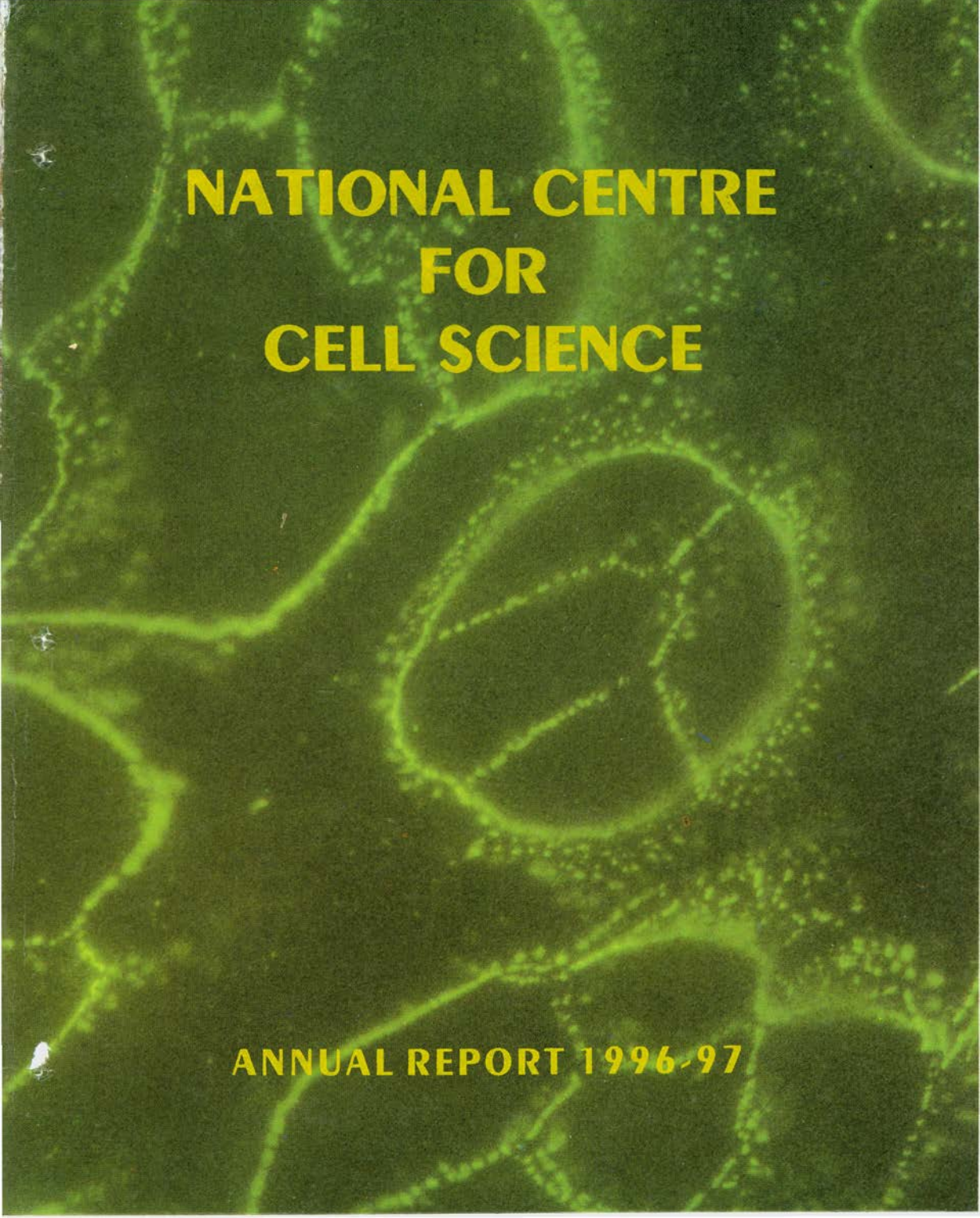
NATIONAL CENTRE FOR CELL SCIENCE



ANNUAL REPORT 1996-97

Dr. Ulhas Wagh, founder director of NCCS, inaugurating the Science Day celebration on 28th February 1997

NATIONAL CENTRE FOR CELL SCIENCE

An autonomous Institution of the Department of Biotechnology
Government of India

ANNUAL REPORT 1996-97

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

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 & 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.

CONTENTS

SUPPORTING UNITS

PREFACE

It is my privilege to present this Annual Report of the National Centre For Cell Science 1996-97). I present here an overview of the activities carried out during the last one year:

Cell Repository:

During 1996-97, a total of 538 cell cultures comprising of 164 cell lines were supplied to 104 research institutes located in 65 different cities. Four hundred individuals have registered for availing the cell supply facility. A total of Rs 64,800/- was received from the users as handling charges during the last year.

Research programmes:

Out of the eleven ongoing research projects, two are about to be concluded. The first one relates to the cryopreservation and revival of haematopoietic cells. 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. The second project being concluded relates to the characterization of mosquito cell lines using mitochondrial DNA RFLP. Here, a highly sensitive method using heteroduplex analysis and restriction fragment length polymorphism of the mitochondrial DNA has

been established for the identification and characterization of mosquito cell lines.

Earlier, we had prepared bio-equivalent skin for transplantation in burns, nevi and vitiligo cases. These cultured epithelia are fragile and have to be handled with a lot of skill. To improve graft takes, bio-artificial matrix, prepared from bioartificial matrix cultured cells will help in improving the graft takes and encourage earlier healing in these cases. Extensive clinical trials of cultured epithelia would be carried out in collaboration with Sion hospital, Mumbai Moreover, six centres will be set up in three cities to take up this activity further.

Development of cell cultures from commercially important invertebrates and vertebrates is another area being actively persued. A collaborative programmed to establish in vitro cultures of epithelial cells derived from the mantle tissue of the pearl oysters has been undertaken. Cultured fetal hepatocytes hold the potential for preparation of bio-artificial liver support device in case of acute and chronic liver failure.

Studies on endemic and non-endemic sera for IgG4 antibodies to Starrier digital antigen have indicated its importance in identification of human filaria due to *W. bancrofti* infection. Efforts are in progress to establish a collaboration with industries to develop this novel concept into a diagnostic kit for filaria.

Studies on the identification of melanoma oncogenes) have yielded promising results and suggest the generation of subclone of M3 transfectants from the cDNA library which exhibits features of malignant melanoma. Erythropoietin was found to induce secretion of TGF-beta-1 from bone marrow mononuclear cells in a dose-dependent manner. The released TGF-beta-1 was responsible for bringing about endothelial characteristics in bone-marrow derived stromal cells and also induced adhesive properties in them.

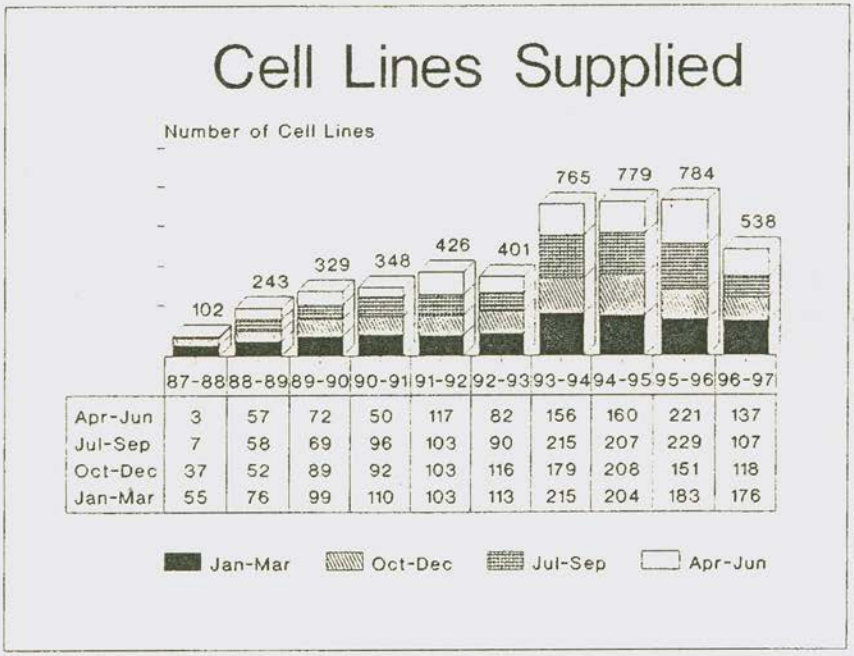
The neuroblastoma cell lines, such as Neur-2a, are a useful model to study differentiation. The changes in adhesion to collagen-I have been studied after induced differentiation in these cells. The differentiated cells exhibited increased binding to collagen as compared with undifferentiated cells.

The events occurring during epithelial-mesenchymal transition are being evaluated using an *in vitro* wound healing model. Cells lining the wound respond to addition of serum or growth factors by migration into the wound. Progression of the cell division cycle in these migrating cells appears to be reversibly arrested.

In vitro studies on molecular and cellular interaction lipopolysaccharide with human intestinal mesenchymal cells have indicated that a 75 kDa protein of the lamina propria fibroblasts is a probable candidate molecule for the LPS recognition and signal transduction.

CULTURE REPOSITORY

In 1996-97, 538 cultures comprising 164 different cell lines were supplied to 104 research institutes located in 65 different cities (Fig. 1). Four hundred individuals have registered for availing the cell supply facility. In 1996-97, Rs. 64,800 was received from the user as handling charges.



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

Fig. 1. Cell lines supplied during 1996-97.

MEDIA REQUIREMENTS 01/04/96 TO 31/03/97

MEDIUM	QUANTITY IN LITRES
BGJB	0.25
BME(E)	7.25
DMDM	121.7
Grace's insect medium	0.5
Ham's F-10	9.7
Ham's F-12	5.9
IMDM	32.0
L-15	6.0
M-16	0.5
M-199(E)	0.75
M-199(H)	1.0
M-2	0.5
MCDB 153	1.25
MEM	4.5
MEM(E)	113.85
MEM(H)	2.45
MM	10.2
MM+VP-12	0.95
McCoy's 5a	2.75
NCTC 135	0.25
Opti-MEM	0.25
RPMI 1640	44.75
RPMI 1640(H)	57.4
SBCM	4.25
Schneider	1.25
TNM-FM	1.75
William's	0.1

REAGENT	QUANTITY IN LITRES
EDTA	0.1
HBSS	9.1
PBS	65.3
TPB	0.2
TPVG	29.19
Trypsin (2%)	4.52

SERUM	QUANTITY IN LITRES
HOS	1.8
FBS	3.65
FCS	26.5

Culture of human skin keratinocytes and their 3-D epitheliation for transplantion to burns, non-healing ulcers & vitiligo cases

Participants: Dr. Manoj Mojamdar
Pallavi Kulkarni
Vaishali Chaubal

Expected date of completion: 1998

Background:

Current modalities of treatment of burns are not satisfactory. The skin performs two major functions:

1)To prevent water loss

2)To prevent infections.

Presently the methods used to cover the burn wounds can carry out either one of the functions and hence there is a need to develop system that can do both the functions at least temporarily, till the wound heals.

The incidence of burns, either chemical, electrical or fire is increasing .While up to 20% burns are handled well by burns wards, people with more than 20% burns die either due to water loss or due to septicemia. Bio-artificial skin is therefore the most viable alternative that needs to be developed.

Summary of the previous work:

Human skin biopsy obtained from patients have been successfully cultured, 3-D epithelia have been grafted with good results in burns, nevi and vitiligo cases. Several cell lines have been tested for growth of human skin Keratinocytes. During the case of these studies we have observed that lot of work remains to be done on immobilization of the cultured skin so as to enable us to transport the epithelia and graft it on to burns cases.

Work carried out during the current year:

In order to immobilize human skin keratinocytes and the preparation of skin equivalents lattice matrix made of collagen, chitosan, gellose agarose were prepared. Fibroblasts were found to grow well on these matrix.

Keratinocytes & Melanocytes were successfully grown as explant cultures on collagen gels.

Extensive clinical trials of cultured trials of cultured epithelia for treatment of burns in collaboration with Sion Hospital , Bombay.

Outcome of the project:

1) Patents (Indian): Nil
(International): Nil

2) Development of marketable technology/products : Two products are bio- artificial matrix & cultured epithelia for burns treatment can be marketed .

3) Publications : Nil.

4) Six centers are being set up ; out of which three have been identified

Regulation of skin pigmentation: a) regulation of pigment transfer; b) regulation of melanogenesis.

Participants: Dr. Manoj Mojamdar
Dr. G. Raman, *HLRC, Bombay*
Pallavi Kulkarni
Vaishali Chaubal

Date of initiation: 1993
Expected date of completion: 1998.

Background:

Skin pigmentation is a prominent social marker. For normal skin pigmentation to take place a co-ordinated cascade of events have to function. These include melanin synthesis inside the melanocyte and its transfer to keratinocytes.

Pigmentary disorders are cosmetically disfiguring disease. Scientific understanding of the normal biochemistry and physiology of skin pigmentation is a prerequisite to understand the process of its impairment leading to pigmentary disorders.

Targets defined and work carried out:

1st year: Culture of normal human melanocytes for transplantation to vitiligo cases.
2nd year: keratinocyte-melanocyte interactions.
3rd year: Role of phosphorylation in melanogenesis.

4th year: To investigate the effect of some plant compounds that are known to be useful for pigmentation.
5th year: Identification of factors released by keratinocytes under normal and UV-irradiated conditions.

Work done till date:

Culture of melanocytes from uninvolved skins of vitiligo patients were successfully cultured and transplanted. Studies on melanocyte and keratinocytes *in vitro* have revealed that keratinocytes could be producing dendrite extension factors and dendrite inducing factors.

Work carried out in the current year:

1) Lawsone, a quinone isolated from mehndi (Heena) plant has been mentioned in Ayurveda as a drug therapy for vitiligo. Other reports however show its action as tyrosinase inhibitor. We tested lawsone for its action on cell free tyrosinase and for its action on B16 melanoma cells.

Lawsone has been found to increase the initial rate of reaction when tested in cell free tyrosinase assay system with L-Dopa as substrate.

On cells however Lawsone is showing dual mode of action. In melanotic B-16 melanoma cells lawsone depleted the tyrosinase activity. In amelanotic melanoma cells lawsone increased tyrosinase activity as well as initiated differentiation by 48 hours of lawsone treatment. The response has been found to be dose dependent. Thus our

experiments confirmed that lawsone has dual function on B-16 melanoma cells.

2) Culture of normal human keratinocytes and melanocytes were set up using circuscion skin.

The keratinocytes and melanocytes were cultured on petri plates in such a manner that the two cell types were isolated from each other. The keratinocytes were irradiated and the effect was observed on melanocytes. It was seen that in response to keratinocyte irradiation the melanocyte dendricity and melanin production had greatly increased. This clearly indicates that keratinocytes make certain factors in response to UVR. which influence melanocyte dendricity and melanogenesis. The results also indicate that contact between two cell types is not necessary for induction of melanogenesis and dendricity by UV.

3) Treatment with sodium periodate has been found to increase melanogenesis. Further studies are being carried out to clarify the oxidative melanogenesis by sodium periodate.

4) Using B-16 melanoma cells cultured in high and low phosphate medium, it has been found that active form of tyrosinase may be the dephosphorylated form and the phosphorylated form may be the inactive form.

Outcome of the project:

1) Patents: Indian - possibility exists.
International - possibility exists.

2) Development of marketable technology / products: Possibility exists.
3) Publications: Manuscript in preparation.

Studies on cryopreservation and revival of Haematopoietic cells.

Participants : Dr. L. S. Limaye
Dr. S. G. A. Rao.
Cancer Research Institute, Bombay.
Dr. R. L. Marathe,
Jehangir Hospital, Pune.

Date of initiation : June 1990.
Expected date of completion : December 1997.

Background :

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 till date :

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. So far we have cryopreserved and revived a total of 60 mouse bone marrows, 60 adult human bone marrow and 60 fetal liver haematopoietic cells.

Details of the work :

The work was held up for a period of seven months between June'96 to Dec.'96 due to shifting to new campus and setting up of new labs.

Our recent studies have shown that response of cryopreserved cells to different concentrations of growth factors in CFU assays is not the same as fresh cells. In mouse studies it was observed that when mouse bone marrow cells were frozen with antioxidants in the freezing mixture the poor growth factor response of cells is slightly restored (Fig. 2). Catalase was found to be particularly effective here (Fig. 3).

It is hypothesized that probably oxygen free radicals damage the CSF receptors during freezing and this damage is reduced to some extent by antioxidants. We have used two cell lines i.e. HL 60 and KG1a as models of haematopoietic cells and have done some experiments with them. It is observed that levels of MDA is higher in frozen cells as compared to fresh cells. The MDA level was estimated by spectrophotometric measurement of the coloured product it forms with TBA. Thus our observations do show that oxygen free radicals are formed during

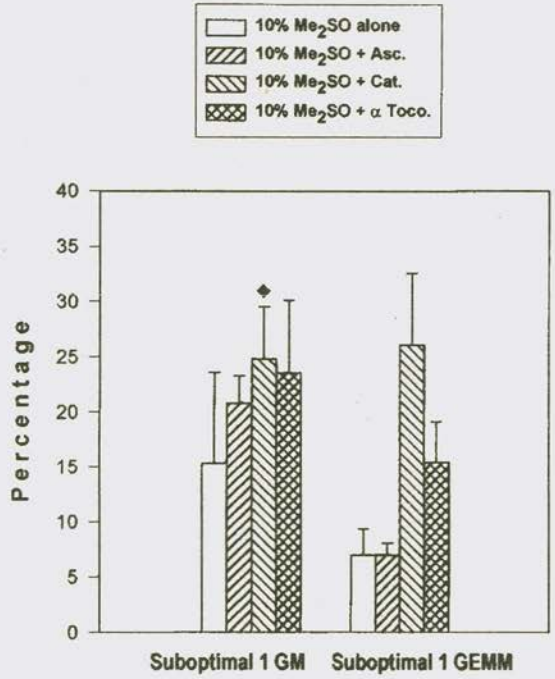


Figure 2. Response of mouse bone marrow cells cryopreserved with or without antioxidants to suboptimal GM and GEMM assay.

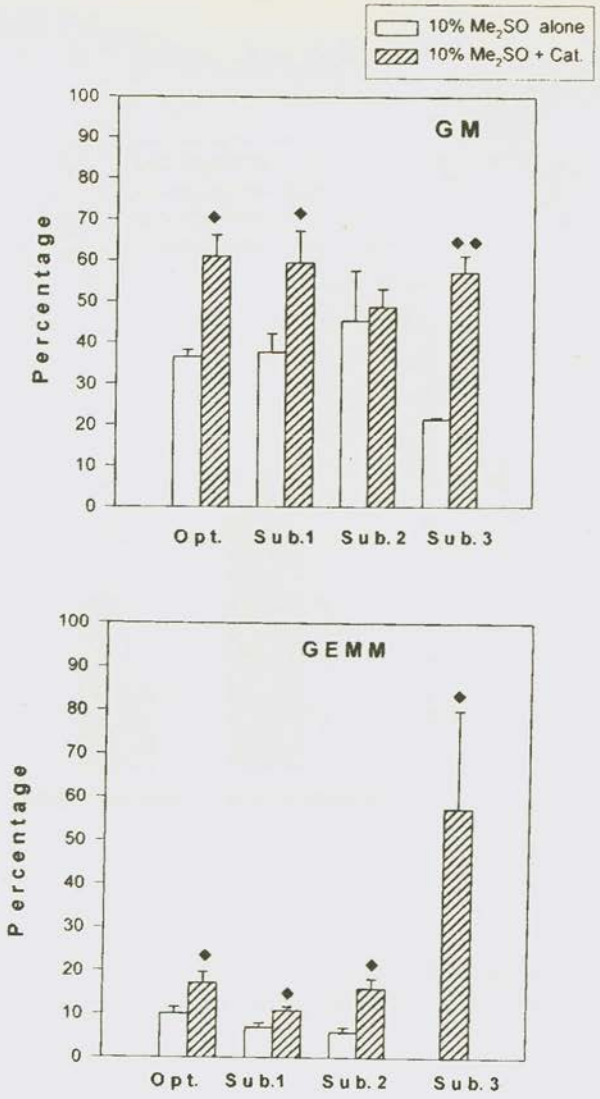


Figure 3. Response of mouse bone marrow cells cryopreserved with or without catalase to suboptimal GM and GEMM assay.

freezing. Cryopreservation of Fetal liver haematopoietic cells Refer to Dr. Kale's report.

Outcome of the project :

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

Patents : NIL.
Development of marketable technology : NIL
Publications: One manuscript communicated to Stem Cells; Two manuscripts are under preparation.

Stromal cell Biology: *a) Definition of factors triggering cell-cell interactions in the haematopoietic cells; b) Analysis of signals generated in the hematopoietic cells as a consequence of cellular interactions.*

Participants: Dr.(Mrs.) Vaijayanti Kale.
Dr. L. C. Padhy.

Date of Initiation: January 1994.
Expected date of completion: a) Nearing completion.
b) December 1998.

Background:

Blood cell formation or haemopoiesis 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 pleuripotential stem cell and culminating in fully differentiated end products are broadly understood, several important aspects of this process still remain elusive.

Stem cells are found in intimate association with Stromal cells in the hematopoietic microenvironment in vivo as well as in vitro. Several experiments have documented the importance of hemopoietic environment consisting mainly of bone marrow stroma. The bone marrow micro environment is defined by the Stromal cells and the ECM component surrounding them. In long term bone marrow culture experiments, it has clearly been shown that bone marrow derived Stromal cells play a crucial role in colony formation. Several experiments have underscored the need for cell-cell interactions that allow effective interaction or cross-talk between the differentiating hemopoietic 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 various stages of differentiation process. The role of various cytokines, interleukins etc. in this process is reasonably understood to allow designing of more precise experiments. 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 hemopoietic cells?" This undoubtedly is a central issue, because of the deterministic factors modulating specific cell to cell contact could be identified, it may pave the way to modulate haemopoiesis in a pre-determined manner, at least, in cell cultures. Secondly, this understanding may aid in the identification of defective steps in human disease process.

Work carried out:

We previously reported that bone marrow derived mononuclear cells released soluble factor(s) in the medium (CM) in response to Erythropoietin (Epo) in a dose-dependent manner. This CM was shown to induce adhesive properties on Stromal cells. One major constituent of the CM was identified as TGf using neutralizing antibody experiments, heparin agarose depletion and Mink Lung cell inhibition assay. $TGfb_1$, secreted by MNC was further shown to be responsible for heterotypic adhesion of CD34+ stem cells from bone marrow leading also to enhanced colony formation. fibronectin receptor ,namely a_5b_1 was shown to be involved in these cellular interactions. We also found that the treatment of Stromal cells with active CM as well as purified $TGfb_1$ induced an endothelial phenotype on them (Fig. 4, Plate I and Fig. 5, Plate II).

Our next goal was to identify the signals generated in the Stromal cells as a result of treatment with CM as well as with purified $TGfb_1$. We carried out a set of experiments using pharmacological reagents to modulate the PKC and intracellular Ca2+; as these are the most common signaling molecules of many pathways. When cells were treated with phorbol myristate, we got an enormous enhancement of colony formation (Fig. 6, Plate III). Moreover, when the Stromal cells were incubated for 48 hours with phorbol dibutyrate, a treatment known to down regulate PKC, the response on Cofl was totally abrogated indicating that PKC was involved in the process. This observation was further substantiated using specific agonists of PKC namely Indolactam V(-) and Diacylglycerol.

Treatment of Stromal cells with these reagents also resulted in enhanced CFU. The control compounds namely, Indolactam V(+) nd Dioctoylglycerol had no effect. In order to assess whether PKC acted downstream of TGf signaling, the Stromal cells treated with TGf were also treated with Myristoylated Octapeptide (BACHEM), a very specific PKC inhibitor (IC 50 = 50mM). Such cells failed to support CFU formation indicating that PKC indeed was an important downstream signaling molecule of TGf response.

Similarly Stromal cells treated with $TGfb_1$ / CM were treated with BAPTA-AM (Molecular Probes) to buffer the intracellular Ca2+, failed to support colony formation. Half-BAPTA used as control did not have any effect on the competency of Stromal cells. This experiment demonstrated that intracellular Ca^{2+} also formed an important downstream signal along with PKC. Blocking of either signal abrogated the response. We also used Thapsigargin, a known agonist to elevate intracellular Ca^{2+} to treat the Stromal cells. Such Stromal cells also were found to induce enhanced colony formation. Our earlier experiments had indicated involvement of integrins in the cellular cross-talk. Secondly, our experiments with pharmacological reagents indicated the role of PKC and Ca^{2+} which are known downstream effectors of integrin stimulation and also the fact that TGf is a known modulator of integrin expression, we examined the role of various integrins using synthetic peptides against a_5 b_1 and $apbb_3$ integrins. The results showed that a_5b_1 was more potent stimulator of CFU formation than $apbb_3$.

Outcome of the Project:

The study is likely to throw light on the yet unresolved problem in hematopoietic research, namely the signals influencing the self-renewal as against differentiation of the stem cells.

Publications: Data of part a) in being consolidated for manuscript preparation.

Proposed Work:

Experiments carried out in the first phase of the project indicated that the activated or competent Stromal cells delivered a signal to CD34+ cells and this signaling resulted in a rapid self-renewal of these cells .We propose to delineate completely the signaling pathway in Stromal cells as well as in the stem cells, using specific probes. We also propose to carry out the characterization of this amplified population in terms of its reconstitution ability using in vitro as well as in vivo models.

Fetal Liver Infusion Its mode of action efficacy of cryopreservation cells and its potential application in the management of cancer

Participants Dr. (Mrs.) V. P. Kale
Dr. (Mrs.) L. S. Limaye
Dr. Smita Sivraman

Background:

This project has been undertaken as a collaborative study with Dr. Vinod Kochupillai, AIIMS, New Delhi, to investigate the mechanism of autologous bone-marrow recovery, observed in several patients of aplastic anemia, acute leukemia after fetal liver infusions. The results of their clinical trial as well as the results reported by several authors indicate that fetal liver cells have the capacity to stimulate patients stem cells without the use of preconditioning regimen. However, the mechanism of this autologous recovery is not clear at present.

The proposal has been designed with the following objectives

i) To study the proliferative capacity of fetal liver hematopoietic stem cells compared to adult bone marrow.

ii) To study the mechanism of hematopoietic recovery induced by fetal liver hematopoietic cells, using coculture studies *in vitro.*
iii) To standardise the method of cryopreservation of fetal liver cells.

iv) To assess the efficacy of cryopreserved fetal liver cells using committed stem cell assays.

v) To assess the possible application of fetal liver hemopoietic stem cells in the management of cancer, if above studies succeed.

Work carried out:

We reported previously that fetal liver cells secrete factor(s) in the conditioned medium (FL.CM) and the CM was found to be responsible for enhanced colony formation of adult Bone Marrow cells in a co-culture system.

During this year experiments were carried out to identify the factor(s) in the FL.CM, responsible for enhanced CFU. Since mid trimester fetal liver is a very rich source of TGF-beta, we specifically looked for the presence of this factor in the CM. In the first set of experiments a neutralizing antibody (Genzyme) was used to neutralize the TGF in the FL.CM if present. It was observed that when such neutralized CM was used in the CFU assays the stimulation Index dropped down significantly (from S.I.= 4 to S.I. = 1.7). Since the response was not totally abrogated, the results indicated presence of the factor(s).

FL.CM was then passed through heparin agarose column and the heparin binding fraction was eluted with 2M NaCl. This fraction was dialyzed, concentrated and run on SDS-PAGE. The resolved proteins were then electrophoretically transfered to PVDF membrane. The membrane was blocked with 3% BSÁ and TGF-beta was immunodetected using MoAb (Genzyme) as well as polyclonal antibody (Santacruz Biotech) against TGF-beta-1 with appropriate secondary antibody conjugates and substrates.

The FL.CM was also screened for of the growth factors, namely GM-CSF, IL-3, Epo and SCF using ELISÁ Kits (R & D systems). It was found that FL.CM contained significant amounts of SCF ranging in 400-500 pg/ml.

The Fetal Liver Heamatopoietic cells were thus shown to secret two important modulators of hematopoiesis namely SCF and TGF-beta-1. Further experiments using these two factors alone or in combination are in progress.

Publication / Patents / Conferences:

Colony stimulating activity (CSA) secreted by fetal Liver and normal adult bone marrow hematopoietic cells role in engraftment after transplantation. Abstract accepted for platform presentation in 38th Annual Meeting of American Society of Hematology, December 6-10, 1996, Orlando, USA. Abstract in Blood, Dec. 1996 (Ab. No.3760). Manuscript in preparation.

Regeneration of pancreatic Beta cells

Participants : R. R. Bhonde
Anandwardhan Hardikar
Yogita Shewade

Date of initiation : December 1993
Probable date of completion : December 1999.

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. 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 maintainance 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 mimicing intra-uterine environment.
5. To ascertain the role of nutrition on induction and prevention of experimental diabetes.
6. To ascertain the role of immuno-supression and immuno-stimulation in modulation of the course of IDDM.

Work carried out :

In vivo studies:

In order to find out the possible role of pancreatectomy on reversal of diabetes, streptozoticin (STZ) induced diabetic mice were subjected to subtotal pancreatectomy (TPx).

Eight to ten week old BALB/c mice were grouped under 4 groups *viz.* control; non-diabetic sham operated (CS), Control; non-diabetic TPx (CTPx), diabetic sham operated (DS) and diabetic TPx (DTPx). Body weight, blood sugar levels, urine sugar and ketone levels, serum as well as pancreatic insulin levels were estimated at pre-defined time intervals for a long term period of 90 days. The body weight as well as blood sugar profiles of CTPx and DTPx clearly reflect a diabetic condition in the initial phase followed by an improvement towards normoglycemic status as compared to their sham operated controls (Fig. 7). The STZ-induced diabetic animals (DS) however fail to show diabetes reversal (Fig. 8). The serum insulin levels show a steady improvement in the insulin levels monitored from 6 hours post-op. (Fig. 9) and this is also evident from the progressive increase in our pancreatic insulin content data (Table 1.). Our data clearly indicates the process of nesidioblastosis after pancreatectomy in the diabetic animals and supports the theory that islet-stem cells are present in the pancreatic duct. A fool-proof evidence for the same comes from our histological sections (Fig. 10, Plate IV) which show neoislets budding out from the pancreatic duct. This is the first report that highlights a new model in diabetic research and also proposes a novel approach towards understanding of this very basic process of regeneration in diabetic animals which may prove a highly potent alternative to diabetes cure.

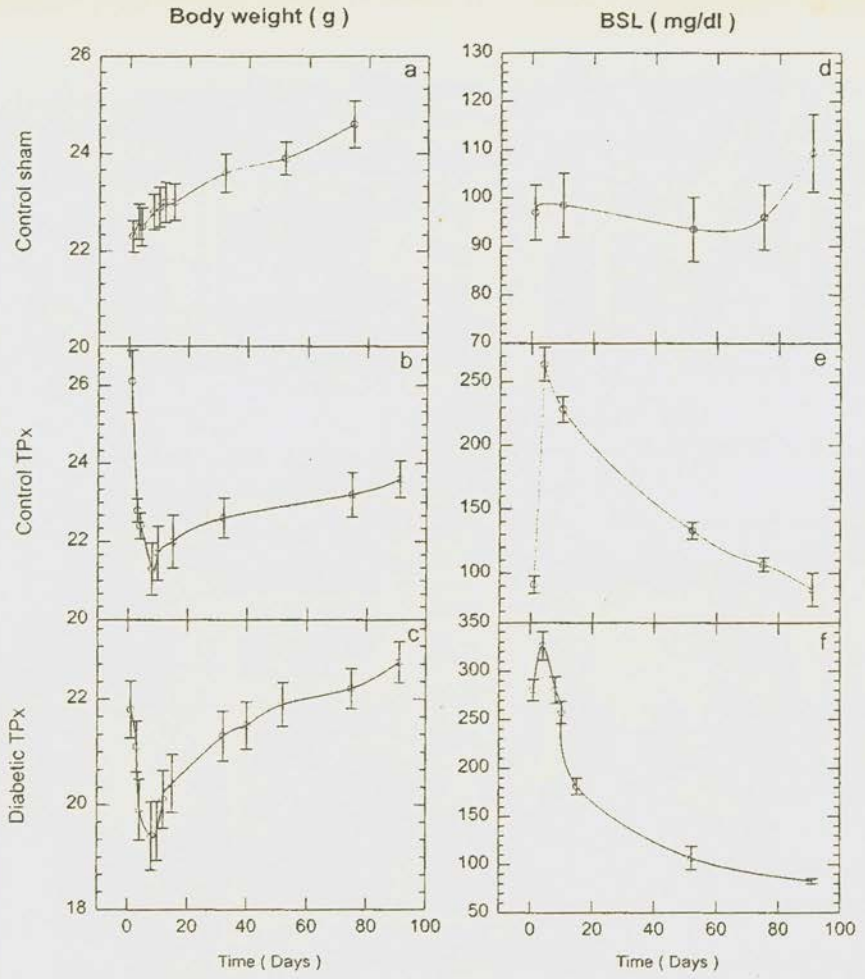


Figure 7. Body weight and blood sugar profiles throughout the period of study. Panels a, b and c represnt the body weight in grams of the CS (n = 7), CTPx (n = 7) and DTPx animal (n = 15) animal groups, respectively. Panels d, e and f represent the blood sugar levels in mg/dl of the CS (n = 7), CTPx (n = 7) and DTPx (n = 15) animal groups. In all the operative groups, zero day period represents the operative day. Data expressed as mean ± SEM.

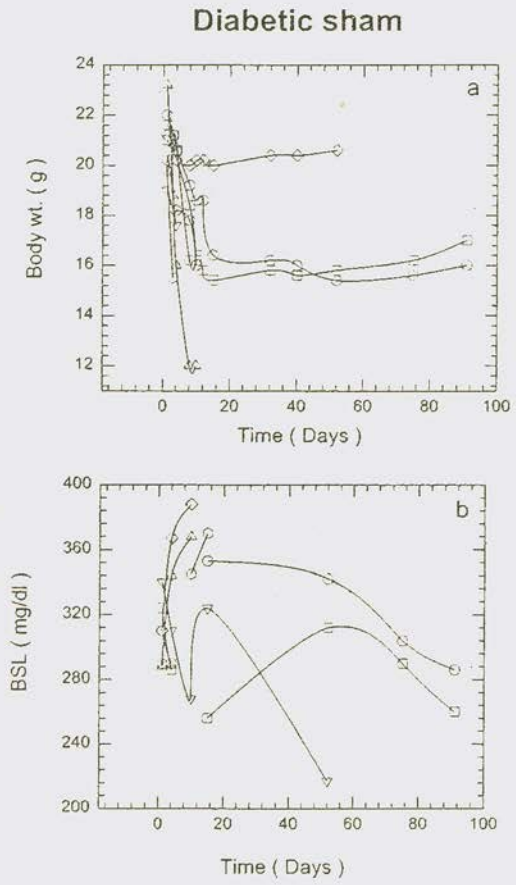


Figure 8. Body weight and blood sugar levels of individual diabetic, sham operated animal in the DS group (n = 9). Body weight and glycemic status of each animal is shown separately.

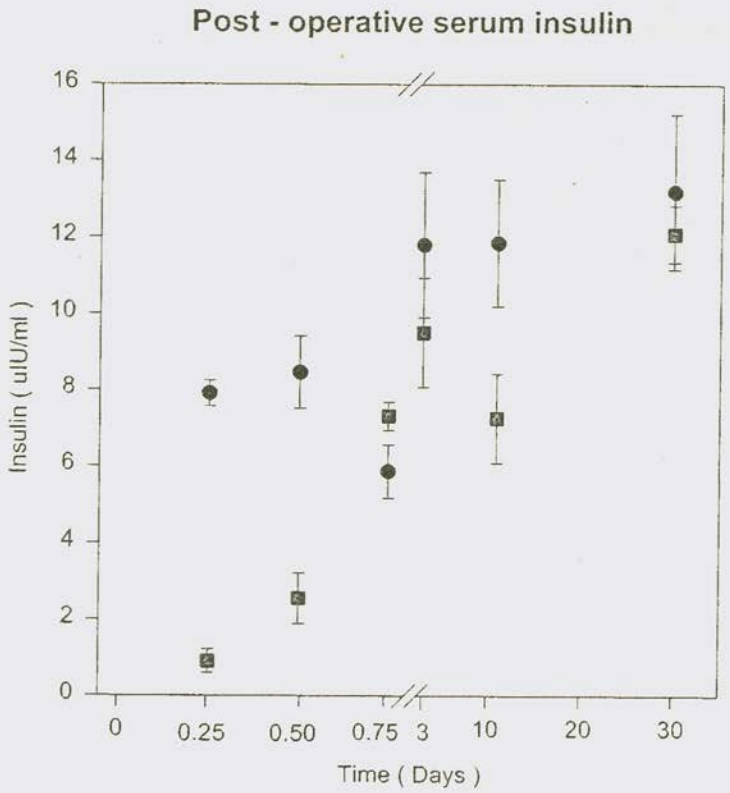


Figure 9. Post-operative serum insulin levels in the DTPx ( ) and the CTPx (o) groups starting at 6 hours after TPx. Data represented as mean + SEM. Serum insulin for equal number of animals in each group is compared (n = 7).

Post operative days	15	20	25	50
Operative groups				
CTPx	9.45	15.44	21.5	24.8
DTPx	14.39	17.68	22.08	25.24
DS	3.8	1.7	-	-

Table 1. Post-operative pancreatic insulin content (ulU/mg pancreas). Post-operative insulin was estimated by radio-immune assay. Data is expressed as ulU of insulin/mg pancreas. Insulin content in DS pancreas could not be estimated after 25 days due to high mortality in this group. Control animals always showed a value similar to that shown by the CTPx animals, 50 days after TPx.

In vitro studies:

(a) Islet cultures

The existing methods of islet isolation yield poor number of islets which are not sufficient to reverse diabetes on transplantation. Since it is essential to obtain large number of islets from a single host we modified existing technology so as to give higher number of islets. We modified conventional technology to obtain high yield of islets. The alteration was made in dissociation medium by supplementing with soyabean trypsin inhibitor (STI) & bovine serum albumin (BSA) in addition to collagenase. The islets were cultured for 48 hours before isolation. This protocol yielded 1600-2000 islets as evidenced by the actual counts. In order to optimise culture conditions for long

Post operative days	15	20	25	50
Operative groups				
CTPx	9.45	15.44	21.5	24.8
DTPx	14.39	17.68	22.08	25.24
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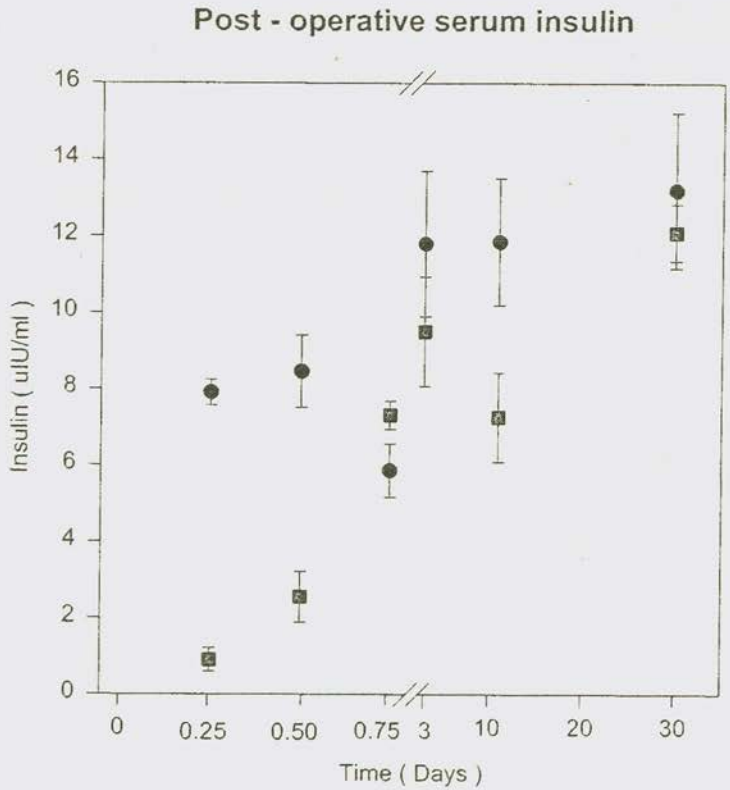

Figure 9. Post-operative serum insulin levels in the DTPx ( ) and the CTPx (o) groups starting at 6 hours after TPx. Data represented as mean + SEM. Serum insulin for equal number of animals in each group is compared (n = 7).

term survival of islets different nutrient media & incubation temperatures were tested. The parameters looked at were islet viability, morphology, attachment & insulin production. Amongst the media used RPMI-1640 & L-15 proved to be better than MEM. Amongst the different temperatures tested, 28° C permitted free floating culture of islets whereas 37° C permitted better islet attachment and proliferation. These results suggest that status of islet culture can be monitored as desired by modulating culture conditions .

(b) Embryo culture

With a view to find out the effect of different concentrations of glucose on early embryonic development, chick embryo cultures were set employing New's technique . Lower stages (16 to 24 hrs incubated) & higher stages (30 to 40 hrs incubation) of developing embryo were exposed to 16, 32 & 55 mM of glucose for 24 hrs. Control cultures were treated with PC-saline. It was observed that glucose induced abnormalities in the developing chick embryo, in a dose dependent manner. The abnormalities induced failure of neuropore closure, failure of heart primordia fusion, less number of somites & delayed development. All these were more pronounced in the lower stages whereas a similar glucose treatment regime induced enhanced development of brain & heart. Thus, developmental changes induced by high glucose seem to be detected by the stage of development. Biochemical studies are in progress.

Outcome of the project :

The proposed project would enhance our understanding of the pathogenic mechanisms involved in the induction of NIDDM and IDDM. This would indirectly help in planning strategies for prevention of diabetes, probably through promotion and protection of β-cells from diabetogenic insults. The study would aid in changing the therapeutic approach in control and cure of diabetes by enhancing endogenous β-cell regeneration as an alternative to islet transplantation.

Investigation into Beta cell protective mechanisms as means of reducing incidence of diabetes

Participants: R. R. Bhonde
Sandhya Sitasawad

Date of initiation: 1996
Expected date of completion: 1998

Background:

Genetic as well as environmental factors play a key role in both the insulin dependent diabetes mellitus (IDDM/Type I) and Non-insulin dependent diabetes mellitus (NIDDM/Type II). Oxidative stress is known to play a critical role in the etiology and complications of diabetes. The insulin producing beta cells are killed by the production of reactive oxygen species (ROS) such as, superoxide ($O_2^{\cdot -}$), Hydroxyl radicals ($^{\cdot}OH$), nitric oxide ($NO^{\cdot}$), hydrogen peroxide (H_2O_2) etc.

In normal situation there is a fine balance between the production of the free radicals and the antioxidant defense.

$$2O_2^{\cdot -} + +2H+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (1)$$

$$2H_2O_2 \xrightarrow{Catalase} H_2O + O_2 \qquad (2)$$

$$H_2O_2 + 2GSH \xrightarrow{GSHP} 2H_2O + GSSG \quad (3)$$

However, if the production of free radicals is more and if the antioxidant enzymes are low or defective, it leads to the disease process. Superoxide is the first radical produced and is the key molecule in the disease process. Low levels of or defective synthesis of the superoxide scavenging enzyme superoxide dismutase SOD) can lead to increased production of superoxide. Therefore to find out mutations if any in this enzyme and their correction would be the first line of therapy. In *in vivo* situation superoxide does not kill the cells on its own. It attacks the iron transport and storage proteins transferrin, ferritin etc. and release the iron which in turn forms more reactive hydroxyl radicals by the Haber-Weiss/Fenton reaction that kills the cells.

$$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+} \quad (4)$$

Therefore, measurement of iron released by the diabetogenic agents and thus its prevention by using iron-chelators would be another therapeutic approach in the treatment of diabetes.

Since nitric oxide (NO) participates in physiologically significant free radical reactions[1,2] and nitric oxide synthase is present in pancreatic beta cells, it has generated great deal of interest in the etiology and complications of

diabetes[3,4]. Nitric oxide, a transient species with a half-life of about five seconds, can exist in redox state NO· and NO^+ (nitrosonium ion). The toxic effect of nitric oxide results from its reaction with superoxide radical, to produce peroxynitrite (ONOO-) (reaction 5) which can lead to the oxidation and nitration of biological molecules. Peroxynitrite itself decompose into toxic compounds including nitrogen dioxide (NO_2·), ·OH and the nitronium ion (NO^{2+}) (raction 6, 7)

$$O_2^{\cdot -} + NO\cdot \quad ONOO^- \qquad (5)$$

$$ONOO^- + e^- + H+ \quad OH^- + NO_2\cdot \qquad (6)$$

$$ONOO^- + e^- + H+ \quad NO_2\cdot + OH^- \qquad (7)$$

$$HOCl^- + O2^{\cdot -} \quad Cl^- + O_2 + \cdot OH \qquad (8)$$

Apart from one electron reduction of peroxynitrite, the reaction of superoxide radical, which hypochlorous acid ($HOCl^-$) is also a likely source of hydroxyl radicals[5]. Nitric oxide in the presence of oxygen is also known to release superoxide anion. Recent findings described the formation of free radicals from nitric oxide and their role in the killing of pancreatic beta cells during the development of Type I diabetes which occurs spontaneously [6,7] or induced by STZ[8].

It has been observed that STZ contains a methylnitrosourea moiety attached to carbon two of glucose and may generate NO· by processes analogous to those for the ·OH-releasing

drug nitroprusside[4] or by local liberation of NO within beta cells. So, although the role of NO· in diabetes mellitus is highly emphasized and STZ's action by generation of NO· is highlighted, the action of other diabetogenic agents e.g. alloxan has not been discussed. It is important to check this aspect experimentally. NO· is formed by a calcium-dependent nitric oxide synthase (Nos) during the conversion of arginine to citrulline. i.e samall amounts of NO is rapidly triggered by agonists that elevate Ca^{2+}. Increased intracellular Ca^{2+} alters the conformation of calmodulin, which binds to nitric oxide synthase (NOs) to activate NO· production. Use of calcium/calmodulin antagonists would therefore be a good therapeutic approach lin the treatment of diabetes. It was therefore felt desirable to study these important aspects.

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 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.

Work carried out during last year:

Studies were carried out to test the efficacy of D-glucose and aqueous extract of bittergourd in reducing the incidence of STZ induced diabetes. In this study, lipid peroxidation was measured using Malondialdehyde (MDA) as a parameter of lipid peroxidation.

Work carried out till date :

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. Experiments were also carried out to test the efficacy of promethazine as an inhibitor of NO· production.

RINm5F cells were treated with different concentrations of SNP (0-1 Mm). It produced NO· formation (pmole nitrite/cells) in a concentration dependent manner (Fig. 11), however, there was no consistency in the results obtained by MTT assay (results not shown)

RINm5F cells were treated with 1 mM STZ, 1 mM alloxan, 1 mM H_2O_2 and 50 mM SNP. Nitrite prodction was very high in the SNP treated cells, moderate in STZ treated cells, negligible in alloxan treated cells and nil in the H_2O_2 treated cells (Fig. 12). In this case also there was no consistency in the results obtained by MTT assay (results not shown)

RINm5F cells were treated with 1mM STZ, alloxan, H_2O_2 and 50 mM and 100 mM SNP and different concentrations of PMZ (0-100 mM). In all the above cases PMZ at a concentration of 5 mM was found to be effective in inhibiting the nitric oxide production, whereas in both the 50 mM and 100 mM SNP treated cells the nitric oxide inhibition was concentration dependent (Fig. 13-16).

Publications: Nil

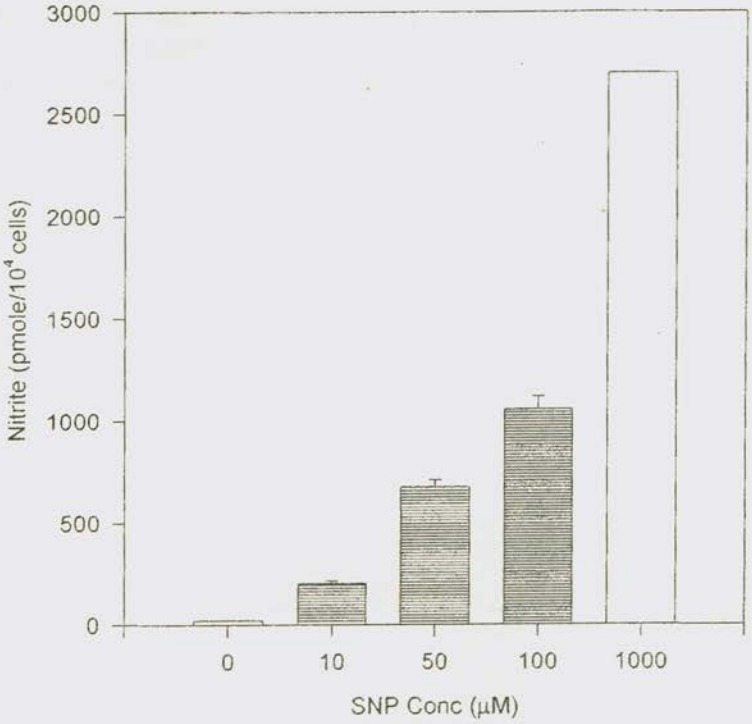

Fig. 11. RINm5F cells (10^4) were treated with different concentrations of SNP (0-1 Mm) for 24 hrs. and the production of NO· (pmole nitrite/cells) was measured. Each value represents an average of 10 samples ± Standard deviation.

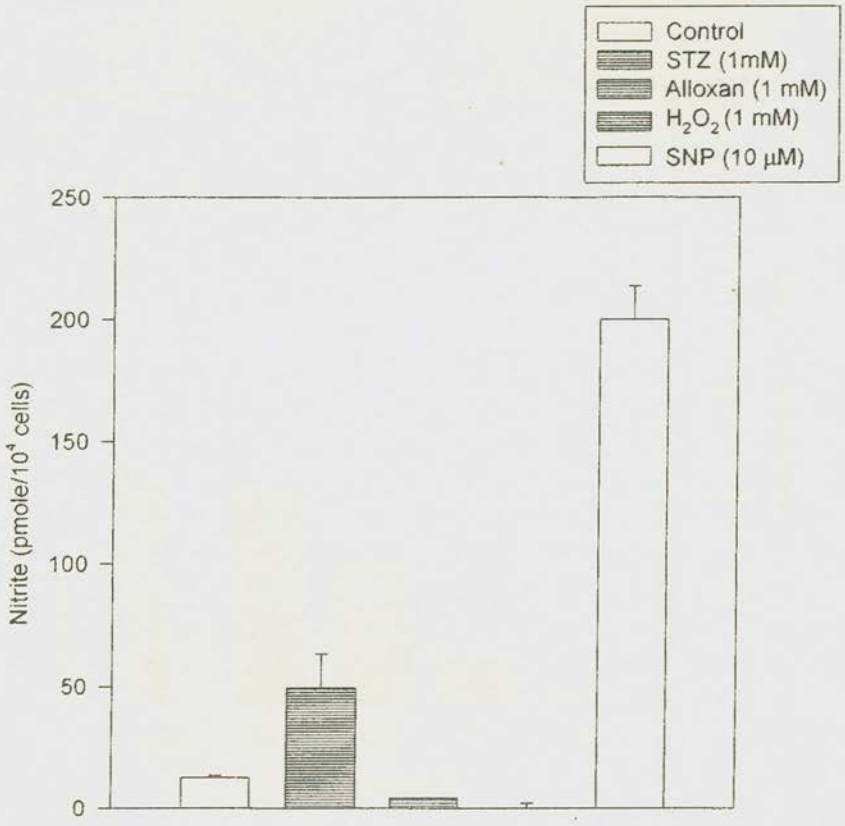

Fig. 12. RINm5F cells (10^4) were treated with 1 mM STZ, 1 mM alloxan, 1 mM H_2O_2 and 50 mM SNP for 24 hrs. and the production of NO· (pmole nitrite/cells) was measured. Each value represents an average of 10 samples ± Standard deviation.

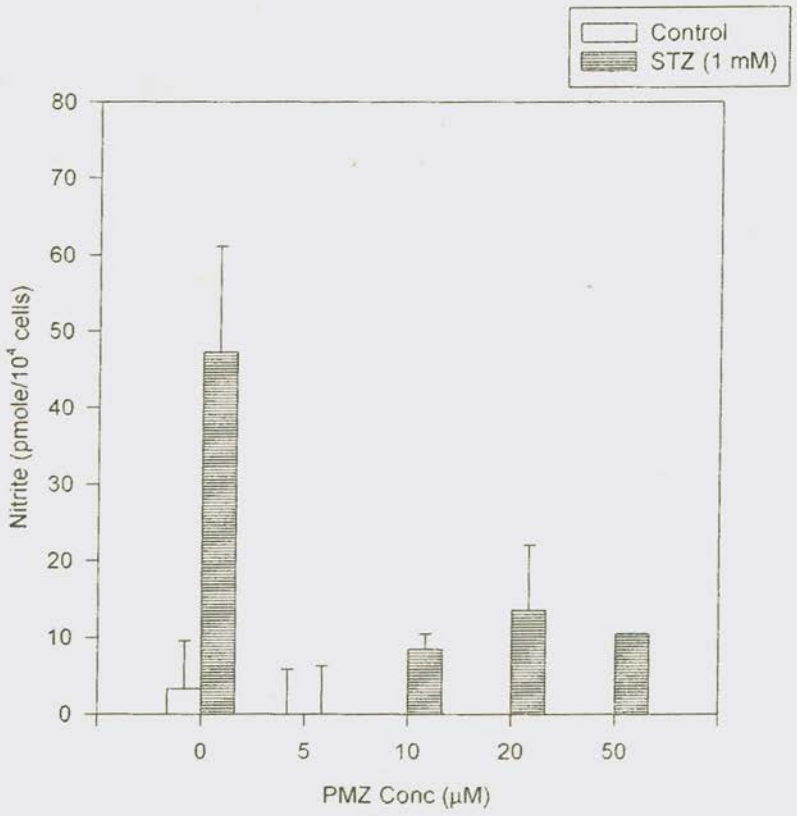

Fig. 13. RINm5F cells (10^4) were treated with 1mM STZ, and different concentrations of PMZ (0-50 mM) for 24 hrs. and the production of NO· (pmole nitrite/cells) was measured. Each value represents an average of 10 samples ± Standard deviation.

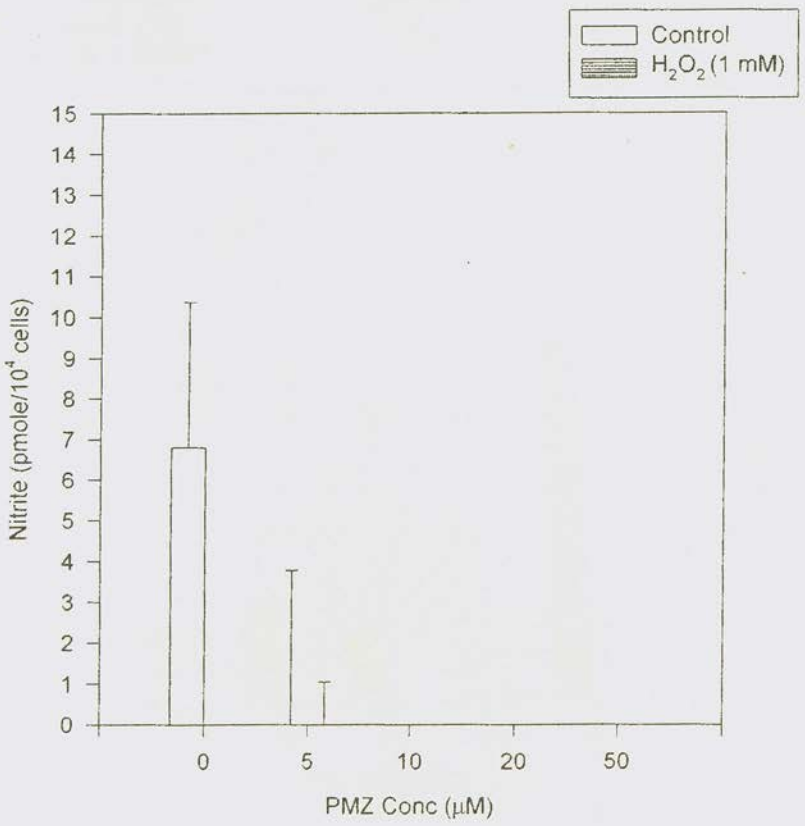

Fig. 14. RINm5F cells (10^4) were treated with 1mM H_2O_2, and different concentrations of PMZ (0-50 mM) for 24 hrs. and the production of NO· (pmole nitrite/cells) was measured. Each value represents an average of 10 samples ± Standard deviation.

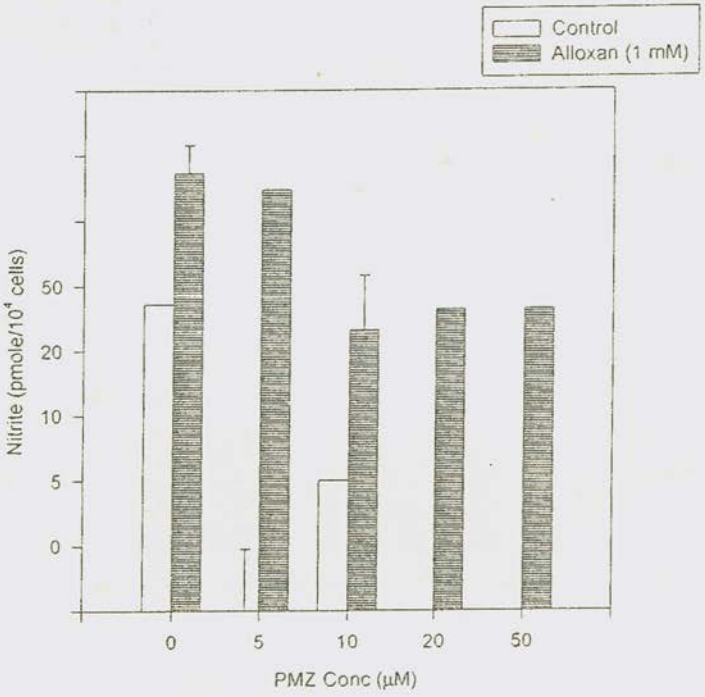

Fig. 15. RINm5F cells (10^4) were treated with 1mM Alloxan, and different concentrations of PMZ (0-50 mM) for 24 hrs. and the production of NO· (pmole nitrite/cells) was measured. Each value represents an average of 10 samples ± Standard deviation.

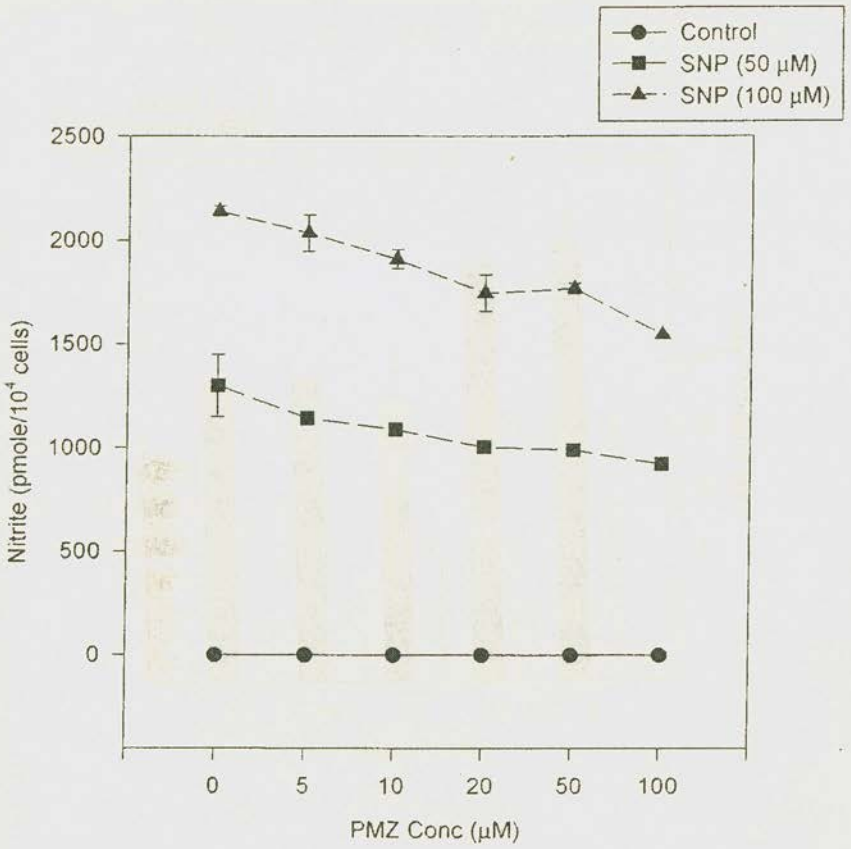

Fig. 16. RINm5F cells (10^4) were treated with 50 mM and 100 mM SNP and different concentrations of PMZ (0-100 mM) for 24 hrs. and the production of NO· (pmole nitrite/cells) was measured. Each value represents an average of 10 samples ± Standard deviation.

Development of cell cultures from commercially important invertebrate and vertebrates

Investigator : R. R. Bhonde.

Date of initiation: October 1995
Date of completion : December 1999

Background :

(a) Invertebrate cell cultures:

Invertebrate and fish cell cultures have been demonstrated to be important for the characterization of viruses originated from these groups. Invertebrate and lower vertebrate tissue culture is gaining importance because of developments in aquaculture technology. Aquaculture requires large scale production of prawns, bivalves, fish etc. as an alternative food source. There is a direct need to develop cell cultures from invertebrates and fish of commercial importance, so as to employ them for virus propagation of selected variety that causes heavy economic loss. The bivalves; especially pearl oysters are of commercial importance due to their use in pearl industry.

(b) Vertebrate cell cultures:

The annual cost of injured or failed tissues or organs runs to billions of dollars. Over the past 50 years remarkable progress has been made in restoring the structure and

function of the damaged / dysfunctional tissues through bionic devices and organ transplants. Such replacement of parts is not useful in all situations. However with the advent of advances in tissue culture technology, knowledge and importance of ECM in attachment of cells and encapsulation of cells, it is possible to venture for preparing bio-artificial organs. One of the greatest advantages of such devices is that they need not be implanted into the body but could be connected to the blood vessel to detour the blood circulation. This dispense with the problem of tissue matching and graft rejection. Hence it is worthwhile attempting to develop new devices that will serve as bio-artificial organs, to replace the damaged or dysfunctional tissues. Moreover, the cell cultures derived from human conjunctiva and corneal endothelial cells are of importance as *in vitro* models for host-virus interactions and transdifferentiation studies.

Objectives:

* To optimize conditions for high yield preparation of (human fetal) hepatocytes, vascular endothelial cells and corneal endothelial cells.
* To study feasibility of preparation of bio-artificial organs especially liver, pancreas and vascular endothelial cells by seeding culture cells onto prosthetic membrane / hollow fiber reactors.
* To develop cell cultures from mantle tissue of pearl oyster and hepato-pancreas of crustaceans.

* To employ developed cell culture systems for toxicological and carcinogenecity testing.

Work carried out :

Invertebrate cell cultures:

A project on 'Tissue culture in Pearl Oysters' is undertaken at Tuticorin Research Centre of CMFRI, in collaboration with NCCS. As a part of this project attempts were made to culture mantle tissue by employing primary culture and organ culture techniques. Different tissue culture media in 2X concentration in combination with or without sea water and fetal calf serum were tried. Cultures were incubated at 28° C. It was observed that ciliary epithelial cells of mantle could be cultivated either as single cells or explant culture exhibiting ciliary movement for 12 days so far. The experiments were terminated due to protozoon contamination. Another batch of cultures set at Tuticorin laboratory, incorporating medium M-199, PF-35, FCS and sea water exhibited epithelial outgrowth from the mantle tissue within 24 hours. This culture formed monolayer and lasted for two months. The epithelial cells also synthesized organic crystals which were visible after 30 days. The cultures are being continued and characterized at Tuticorin.

Vertebrate cell cultures:

In order to study the feasibility of establishing and maintaining fetal hepatocyte cultures for making bio-artificial liver support devices, attempts were made to initiate hepatocyte cultures from human fetal liver. After initial standardization, it was possible to obtain hepatocyte monolayer. It was found that a nutrient mixture supplemented with insulin and bovine serum albumin supported growth and proliferation of hepatocytes. The cultures are being characterized for their morphological and functional markers.

Outcome of the project:

The proposed project will provide alternative to animal experimentation in toxicology and carcinogenecity testing of compounds with special reference to hepatotoxicity and endothelial injury. Large scale production of these cells would be of immense value in developing bio-artificial devices to replace the damaged parts. The cell cultures developed from the marine invertebrates may yield useful biologicals of commercial importance.

Publications:

Bhonde R.R. (1996) Dietary management of β-cell homeostasis and control of diabetes. *Medical Hypothesis* **46:** 357 - 361.

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Bhonde R.R. (1996) Spontaneous regeneration of pancreatic β-cells in EMC-D virus induced diabetic mice and reversion from diabetic to normal state. *Current Science* **71,** 401 - 404.

Bhonde R.R. (1997) Factors influencing induction and reversal of virus induced diabetes. *Indian J. Expt. Biol.* **35,** 267 - 271.

Conferences and seminars :

1. Bhonde R.R. Large scale isolation of islets from adult mouse pancreas. Presented at *National symposium on Developmental Biology* at Utkal University, Bhubaneshwar, Orissa, Feb. 21 - 23 1997.

2. Shewade Y.M., and Bhonde R.R. Tissue specific *in vitro* reflection of *in vivo* diabetic complications. Presented at *XX All India Cell Biology Conference and Symposia* at CRI, Mumbai, during Jan. 13 - 15, 1997.

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Identification of Melanoma Oncogene/s and characterization of the melanoma oncogene protein product.

Participants: Dr. Anjali Shiras
Varsha Shepal
Dr. L. C. Padhy, *TIFR, Mumbai.*

Date of initiation: May 1994
Probable date of completion: April 1999

Background:

Melanoma, a predominant form of cancer, arises from the melanocytes in the basal layer of epidermis. A number of cellular abnormalities have been observed, such as growth factors, angiogenesis mediators, proteases, ECM proteins all seem to be involved in invasiveness that contribute towards the malignant phenotype. The project undertaken concerns with study of the genes that could be implicated in the transformation process for Cloudman Melanoma.

Project querries:

(i) Analysis and Characterization of the transforming genes with reference to melanoma using the Cloudman melanoma cell-line Clone M3 as the model system by expression cloning. (ii) Elucidate the key events involved in transformation pathway with reference to the gene/s identified.

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Work carried out during the current year:

In our earlier work, we have reported the construction of an eukaryotic cDNA expression library from Clone M3 cells using the Automatic Directional Cloning approach. The cDNA library was characterized by transfection into NIH3T3 cells for selection of G418® clones. Clones that displayed novel morphological features and transformation ability in nude mice were selected for study.

The insert cDNA was fished out from the G418® clones selected for study by PCR using the primer set of SP6 and T7 promoters. The present work reported here revolves around the use of only one cDNA clone named NG3 with an insert size of 1.3 Kb. This insert cDNA was subcloned into the plasmid CEV27 using Sfi1 adapters, ligation and transformation into DH5 *E. coli*. The Amp® transformant colonies were checked for presence of 1.3 Kb insert by Sfi1 digestion. Due to the strategy applied for subcloning we obtained almost 100% colonies expressing the appropriate insert.

Since, we are interested in the functional expression of the gene in eukaryotic cells, we transfected the subcloned plasmid DNA by lipofection into NIH3T3 cells and we obtained successful generation of G418(r) clones with an almost efficiency of 50-60%. The clones besides being G418(r) also displayed novel phenotypic characters like appearance of ball shaped structures which were anchorage independent and promoted cell-outgrowths as depicted in the photographs (Fig. 17, Plate V). Besides NIH3T3, we also looked into the ability of subcloned plasmid DNA to

transform cell-lines from other species i.e. rat and human cells. As a prototype we selected Rat-2 cells from rat species and WI38 from human species for transfection studies. Interestingly, we obtained appearance of the same phenotypic characters amongst transformants from all species confirming our observation that the subcloned PCR product is capable of inducing same unique characteristics amongst transfectants in all the three species studied (Fig. 18 and 19, Plate V).

We have confirmed the transforming ability of NIH3T3 transofectants by tumorigenicity studies in nude mice. Two of the transformants induced large tumors in nude mice which have been characterized histopathologically and found to be pleomorphic in nature, displaying high grade malignancy.

The insert DNA has a sequence different from the plasmid CEV stuffer fragment and this was confirmed by restriction enzyme digestion with Bgl2, Hind and EcoR1. Also, we did Southern blotting and hybridization with CEV PCR product as a probe for confirmation about a completely different sequence of the NG3 PCR product.

We are presently studying the status of this gene in terms of its RNA expression profile in various other melanoma cell-lines and related cancers. This study will enable us to understand the significance of this gene in the transformation pathway and elucidate the mechanism of neoplasia.

Outcome of the project:

The complete characterization of our gene that is displaying various novel features characteristic of transformation and its ability to induce them in isolation makes it an interesting candidate gene for melanoma. Its study at the molecular level along with its effect at the phenotypic level in vitro may help in understanding certain steps of transformation.

Identification of factors in supernatants from stimulated lymphocytes on growth and differentiation in neuroblastoma cells.

Participants: Padma Shastry
Nagarthna
R. N. Damle

Date of initiation: November 1993
Probable date of completion: November 1998

Background:

Neuroblastoma (NB) is the most common childhood tumour. Surgery, irradiation and chemotherapy have been long employed as different modalities of treatment and management of neuroblastoma for many years. These lines of treatment have had some success and given temporary relief and lead to recurrence of the disease. Therefore search is on for development of potential therapies in management of NB.

One of the striking features of NB is its ability to undergo spontaneous differentiation into a benign form of ganglioneuroma. It is also believed that NB originates from neural crest due to the failure of signals or response to them to differentiate or to undergo apoptosis. Hence there has a growing interest in exploring the approaches for induction of NB cells as treatment regime.

Work carried out during the current year:

In experimental systems NB cell lines are excellent models for studying such mechanisms. In the initial experiments, procedures were standardized for growth kinetics, proliferation, and morphological evaluation using murine NB cell lines Neuro-2a and NB41A3 and human cell line SK-N-MC for studying the effect of cytokines and a protein kinase inhibitor - staurosporine.

In the subsequent studies, role of specific cytokines IL-6 and TNF-x were in inhibition of proliferation, differentiation and apoptosis in SK-N-MC were done. Further studies are underway to investigate the modulation of receptors to TNFx by IL-6. The extracellular matrix components play an important role in cell survival, differentiation and apoptosis of cells. The characterization of cells differentiated by treatment with staurosporine was done by morphological evaluation, neurite extension, enzyme sites and binding to ECMC specifically to collagen I. The changes associated with it is evaluated with respect to interaction with extracellular matrix. The matrix. The data demonstrated an increased binding to collagen I by staurosporine treated cells.

B1 integrins constitute a family of molecules which mediate interaction between cells and ECM. They are linked to x subunits which confer ECM specificity on cells. We examined the integrin expression of B1, x2 and x3. There was no significant difference (27% in treated vs 22% in control) in the B1 expression as assessed by flow cytometry analysis. There was no difference in x2 and x3 expression also. Gangliosides serve as functionally important molecules

in cell-substratum interaction. NB cells express GD2 as major ganglioside. Using a monoclonal antibody MAb-126 the expression of GD 2 was quantitated in neuro-2a cells. The staurosporine induced differentiated cells demonstrated 32% increase in GD 2 expression compared to control cells.

Outcome of the work:

As induction of differentiation of actively dividing and metastazing neuroblastoma cells is current area of interest world wide, the data from these studies will contribute to mechanisms and the role of apoptotic factors like BCL-2 in differentiation and apoptosis.

Publications: Nil.

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

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

Date of Initiation: June 1996
Probable date of completion: Dec 1999

(a) *Epithelial mesenchymal transition:*

Background:

Epithelial cells form a tight continuous layer of cells covering a body surface or lining a body cavity. However, cohesiveness of epithelial cells gets modulated at times and they are induced to dissociate, disperse and migrate. Cell migration plays a central role in many physiological processes such as tissue formation and remodeling 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. The search for inducer molecules including chemokines and growth factors. 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 tumor cells. Some types of tumor cells are reported to produce growth factors receptors at various levels, thereby increasing their reactivities.

One of the most striking examples of cellular transformations leading to cell motility is provided by EMT. 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 interaction of various transduction systems. It is important to understand the mechanism that trigger 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 signaling 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 cellular response to a given growth factor.

Work done during the current year:

Preliminary work carried out to establish an in vitro wound healing model using SiHa (cervical carcinoma cells) was described previously. further, AV-3 (amnion) and MDBK (kidney) cell lines were used for the experimental studies. The cellular response (both early and late) were evaluated with respect to morphological and immunocytochemical features.

In order to ascertain the effect of the induced migration on the progression of the cell cycle, the duration of G1 phase was estimated in SiHa cells synchronized in early G1 phase. Incorporation of bromodeoxyuridine (BrdU) in interphase nuclei during the short pulse (10 minutes) labeling was visualized by indirect immunoofluorescence. The results indicated that the cells lining the wound 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. This temporal dissociation of cell cycle progression and cell migration is being analysed further.

(b) Interphase nuclear 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 organised 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 that 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." (Spector, D L, Macromolecular domains within the cell nucleus" In: Ann. Rev. Cell Biol., 1993, 9: 265-315).
Year-wise

Work carried out during the current year:

As proposed earlier, preliminary work has been initiated employing anchorage-dependent cells to study the organization of interphase nuclei. Experimental protocols for simultaneous visualization of nuclear and extranuclear

components using one-and two-colour fluorescence are being standardized. Localization of the following antigens has been carried out: alpha- and beta-tubulin, desmosomal proteins, splicing factor (SC-35).

Morphometric estimations on two-dimensional images are also being standardized.

Publications:

J. M. Robinson, J. M. Chiplonkar and Z. Luo (1996). A method for co-localization of tubular lysosomes and microtubules in macrophages: fluorescence microscopy of individual cells. *J. Histochem. Cytochem.* **44**(10): 1109-1114.

Molecular Cross-talk Between Pathogenic Bacteria / Bacterial Antigens with Human Mucosal Epithelial and Submucosal Mesenchymal Cells System Using Cell Culture as a Model: *Molecular and Cellular Interaction of Lipopolysaccharide with Human Intestinal Mesenchymal cells*

Participants: K S Nanda Kumar
Dipshikha Chakravortty

Date of initiation:1994
Expected date of completion:1999

Background :

Gram-negative bacteria are important pathogenic organisms responsible for the development of various diseases in human. Among them, *E. coli* and *Salmonella* are associated with the pathogenesis of diseases like gastroenteritis, diarrhea, urinary tract infection, food poisoning, enteric fever etc. leading to morbidity and mortality. One of the important bacterial antigens of *Salmonella* and *E. coli* which has been extensively studied is Lipopolysaccharides (LPS) . This toxin substance produced by *E. coli* has been found to exert a wide variety of biological action on mammalian cells, which are important causative agent for the initiation of the disease. Intestinal epithelial cells have been found to play a significant role in the pathophysiology of diarrhea and

dysentry . It is known that normal cell physiology of intestinal epithelial cell is controlled by the underlying submucosal mesenchymal cells. The invasive nature of *E. coli* and *Salmonella* provides an opportunity for the bacteria /bacterial antigens/toxins like LPS to interact with various cell types of the submucosal regions of the intestine. Among the submucosal mesenchymal cells, fibroblasts exist as a major resident cell population of all organs playing vital role in cell-cell interaction , cell growth, cell proliferation, cell adhesion by secreting many of the matrix components and growth factors. Hence, it is possible that LPS could directly act on the mesenchymal fibroblasts thereby, leading to the cascade of immunobiochemical reactions resulting in the alteration in the intestinal physiology by affecting the mucosal epithelial and submucosal cells during musocal infection by these bacteria. Hence, the present study was aimed to reveal the molecular and cellular events involved in LPS action on this vital cell population of the small intestine.

Summary of the previous work:

Lamina propria mesenchymal cells were cultured from 18 week old human fetuses (n=40). The cell populations were characterized morphologically and immunocytochemically and confirmed homogenous population of fibroblasts. Production of various extracellular matrix components (ECM) by the fibroblasts was determined. Passaging efficiency, cryopreservation and revival capacity of this cell population were also assayed. LPS from *E. coli* O55.B5 was purified and characterized biochemically and immunochemically. LPS at different concentrations at

various experimental duration was found to rearrange cytoskeletal architecture of fibroblasts. LPS was also found to induce proliferation and growth of the fibroblasts.

Work done during 1996-97:

Activation of mammalian cells by bacterial lipopolysaccharide (LPS) plays a key role in the pathogenesis of gram-negative infection and inflammation. The 55 KDa CD14, lipopolysaccharide binding protein (LBP), 70 KDa cell albumin and hemoglobin are known to bind LPS and initiate cell activation. However, there must be some other putative LPS receptors in the cells of nonmyeloid lineage as they are not generally known to express CD14 . Searching for the potential receptors in human small intestinal lamina propria fibroblasts for LPS of enteropathogenic *Escherichia coli*, we investigated binding of purified LPS (*E. coli O55:B5*) to membrane, cytosolic proteins and whole cells. Membrane, cytosolic and whole cell samples were electrophoresed and transferred to nitrocellulose and exposed to biotinylated LPS which were visualized by streptavidin- alkaline phosphatase and BCIP-NBT . In the presence of serum, LPS preferentially bound to approx. 75 KDa membrane protein. We have also observed that LPS could induce DNA synthesis and enhance collagen production in lamina propria fibroblasts. LPS induced DNA synthesis was dose dependent and was inhibited by polymyxin B sulphate. The proliferation of fibroblasts by LPS may be mediated by inhibition of NO production, as in our culture system basal level of NO output was suppressed upon LPS exposure whereas a combination

of LPS and IFN g enhanced NO production which resulted in decreased DNA synthesis. Using anti-inducible nitric oxide(iNOS) antibody, the production of NOS in intestinal fibroblasts was also determined. Aminoguanidine, NMMA and L-NAME could inhibit the LPS-IFN g induced production of NO in fibroblasts. LPS was also found to stimulate total collagen synthesis studied over a period of time and pretreatment of LPS with polymyxin B sulphate completely abolished LPS induced collagen synthesis. Reconstitution or co-culture experiments showed that LPS treated fibroblasts were able to support the proliferation of DNA synthesis in small intestinal epithelial cells. From these results we conclude that the 75 KDa protein of lamina propria fibroblasts is a probable candidate molecule for the LPS recognition and signal transduction. The proliferation and enhanced collagen synthesis in lamina propria fibroblasts and maintenance of epithelial cells by LPS treated fibroblasts suggest its repairing mechanism, construction of the basement membrane of the injured tissue thereby participating in the intestinal disorders caused by bacteria.

Publications/Conference attended/patent

1. Dipshikha Chakravortty and K S Nanda Kumar, 1996, Lipopolysaccharide induced production of proinflammatory cytosine in lamina propria fibroblasts. *J Endotoxin Res.* **3**:59

2. Dipshikha Chakravortty and K S Nanda Kumar, 1997 Microplate assay for the detection of bacterial contamination in tissue culture reagents. *Id J Exp. Biol.* **35**:86

Use of mitochondrial DNA restriction fragment length polymorphism for characterisation of mosquito cell lines

Participants: M. S. Patole
Yogesh S. Shouche
Supriya G. Kshirsagar

Date of initiation: January 1995
Date of completion: April 1997 (Concluded)

Background:

a) Heteroduplex analysis for characterisation and authentication of insect cell lines :

Insect cell lines are becoming popular hosts for production of recombinant proteins because of several advantages they offer over the mammalian cell lines. There are very few methods available for the characterisation of these cell lines. Recent advances in molecular biology and development of fingerprinting probes have revolutionised this filed but unfortunately these methods can not be applied to insect cell lines. In our studies we have filled this gap using heteroduplex analysis.

This method is based on the fact that mismatches, insertions/deletions of nucleotides induce structural deformations in double stranded DNA which result in reduction in the mobility in the non denaturing

polyacrylamide gels. The technique is sensitive enough to detect 1-2% changes. In our studies we used sequence variation in mitochondrial rRNA genes.. These genes are considered and "molecular chronometers" and used widely in molecular taxonomy and phylogeny. In our studies we used this information for the authentication and characterisation of insect cell lines.

Insect specific primers were used to amplify 16S and 12S ribosomal RNA genes of the larvae and cell lines under study. These products were then subjected to heteroduplex analysis. If the PCR fragment of the cell line yields a single band of homoduplex with the larvae of the known species then they are conspecific. If the larvae and cell line originate from different species, then the analysis yields multiple bands indicative of heteroduplex formation.

Figure 20 (Plate VI) shows results of a typical analysis. Cell line derived from *Anopheles stephensii* mosquitoes when analysed with corresponding larvae gave heteroduplex formation where homoduplexes were expected. In order to analyse this further, we tested this cell line with other and found that it gives homoduplex with that derived from *Aedes albopictus* mosquitoes. Other cell lines those were found to be mislabelled using this technique were *Culex thileri* and *Aedes aegyptii*. Sequencing the PCR fragments confirmed this observation, thus confirming that Anopheles stephensii cell line was probably mislabelled. Further studies were carried out to determine the efficiency of this method to determine the cross contamination and we found that it can detect the cross contamination up to 5%. Similar efficiencies were observed for mammalian cells by fingerprinting of by

the use of Single Locus Probe, but these methods involved use of radioactivity and took longer time to deliver results.

At present the studies are underway to extend these studies to mammalian cell lines.

b) Sequencing of mosquito mitochondrial rRNA fragments for phylogenetic analysis:

Studies were continued to fill the gaps in the sequences already done, but the work was delayed due to late arrival of primers.

Molecular biological studies on mosquitoes

Participants: Yogesh S. Shouche
M. S. Patole
Mr. Vyankatesh Pidiyar

Date of initiation: April 1997
Date of completion: March 2000

Background:

Mosquitoes are responsible for transmission of several human diseases including malaria, filaria, Japanese encephalitis, dengue, yellow fever. Development of insecticide resistance strains has resulted in reappearance of these diseases in worrying proportions and genetic methods are being tried to control mosquito populations. This necessitates the need for complete understanding of molecular mechanisms and control circuits. With this goal in mind detailed molecular biological studies on mosquitoes are being undertaken. Initially some housekeeping genes and there control elements are being investigated and primer have been designed for the amplification of hexokinase gene. These will now be used to pick up the gene from cDNA library for further studies.

An alternative to generate genetically engineered mosquitoes is to manipulate their endosymbionts. Several species of mosquitoes are known to harbor endosymbionts belonging to *Wolbachia sp.* We screened the local

populations of mosquitoes using PCR primers that specifically amplify *Wolbachia* and they were found to be positive (Fig. 21, Plate VI). We plan to carry out detailed analysis of these endosymbionts and their association with mosquitoes.

Mosquitoes harbor a large number of bacteria in their midgut. As this the first site any pathogen enter after entry in the mosquito body through blood meal, this becomes 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 we have isolated thirty different types of bacteria based on colony morphology and gram characters, eight of which are haemolytic. Presence of these is significant as they may have a role to play in the digestion of blood meal. Identification of these by biochemical and other tests is underway.

All the attempts to culture organisms from the natural environments have underlined the limitations to culture the bacteria from these. It is proposed that as little as 10% of the total flora is unable to grow under the controlled laboratory conditions. In order to ensure the survival of 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 ribisomal RNA (SSU 16S rRNA) sequence analysis has been popularly used for this. A library of PCR amplified eubacterial SSU rRNA genes from total female mosquito gut DNA has been prepared and at present it is being characterised.

First year : Generation of cDNA and genomic libraries, screening for endosymbionts, isolation and identification of gut flora.

Second year : Screening of the libraries for specific clones and characterisation of these clones, generation of library of PCR amplified rRNA genes from female and male guts, purification of endosymbionts.

Third year: Identification of regulatory sequences and development of expression vectors. Complete characterisation of gut flora with help of isolates and library. Generation of endosymbionts specific genomic DNA library.

Publications:

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

2. Supriya G. Kshirsagar, Milind S. Patole and Yogesh S. Shouche. Characterisation of insect cell lines: Heteroduplex

analysis employing mitochondrial 16S ribosomal RNA gene fragments. *Analytical Biochemistry* (In press).

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

Identification and characterization of protective antigens in lymphatic filariasis

Participant: P B Parab

Date of initiation: 1993
Date of completion: December 1998

Background:

Lymphatic filariasis is most important vector borne disease prevalent in the tropical and subtropical regions of the world affecting nearly 100 million people. In India, *Wuchereria bancrofti* and *Brugia malayi* nematodal infections are the causative agents of this disease. Owing to difficulties in obtaining human filarial parasites and lack of animal model, we have tried to analyze *Setaria digitata* bovine filaria antigens which could be useful for diagnosis and prophylaxis in lymphatic filariasis.

Work done:

1. In lymphatic filariasis, differential diagnosis based on the presence of IgG4 antibody was reported recently. In our studies we found high levels of Setaria digitata specific antibodies in microfilaraemics as compared to the endemic normals and chronic pathology sera. Microfilaraemic sera predominantly recognised 40 kDa protein by Western blot analysis.

Sera were collected from patients suffering from malaria, tuberculosis, leprocy, ascaris, *D. medinensis* and *E. Granulosus* infection. IgG4 levels in these sera were estimated in ELISA using *Setaria digitata* antigen.

2. Immuno-screening of B. malayi L3, cDNA expression library with rabbit antisera.

For immunoscreening the library antisera to B. malayi infective larvae and *W. bancrofti* infective larvae were prepared and tested in ELISA. Antisera were aliquoted and stored at -20oC

2 Development of monoclonal antibodies to co-stimulatory molecules present in the surface of activated B cells and on macrophages.

Monoclonal antibodies against the co-stimulatory molecules on antigen presenting cells would facilitate the studies on regulation of immune responses. Heterohybridomas were produced against mouse B1, B3 and M150 co-stimulatory molecules using mouse myeloma and immunised hamster spleenocytes. Attempts are also being made to produce hybrids using rat myeloma and immunised rat splenocytes. The clones were tested and frozen in liquid nitrogen.

Wistar rats were immunised with M150 membrane protein Sera were tested for antibody titre in ELISA against M150 antigen. Splenocytes of the animal showing highest titre were fused with Y3Ag123 rat myeloma cells. Forty six hybrids obtained were again tested for antibody secretion 382 and 3832 hybrids were further cloned and off clones

isolated out of these three clones found to be non-secretary by ELISA against M150 antigen. Two clones are still to be tested.

Publications:

P. B. Parab (1996) IgG4 antibodies in microfilaraemic (lymphatic filariasis) sera detects specific cross reactive antigens in *Setaria digitata* worm extract. J. Parasit. Dis. 20(1): 79 (Abstract).

Conference:

Attended International Conference on Vectors and Vectors Borne diseases. Goa, 16th to 20th March 1997.

Role of haemozoin in immune response

Participnts: Prakash Deshpande
Padma Shastry

Date of initiation: 1995
Date of completion: December 1997

Background:

World Health Organization estimates that there are 270 million new cases of malaria each year, about half caused by *Plasmodium falciparum*, the most dangerous of human malarious, with about 2 million fatalities per year.

Intraerythrocytic malarai parasite uses hemoglobulin as major nutrient source of amino acids. However, parasites are unable to degrade hemoglobulin heme and instead polymerize it to an insoluble dark brown pigment called haemozoin. haemozoin is released in to the blood circulation along with merozoites as schizont infected erythrocyte brusts.

Impairment of phagocytic function of macrophages by haemozoin is reported. Moreoveer, drastic changes in peripheral blood mononuclear cells induced by *P. falciparum* have ben documented. We are investigating role of haemozoin in peripheral blood mononuclear cells proliferation.

Work carried out during the year:

P. falciparum parasites (strains SO-HS, FAN 5HS, MP14) were maintained in cultures according to the method of Trager and Jensen using O+ erythocytes in medium RPMI 1640 supplemented with 21 mM sodium bicarbonate, 25 mM HEPES buffer, 10 mg/ml gentamycin and 10% O+ human serum or plasma. haemozoin pigment was isolated from trophozoites rich parasitized erythrocytes by osmotically lysed using distilled water. Protein concentrations was determined by Bradford method.

Peripheral blood mononuclear cells were isolated from healthy individuals. PBMC proliferation were studied with different concentrations of haemozoin. Our preliminary results indicate hat the hemozin is not toxic to the cells. Decrease in PBMC proliferaion with increasing concentration of haemozoin have been observed. haemozoin inhibit proliferation of PHA stimulated cells. Further studies are in progress.

Publications: Nil.

LIBRARY AND DOCUMENTATION FACILITY: Information services for scientific research.

N. V. Ramakrishnan

For sustained advancement of research and development programmes, easily accessible comprehensive information is an absolute prerequisite. With this objective in view, NCCS library and documentation facility over the years has developed necessary infrastructure and facilities to meet the information requirement of the centre. The library has been gearing itself upto the developments in information technology for improving its services. Besides introducing electronic information services such as CD-ROM and On-line databases, the library has established CD-NET working recently to provide instant access to multiple CD-Databases. Aditional facilities at NCCS library include local area net work confined to library activities, a number of CD-ROM databases including bibliographic, full text and factual databases, on-line access to Knight ridder (DIALOG) through (VSNL/GPSS & Internet). The library has a good collection of books and subscribes to journals and news letters related to Biotechnology and its allied field. The novel feature of the library is that, it is continued to provide electronic mail, Internet access to user's community.

Library has continued to extend its information consolidation services such as press vision/Biovision having relevance to Biotechnology on front line R & D activities.

DATABASE SEARCH SERVICES:

CURRENT INFORMATION SERVICES:

These are alerting services reporting new developments.

CURRENT AWARENESS SERVICE (CAS):

Library provides periodically, up to date references with abstract of recently published literature having relevance to NCCS activities using databases at the centre.

BIO-VISION:

Biovision aims at creating an awareness among scientists/technocrats in the field of biotechnology having relevance to NCCS activities. It is an abstracting publication issued bi-monthly. The coverage for this publication is from various primary and secondary periodicals.

PRESS VISION: A monthly publication confined to topical press information in the area of biotechnology and it's allied field.

Forthcoming scientific events: A-bimonthly publication confined to conference proceedings, seminars, symposiums etc. in the frontier areas of biotechnology.

ON-DEMAND INFORMATION SERVICES:

CD-ROM DATABASE SEARCH SERVICE (CDRS):

This is a retrospective search carried out on the CD-ROM databases subscribed at library in biotechnology research.

ON-LINE SEARCH SERVICE (OSS):
The library is online with online hosts like DIALOG, DATA-STAR providing access to several hundred databases in ALL areas of science and technology.

INTERNET SEARCH SERVICE (ISS):

Library is actively pursuing the exploitation of internet, for the benefit of the research community. Some efforts include identification of key internet sources in Biotechnology for pertinent information to users.

DOCUMENT SUPPLY SERVICES:

Document delivery service (DDS):

The library provides photocopies of articles from various sources including from National as well as overseas agencies BLDSC, UK, Genuine articles of ISI, laser print copies of articles are also made available from full-text CD-ROM ADONIS. (Biomedical, chemical and pharmaceutical).

Publications:

N.V.Ramakrishnan Comparative Evaluation of On line and CD-ROM searches in Biomedical Sciences: Á preliminary study. Malasian Journaìl of Library and Information Science Vol. No. ,July 1997

Animal House

The facility for small laboratory animals has been equipped and started functioning in the new environmentally controlled animal house. 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 infestation.

The breeding of laboratory animals has been planned to meet the needs of scientists for various animal experiments. The details of the animals procured from various sources, bred in the animal house and supplied for various R & D activities are given in Table 2.

Species / strain	Animals procured	Animals bred	Animals supplied	Stock available
Rats				
Wistar	13	56	19	41
Mice				
C57BL		379	85	212
Balb\c	250	656	472	525
Swiss	60	147	56	144
Nude	25		25	
BcC6		30	2	47
Mastomys		45		33
Rabbit	10		10	10
Hamster			2	3

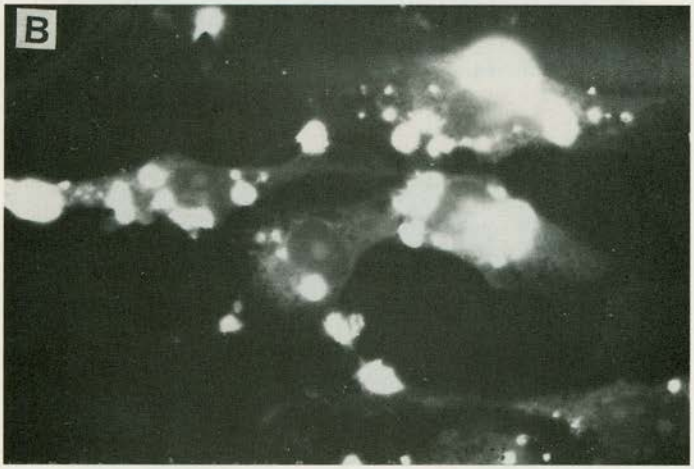
Table 2: Summary of Animals supplied for R & D activities.

Plates

Plate I.

Figure 4. Human bone marrow derived stromal cells stained with biotin labelled UEA-1, a specific marker for human bone-marrow derived endothelial cells. (A) Control; (B) CM-treated. (x1000).


A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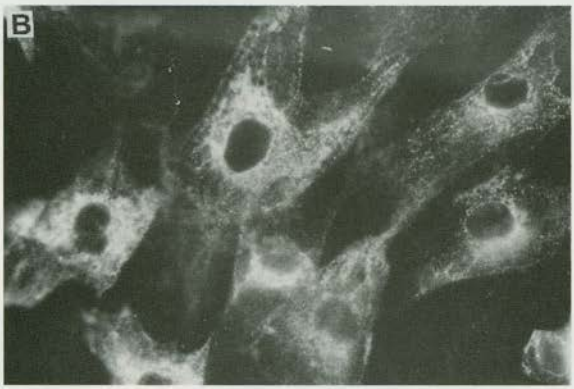
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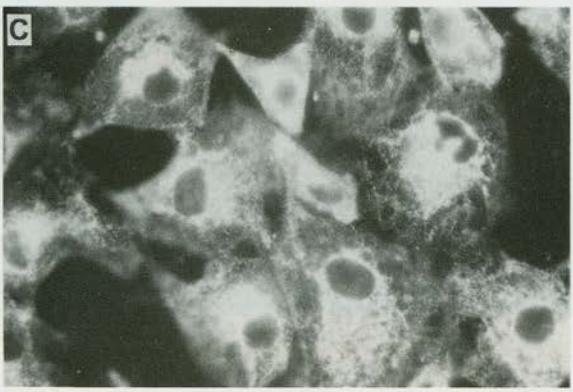
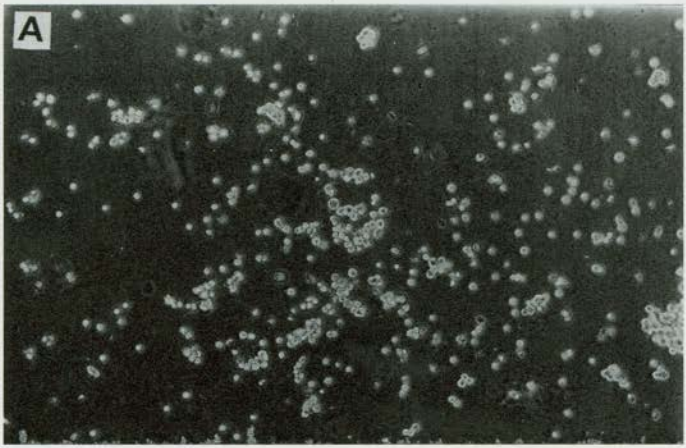

C

Plate II.

Figure 5. Human bone-marrow derived stromal cells stained with antibody against human endothelial differentiation marker, BMA.1. (A) Control; (B) CM-treated; (C) TGF beta-1 treated. (x1000).

Plate III.

Figure 6. Colony formation from adult human bone marrow cells in response to the stromal cells. (A) Control stromal cells; (B) Phorbol myristate treated stromal cells. (x60).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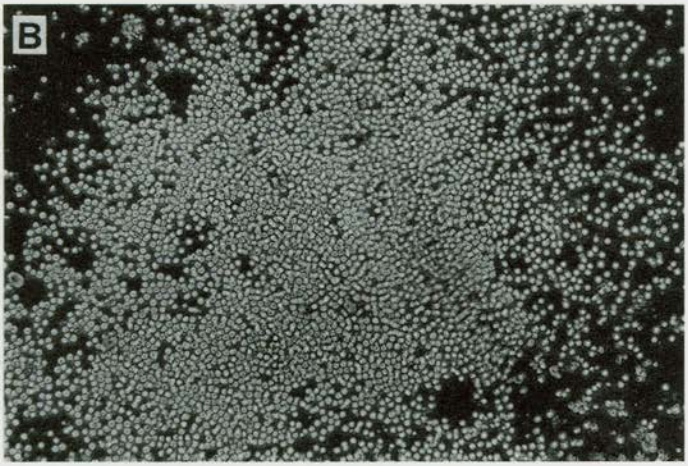
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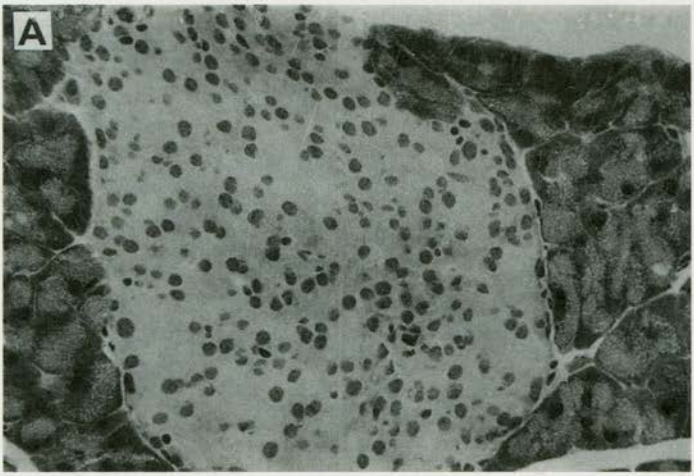
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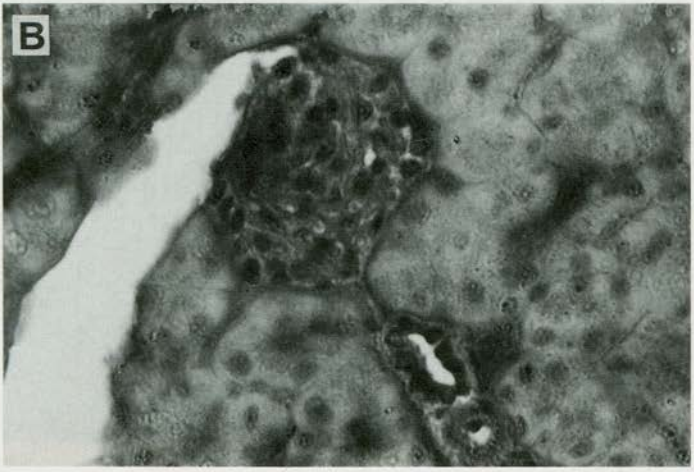

B

Plate IV.

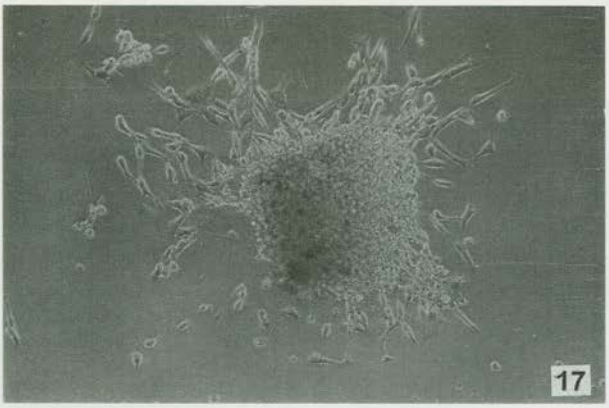
Figure 10. Mouse pancreas from (A) control group and (B) regenerating pancreas. Note the budding of endocrine cells (arrow) from a small terminal pancreatic duct (x360).

Plate V.

Figure 17. G418(R) clones arising from transfection of plasmid # 4 DNA into the mouse cell line, NIH 3T3.

Figure 18. G418(R) clones arising from transfection of plasmid # 4 DNA into the rat cell line, Rat-2.

Figure 19. G418(R) clones arising from transfection of plasmid # 4 DNA into the human cell line, WI-38.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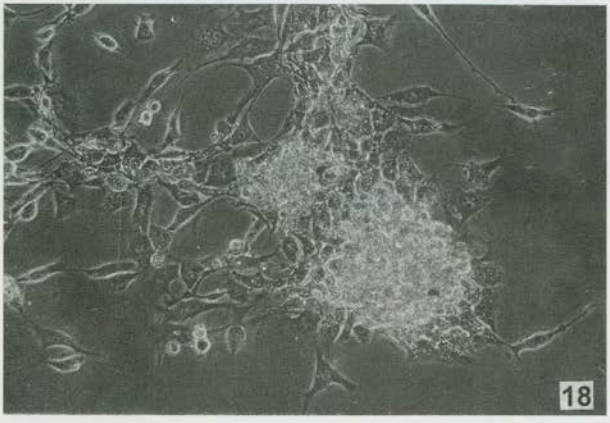
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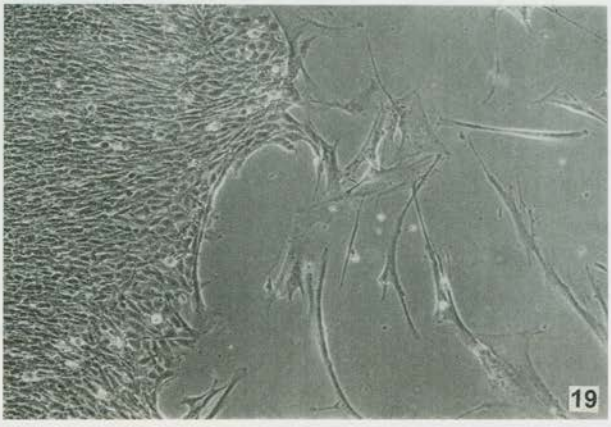
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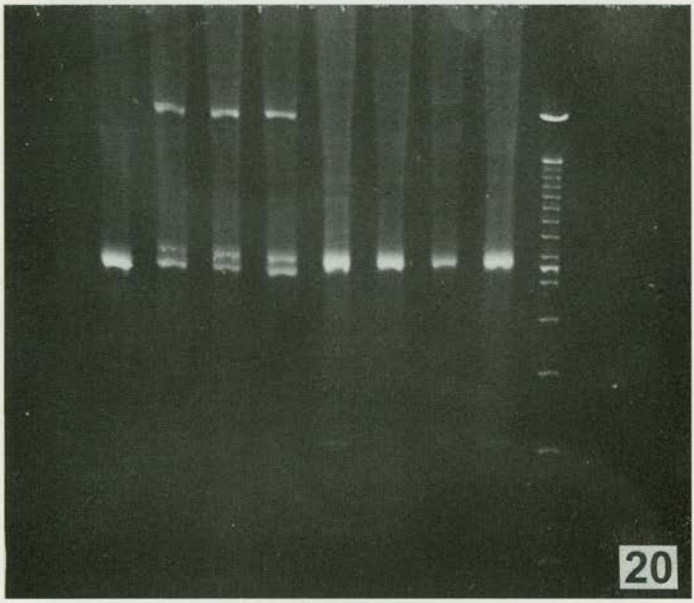
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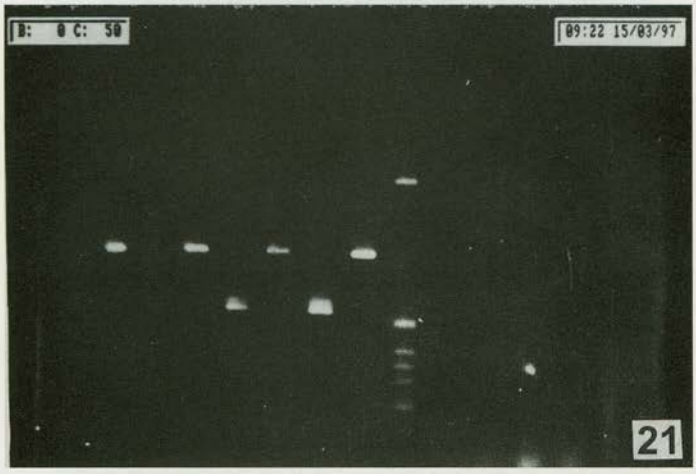
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21

Plate VI.

Figure 20. 16S rRNA PCR products were mixed and subjected to heteroduplex analysis. The products were resolved on PAGE and visualised by EtBr staining. The lanes represent PCR products from a) *An. stephensi* larvae; b) and c) *An. stephensi* larvae and *An. stephensi* cell line; d) *An. stephensi* larvae and C6/36 cell line; e) and f) C6/36 cells and *An. stephensi* cell line; g) C6/36 cells and ATC-11 cell line; h) C6/35 cell line; i) 100 bp ladder (marker).

Figure 21. Using mitochondrial 16s rRNA and *Wobacia* rRNA specific primers rDNA genes were amplified from ovaries of 4 wild mosquitoes. *Wobachia* rDNA gene fragments of approximate 1000 bp is shown in lane 2,4,6 and 8. Mitochondrial rDNA fragments of approximately 550 bp is shown in lane 1,3,5 and 7. Lane 9 represents pBR *Hinf I* digest (marker).

B.L. PHATAK & CO.

CHARTERED ACCOUNTANTS

1, JAY APARTMENTS, SUNDERRAO REGE MARG, 64/3 ERANDWANE, 14TH LANE, PRABHAT ROAD, PUNE 411 004

AUDIT REPORT

We have examined the Balance Sheet of National Centre for Cell Science (Formerly National Facility for Animal Tissue & Cell Culture),Pune as at 31.03.1997 and the Income and Expenditure Account for the year ended on that date which are in agreement with the books of account maintained by the said Trust.

We have obtained all the information and explanations which to the best of our knowledge and belief were necessary for the purpose of the audit. In our opinion, proper books of account have been kept by the Trust so far as appears from our examination of books, subject to the comments given below :

- See our General Remarks as per Annexure 1 and Significant Accounting Policies followed as per Annexure 2.

In our opinion and to the best of our information and according to explanations given to us, the said accounts give a true and fair view,

i) In the case of Balance Sheet, of the state of affairs of the above named Trust as at 31.03.97 AND
ii) In the case of Income and Expenditure Account, of the deficit for its accounting year 31.03.97.

For B.L.Phatak & Co.
Chartered Accountants

PUNE
DATED: 8.7.97

Sd/-
Partner

B. L. PHATAK & CO.,
CHARTERED ACCOUNTANTS
1, JAY APARTMENTS,
SUNDERRAO REGE MARG,
14TH LANE, PRABHAT ROAD,
PUNE - 411 004

Registration No. F-5282/Pune

REPORT OF AN AUDITOR RELATING TO ACCOUNTS AUDITED UNDER SUB-SECTION (2) OF SECTION 33 & 34 & RULE OF 19 OF THE BOMBAY PUBLIC TRUST ACT.

Name of the Public Trust : NATIONAL CENTRE FOR CELL SCIENCE (NCCS), PUNE. (Formerly National Facility for Animal Tissue & Cell Culture (NFATCC)

For the year ending : 31.3.97

(a)	Whether accounts are maintained regularly and in accordance with the provision of the Act and Rules	YES
(b)	Whether receipts and disbursements are properly and correctly shown in the accounts	YES
(c)	Whether the cash balance and vouchers in the custody of the manager or trustee on the date of audit were in agreement with the accounts	YES
(d)	Whether all books, deeds accounts, vouchers or other documents or records required by the auditor were produced before him	YES

continued ...

(e)	Whether a register of movable and immovable properties is properly maintained, the changes therein are communicated from time to time to the regional office and the defects and inaccuracies mentioned in the previous audit report have been duly complied with	Registers of Movable and Immovable properties are maintained
(f)	Whether the manager or trustee or any other person required by the auditor to appear before him did so and furnished the necessary information required by him	YES
(g)	Whether any property or funds of the Trust were applied for any object or purpose other than the object or purpose of the trust	NO
(h)	Theamounts of outstandings for more than one year and the amounts written off, if any	Amount outstanding for more than one year: Motor cycle advance Rs. 2,59,584.00 paid to employees
(i)	Whether tenders were invited for repairs or construction involving expenditure exceeding Rs. 5000/-	YES

continued ....

(j)	Whether any money of the public trust has been invested contrary to the provisions of Section 35.	NO
(k)	Alienations, if any, of the immovable property contrary to the provisions of Section 36 which have come to the notice of the auditor	NIL
(l)	All cases of irregular, illegal or improper expenditure or failure or commission to recover monies or other property belonging to the public trust or of loss or waste of money or other property thereof and whether such expenditure, failure, commission, loss or waste was caused in consequence of breach of trust or misapplication or any other misconduct on the part of the trustees or any other person while in the management of the trust	NIL
(m)	Whether the budget has been filed in the form provided by the rule 16 A	YES
(n)	Whether the maximum and minimum number of the trustees is maintained	YES
(o)	Whether the meetings are held regularly as provided in such instrument	YES
(p)	Whether the minute book of the proceedings of the meeting is maintained	YES
(q)	Whether any of the trustees has any interest in the investment of the trust	NO
(r)	Whether any of the trustees is a debtor or creditor of the trust	NO
(s)	Whether the irregularities pointed out by the auditors in the accounts of the previous year have been duly complied with by the trustees during the period of audit	Please see our General Remarks as per Annexure - I

(t)	Whether list of members is maintained and is kept up-to-date	YES
(u)	The name and address of the present Secretary to the Society with whom the communication is to be made	Dr. G.C.Mishra, Director, NCCS, (Formerly NFATCC) Pune University Campus,Ganesh Khind Road,Pune - 411 007
(v)	If the rules of the Society prescribe any mode of investment of the money of the society and whether the investments is as per such rules	Trust holds no investments
(w)	Any special matter which the auditor may think fit or necessary to bring to the notice of the Deputy or Assistant Charity Commissioner	Please see our general remarks as per Annuexure - I and significant accounting policies followed as per Annexure - 2

For B.L. Phatak & Co.
Chartered accountants

Pune:
Dated: 8.7.97

Sd/-
Partner

ANNEXURE - `1'

NATIONAL CENTRE FOR CELL SCIENCE, PUNE

Generel Remarks for the year ended 31.03.1997

1. We have conducted the audit of NCCS (formerly NFATCC) as per our acceptance letter dated 14.02.96 to our appointment as auditors.

2. Out of grants received during the year from Government of India, Ministry of Science & Technology, Department of Biotechnology, grants of Rs. 6,29,00,000/- received for incurring capital expenditure are added directly to opening balance of Capital Grants from Department of Biotechnology under the head `Other Earmarked Funds'.

During the year ended 31.3.97, the Trust has received bank interest of Rs. 32,97,771/- on deposits made out of capital grants. It is added directly to `Other Earmarked Funds' as it is considered as capital grant to be adjusted against future Capital Expenditure to be reimbursed by way of capital grants from Government of India.

3. It is informed to us that as per Memorandum of Understanding between NCCS (Formerly NFATCC) and University of Poona, the University of Poona has allotted land measuring about 10 acres for construction of the NCCS (Formerly NFATCC) complex including laboratories and residential accommodation of NCCS's staff members, students, fellows and visitors. Title of the land allotted

by the University of Poona for construction of the NCCS buildings will continue to be vested in the University of Poona (Government of Maharashtra).

NCCS will enjoy the land privileges throughout its existence. Construction & Service Group of Department of Atomic Energy, Government of India is constructing the NCCS Complex on the amounts deposited by the trust. The aggregate amount so deposited with C & S Group, Department of Atomic Energy alongwith other payments made is carried forward as capital Work-in-progress.

4 Following project grants are carried forward in the Balance Sheet as liabilities as these grants are

received for specific projects :-

a) CSIR Project	:	Rs. 2,33,072.00
b) Biotech Project	:	Rs. 15,888.10
c) H.L.R.C. Project	:	Rs. 24,489.00
d) Fetal Liver	:	Rs. 6,15,569.00

Expenditure whenever incurred on these projects deducted from the said grants.

For B.L.Phatak & Co.
Chartered Accountants

Pune:
Dated: 8.7.97

Sd/-
Partner

ANNEXURE `2'

NATIONAL CENTRE FOR CELL SCIENCE, PUNE

SIGNIFICANT ACCOUNTING POLICIES FOLLOWED :

1. The financial statements are prepared on cash basis.

2. Fixed Assets are carried at cost. No depreciation is provided on Fixed Assets upto 31.03.1997

3. As per the system consistently followed, entire expenditure on consumables is charged to Income & Expenditure Account Opening and Closing Stocks of consumables are not accounted for.

4. Contributions to Provident Fund are charged to Income & Expenditure Account.

5. Foreign Exchange transactions are recorded at the rates prevailing on the dates of payment.

For B.L. Phatak & Co.
Chartered Accountants
Sd/-
Partner

Pune :
Dated : 8.7.97

NATIONAL CENTRE FOR

INCOME AND EXPENDITURE ACCOUNT FOR

For the year ended31.03.1996	Expenditure	For the year ended 31-03-97
	To Expenditure in respect of properties	
166600.00	Municipal Taxes	
---	Establishment expenses	
162555.00	To Remuneration to Trustees	201441.00
2500.00	Honororium to non-official member of Governing Body	
3000.00	To Audit fees	3000.00
	To Amount written off	
	Reserve or specific fund	
304411.00	To Liquid Nitrogen Fund	
15507591.11	To Expenditure on objects of Trust (as per Schedule I)	19126450.20
	Surplus	750328.80
16146657.11	Total Rs.	20081220.00

As per our report of even date

For B. L. PHATAK & Co.
Chartered Accountants
Sd/-
Partner

Pune:
Dated: 8.7.97

NATIONAL CENTRE FOR
BALANCE SHEET

As on 31-03-96	Funds and Liabilities		As on 31-03-97
	Other Earmarked Funds:		
242199810.71	As per Schedule 2		308397581.71
	Project Grants:		
396000.00	Plasticware Project	Nil	
	(Balance as per last Balance Sheet)		
	Fetal Liver		
327267.00	balance	327267.00	
	Add. Additions	320000.00	
	Less Exp. during the year	31698.00	
			615569.00
	(Capital Grants 2,00,000.00)		
	CSIR Projects	119547.00	
	Add. Additions	249985.00	
	Less Expend	136460.00	
119547.00			233072.00
15888.10	Biotech Associates		15888.10
	H.L.R.C. Project		28489.00
	Amt. Rec. 55000.00		
	Less Exp. 26511.00		
1032073.75	Amount payable to Dept of Atomic Energy, C & S Group.	Nil	

CELL SCIENCE, PUNE

CELL SCIENCE, PUNE

THE YEAR ENDING 31ST MARCH, 1997

For the year ended 31-03-96	Income	For the year ended 31-03-97
569.00	By Interest on vehicle advance to employees	310.00
15700000.00	By Grants (Govt. Grants) DBT	19935000.00
---	Revenue Recurring N.S.D. Grants	25000.00
304411.00	By Liquid Nitrogen Receipts	
15576.00	By Consultancy	15200.00
	By Cell line handling charges	69983.00
	By Misc. Receipts	35727.00
126101.11	Deficit carried over to Balance sheet	
16146657.11	Total Rs.	20081220.00

National Centre for Cell Science, Pune

Sd/-
T. G. R. Pillai
Sr. Acc. Officer

Sd/-
Dr. G. C. Mishra
Director

CELL SCIENCE, PUNE
AS ON 31.3.1997

As on 31-03-96	PROPERTY AND ASSETS		As on 31-03-97
	Immovable Properties:		
	As per Schedule 3		
12924034.55	Building		12940819.55
144497923.31	Work-in-Progress		149018697.46
Nil	Investments		Nil
	Furniture & Fixture & Other assets		
68783692.35	As per Schedule 4		102871452.45
Nil	Loans (Secured or Unsecured)		Nil
	Advances:		
	As per Schedule 5		
404670.00	Employees	294,484.00	
125150.00	Deposite	195,150.00	
			489634.00

Continued ...

As on 31-03-96	Funds and Liabilities	As on 31-03-97
	Amt. rec. on behalf of P.F.	87261.00
	Canteen Deposit	5000.00
Nil	Income & Expenditure	558700.64
	As per last Balance Sheet	
	Surplus 750328.80	
	Less Deficit 191628.16	
244090586.56	Total Rs.	309941561.45

* Income outstanding: Nil. (If accounts are kept on cash basis).

a) Significant Accounting policies followed

b) The above Balance sheet to the best of our belief and of the property

As per our report of even date

For B. L. Phatak & Co.
Charterd Accountants
Sd/-
Partner

Pune:
Dated: 8.7.97

Nil	Income Outstanding	
	Cash & Bank Balances	
17163488.19	As per Schedule 6	44620957.99
	Income & Expenditure Accounts:	
19162816.00	Balance as per last Balance sheet	
244090586.56	Total Rs.	309941561.45

are as per Annexure 2 to Audit report.
contains a true accounts of the Funds and liabilities and assets of the Trust.

For National Centre for Cell Science

Sd/-	Sd/-
T.G.R.Pillai	Dr. G. C. Mishra
Sr. Accounts Officer	Director

NATIONAL CENTRE FOR CELL SCIENCE, PUNE

SCHEDULE I

EXPENDITURE ON THE OBJECTS OF TRUST

349,229.60	Printing & Stationery	210,567.20
740,264.00	Advertisement	343,157.00
3,385.90	Newspapers & Periodicals	5,395.20
222,524.90	Telephone Telex	294,367.00
31,483.50	Postage	33,516.00
168,395.69	Meeting & Misc. Expenses	126,757.45
75,629.26	Vehicle Expenses - Petrol	110,436.50
55,417.80	-Repairs	95,585.50
55,900.00	Rent Rates & Taxes	37,556.00
32,845.00	Carriage Expenses	38,186.00
1,272,170.00	Water & Electricity	4,060,844.00
12,845.60	Labour Charges	79,088.70
78,465.52	Misc. Purchases	76,009.20
12,808.70	Conveyance	8,515.05
32,219.81	Repairs & Maintenance	124,727.50
42,750.00	Membership Fees	5,450.00
4,000.00	Inspection Fees	18,000.00
16,540.00	Professional Fees	52,816.00
15344986.11	c/f	18,986,881.20

As on 31.03.1996	Particulars	As on 31.3.97
15344986.11	b/f	18986881.20
2810.00	Contribution to Charity	6411.00
	Commissioner	
143565.00	Maintenance Contract	89185.00
1350.00	Life 2000 Project - Exp.	--
14880.00	CSIR Associateship	
	Bank Charges	973.00
	National Science Day	25000.00
	U.G.C. Project	18000.00
15507591.11	Total	19126450.20

NATIONAL CENTRE FOR CELL SCIENCE, PUNE
SCHEDULE 2
OTHER EARMARKED FUNDS

a)	Capital Grants from DBT New Delhi:	
	Balance As per last Balance Sheet	241356409.71
	Grants for Capital Expenditure	62900000.00
	Bank Interest to be adjusted against future capital grants	3297771.00
b)	Vehicle fund:	
	Balance as per last Balance Sheet	400000.00
c)	Liquid Nitrogen Fund	443401.00
	Balance as per Balance Sheet	
	Total Rs.	308397581.71

NATIONAL CENTRE FOR CELL SCIENCE, PUNE

SCHEDULE 3

IMMOVABLE PROPERTIES AS ON 31.03.97 (AT COST)

Particulars	As on 01.04.96	Additions during the year	Total as on 31-03-97
A] Building:			
1) Jopasana	6026554.30		6026554.30
2) Jidnyasa	6897480.25	16785.00	6914265.25
Total	12924034.55	16785.00	12940819.55
B] Capital work in progress NCCS complex at Pune University Campus			
1) Amt. deposited with C & S Group, Dept. of Atomic Energy, Govt. of India	130900000.00	-	130900000.00
2) Other payments	13597923.31	4520774.15	18118697.46
Total	144497923.31	4520774.15	149018697.46

NATIONAL CENTRE FOR CELL SCIENCE, PUNE

SCHEDULE 4

FURNITURE, FIXTURES AND OTHE RASSETS AS ON 31.03.97

Particulars	As on 01.04.96	Additions during the year	As on 31.03.1997
A) Furniture & Fixture	9953935.73	3128442.00	13082377.73
B) Library Books	5106727.25	2288314.10	7395041.35
C) Equipments	53108955.37	28671004.00	81779959.37
D) Vehicles	414074.00	-	414074.00
E) Equipments under Fetal Liver Project	200000.00	-	200000.00
Total	68783692.35	34087760.10	102871452.45

NATIONAL CENTRE FOR CELL SCIENCE, PUNE
SCHEDULE 5
ADVANCES

As on 31.03.1996		As on 31.03.1997
	A) To Employees:	
349930.00	Motor Cycle Advance	283504.00
10740.00	Festival Advance	10980.00
44000.00	TA/DA Advance	
---	Advance for purchases	---
404670.00	Total A	294484.00
	B) Deposits:	
108000.00	Telephone Deposit	168000.00
17150.00	Gas Deposits	27150.00
125150.00	Total B	195150.00
529820.00	Total A + B	489634.00

NATIONAL CENTRE FOR CELL SCIENCE, PUNE

SCHEDULE 6

CASH AND BANK BALANCES

As on 31.03.1996		As on 31.03.1997
	Balance with Bank of India	
16120414.44	- in Current Account	44609957.99
1000.00	- in Fixed Deposit	1000.00
1.032,073.75	- Saving Account	
10000.00	Petty Cash in Hand	10000.00
17163488.19	Total Rs.	44620957.99

NATIONAL CENTRE FOR

RECEIPTS AND PAYMENTS FOR THE

RECEIPTS		AMOUNT
To Opening Bank Balance as on 01.04.96		
Petty Cash		10000.00
Bank of India		16120414.44
Bank of India Fixed Deposit		1000.00
Bank of India Savings A/c		1032073.75
To Government grant (Dept. of Biotechnology, Misnistry of Science & Technology, Govt. of India)		82835000.00
Capital 62900000.00		
Revenue 19935000.00		
To Interest to be adjusted against future capital grants		3297771.00
To Interest on Vehicle Loans		310.00
To recovery of advances		
Vehicle advance		91906.00
LTC advance	--	
Purchase Advance	--	
Festival Advance		19560.00
TA/DA Advance		44000.00
TO Grants-in-Aid Project Fetal Liver		320000.00
CSIR Proj. a) JRF/SRF/RA		114985.00
b) Prof. Iqbal		135000.00
National Science Day		25000.00

CELL SCIENCE, PUNE

THE YEAR ENDED 31ST MARCH, 1997

PAYMENTS	TOTAL
BY CAPITAL EXPENDITURE:-	
a) Building (Jidnyasa)	16785.00
b) Building (Jopasana)	-
c) University campus	
i) University campus bldg.	4,520,774.15
Furniture	3128442.00
Equipment	28671004.00
Library books	2,288,314.10
Deposit (L.P.G. & Tel,)	70,000.00
BY OTHER EXPENDITURE:-	
1. Salaries / Allowances	6322741.23
2. Remuneration to Director	201441.00
3. Works on contract	1440468.00
4. Consumables / Overheads	4700905.42
5. Travel	701759.25
6. Consultancy	91633.00
BY CONTINGENCIES	
1. Labour charges	79088.70

RECEIPTS	AMOUNT
To Liquid Nitrogen	
Consultancy	15200.00
To .Amount received from C&S Group of Dept. of Atomic Energy for interest	34367.00
To Cell line handling charges	69983.00
TO H.L.R.C. Project	55000.00
To Amt. received from Ferro Scrap Lt. forP. F. disbursement	87261.00
To Misc. Receipts	35727.00
To Canteen Deposit	5000.00

PAYMENTS	TOTAL
2. Postage	3516.00
3. Vehicle	
Petrol	110436.50
Repairs	95585.50
4. Contribution to Charity Commissioner	6411.00
5. Maintanance Contract	89185.00
6. Rent Rates Taxes	37556
7. Carriage Expenses	38,186.00
8. Advertisement	343157.00
9. Printing & Stationary	210567.20
10. Telephone/Telex	294367.00
11. Water/Electricity	4060844.00
12. Inspection fees	18000.00
13. Audit fees	3000.00
14. Other fees	52,816.00
15. Misc. purchases	76,009.20
16. Repairs & Maintenance	124727.50
17. Newspaper & Periodicals	5395.20
18. Conveyance	8515.05
19. Meeting & Misc. Expenses	126757.45
20. Membership fees	5450.00
21. Bank charges	973.00
22. Consultancy	8400.00
BY PROJECT	
CSIR (JRF/SRF/RA)	72519.00
CSIR (Emeritus Scientist)	63941.00

RECEIPTS	AMOUNT
Total	104349558.19

As per our report of even date
For B. L. Phatak & Co.
Chartered Accountants

Sd/-
Partner
Date: 8.7.97

PAYMENTS	TOTAL
H.L.R.C.	26511.00
National Science Day	25000.00
U.G.C.	18000.00
DBT (Bio-Reactor)	396000.00
BY PROJECT	
Fetal Liver Project	31698.00
BY VEHICLE ADVANCE	25480.00
BY FESTIVAL ADVANCE	19800.00
BY Amount disbursed on behalf of C&S Group DAE	1066440.75
BY CLOSING BALANCES	
a) Balance with Bank of India, NCCS	44609957.99
b) Saving A/c DAE	--
c) Fixed Deposit	1000.00
d) Cash in hand	10000.00
Total	104349558.19

Sd/-
Dr. G. C. Mishra
Director
NCCS, Pune.

Sd/-
T. G. R. Pillai
Accounts Officer
NCCS, Pune.

Visitors at the Science Day Exhibition at NCCS
(28th February to 3rd March 1997).