the hypermelanization phenomenon.

Tyrosine increased the γ-GTP activity as well tyrosinase activity and glutamine increased the tyrosinase activity as well as the γ-GTP activity in treated as well as sham treated cells. Thus, the hypermelanization did not appear to be due to variations in substrate uptake.

Treatment with cycloheximide (10^{-6} M) resulted in 50% inhibition of cell proliferation. Despite this the tyrosinase activity increased in the treated cells. Thus there appeared to be preferential synthesis of tyrosinase over that of general proteins.

Another selective inhibitor of γ-GTP, acivicin did not allow the complete recovery of γ-GTP levels for up to 72 hrs. Also the hypermelanization did not occur in this period. Further work on the relation of these two enzymes in melanogenesis is in progress.

2. Regulation of dendritogenesis:

Melanocytes have dendrites all around in pure cultures in contrast to *in vivo* situations where the dendrites are extended only towards keratinocytes. As mentioned earlier we have devised a system of studying keratinocyte-melanocyte interactions.

It was observed that the melanocytes sent out dendrites towards the irradiated keratinocytes and there was withdrawal of dendrites from the other side. Image analysis of these cultures is in progress.

3. Role of phosphorylation in melanogenesis:

Earlier we had reported that B-16 melanoma cells cultured in low phosphate medium were highly pigmented. Phorbol esters, known activators of PKC, are a mandatory requirement for the growth of melanocytes *in vitro*. Hence, we have investigated the levels of PKC and tyrosinase in B-16 melanoma cells. The levels of PKC were found to be inversely proportional to the levels of tyrosinase activity and melanin content.

4. Melanogenic enzymes during phenotypic drift in B-16 melanoma cells:

B-16 melanoma cells are known to become amelanotic when cultured continuously. This phenomena is attributed to phenotypic drift. We have observed that B-16 melanoma cells in continuous culture undergo cyclic amelanization and remelanization. There are three enzymes known to play a role in melanin synthesis. During phenotypic drift, one of the enzymes, dopachrome tautomerase is seen to be present under both amelanized and melanized conditions. Since dopachrome tautomerase is also known to be present in the melanoblast stages, we propose that the melanoma cells in culture go through a phase of diferentiation and dedifferentiation. It was found by western blotting that the other two enzymes tyrosinase and TRP-1 were not expressed under the amelanized conditions. Investigations on the status of mRNA's of these proteins in the amelanotic B-16 melanoma cells are in progress.

5. Transient expression of a high molecular weight form of tyrosinase in B-16 melanoma cells:

We have also observed that during remelanization, the cells expressed a high molecular weight form of tyrosinase that was trypsin resistant but heat sensitive. This transiently expressed form of tyrosinase appeared to be a dimer. We have proposed that this form of enzyme was expressed to enhance substrate utilization by this melanogenesis regulatory enzyme for rapid melanization of the cells. We are testing this hypothesis by using agents that enhance melanogensis in amelanotic and melanotic cell lines.

Outcome of the project:

Publications: Nil.
Patents: Nil.
Products: Nil

Studies on cryopreservation and revival of Haematopoietic cells

Participants: L. S. Limaye

Collaborators: S. G. A. Rao, Head, Stem Cell Biol. Division, Cancer Research Institute, Mumbai
R. L. Marathe. Head, Haematology Division, Jehangir Hospital, Pune.

Date of initiation:
Expected date of completion: December 1999

Background:

This project was initiated with two objectives:
a) Research in the area of haematopoietic stem cell cryobiology.
b) Standardization of the known technology of bone marrow cryopreservation and transfer of the technology to hospitals that required it. Part (b) of the project i.e. standardization of the technology is over and the technology is now ready for transfer. Part (a) of the project is being continued.

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:

In this project our aim is to improve the efficacy of cryopreservation. We have used certain additives to the freezing solution such as antioxidants, membrane

stabilizers, antifreeze proteins and glycoproteins and methylcellulose of different viscosities. These were used in combination with 10% DMSO. Some of the additives have given improved recovery post freezing as compared to 10% DMSO alone.

Haematopoietic stem cell cryobiology:

Here our immediate goal was to improve the recovery of haematopoietic cells in terms of viability and functionality as assessed by *in vitro* colony forming assays, CFUs assays and engraftment potential of the frozen marrow. For this we have mainly used mouse bone marrow and repeated some crucial experiment on human bone marrow. Our work has so far shown that addition of bioantioxidants and membrane stabilizers do improve the post thaw recovery. The data on antioxidants as additives has been published.

The experiments on Trehalose (membrane stabilizer) were carried out further. Fresh and frozen mouse bone marrow cells were injected in lethally irradiated mice and the *in vitro* CFU - S assay was done. Results showed that when the bone marrow was frozen with Trehalose the colonies formed on spleen were more than those formed by cells frozen with DMSO alone (Fig. 3). *In vitro* assays for primitive haematopoietic progenitors such as high proliferative potential colony forming cells (HPPCFC) & long term culture initiating cells and (LTC-IC) been standardized and the results indicated that Trehalose indeed was a useful additive for freezing of haematopoietic cells. Engraftment studies on fresh and frozen mouse bone marrow have been initiated and the results were encouraging. However the experiments need to be repeated to get reproducible results. FACS analysis of fresh and frozen bone marrow for surface antibody staining using a panel of MAbs is being carried out. The profile of different surface markers before and after freezing is being studied.

Proposed work for the next year:

Studying the behaviour of haematopoietic stem cells before and after cryopreservation with special reference to differentiation and engraftment potential.

Outcome of the project:

Publications:

Limaye L. S. (1997) Bone Marrow Cryopreservation: Improved Recovery Due to Bioantioxidant Additives in the Freezing Solution. *Stem Cells* **15**: 353-358.

Patents: Nil.
Products: Nil.

Conferences:

Attended as participant in "International Workshop on Molecular Biology of Stress responses." at BHU from 13/19/97 to 18/10/97.

Figure 3: Colony forming units spleen (CFU-S).

1. Irradiated mice infused with fresh cells.
2. Mice infused with frozen cells (10%DMSO alone)
3. Mice infused with frozen cells (10%DMSO + Trehalose)
4. Ctrl Irradiated mice infused with saline.

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

Participants: Vaijayanti P. Kale
L. C. Padhy, Tata Institute for Fundamental Research, Mumbai

Date of initiation: *First phase*: December 1993
Second phase: December 1997
Expected date of completion: *First phase*: December 1997
Second phase: December 1999

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 co-operation with a large number of soluble 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 microenvironment *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 bone-marrow 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 , such as c-kit ligand (SCF),TGF, TNF etc. are required to convey specific signals to the

differentiating progenitor cells at stages of differentiating process. The role of various cytokines, interleukines 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 cell-cell interactions in the haematopoietic cells?" 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, this understanding may aid in the identification of defective steps in human disease process.

Targets defined and work carried out:

We have previously reported that bone-marrow mononuclear cells (BM-MNCS) released a significant amount of TGF β1 in the medium in response to erythropoietin. TGF β1 in turn was found to confer an adhesive phenotype on the stromal cells leading to both homotypic as well as heterotypic interactions. The heterotypic interaction of $CD34^{+}$ stem cells with the stromal cells was found to result in amplification of stem cells in a PKC and Ca^{+2} dependent pathway (AR 97).

Studies of signalling pathway:

Our next goal was to further define the signal transduction pathway involved in the stem cell amplification pathway and also to characterize the amplified population to define its developmental stage.

PC-PLC dependent PKC activation is reported to be involved in the TGF B1 mediated pathway in many systems. We, therefore, used D 609 to inhibit PC-PLC. This agent inhibits PC-PLC and thus secondarily inhibits PKC by preventing the generation of DAG. We observed that D 609 completely inhibited the CFU formation showing the role of PC-PLC dependent activation of PKC in the process.

Characterization of amplified population:

We had demonstrated earlier that the amplified population obtained by treatment of MNCs with TGF β1 treated stromal cells was positive for CD34 while negative for CD 33 and CD 38 indicating its primitive nature. These cells were characterized after a culture period of 7 days to determine the lineage commitment. It was observed that the cells were still CD 34^+, though the intensity was less, but many had become CD 38^+ and also TdT $^+$.CD 38 is a marker for differentiation commitment and TdT is a very early marker for lymphoid commitment. We plan to extend these studies by FACS analysis.

Competency of stromal cells:

We have observed that CM/TGF treatment induced a "competent" state on the stromal cells .We carried out experiments to decide whether the competence of stromal cells *per se* can affect colony formation from a fixed population of BM-MNCs. We exposed a fixed number of BM-MNCs to increasing dose of TGF β1 treated stromal cells and plated these cells for colony formation. It was observed that both control as well as TGF β1 treated stromal cells caused increased colony formation in a dose dependent way. However, the colony formation by the lowest dose of TGF β1 treated stromal cells was significantly higher than the highest dose of control stromal cells, suggesting that the competence of stromal cells could play a deterministic role in the stem cell behaviour.

Sequestration of progenitors:

We carried out a set of experiments to support our earlier observation that the $CD34^+$ cells adhered better to TGF treated stromal cells than to control stromal cells. We "panned" a fixed number of BM-MNC three times over control as well as TGF β1 treated stromal cells and compared the colony formation ability of BM-MNC both before and after "panning". It was observed that the colony formation after panning over control stromal cell was reduced by 23% while that after panning over TGF treated stromal was reduced by 80%.The results indicated that the competent stromal cells specifically sequestered progenitor/stem cells.

Outcome of the project:

The study has underscored the importance of activation state of stromal cells and cellular cross talk in the development of hematopoiesis. The stromal regulation of stem cell proliferation appears to be dependent on activation of PKC and rise in intracellular Ca^{+2} mediated via PC-PLC pathway.

Publications: Nil.
Patents: Nil.
Products: Nil.

presence of stimulatory activity in the fetal liver extracts The stimulatory factor(s), however, have not been identified.

We have initiated these studies with a view to understand the mechanism of autologous recovery of adult hematopoiesis in response to FLI. Our contention is that if the mechanism by which fetal liver cells bring about the stimulation of adult bone marrow cells is understood, then it would pave the way to establish quality control criterion for the " infusion material " and to improve the success rate of FLI therapy.

Effect of co-culture with M-FLHCS on clonal growth of adult MB cells:

We tried to mimic the *in vivo* situation of "Fetal Liver Infusion "by mixing M-FLHCs with adult bone marrow cells to be plated for clonal assays .The FLHCs were treated with Mitomycin C as described in the methods section to inhibit their colony formation . The cells were washed extensively to remove the drug. The dose and time of Mitomycin C treatment was optimised in such a way that the viability of the cells was maintained but their colony forming ability was blocked.

Various numbers of M-FLHCs were mixed with 2×10^5 BM cells and this mixture was plated in semisolid medium with limiting concentrations of 5637 CM and Epo (50 μl and 1 U respectively, N = 6). Number of colonies formed in the plates with and without FLHCs was scored after 14 days and a mean of replicate plates was calculated. The average number of colonies obtained with or without the addition of M- FLHCs was compared to calculate fold increase.

It was observed that M-FLHCs stimulated the colony formation as indicated by 2- 4-fold increase in the plates with M-FLHCs over those without M-FLHCs. The stimulation was evident with as low as 500 M-FLHCs. Figure 4 shows the data from representative experiments carried out independently using different BM cells and M-FLHCs. M-FLHCs never formed colonies under the assay conditions used. Similarly omission of 5637 CM and Epo from the plates resulted in lack of colony formation irrespective of the presence or absence of

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

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

Participants: V. P. KALE
L.S.LIMAYE

Background:

Fetal Liver Infusion (FLI) therapy has been attempted in certain major disorders like immunodeficiency syndrome, aplastic anemia, leukemia and genetic or metabolic disorders. Though the engrafting capacity of transplanted fetal liver cells has been successfully demonstrated in animal models, human subjects have shown extremely variable responses to the treatment, ranging from complete recovery on one hand, to no response on the other, even within a single clinical category. Most of the animal studies ,however, are done with complete pre-transplant immune suppression while in the case of human subjects such preconditioning was not followed routinely. The failure of engraftment, therefore, has been thought to be due to lack of proper preconditioning and in turn unavailability of stromal niches. Recently it was shown that preconditioning may not be absolutely essential for successful engraftment to take place. It is also speculated that there exist histocompatibility as well as ontogenic barriers which interfere with the engraftment. Experimental results indicate that adult bone marrow stromal cells induced apoptosis in FL derived BFU-E, thereby suggesting that FL progenitors may be incapable of differentiating in an adult environment. Though such studies partly explain the failure of engraftment, the mechanism of recovery observed in the patients who responded to the therapy is not well understood. Since the regenerated hematopoiesis is of recipient origin, it is assumed that fetal liver cells secrete some growth factors, which stimulated the patients marrow cells. Several experimental studies carried out in this direction have demonstrated the

FLHCs . The colonies formed were of mixed lineage and no bias towards any particular lineage was observed, as assessed by Wright's - Geimsa staining of colonies picked up from the methyl cellulose.

Quantitation of TGF β1 in FLCM by ELISA:

In earlier experiments, we had detected TGF B1 in the FLCM by western blot and had shown that TGF b1 was the principle factor responsible for the colony stimulation (AR 97). We carried out ELISA experiment to estimate the quantity of TGF ß1 in the FLCM. We used TGF ß1 ELISA kit (Genzyme) for the purpose. The assay was carried out according to the manufacturer's instructions. Four FL-CM preparations tested in this experiment contained TGF β1 in the range of 651 pg /ml to 2387 pg /ml .(after correction for medium values, Figure 5). The effect of these FLCM on colony formation was directly related to the concentration of TGF ß1 in the FLCM. (data not shown).

Effect of purified TGF β1 on colony formation:

Since the results obtained so far indicated the role of TGF β1 in the observed phenomenon ,we carried out experiments to check whether TGF β1 also acts as a stimulator or not. We used commercial preparation of recombinant TGF β1(Bohringer Manheim). at various concentrations covering this range in the assay plates along with 5637 CM and Epo at limiting concentrations. The results of these experiments (N=7) showed that TGF β1 stimulated the CFU-GEMM formation when added in the range of 1-50 pg /ml. (Fig. 6).

We also included SCF in one set of experiment at the concentration of 500 pg /ml along with various concentrations of TGF β1 in CFU assays .It was observed that TGF β1 showed an additive effect on the colony formation when used in the range of 1 pg to 100 pg but was clearly inhibitory when used at 200 pg (Fig. 7).

Our results indicate that fetal liver cells bring about stimulation of haematopoiesis by secretion of TGF β1.

Outcome of the project:

Publications:

Kale, V. P. and Limaye, L. S. Stimulation of adult human bone marrow by factors secreted by fetal liver haematopoietic cells: In vitro evaluation using semi-solid assay system. Communicated to

Patents: Nil.
Products: Nil.

Conferences:

Attended **"INTERNATIONAL WORKSHOP ON MOLECULAR BIOLOGY OF STRESS RESPONSES"** Organized by Banaras Hindu University and German Ministry of Research, Germany. October 14-17, 1997.

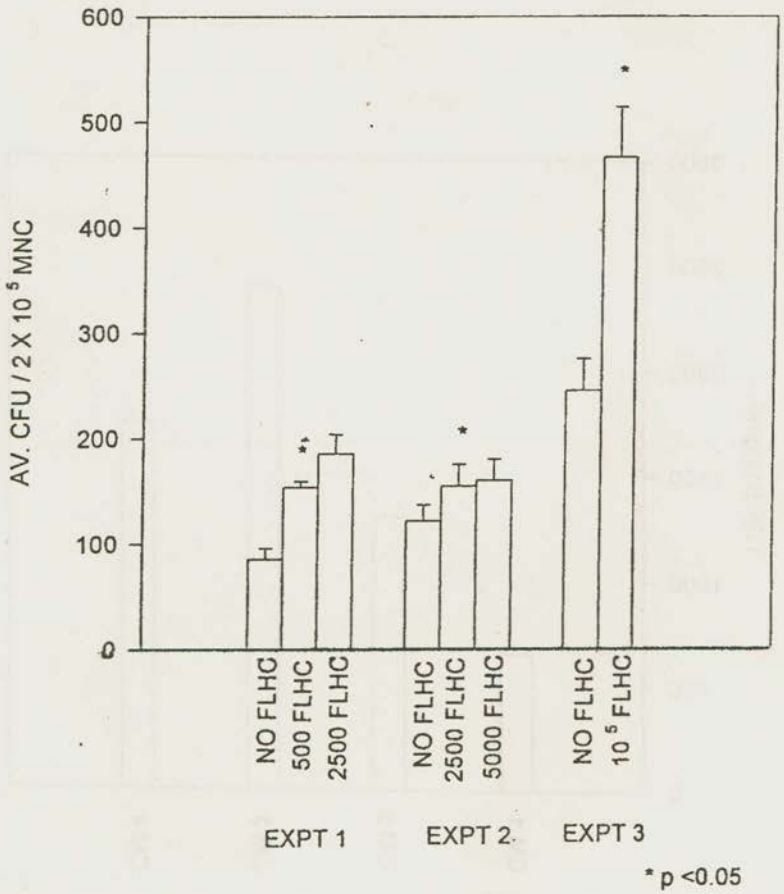


Figure 4: Effect of M-FLHCs on the semi solid colony formation of human bone marrow derived mononuclear cells. Clonal cultures were established in methyl cellulose medium with limiting concentrations of 5637 CM and Epo.2 x 10^5 BM MNCs were mixed with indicated number of M-FLHCs The cellular mixture was plated for colony formation. The results were expressed as mean of replicate plates. Results of three independent representative experiments are shown. * $p < 0.05$

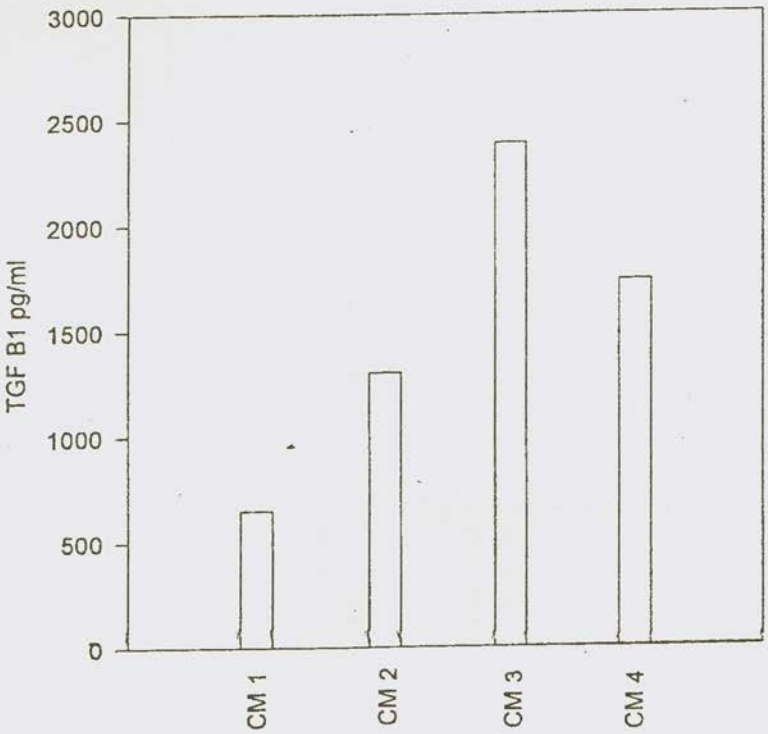


Figure 5: Immuno detection of TGF β1 in FLCM using ELISA. The assay was carried out as per the manufacturer's instructions. Briefly the CM samples were activated with 1N HCL and subsequently neutralized with 1N NaOH. Culture medium of the same batch and treated exactly as the CM was used as control . The corrected values are depicted in the graph.

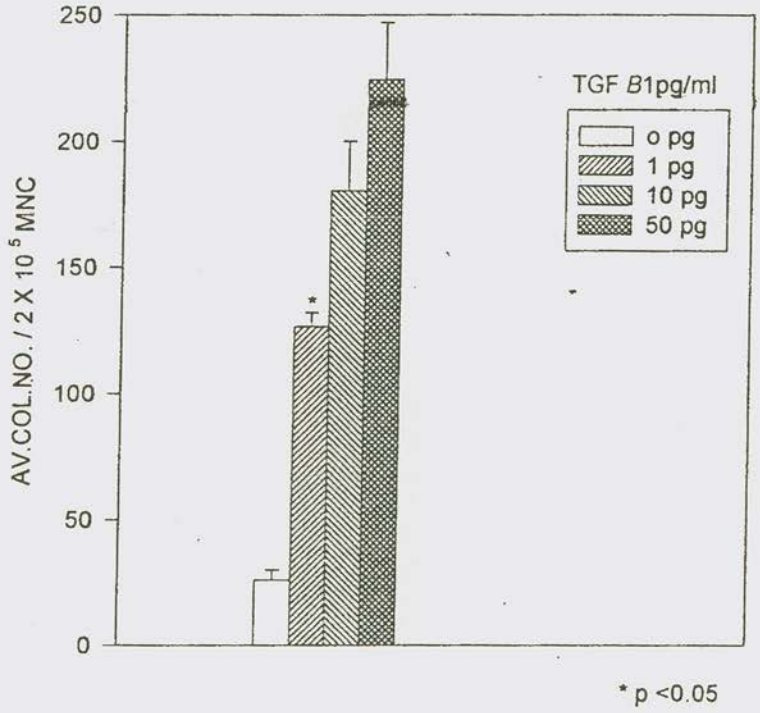


Figure 6: Effect of TGF β1 on colony formation of human BM MNC. Clonal cultures were established with limiting concentrations of 5637 CM and Epo. TGF β1 was added in various concentrations as indicated. Mean of replicate plates was calculated. Figure shows the result of a representative experiment. *p<0.05

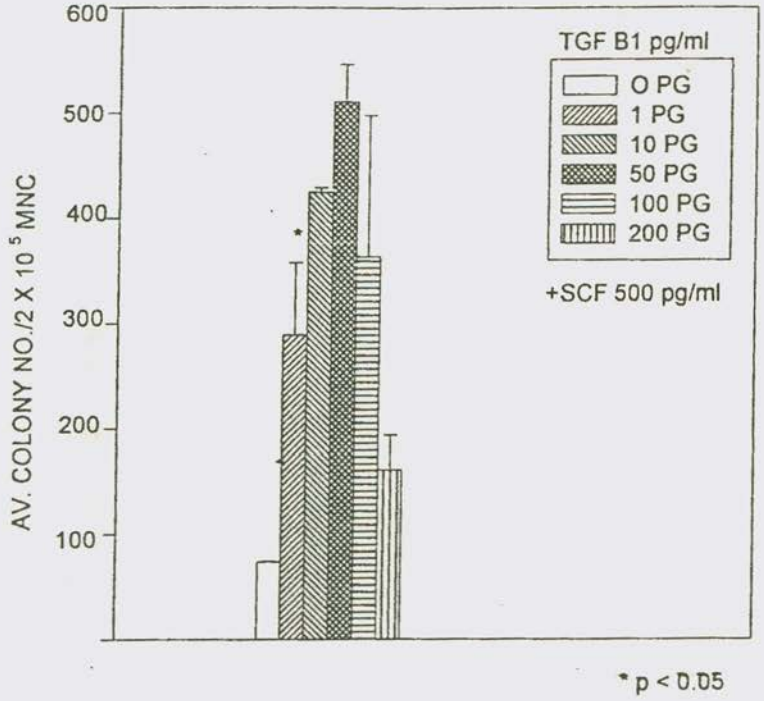


Figure 7: Effect of TGF β1 on colony formation of BM MNC in the presence of SCF. Clonal cultures were established with 500 pg/ml of SCF along with limiting concentrations of 5637 CM and 1 U Epo. TGF β1 was added at various concentrations as indicated. Plates without SCF and without TGF β1 were also set. Mean of replicate plates was calculated. * $p < 0.05$

Regeneration of pancreatic β-cells

Participants: R. R. Bhonde
Anandwardhan A. Hardikar
Savita Kurup
Yogita Shewade
Malati Umrani

Date of initiation: December 1994
Probable date of completion: December 1999.

A. Regeneration of pancreatic β-cells

Background:

The pancreatic β-cell is highly specialized for its vitally important function; the production and release of insulin to regulate the blood glucose levels. The number of functionally intact β-cells in the islet organ is of decisive importance in the development, course and outcome of diabetes mellitus. Elucidation of regenerative potential in experimentally induced diabetic animals would be of interest as an alternate therapy for diabetes. The pathogenesis of diabetes has been viewed by some as balance between the destructive and regenerative processes. The therapeutic goal would then be to down-regulate the destructive processes and to upregulate the regenerative ones. As 3 % of adult islets are still capable of proliferation, a specific factor may be used to enhance β-cell proliferation; in terms of either mass or number and tilt the balance towards regeneration in the diabetics. The general approach of regenerative biology is to identify the cellular and molecular differences that distinguish tissue embryogenesis from wound repair and then to re-create an embryonic (regenerative) environment in an malfunctioning adult tissue. Hence, it is important to define the growth factors responsible for proliferation of islets and islet-stem cells. Such studies would enhance our understanding of the patho-physiology of diabetic pancreas and perhaps offer novel approaches to the cure of diabetes.

Objectives:

1. To develop methodology for large-scale isolation of islets and their long-term maintenance in culture / cryopreservation.
2. To find out the factors affecting the growth of β-cells *in vivo* and *in vitro*.
3. To ascertain the role of exocrine pancreas / liver in promoting β-cell proliferation.
4. To study the effects of hyperglycemia and other environmental insults on early development using chick / mouse embryo culture systems.
5. To ascertain the role of nutrition on the induction and prevention of experimental diabetes
6. To ascertain the role of immuno-suppression and immuno-stimulation in modulation of the course of IDDM
7. To study the cell-matrix and cell-cell interactions during pancreatic development.

B. Chronic Multigenerational Malnutrition and Diabetes

Background:

Chronic multigenerational malnutrition is known to alter the overall fetal development. However its influence on the endocrine pancreatic development and diabetogenic susceptibility have not been documented as yet. We studied two colonies of Wistar rats *viz.* the malnourished (M) colony that was reared for 25 generations on a diet low in protein (12%), fat (<1%), water and calories (50% of *ad libidum* diet). The control colony rats (C) were reared on a diet containing 21% protein, 7% fat and *ad libidum* food and water. In order to study the effect of multigenerational malnutrition on diabetes susceptibility we carried out a STZ dose shift study to test, if any, the altered diabetic susceptibility in the malnourished group.

Work carried out:

- ***In vivo* studies:**
  We compared the two colonies (C and M colonies) of Wistar rats. Animals of either sex at 8 to 12 weeks of age were exposed to different doses of STZ

and then followed for the changes in their plasma glucose and insulin concentrations. Data was obtained to test the effect of different STZ doses in the two colonies.

The M colony rats were lighter and showed fasting hyperinsulinemia despite lower plasma glucose concentration (table 1) suggesting insulin resistance. M rats had smaller pancreas and smaller total pancreatic insulin content. All ($n = 30$) of the M colony animals became diabetic by 48 hours after 60mg/kg STZ injection while none of the C colony animals became diabetic at this dose. Ninety three percent of the C animals became diabetic by 96 h after an injection of 200 mg/kg STZ, none died. At this dose, all the M colony rats died of hypoglycaemia within 24 hours of the injection. Our data suggests that chronic multigenerational malnutrition is associated with hyperinsulinaemia probably due to insulin resistance and that such animals have a lower threshold to diabetogenic action of STZ. Our findings have important implications in explaining the rising epidemic of diabetes in the Indian subcontinent where a large section of the population is chronically malnourished and pancreatic toxins may abound in the diet and environment.

- ***In vitro* studies**:
  Islets were isolated from the M as well as the C colony animals and were tested *in vitro* for their stability to STZ exposure *in vitro*. In another set of experiments, the STZ exposure followed pre-treatment of the islets with a soybean lipid mixture (Boehringer Mannheim, Germany). The islet viability after STZ exposure was quantified after dye exclusion test.

  In vitro exposure to STZ also shows a significantly higher mortality ($p<0.05$) in the malnourished group suggesting the increased sensitivity of the M colony animals to diabetogenic insults. It was also observed that SLM pre-treatment offers a protective effect (Fig. 8) to the diabetogenic action of STZ.

Table 1.

Parameters estimated	N Colony	M Colony
Body weight (g); $n = 7$*	224.4 ± 25.465	164.2 ± 8.319
Snout - tip length (cms) ; $n = 7$	398.4 ± 21.037	374.7 ± 3.037
Body mass index (Kg/m^2) *	1.396 ± 0.049	1.169 ± 0.056
Fasting plasma glucose (mg %) *	103.6 ± 7.82	82.1 ± 3.18
Fasting insulin (μIU/ml) *	13.0 ± 1.36	23.9 ± 1.60
Pancreatic weight (g); n = 6 *	298.7 ± 19.5	166.8 ± 15.9
Total pancreatic insulin (μIU/ml) n = 6	3335.0 ± 271.2	2576.7 ± 153.7
Pancreatic insulin (μIU/ml)/mg pancreas; n = 6	11.16 ± 0.6159	16.56 ± 2.412
STZ dose response : $n = 60$		
200 mg / kg body weight		
% Diabetic	93.33	-
% mortality	0	100
125 mg / kg body weight		
% Diabetic	0	31.33
% mortality	0	68.67
60 mg / kg body weight		
% Diabetic	0	100
% mortality	0	0

Table 1: Data for different parameters tested in the M (malnourished) and N (Control) rat colonies. Data is expressed as Mean ± SEM. The number of animals in each group (n) is given. * indicates a statistically significant difference ($p \leq 0.05$) as compared to respective controls.

C. Intra-uterine protein malnutrition

Background:

Intra-uterine malnutrition is known to program permanent changes in the hormone, vascular and lipid responses. However, the effect of intra-uterine

protein malnutrition on the β cell susceptibility to diabetogens has not been reported as yet. The aim of the study was to see for differences, if any, in the diabetogenic vulnerability in fetuses whose mothers received a low protein (LP) diet in contrast to those receiving a control diet (C), throughout the period of pregnancy.

Work carried out:

- ***In vitro* studies:**
  Wistar females were exposed to 8% protein (isocaloric), low protein diet as compared to the control dams receiving 20 % protein, from the first day of pregnancy. The fetuses were sacrificed for obtaining the fetal islet cultures on 21.5 days of pregnancy. These islets were exposed to different concentrations of sodium nitroprusside (SNP), a nitric oxide donor on 6^{th} day of culture. These islets were then labeled with Ethidium Bromide and Hoechst for visualizing the dead and viable cells respectively. The percent mortality was quantified by scanning these islets on a confocal microscope.

  Our data indicates that mere low protein diet during the period of pregnancy increases diabetes susceptibility of the LP animals by 5 fold (fig. 9) as compared to the controls. Further studies were carried out to see the effect of pre-treatment of these islets with the amino acid, Taurine. Taurine is a β amino acid, which has been well documented for its membrane stabilizing and scavenging action. Our studies show that taurine offers protection to the LP islets, in a dose dependent manner (fig. 10, 11, 12) from the toxic effects of SNP. This is the first report of its kind wherein we show that LP animals have a 5 fold high risk of diabetes and that these effects can be overcome by pre-treatment with taurine. Our studies have important implications especially with reference to the Indian sub-continent wherein a large section of the population is malnourished and diabetogenic agents prevail the environment.

- ***In vivo* studies:**
  In order to examine the effects of diabetogenic susceptibility *in vivo*, we injected Streptozotocin (STZ) at a lethal (70 mg/kg) and sub-lethal (35mg/kg) doses, to 19-day-old Wistar neonates kept on low protein or

control diet. In another set of experiments, STZ injection was preceded by a single intra-peritoneal taurine injection, in order to test if taurine offered similar protection as seen in the *in vitro* studies. The animals were then observed for their glycemic status and insulin concentrations on the 7th day from STZ injection.

The *in vivo* studies have been already initiated and the experiments are still in pipeline. However, initial studies carried out with lethal doses of STZ, show a increased vulnerability of the low protein animals to the diabetogenic action of Streptozotocin. This is in accord with our earlier STZ dose shift studies on the chronic multigenerational malnourished model.

D. Regeneration and Diabetes

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 end of the study. The animals (n=10) were then followed for another 60 days to check for their any diversion from their glycemic status.

Our data indicates a 100 % reversal (n=10) of diabetic status by one month of treatment (Fig. 13). The animals showed a normal GTT (Fig. 14) and also remained normoglycemic throughout the follow-up study.

- ***In vitro* studies:**
  We also studied the effect of an islet-conditioned medium on its nesidioblastotic activity. The medium was also fractionated on HPLC and 8 fractions were obtained. These 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 (Fig. 13) by one month and remained normoglycemic with a normal GTT (Fig. 14) 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.

Outcome of the project:

1. Publications / Presentations:

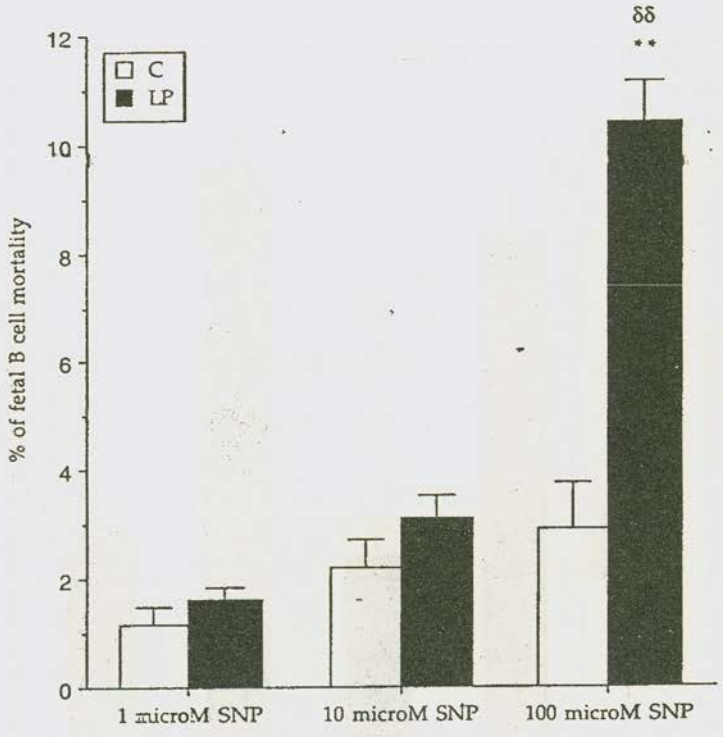
Hardikar AA, Yajnik CS, Karandikar MS and Bhonde RR, Chronic malnutrition increases susceptibility to diabetogenic insult, *presented at AIDSPIT conference, Austria, Jan. 1998 (abstract, Hormone Metabolic Research, January 1998 supplement)*

Hardikar AA and Bhonde RR, Islet neogenesis by subtotal pancreatectomy leads to normoglycemia in diabetic mice *presented at AIDSPIT conference, Austria Jan. 1998 (abstract, Hormone Metabolic Research, January 1998 supplement)*

Hardikar AA, Karandikar MS and Bhonde RR, Pancreatic regeneration in experimental diabetes *Proc. International Symposium on Development, Growth & Differentiation, December 1997, Mahabaleshwar, India*

Bhonde RR, Hardikar AA and Kurup S, Development of bioartificial organ devices to achieve a functional restoration of damaged cells. *Proc. International*

Symposium on Development, Growth & Differentiation, December 1997, Mahabaleshwar, India.



** p<0.01; 100 microM in LP group vs. 100 microM in C group

δδ p<0.01; 100 microM vs. 1 & 10 microM SNP in LP group

Figure 9. Effect of SNP on C or LP fetal islets.

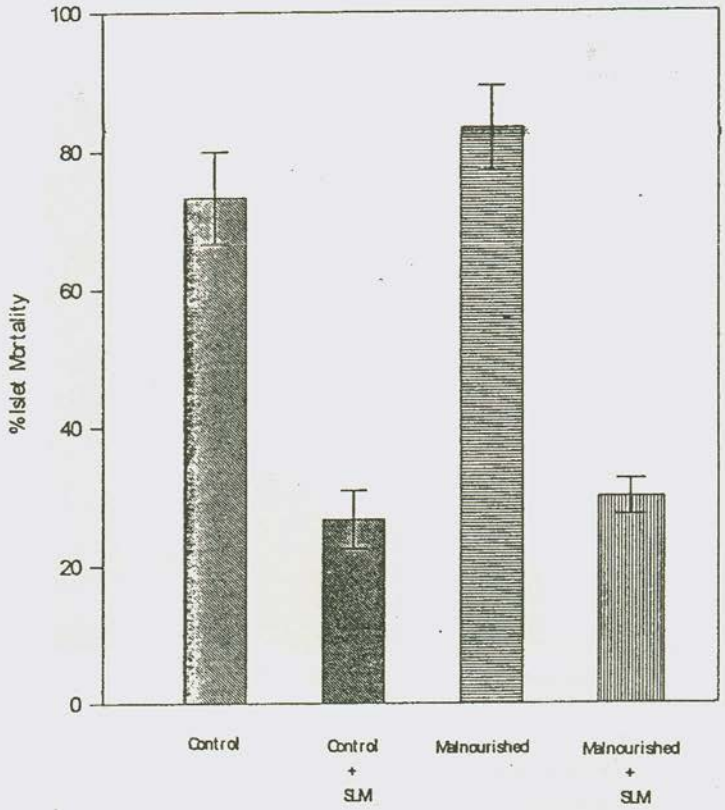


Figure 8. STZ challenged membrane stability.

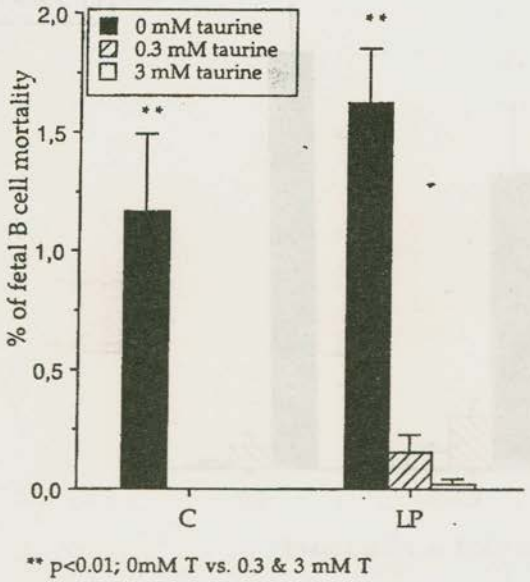


Figure 10. Effect of taurine on 1 microM SNP treated B cells of fetuses from C or LP dams.

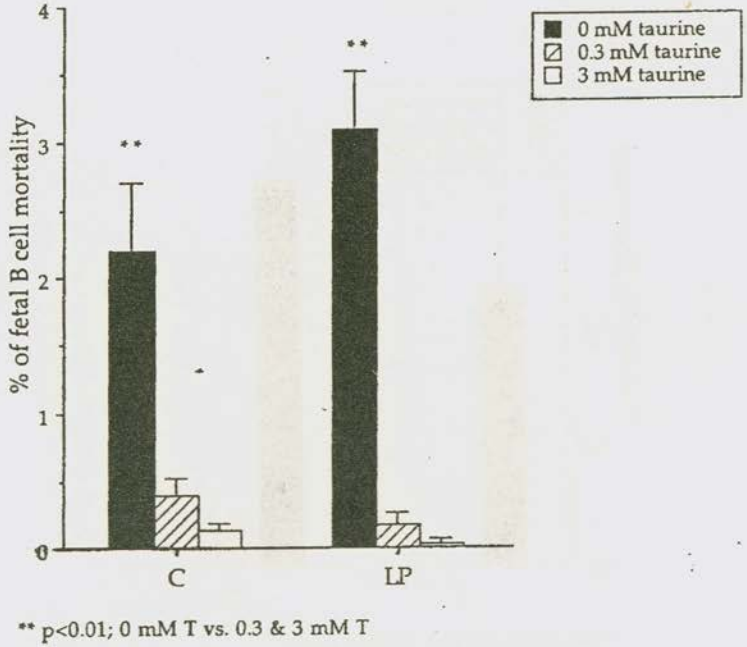


Figure 11. Effect of taurine on 10 microM SNP treated B cells of fetuses from C or LP dams.

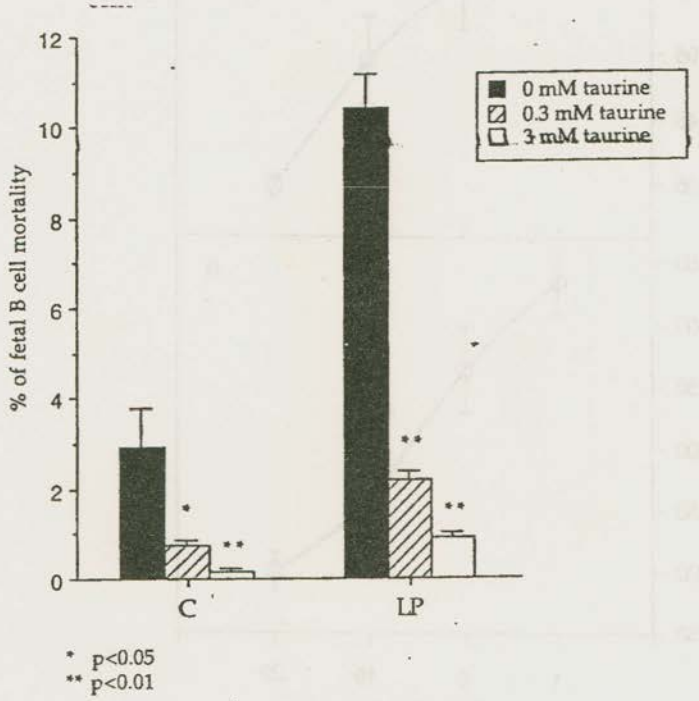


Figure 12. Effect of taurine on 100 microM SNP treated B cells of fetuses from C or LP dams.

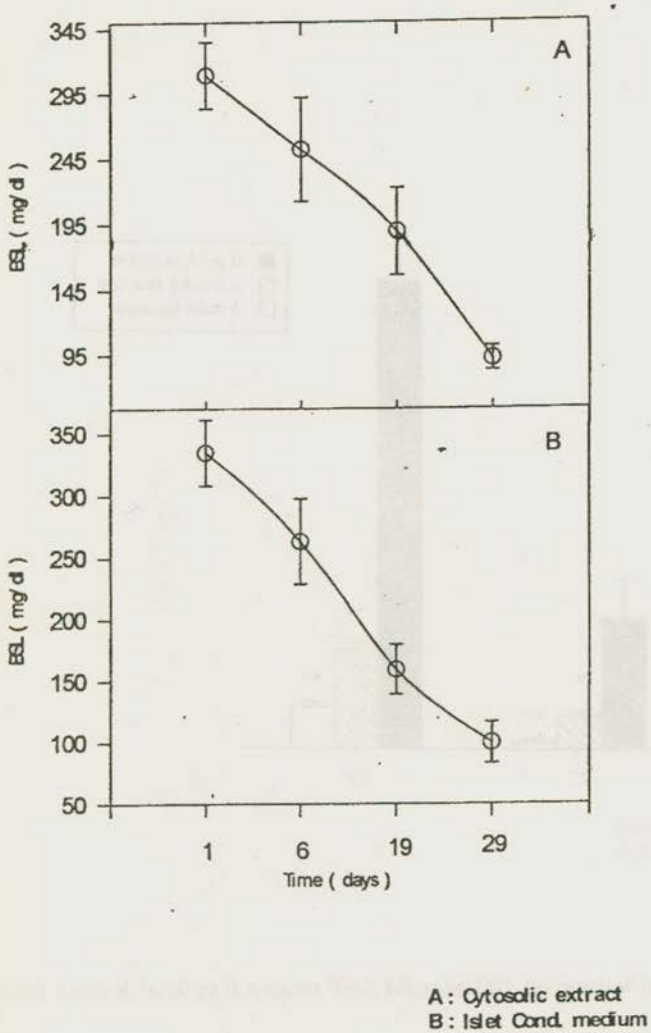


Figure 13. Post treatment blood sugar profile.

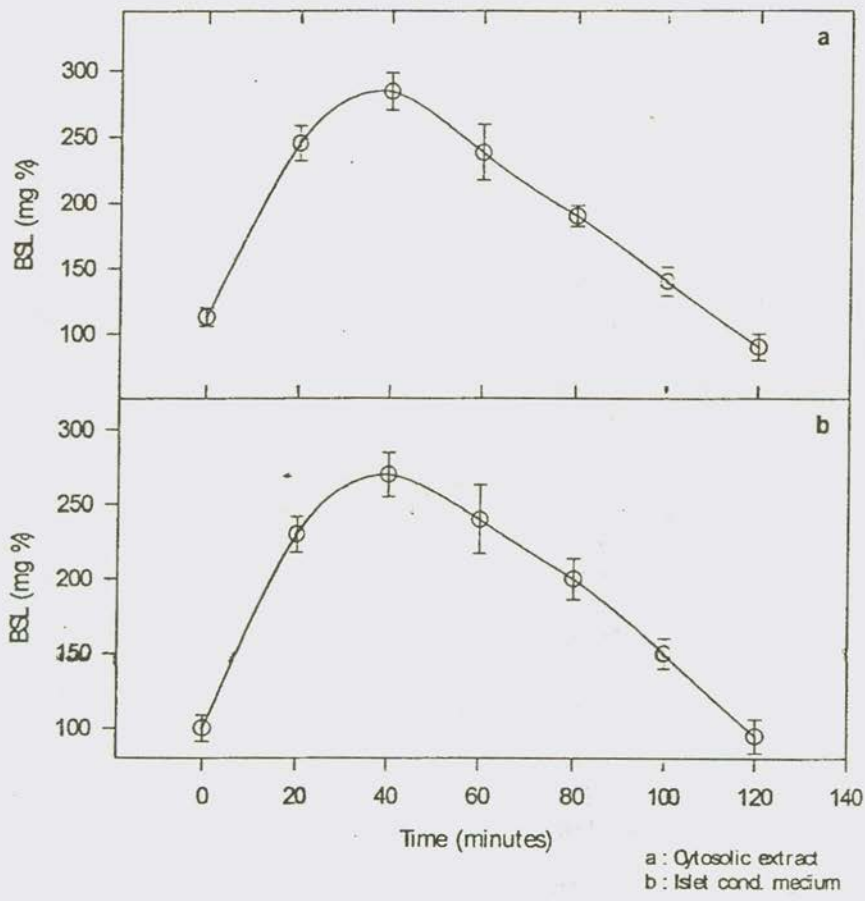


Figure 14. Glucose tolerance test.

Oxidative stress in diabetes mellitus and development of therapeutic strategies

Participants: Sandhya Sitasawad

Date of initiation: January 1997
Expected date of completion: 2000

Background:

Free radicals and oxidative stress may act as a common pathway to diabetes itself (both IDDM and NIDDM), as well as to its later complications. Abnormalities in the regulation of peroxide and transition metal metabolism are postulated to result in establishment of the disease as well as its longer-term complications. Diabetes mellitus is associated with oxidative reactions, particularly those which are catalyzed by decompartmentalized transition metals, but their causative significance in diabetic tissue damage remains to be established cells appear to have an increased sensitivity toward peroxides and low levels of the antioxidant enzymes. Although the mechanism of the frequently used diabetogenic agents Alloxan and streptozotocin (Stz) is unknown, several lines of evidence suggest that they stimulates generation of superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) during the redox cycling which damage ? cells. However, $O_2^{\cdot-}$ and H_2O_2 lack sufficient reactivity to cause oxidative damage and require the presence of a low molecular weight chelated transition metal ion as a catalyst to produce a more reactive oxidant, such as hydroxyl radical ($^{\cdot}OH$) which consequently initiate lipid peroxidation peroxidation and cause DNA damage. The mechanism for the $^{\cdot}OH$ production by $O_2^{\cdot-}$ and H_2O_2 is called superoxide-driven Fenton reaction.

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \qquad (1)$$

Several transition metal ion complexes such as chromium (Cr^{2+}), cobalt (CO^{2+}), titanium (Ti^{3+}) and vanadium (vanadyl) were shown to decompose H_2O_2 resulting in the formation of hydroxyl radical in *in vitro* systems. However, in *in vivo* systems, Fe^{2+} is likely to be more relevant. At physiological pH, iron is known to bind to transport and storage proteins and not available to react with

peroxides. Moreover, bound iron is always in Fe^{3+} form. For the release and the participation in Fenton reaction, iron has to be reduced to Fe^{2+}. Superoxide radical , or other chemical agents which donate electron will release iron from various proteins. Iron is also released in an event of incorrect loading of iron (> 2 mol of Fe^{2+} /mol), or exposure to an acidic pH (e.g. during hypoxia or impairment of mitochondrial function).

A potential intracellular source of the iron involved may be the iron storage protein ferritin. Several previous studies have demonstrated that $O_2^{\cdot}$ or a number of organic radicals release iron from ferritin suggesting that ferritin may serve as a source of iron for oxidative damage in vivo. The physiological reluctant(s) is unknown, but in recent years the ability of numerous toxicological reductants to mobilize ferritin iron has been reported.

Recently an ELISA for rat ferritin showed that the insulin cell contains a surprisingly high amount of ferritin comparable to that of iron storage tissues. Most of the ferritin was located in the beta cell. Taking into consideration the above aspects the present study was planned and work is being carried out accordingly. The aim of the present work is therefore to study the evidence that diabetes mellitus may, in part at least, thus result from oxidative stress catalyzed by 'decompartmentalized' transition metals.

Current evidence suggests that, alloxan toxicity in vitro and in vivo can cause iron release from the iron-proteins and be inhibited by metal-chelating agents, hydroxyl radical scavengers and lipid soluble antioxidants. The mechanism of action of Stz is less well understood. Recent evidence suggest that Desferrioxamine blocks diabetes induced by multiple low doses of streptozotocin suggesting that transition-metal-catalyzed free radical reactions may contribute to Stz toxicity. The role of Stz in the iron theory is studied in the present work.

Importance of the project:

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. This number is likely to increase to 28 millions by the 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.

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. 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 and inhibit the active calcium transport and calmodulin activated protein kinase activity. Some of these aspects are required to be examined and understood experimentally inorder to develop therapeutic as well as preventive strategies.

Targets defined :

1. To study the mechanism of action of different diabetogenic agents viz. Streptozotocin (Stz), Alloxan, and the mediator of tissue destruction H_2O_2 in terms of insulin production
2. To measure the Stz/alloxan induced release of iron from the iron transport and storage proteins, transferrin and ferritin and its inhibition by the iron chelators, EDTA, desferrioxamine and diethylene triamine penta acetic acid (DETEPAC) etc.
3. To find out the mutations if any in the superoxide dismutase (SOD)
4. To study the effect of the calcium/calmodulin antagonists chlorpromazine (CPZ), promethazine (PMZ) and triflupromazine (TFP) which are the inhibitors of $NO^{\cdot}$ as well as other free radicals

Work carried out:

1. Experiments on the role of the diabetogenic agents STZ, alloxan and H2O2 in the production of NO· were carried out in RINm5F cells using sodium nitroprusside as a positive control. MTT assay was done for testing cell viability.
2. Experiments were also carried out to test the efficacy of promethazine as an inhibitor of NO· production.

In the present study, we report that the addition of Stz to the iron storage protein rat liver ferritin resulted in a time and concentration dependent release of ferrous iron as assayed by spectrophotometric detection of ferrous-ferrozine complex formation (Fig. 15, 16). RINm5F cells were treated with Stz (1Mm) and ferritin (200?g) and it was found that viability of the RIN cells as assayed by methylene blue test reduces after the treatment of cells with Stz. This viability was further reduced in the presence of ferritin suggesting that the ferritin iron may be involved in Stz toxicity (Fig. 17).

Further, the iron chelators DETAPAC and Deferrioxamine inhibit this iron release in a dose dependent manner (Fig.18).

Outcome of the project:

Publications: Nil.
Patients: Nil.
Products: Nil.

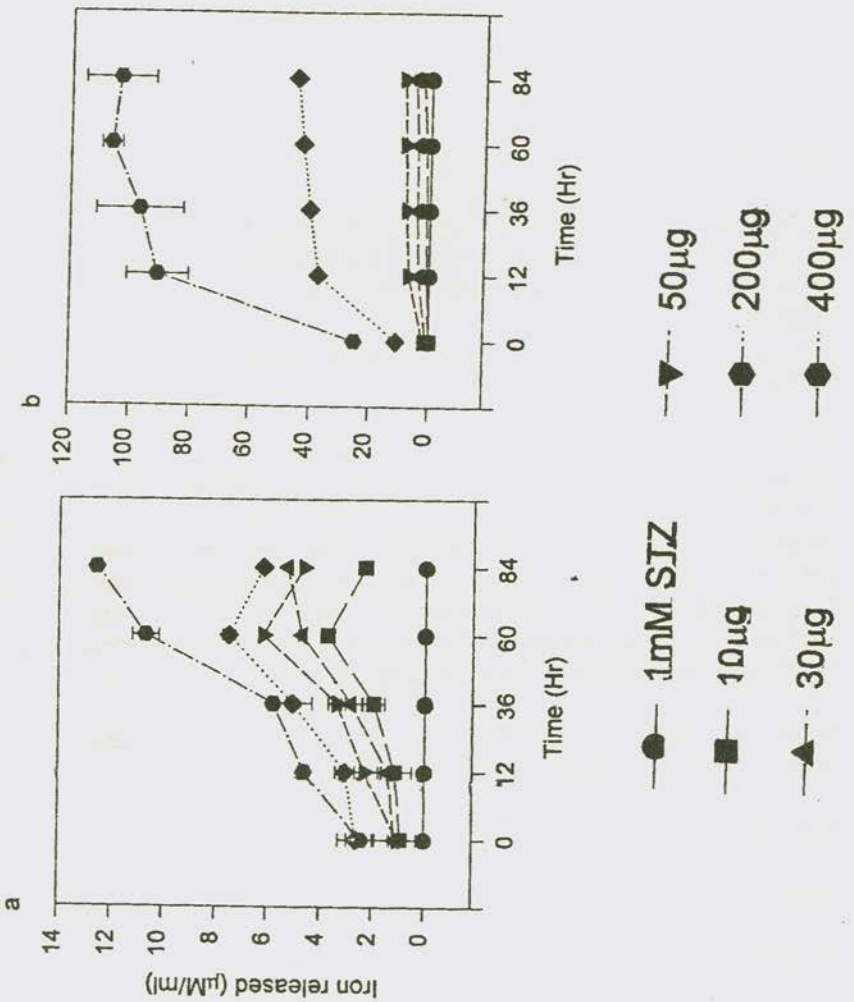


Figure 15: Effect of protein concentration on iron release. Reaction mixtures (1 ml final volume) in 50 mM HEPES, pH 7.0, contained 500 uM ferrozine, rat liver ferritin/hemoglobin and streptozotocin (1 mM). The reaction was initiated with Stz at 37°C for 96 Hr and iron release was determined following the change of optical density at 562 nm. All values are expressed as means ± standard deviation of replicate (n = 6).

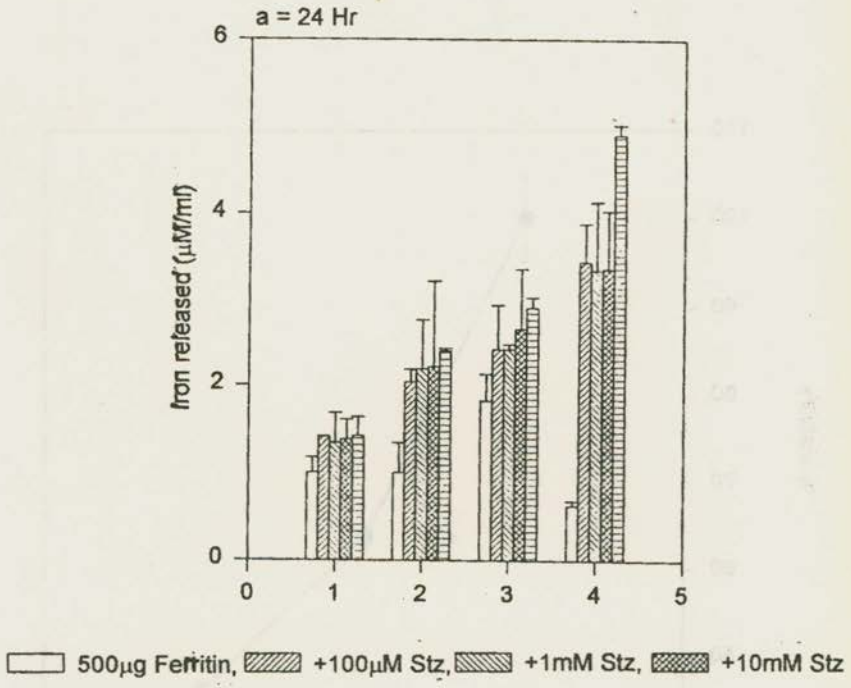


Figure 16: Effect of Stz concentration on iron release. Reaction mixtures (1 ml final volume) in 50 mM HEPES, pH 7.0 contained 500 uM ferrozine, rat liver ferritin (500 ug) and Stz. The reaction was initiated with Stz at 37°C for 24 Hr and iron release was determined following the change of optical density at 562 nm. All values are expressed as means ± standard deveiation of replicates (n = 6).

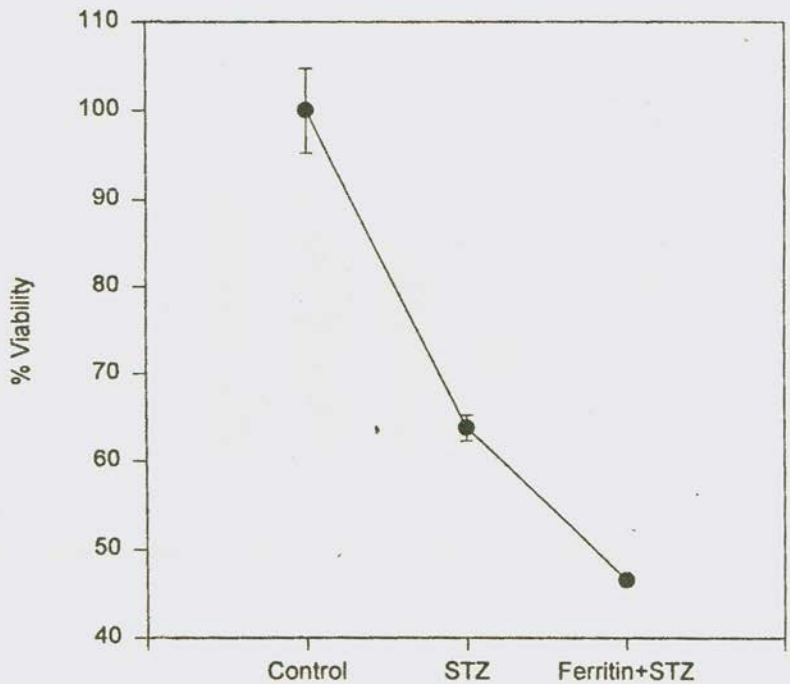


Figure 17. Loss of RINm5F cell viability following exposure to STZ and Ferritin + STZ. Results are expressed as the mean $\pm$ SD (n = 6).

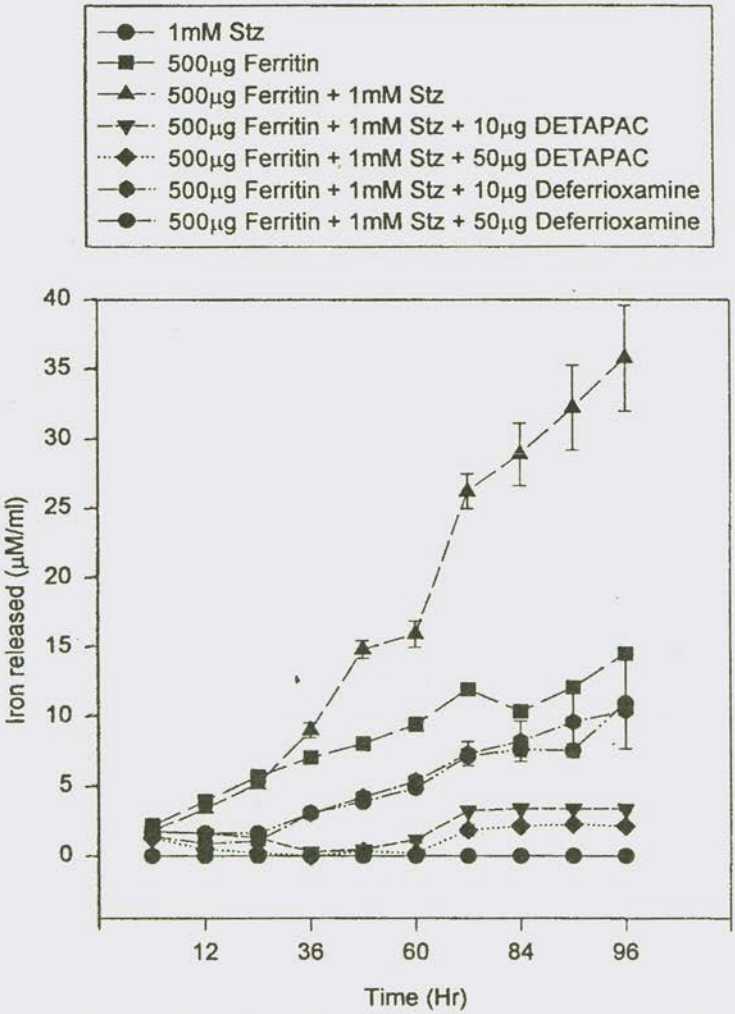


Figure 18: Effect of DETAPAC and Desferrioxamine on Stz-induced iron release from ferritin. Reaction mixtures (1 ml final volume) in 50 mM HEPES, pH 7.0 contained 500 uM ferrozine, 500 uM Fe as rat liver ferritin iron, Stz (1 mM) and the iron chelators DETAPAC and deferrioxamine (10 and 50 ug). The reaction was initiated with Stz, DETAPAC and deferrioxamine and the amount of iron released determined for 96 Hr. Values represented are the means $\pm$ SD for six replicate incubations.

Identification and characterization of oncogenes implicated in melanoma

Participant: Anjali S. Shiras
Varsha Shepal
Mandar Bhonde

Date of initiation: August 1990
Expected date of completion: August 2000

Background:

Oncogenes are implicated in various steps of tumorigenesis. The mechanism of oncogene action could be at the level of cell adhesion wherein various integrins could be involved or it could be at the level of tumor formation, progression or metastasis [Schwartz M A., Schaller M D and Ginsberg M H (1993) *Cancer Res* **53**: 1503-06; Judware R, and Culp L.A. (1997) *Oncogene* **14**, 1341-50].

The project involves the identification of oncogenes implicated in mouse melanomas using Cloudman Melanoma ' Clone M3 ' as an experimental cell system. Using the automatic directional expression cloning strategy, we have cloned a 1.2 kb gene which on transfection into various mouse, rat and human cell systems induces stable transfection and creation of novel phenotypes. Efforts are underway to characterize this gene and understand its significance in cell transformation.

Targets defined and work carried out:

Efforts were done this year to characterize the 1.2 kb gene in details using mRNA expression studies and FACS analyses of transfected cells. We are attempting the sequencing of this gene to understand its role in the signal transduction cascade.

We have generated an expression cDNA library from Clone M3 (a mouse melanoma cell line) cells. The cDNA library on transfection gave rise to about 50 - 60% G418 (+) clones. One of the G418 (+) clones did produce large tumors

in nude mice and satisfied all criteria of transformation. Using PCR and the SP6 and T7 primers we have fished out a 1.2 kb gene product. We have confirmed the expression of this molecule at the mRNA level by Northern hybridization using the PCR product as probe. Also, the transfected clone was studied for the expression of various cell specific markers using FACS. Our experimental results suggest the expression of melanoma specific markers in the clone, like expression of S 100 and PAL-M1 along with change in expression of CAM`s like E-cadherin and BCL-2 (Fig. 19, 20).

We have attempted sequencing this gene by manual sequencing and the partial sequence obtained shows no significant homology to the known genes involved in cell transformation.

Proposed work:

We aim at sequencing the entire gene and understand its role in the signal transduction cascade.

Outcome of the project:

Publications: Nil.
Patents: Nil.
Products: Nil.

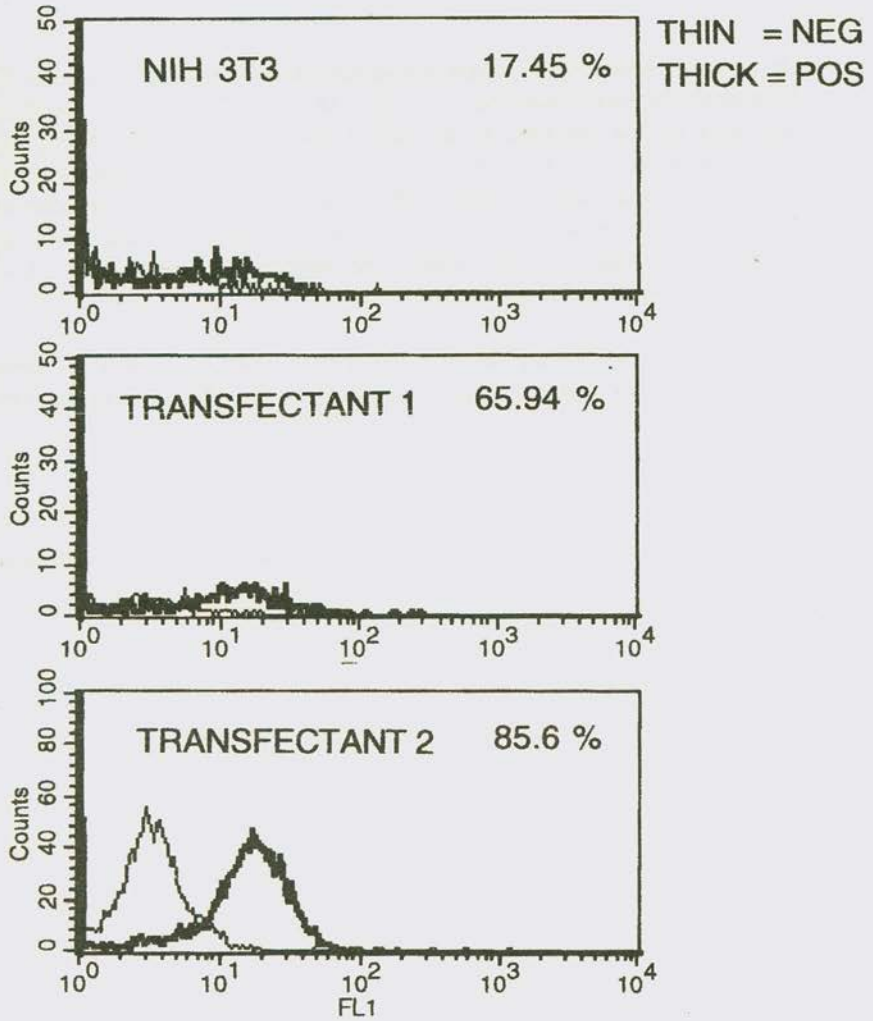


Figure 19. Expression of S-100.

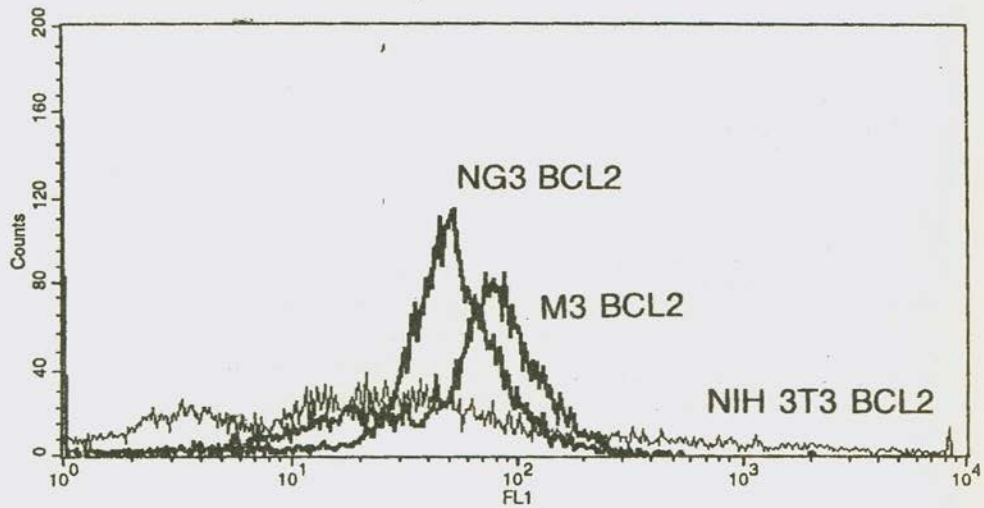


Figure 20. Expression analysis by FACS.

Inducing Differentiation and Apoptosis in Neuroblastoma Cells

Participants: Padma Shastry
Nagarathna
D. R. K. Mohan
Jayashree Jagtap

Date of initiation: December 1993
Expected date of completion: December 1999

Background:

Neuroblastoma (NB) is the most common childhood tumour. 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.

Work carried out:

Cyclohexidmide (CHX), a protein synthesis inhibitor, when given at low doses has been reported recently to delay or prevent neuronal death induced by various insults via mechanisms similar to those of neuroprotective factors. We

examined the effect of CHX in combination with SSP on the growth and differentiation of two murine NB cell lines viz. Neuro2A and NB41A3 (Fig. 21, 22).

In the experimental design, 24-hour cell cultures were treated with SSP and CHX, individually or in combination for 24, 36 or 48 hour periods. The parameters studied were cell number and viability for proliferation, and morphological features — cell spreading, adherence, neurite extension — for differentiation.

The results indicated that CHX enhanced SSP induced differentiation in both cell lines. Neuro2A showed a remarkable increase in the number of neurite bearing cells. Almost all of the cells (95%) were polar and expressed thin, long neurites. SSP treatment alone induced cell spreading, flattening of cells, and short neurites, while individual treatment with CHX resulted in cell clustering and occasional branched neurites. NB41A3 cells, which usually grow as clumps, became spread, polar, and expressed short neurites on combined treatment. SSP alone induced flattening and expression of very few neurites, while CHX alone caused the cells to round up.

Cellular differentiation is associated with the cessation of cell division/growth. Therefore the cell number and viability was assessed in the two cell lines following treatments. There was a significant decrease in the cell number with combined treatment compared with SSP alone at the three time points studied, but not with CHX alone. That this decrease in growth was not due to cell death was confirmed by a viability test. The data indicates that arrest in cell division alone may not be adequate to direct the cells towards differentiation as with CHX additional signals may be necessary for inducing the cells to undergo differentiation with combined treatment.

We conclude that CHX enhances SSP induced differentiation in NB cells. This experimental system could therefore be useful as a model to study the mechanisms involved in neuronal differentiation.

Outcome of the work:

The system using the cycloheximide and staurosporin for differentiation can be used as a model to study and understanding mechanisms in differentiation in

neuronal cells and the explore the possibility of this mode of treatment in other neuroblastoma cell lines including those from human metastatic tumors.

Publications: Nil.
Patents: Nil.
Products: Nil.

Conferences:

Attended International Symposium on Biology and XXI All India Cell Biology conference held in Bangalore - March 21-22 1998.

Presented paper- Cycloheximide enhances staurosporine-induced differentiation in neuroblastoma cell lines, at International Symposium on Development, Growth and differentiation at Mahabaleshwar 17-20 Dec 1997.

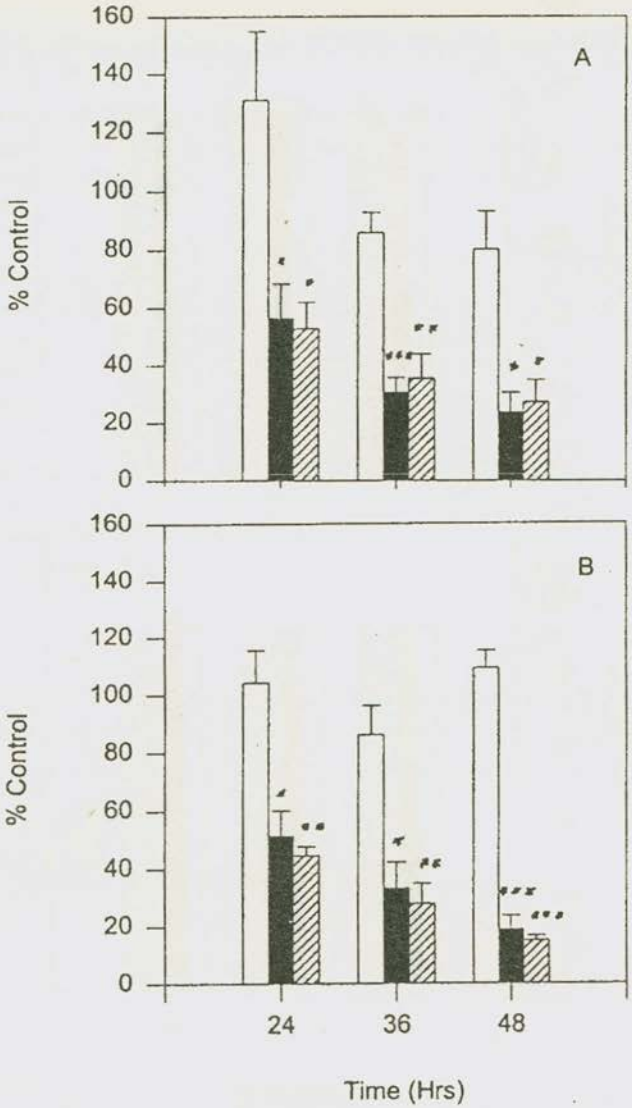


Figure 21: Effect of SSP and CHX on cell number. NB cells N2a and NB41A3 were treated with SSP (open bars), CHX (solid bars) and CHX + SSP (hatched bars) for 24, 36 and 48 H. The results here represent the mean and +/- SEM from 3 different experiments. The viable number was obtained by taking control as 100%.

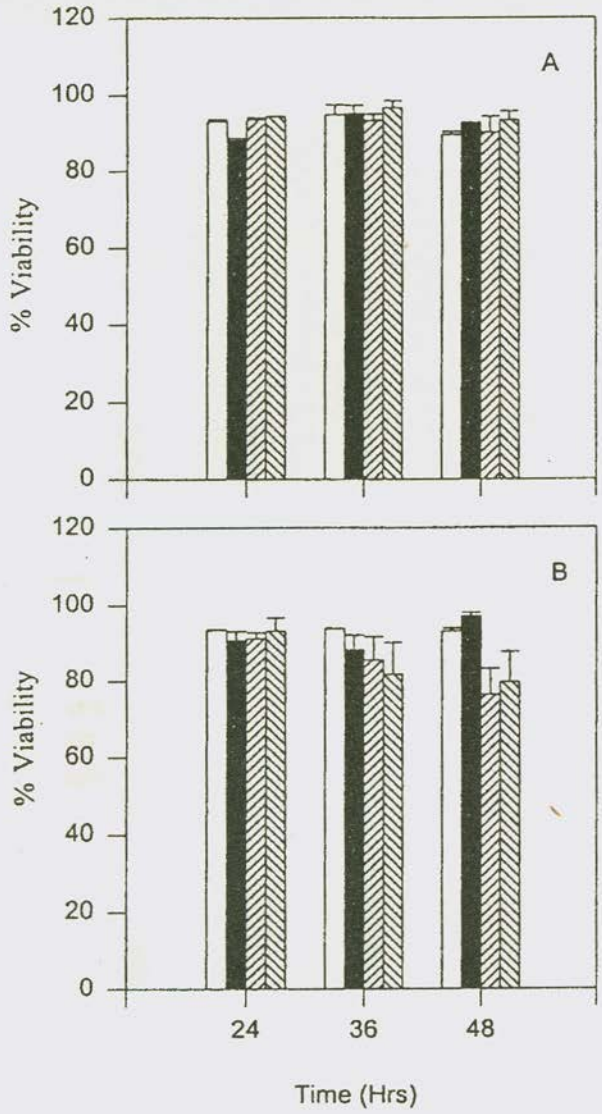


Figure 22: Viability after treatment with SSP and CHX. Percent viability of N2a and NB41A3 cells obtained with control (open bars), SSP (solid bars), CHX (hatched bars, rising up) and CHX + SSP (hatched bars, rising down) after 24, 36 and 48 h of treatment. The results are the mean of 3 different experiments.

Structure function analysis of eukaryotic cells: (I) Epithelial mesenchymal transition; (II) Interphase nucleus organization

Participants: J. M. Chiplonkar
M. R. Vipra
A. Pais

Date of initiation: Part I: June 1996
Part II: September 1996
Probable date of completion: December 2000.

Part I: 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 embryogenesis, 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 motility in vitro. Growth factors are also essential for tissue repair and morphogenesis. They further play an important 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 fibroblastic cells. EMT is a complex process and can result from interaction of more than one regulator. It has been reported that a scatter factor may promote EMT-like changes in vitro: e.g., HGF is a protein which influences 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 a given 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 may 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 cells migrating from the edges of the wound, rather than by cell division.
This leads to the question of how and when the signalling pathways triggered by growth factors diverge in the cell cycle to generate either mitogenic or motogenic response. Therefore it is important to understand the mechanisms that evoke the specificity of the cellular response to a given growth factor.

Targets defined:

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

Work done:

As previously reported, 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, hormones, etc. Besides the cervical carcinoma-derived cell line (SiHa) as described earlier, MDBK (of normal origin) and AV-3 (of embryonic origin) were used for further experimental studies. The cellular responses (both early and late) were evaluated using morphological and immunocytochemical markers for epithelial and

mesenchymal cells.

MDBK:

Cytokeratins are the markers of the cells of epithelial origin, whereas vimentin is found in the cells of mesenchymal origin. MDBK cells constitutively express both keratins and vimentin; however, the migrating cells typically showed down regulation of cytokeratin 18 and 19 with corresponding increase in the expression of vimentin. The process appeared to be temporary and reversible. Another epithelial specific marker, desmosomes also disappeared from cell-cell boundaries, as cells began to migrate. These were then internalized and redistributed over the cytoplasm in migrating cells and eventually disappeared. These observations typically describe the process of epithelial mesenchymal transition. As ECM components play a critical role in migration, different ECM components were checked for. They were fibronectin, laminin, collagen IV A-CAM, etc. These cells do not express collagen and A-CAM. Laminin is present in only small amounts, but fibronectin was secreted in large amounts. However, it was distinctly absent under the migrating cells, up to 3 to 4 layers behind the wound edge. Focal contacts with the substratum are important during migration of cells. Vinculin and talin are the intermediate filaments associated with focal contacts. These were stained and their changing patterns were recorded.

To study the progress of cell cycle in cells undergoing EMT, the incorporation of bromodeoxyuridine (BrdU) in interphase nuclei during short pulse labeling was visualized by indirect immunofluorescence. The results indicated that cells lining the wound (undergoing EMT) required longer time to enter S phase as compared to those within the monolayers. These results suggest that progression of the cell cycle is at least temporarily and reversibly arrested in the cells, which have been induced to migrate. To further analyze the temporal dissociation of cell cycle progression and cell migration, starved, wounded cell monolayers were pulsed only for a short time with the stimulus. The cells were then followed to study their migratory as well as mitogenic response. Two different stimuli were used, FCS and EGF (10 ng/ml). FCS exposure as short as 15 minutes could stimulate cells to enter cell cycle as well as migrate. EGF alone could induce these cells to enter cell cycle even after a brief exposure but continuous presence of EGF was required for migration.

SiHa:

The approach used to study these cells was the same. The synchronization of cells in early G1 was achieved using thymidine and serum starvation. The process of EMT was clearly evident in these cells. There was a decrease in the expression of K18, K19 and particularly K8. Vimentin was more in these cells. An important epithelial specific marker, epithelial specific antigen (ESA) disappeared as the cells started migrating and was completely absent in migrating cells. Fibroblast specific marker was upregulated during the transition. SiHa cells do not show collagen, A-CAM and fibronectin. Laminin is synthesized but not seen under cells undergoing EMT. Vinculin and talin staining was also carried out.

AV-3:

These cells responded to the exogenously added MRC-5 conditioned medium (which was used as the source of HGF). Keratin expression was lowered in the migrating cells. These cells synthesized A-CAM.

Part II: 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 organi8zed in the volume of the nucleus. This is so partly because very little structural specialties 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 of a 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". (Spencer, D L, Macromolecular domains within the cell nucleus" In: Ann. Rev. Cell Biol., 1993, 9: 265-315).

Targets defined:

1. Cell culture and related aspects.
2. Standardization of cytochemical techniques.
3. Standardization of immunocytochemical techniques.
4. 2-dimensional imaging and analysis.
5. Standardization of fluorescence in situ hybridization.
6. 3-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.

Outcome of the project:
Publications: Nil.
Patents: Nil.
Products: Nil.

Molecular Cross-talk Between Pathogenic Bacteria / Bacterial Antigens with Human Mucosal Epithelial and Submucosal Mesenchymal Cell Systems using Cell Culture as a Model

Participants: K S Nanda Kumar
Dipshikha Chakravortty (Ph D thesis submitted)
Jitender Singh Cheema

Project 1: Molecular and Cellular Interaction of Lipopolysaccharide(LPS) with Human Intestinal Mesenchymal Cells

Date of Initiation: 1994
Expected date of Completion: 1999

Summary of the work done (96-97):

1.Identified the existence of a putative receptor of about 80Kda on the intestinal lamina propria fibroblasts for LPS of E. coli

2.LPS was found to enhance DNA synthesis ,decrease population doubling time and increase in S phase cells.

3.LPS induced collagen synthesis in the intestinal lamina propria fibroblasts

4.LPS induced DNA synthesis and collagen production in these cells were related to the down regulation nitric oxide (NO) production (Fig. 22)

Summary of the work done (97-98)

Fibroblasts are important effector cells having potential role in augmenting the inflammatory responses in various diseases. In infantile diarrhea caused by

enteropathogenic E. coli (EPEC) the mechanism of inflammatory reaction at the mucosal site remain unknown. Although the potential involvement of fibroblasts in the pathogenesis of cryptococcus induced diarrhea in pigs has been suggested ,the precise role of lamina propria fibroblasts in cellular pathogenesis of intestinal inflammation caused by EPEC requires elucidation. Therefore, part of the investigation was directed to determine profile of cytokines and adhesion molecules in the cultured and characterized human small intestinal lamina propria fibroblasts(n=48)in relation to neutrophil adhesion and migration in response to LPS extracted from EPEC055:B5. Upon interaction with LPS (1mg-10mg /ml) ,lamina propria fibroblasts produced high levels of proinflammatory mediators, interleukin (IL)-1a,1b, IL-6, IL-8, umor necrosis factor (TNF) alpha and cell adhesion molecuales (CAM) like intracellualr adhesion molecule (ICAM), A-CAM, N-CAM and vitronectin in a time dependent manner. LPS induced cell associated IL-1a and IL-1b ,whereas IL-6,IL-8 and TNF-a as soluble form in the supernatant. Apart from ICAM, vitronectin, A-CAM and N-CAM proteins were strongly induced in lamina propria fibroblasts by LPS .There was ICAM dependent adhesion of PBMC to LPS treated lamina propria fibroblasts .Conditioned medium of LPS treated fibroblasts remarkably enhanced the neutrophil migration .The migration of neutrophils was inhibited by anti-IL-8 antibody. Co-culture of fibroblasts with neutrophils using polycarbonate membrane filters exhibited time dependent migration of neutrophils. These findings indicate the coordinate production of proinflammatory cytokines and adhesion molecules in lamina propria fibroblasts, which do not classically belong to the immune system can influence the local inflammatory reaction at the intestinal mucosal site during the LPS interaction.

Outcome of the project:

Publication:

Dipshikha Chakravortty and K S Nanda Kumar (1997) Induction of cell proliferation and collagen synthesis in human small intestinal lamina propria fibroblasts by lipopolysaccharide: Possible involvement of nitric oxide. ***Biochem. Biophys. Res. Commun***. **240**: 458-463.

Patents: Nil.
Products: Nil.

Recognition and awards:

1. **Best Research Work Award** 1997 by the IAMM conference, Maharashtra Chapter to Dipshikha Chakravortty.
2. **SERC Young Scientist Award** to D. Chakravortty from the DST ,Govt. of India as Principal Investigator.

3. **DAKO INTERNATIONAL RESEARCH FELLOWSHIP** for Postdoctoral work in Japan.

Molecular biological studies on mosquitoes

Participants: Yogesh S. Shouche
M. S. Patole
Vyankatesh Pidiyar
P. Cyril Jaykumar
Suraj S. Tirth

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. 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 their control elements are being investigated and primer has been designed for the amplification of hexokinase gene.

An alternative to generate genetically engineered mosquitoes is to manipulate their endosymbionts. Several species of mosquitoes are known to harbour endosymbionts belonging to Wolbachia sp. We screened the local 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 harbour 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 a promising 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 the digestion of the blood meal.

All the attempts to culture organisms from the natural environments have underlined the limitations to culture the bacteria form 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 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 been popularly used for this.

Work done:

For the studies of hexokinase gene, RNA was extracted from cultured Aedes aegypti cell line as well as larave 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 this clone was done.

The 32 isolates were taken up for the complete characterisation. Biochemical tests were unable to give definitive identification of most of the isolates, hence partial rRNA sequencing was done for these. The partial sequence indicated that these belong to genera as diverse as Orienetia, Pseudomonas, Sprioplasma, Merhylophylus, Acinetobacter, Xylela, Proteus, Cyanidium and Staphylococcus. Complete characterisation of the haemolytic isolate H was undertaken. Its biochemical tests and rRNA sequencing showed highest similarity to genus Aeromonas, but its GC contents by HPLCwas very different from this genus. Presently attempts to find out GC content by Tm measurements are underway and also complete sequencing of rRNA gene.

This isolate was found to contain an approximately 6 kb plasmid, curing experiments indicated that it may be responsible for haemolysis and production of haemolysin. This plasmid is being characterised at present.

A library of PCR amplified eubacterial SSU rRNA genes from total female mosquito gut DNA was prepared and the clones were characterised RFLP of genes. All the 28 clones were independent clones representing different rRNA gene. These are being further investigated, but at the same time efforts are being made to generate another library as 28 clones are not sufficient for it to be a representatvie library.

Proposed work:

The cloned PCR product will be used for picking up cDNA clone from Aedes aegypti larval expression library and will be further analysed. The identification of all the isolates will be completed, for the isolate H in addition to this the plasmid will be completely characterised as it has the potential for being used as cloning vector for the expression of recombinant proteins in mosquito midgut. A better library of PCR amplified eubacterial rRNA genes from the midgut will be prepared.

Outcome of the work:

Publications:

1. Supriya G. Kshirsagar, Milind S. Patole & Yogesh S. Shouche. (1997) Characterisation of insect cell lines: Heteroduplex analysis employing mitochondrial 16S ribosomal RNA gene fragments. *Analytical Biochemistry* **253**, 65-69.

2. Dighe, A., Yogesh S. Shouche and Ranade, D. R., Isolation of Selenomonas lipolyticum sp. nov., an obligately anaerobic bacterium possessing lipolytic activity. Accepted in *International Journal of Systematic Bacteriology*

3. Supriya G. Kshirsagar, Milind S. Patole & Yogesh S. Shouche Insect Cell line authentication by DGGE. Accepted in *In Vitro.*

4. Vyankatesh Pidiyar, Milind Patole & Yogesh S. Shouche. Ribosomal RNA based phylogenies and molecular ecology. Submitted to *J. Biosci.*

Patents: Nil.
Products: Nil.

Conferences:

1. Vyankatesh Pidiyar, Milind Patole and Yogesh S. Shouche Bacterial community structure of mosquito midgut. Annual Meeting of Society of Biological Chemists, Vishakhapatbam, India !997).

2. Vyankatesh Pidiyar, Milind Patole amd Yogesh S. Shouche Detection and identification of endosymbionts in mosquitoes. Annual Meeting of Society of Biological Chemists, Vishakhapatbam, India !997).

3. Insect cell line characterisation and authentication: methods based on sequence variation In mitochondrial rRNA genes. International Symposium on Biology in 21st Century and XXI All India Cell Biology Conference, Bangalore, India (1998).

Identification and characterisation of protective antigens in lymphatic filariasis

Participant: P. B. Parab

Date of initiation: 1993
Expected date of completion: 1998

Background:

Lymphatic filariasis is most important vector borne disease prevalent in tropical and subtropical regions of the world affecting nearly 100 million people. In India, W. bancrofti and B. malayi nematodal infections are causative agents of this disease. Owing to difficulties in obtaining human filarial parasites, studies were initiated to analyse Setaria digitata (bovine filaria) antigen, which could be useful for diagnosis and prophylaxis in lymphatic filariasis.

Targets defined and work carried out:

Earlier we have identified 40-kDa antigen of Setaria digitata adult worm as a candidate antigen to detect active infection in lymphatic filariasis. Further we have estimated IgG4 antibody levels specific to W. bancrofti microfilarial excretory secretory antigen in sera of filarial patients. IgG4 levels were found to be very high in microfilaraemics.

Proposed work:

Antibody levels of other subclass will be studied in lymphatic filariasis sera reactive to ESM.

Outcome of the project:

Publications: Nil.
Patent: Nil.
Product: Nil.

Immune reactions in streptozotocine (STZ) induced autoimmune diabetes

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

Date of initiation: January 1998
Expected date of completion: January 2001

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 the insulin producing B cells. Low doses STZ induced autoimmune diabetes model in mice simulate type I (IDDM) diabetes with similar immunopathologic profile to human disease.

Targets defined and work carried out:

Low dose STZ induced autoimmune diabetes model in Balb/c mice was established. A microassay to detect glucose levels was developed. STZ induced diabetic mice were injected with islet culture medium for 20 days. Glucose tolerance test was carried out in these animals. Results showed impairment of glucose metabolism in these animals. In two out of four animals glucose levels dropped to basal levels to hours after the glucose challenge. Detail studies are in progress.

Proposed work:

To standardize method for obtaining islet supernatant and identify active molecule.

Outcome of the project:
Publications: Nil.
Patents: Nil.
Products: Nil.

Role of hemozoin in immune response

Participants: Prakash Deshpande
Padma Shastry

Date of initiation: 1996
Expected date of completion: 1998

Background:

World Health Organization estimate that there are 270 million new cases of malaria each year, about half caused by *Plasmodium falciparum*, the most dangerous of the human malarias, with about 2 million fatalities per year. Intraerythrocytic malaria parasite uses hemoglobulin as major nutrient source of amino acids. However, the parasites are unable to degrade hemoglobulin - heme and instead polymerize it to an insoluble dark brown pigment called hemozoin. Hemozoin is released in to blood circulation along with merozoites as schizont infected erythrocyte bursts.

Impairment of phagocytic function of monocyte / macrophages by hemozoin is reported. Moreover, drastic changes in peripheral blood mononuclear cells indused by P. falciparum have been documented.

Work done:

Malarial parasites (*P. falciparum*) were cultured and maintained in the laboratory according to Trager and Jensen method. Insoluble hemozoin pigment was isolated from trophozoite rich parasitized erythrocytes (>10%) by osmotically lysed using distilled water. Protein concentration of the pigment was determined by Bradford method. The pigment was stored at -70oC until use. Protein free pigment was obtained after treating the hemozoin with enzymes. Peripheral blood mononuclear cells were obtained from heperinized whole blood (from healthy donors) by histopaque density gradient. PBMC proliferation were studied with different concentrations of hemozoin. The results show that there was an increase in the PBMC proliferation with increasing concentration of the insoluble pigment. The pigment was not toxic to the cells at the highest dose tested. Hemozoin inhibited the proliferation of PHA

stimulated PBMCs. However, hemin chloride and zymosan does not inhibited the proliferation of PHA stimulated cells.

Proposed work:

PBMC proliferation assay with malaria soluble antigen fractions, hemozoin, protein free zemozoin in the presence or absence of growth factors / mitogen(s). Hemozoin phagocytosis by monocyte / macrophages - PBMC proliferation. Effect of hemozoin on the blast cells.

Outcome of the project:

Publications: Nil.
Patents: Nil.
Product: Nil.

Supporting units

Library and documentation:

The Library and Documentation services unit of the Centre added to its stocks 600 documents including books, serial monographs and bound volume periodicals. The library is equipped with a dedicated LAN of 3 terminals and is maintaining a number of databases. The library continued to provide services like electronic mail and internet access to the user community. The library has continued to suscribe for online access with online hosts like DIALOG, DATA STAR for databases in all areas of science and technology.

Publication:

N. V. Ramakrisha, N. B. Pangannaya. Quantitative analysis of animal cell culture literature to identify core journals supporting multidisciplinary R & D: A study based on The Animal Cell Biotechnology. Communicated to Scientometrics, The Netherlands, as an invited paper.

Animal House:

The facility for small laboratory animals has been equipped and started functioning in the new environmentally controlled experimental animal facility. The temperature, humidity and ventilation is regularly monitored and maintained. Health monitoring is done by faecal, blood and skin sample examination. All the animals used for various experiments were healthy and free from any infection.

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 Centre.

The necessary help has been exrtended to scientists / research scholars in handling, collection of samples and procurement of animals by the facility staff.

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 procured from various sources, bred in the facility and supplied for various R & D activities are given below:

Sr. No.	Strain / species	Animals procured	Animals bred	Animals supplied	Animals culled
1	Rats / Wistar	--	199	44	51
2	Mice BALB/c	410	5055	2642	1638
3	Mice C57BL/6	--	740	430	235
4	Mice swiss	--	782	463	217
5	Mice nude	30	--	30	--
6	Mastomys	3	151	11	16
7	Hamster	24	--	13	11
8	Rabbits	2	15	2	16

Media requirements:

Tissue culture media / sera requirements during 1-4-1997 to 31-3-1998

MEDIUM	QUANTITY (ml)
BME (E)	22000
CLICKS	1000
DMEM	118000
DMEM + Ham's F-12	8000
Grace's insect cell culture medium	2000

H-Y MEDIUM	5000
Hos	3000
Ham's F-10	12000
Ham's F-12	7000
Ham's F-12 K	2000
FCS	37000
GS	100
IMDM	50000
L-15	8000
M 199 (E)	1000
M 199 (H)	2000
MEM (E)	140000
MM	7000
McCoy's 5 A	4000
NBCS	2000
Opti MEM	2000
RPMI 1640	172000
Schneider	4000
TNM-FH	5000
Waymouth's medium	2000
William's medium	4000

Publications and conferences

Publications:

1. Javed N. Agrewala, Susmit Suvas, Aneesh Joshi, Archana Bhatnagar, Dass S. Vinay and Gyan C. Mishra (1998). 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. 18: 297-304.
2. Javed N. Agrewala, Susmit Suvas, Rakesh K. Verma and Gyan C. Mishra (1998). Differential effect of anti-B-7 and anti-M150 antibodies in restricting the delivery of costimulatory signals from B cells and macrophages. J. Immunol. 160: 1067-1077.
3. Limaye L. S. (1997) Bone marrow cryopreservation: Improved recovery due to bioantioxidant additives in the freezing solution. Stem Cells 15: 353-358.
4. Kale V. P. and Limaye L. S. Stimulation of adult human bone marrow by factors secreted by fetal liver haematopoietic cells: In vitro evaluation using semi-solid assay system. (Communicated).
5. Chakravortty Dipshikha and Nand Kumar K. S. (1997) Induction of cell proliferation and collagen synthesis in human small intestinal lamina propria fibroblasts by lipopolysaccharide: Possible involvement of nitric oxide. Biochem. Biphys. Res. Commun. 240: 458-463.
6. Kshirsagar Supriya G., Patole Milind S and Shouche Yogesh S. (1997) Characterization of insect cell lines: Heteroduplex analysis employing mitochondrial 16S ribosomal RNA gene fragments. Anal. Biochem. 253: 65-69.
7. Dighe A., Shouche Yogesh S. and Ranade D. R. Isolation of Selenomonas lipolyticum sp. Nov., an obligately anaerobic bacterium possing lipolytic activity. Internatl. J. Systematic Bacteriol. (Accepted for publication).
8. Kshirsagar Supriya G., Patole Milind S. and Shouche Yogesh S. Insect cell line authentication by DGGE. In Vitro. (Accepted for publication).
9. Pidiyar Vyankatesh, Patole Milind S. and Shouche Yogesh S. Ribosomal RNA based phylogenies and molecular ecology. (Communicated to J. Biosci.).
10. Ramakrishna N. V. and Pangannaya N. B. (1997) Quantitative analysis of animal cell culture literature to identify core journals supporting

multidisciplinary R & D: A study based on the animal cell biotechnology. (Manuscript submitted: Invited paper for Scientometrics, The Netherlands).

Conferences:

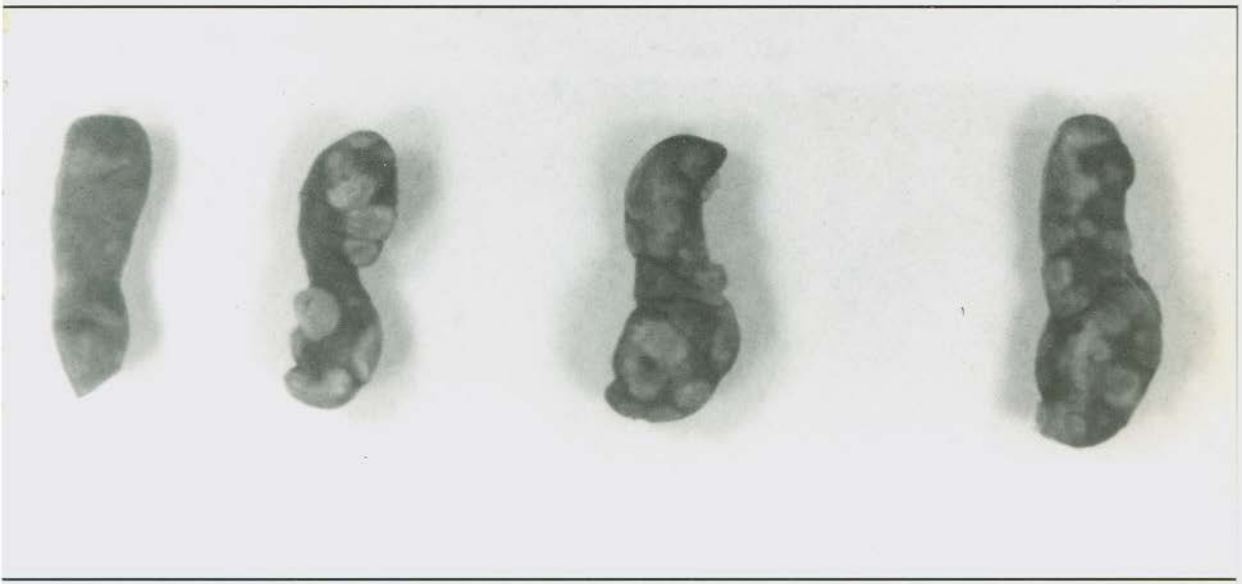
1. Padma Shastry. Cycloheximide enhances staurosporin induced differentiation in murine NB cells. International Symposium on Development, Growth and Differentiation. Mahabaleshwar, December 1997.
2. Padma Shastry. International Symposium on Biology in the 21st Century & XXI All India Cell Biology Conference, IISc, Bangalore. March 1998.
3. Hardikar A. A., Yajnik C. S., Karandikar M. S. and Bhonde R. R. Chronic malnutrition increases susceptibility to diabetogenic insult. AIDSPIT Conference, Austria. January 1998. (Abstract in Hormone Metabolic Research, January 1998 supplement).
4. Hardikar A. A. and Bhonde R. R. Islet neogenesis by subtotal pancreatectomy leads to normoglycemia in diabetic mice. AIDSPIT Conference, Austria. January 1998. (Abstract in Hormone Metabolic Research, January 1998 supplement).
5. Bhonde R. R., Hardikar A. A. and Kurup S. Development of bioartificial organ devices to achieve a functional restoration of damaged cells. Proc. Internatl. Symp. Development, Growth and Differentiation. Mahabaleshwar. December 1997.
6. Pidiyar Vyankatesh, Patole Milind S. and Shouche Yogesh S. Bacterial community structure of mosquito midgut. Annual Meeting of Society of Biological Chemists, Vishakhapatbam, 1997.
7. Pidiyar Vyankatesh, Patole Milind S. and Shouche Yogesh S Detection and identification of endosymbionts in mosquitoes. Annual Meeting of Society of Biological Chemists, Vishakhapatbam, 1997.
8. Kshirsagar Supriya G., Patole Milind S. and Shouche Yogesh S. Insect cell line characterization and authentication: methods based on sequence variation in mitochondrial rRNA genes. International Symposium on Biology in the 21st Century & XXI All India Cell Biology Conference, IISc, Bangalore. March 1998.
9. Limaye, L. S. International Workshop on molecular biology of stress responses. Banaras Hindu University and German Ministry of Research, Germany. October 14-17, 1997.

10. Kale V. P. International Workshop on molecular biology of stress responses. Banaras Hindu University and German Ministry of Research, Germany. October 14-17, 1997.

PLATES

Plate I

Figure 2. Western blot of anti-M-150 antibody. Lane1: Standard molecular weight markers; Lane 2: Membrane preparation of macrophages; Lane 3: Membrane preparation screened with hamster Ig (negative control); Lane 4: Membrane preparation screened with hamster serum showing positive specific reactivity to M-150 (150 kD).



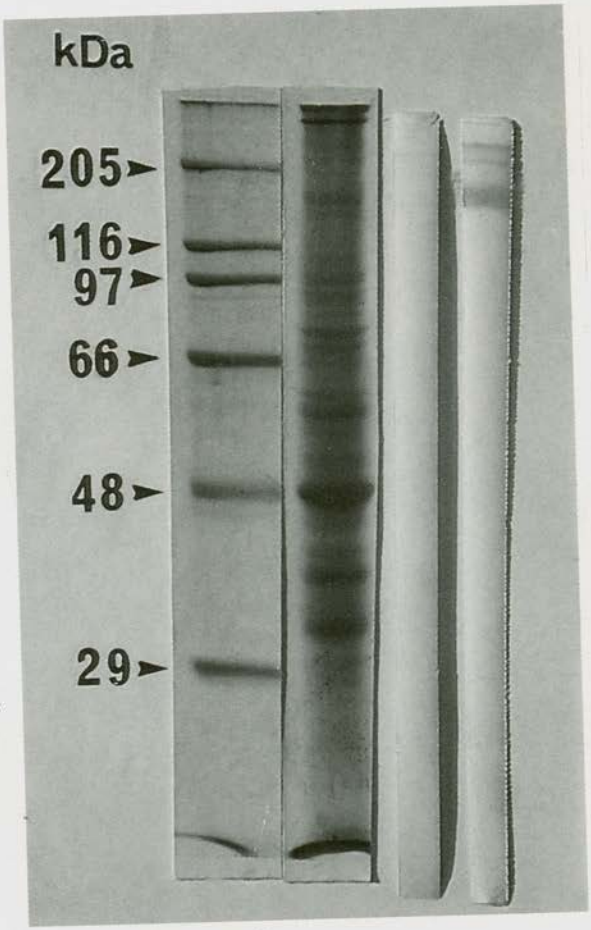
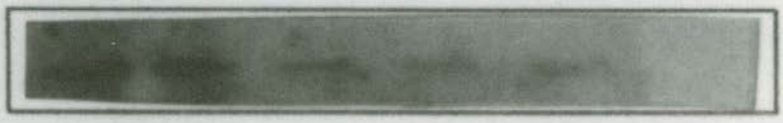

kDa
205
116
97
66
48
29

Plate II

Figure 3. Colony forming units spleen (CFU-S). 1. Irradiated mice infused with fresh cells; 2. Mice infused with frozen cells (10% DMSO alone); 3. Mice infused with frozen cells (10% DMSO + Trehalose); 4. Ctrl irradiated mice infused with saline.

Plate III

Figure 23. Detection of Inos (inducible nitric oxide) in HSILPF (human small intestinal lamina propria fiboblasts) by Western blot. Cells were seeded at a density of 106/ml, under different experimental conditions, with or without inhibitors. Cell contents were lysed and subjected to 10% PAGE. The proteins were electrophoretically separated and transferred to PVDF membrane. Membranes were reacted with antibody to iNOS enzyme followed by HRP conjugated anti-mouse IgG. The blots were developed by an ECL kit as per manufacturer's instructions (Amersham). Data taken from BBRC, 240: 458-463 (1997).



Medium	+	+	+	+	+	+
LPS	-	+	+	+	+	+
IFNγ	-	+	-	+	-	+
L-NMMA	-	-	-	+	+	-
AG	-	-	-	-	-	+