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The Relevance of Medical Education for Overall Health care

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A systematic approach to planning and management of innovative educational program should take into account the changing needs of the society and the student community, the emerging educational technology besides governance and leadership issues to tackle resource mobilization. This paper examines the current deficiencies in the curriculum and offers some general guidelines to adopt innovative approaches to the teaching and assessment of students in medical education. It is argued that curricular reforms must be accompanied by proper faculty development mechanisms and production of learning resources which are vital for sustaining changes.

Curriculum – Some concerns:

Medical education has been patterned after the traditional British System with a strong disciplinary structure. Training in medicine takes place in a tertiary care hospital, alienated from the hard realities of rural life. The curriculum has been stuffed with the information about rare diseases, esoteric syndromes, not in tune with the common health problems of the community. The development of skills – communication skills, managerial skills, and ethical aspects of patient care have taken a back seat. How to develop and assess the full range of professional competence has been a question mark. The instruction in medical colleges has been didactic and largely "teacher centered". This has killed the initiative on part of the learners to pursue self-directed learning, ability to think, analyze facts, interpret data, and solve problems. Assessment system has been a major culprit as it lacked validity, reliability and objectivity.

Staff development in medical colleges is inadequate if not absent. A medical teacher is inadequately prepared for teaching. The main criteria for the selection or promotion are based on research publication. There is also a felt need for institutionalizing staff development activities at various levels.

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Directions of change:

There has been a paradigm shift in the emphasis from tertiary care to the primary care; from disease orientation to a community orientation; from disciplinary structures to integrated structures; and from acquisition of information to the solving of problems. This has resulted in far reaching implications on the content as well as the methodology of teaching-learning. Problem based learning (PBL) has emerged as an alternate pathway to the disciplinary set up. But competency based learning, objective assessment and the application of educational technology can be harnessed effectively.

Mechanisms need to be installed to ensure development of essential skills and provide opportunities for self directed learning and self assessment. It is therefore necessary to think of a two tiered curriculum which consist of a) core curriculum which constitutes the essential knowledge and skills which must be mastered by all learners and b) optional modules which should be accessible to all students for pursing self learning at their pace. The core curriculum should form the focus of evaluation, while the optional modules and assessment systems should be accessible to all learners.

Approach to instruction:

Criticism against traditional didactic teaching has led to more emphasis on participatory interactive techniques. The exploding body of scientific knowledge cannot be handled by traditional approach especially in the backdrop of increase in the enrollment. Information and communication technology (ICT) is likely to have far reaching implications. A medical teacher of 21st century is expected to move away from the role of the subject expert to a facilitator of learning. He is expected to utilize the whole range of participatory methods of learning including small group discussion, case study field work, role play, simulated patient management problem, and computer assisted learning.

Role of ICT:

Thanks to the revolutionary developments in the field of ICT, the educational media have heralded a new era in which students can access information and engage in self directed learning. The application of digital techniques, video, computers, multimedia, internet, teleconferencing and tele-medicine have promised unlimited possibilities in terms of providing exciting learning environments and opportunities, in class rooms, laboratories, and field. It is possible to utilize tele-conferencing methods, for bringing together all innovative techniques, which will help in conserving resources, avoiding duplication and make optimum utilization of limited resources. For example, the EDUSAT launched by the Government of India, may be utilized for reaching out to the target group. The production of learning resources in various media, viz. print form, electronic form etc., are challenging, but once created, they can be beneficial to the whole country.

Beginning with the use of overhead projectors, slides and video, and going on to computers, multimedia and internet, we have traveled a long way in harnessing the use of educational media. Due to the availability of high speed computers, networking, satellite communications, CD-

ROM technology and interactive CDs, instantaneous access to information from any part of the world has become a real possibility. Multimedia presentations and computer simulations are very effective for presenting bio-medical information in an illustrative manner.

The field of telemedicine too has opened up unlimited possibilities in terms of delivery of health care and the exchange of health care information across distances using telecommunication technology. It is used for the transfer of basic patient information, the transfer of images such as radiographs, CT scans, MRIs, Ultrasound studies, pathology images, video images of endoscopic or other procedures, patient interview and examinations, consultation with medical specialists and health care educational activities. Tele medicine is of tremendous advantage in providing consultation facility from centres of expertise to remote areas in matters such as diagnosis, treatment and prognosis besides, providing health education to the rural community or any other target audience in a phased manner.

Rational assessment strategy:

Any attempt to reform education must begin with a reform in assessment strategy. Because, assessment determines the stule of learning and gives credibility to the system. Traditionally, the examinations have witnessed predominance of essay type questions in theory and long cases in clinical examinations. They need to be supplemented with problems based questions of various types in theory and modalities such as objective structured clinical examinations and structured viva examinations for a comprehensive assessment of students learning.

Faculty development and role of medical education units:

Faculty development is the corner stone for ensuring quality of training. At present opportunities for training teachers are limited. It is necessary to strengthen the existing centers to organize faculty development, besides opening medical education unit (MEU) in each college, for providing leadership in medical education at the institutional level. The unit can organize activities in the form of in-house workshops, and facilitate changes in the curriculum. Such a unit would have interdisciplinary faculty, supported by technical staff, equipment and resources.

Active participation is often linked with the question of recognition and incentives to the faculty who contribute to the reform and similarly authority to bring to book those who are incompetent or unwilling to change. A good leader anticipates these potential obstacles and creates a healthy environment.

The resource allocation is a vital component for the success. It should be done in a fair way considering the relevance quality, equity and cost effectiveness.

Governance and academic leadership

Good governance implies, clear vision, setting up goals, allocation of resources, and organize strategies for effective implementation. Delegation and decentralization is highly desirable in a vast country like India. Resistance to changes is inherent in any organization. Leadership

and communication are vital tools for introducing changes. The leader must build teams, take everyone into confidence, including students community before introducing the changes. A lot of inter-departmental coordination is required horizontally (communication should flow across departments) and vertically (from top to bottom) and vice versa. The leader should be a role model, whose integrity and honesty are beyond doubt. He should be transparent and accountable to the stakeholders.

The positive changes in the methodology of teaching have however, remained confined to a few elite centers of learning. A majority of the medical colleges suffer from a chronic shortage of resources, infrastructure, equipment and what is of critical importance – an adequately trained and qualified faculty support. Though the Medical Council of India has stressed upon the need for introducing both horizontal and vertical integration, attempts to introduce an integrated curriculum have not really succeeded. Instead of the encouragement of a participatory, inter-active process, teaching remains didactic. Lectures still dominate instruction, hence curbing the initiative on part of the students to pursue self directed learning, critical analysis of facts, interpretation of data and problem solving. The 3 Cs, which are of utmost importance to the development of a humanistic approach to patient care – communication skills, compassion, and care – have unfortunately remained at the periphery of classroom learning. The public-private dichotomy and divide can be seen in the financial constraints being faced by the public sector medical colleges and the commercialization of private medical colleges.

Providing a high quality of medical education has been an important national agenda, but though India has over 170 medical colleges, there is a perceptive lack of well defined standards, and absence of a strategy to link quality assurance with faculty development. Quality assurance is a holistic concept which includes accountability, development of standards, providing facilities, institutional self evaluation and peer evaluation, besides faculty development as an ongoing activity. One of the foremost requirement for quality assurance is the setting up of a national Quality Assurance Body which would primarily be responsible for providing quality assurance by commissioning visits by review teams and accreditation of programs/ institutions for a specifically defined period. The new health policy 2002 envisages the setting up of a medical grants commission to reduce the problem on account of uneven standards and proliferation of medical and dental colleges in various parts of the country.

Radical changes in medical education are possible but only if there is a change in the role of various stakeholders, namely the central and state governments, private sector, non-governmental organizations and other institutions of civil society. The new national health policy 2002 identified the need to modify the medical curriculum to make it need based and skill – oriented in order to enable fresh graduates to contribute to primary health services. The policy also talks about the need to introduce post graduate courses in family medicine. This indeed is a welcome change! Having

more specialists in public health and family medicine is a long forgotten agenda. Though over the past few decades public health has emerged as a speciality in the developed world, it has remained neglected in India. At present the study of public health is limited to the curriculum of community medicine. The scope needs to be broadened to incorporate areas of current importance such as epidemiology, health policy analysis, gender studies and environmental sciences.

Networking and sharing of knowledge and resources

Modern technologies have capabilities to establish networking of individuals and institutions for sharing information and resources. This will enhance cost effectiveness and provides opportunities for introducing changes on a large scale. Universities and centres of higher learning should come together and come out with proposals for networking educational efforts. The responsibility of coordination should be entrusted to a reputed institution with resource allocation.

There is a need for the under graduate to understand the epidemiological and demographic transition that this country is going through, while we are still far from having wiped out the twin curse of infectious diseases and nutritional deficiencies, we are fast heading towards having the largest number of patients having life style diseases such as diabetes, coronary heart disease and degenerative diseases. The information that has become relevant to the medical graduate has expanded further due to a double revolution that medicine is going through. One part of this revolution is the post genomic stimulus to gene therapy and predictive medicine. The other part of the revolution is at the holistic level. Realizing the role of mental stress in disease, and that of mental peace in self healing and finding scientific support for mind body relationship in psychoneuro-immunology, there has been a tendency to turn to ancient wisdom. Ancient disciplines, such as yoga, and Ayurveda have evinced keen interest. With ancient disciplines on their way to becoming part of mainstream scientific medicine, the range of subjects which a doctor will have to know is indeed mind boggling while medical curricula are not likely to be affected by these futuristic trends very soon, a doctor graduating today may well be practicing medicine 50 years from today. Therefore, it has become all the more important to remember that Sri Aurobindo called the first principle of teaching, that nothing can be taught! The student learns only what he wants to learn, not what the teacher teaches. So just create in the student the desire to learn, the capacity to learn, and the ability to judge what area of medical knowledge he wishes to concentrate on.

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Computer Modeling for Drug Development

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SUMMARY

With the advent of powerful computers and high resolution graphics, the time scale of drug discovery and development, as well as the cost of development of new drugs has been reduced very significantly.

We present here a bird's eye-view of the essential steps involved in the drug discovery and development process starting from identification and characterization of the drug target, the search for a 'hit' or a 'lead compound', the detailed study of the binding of the lead compound to the target, the strategy to improve and optimize the binding, and the final iterative cycle of refinement and testing to yield a suitable drug.

Finally, we describe briefly some of our research efforts in developing novel inhibitors of HIV-1 reverse transcriptase, and of protein tyrosine phosphatase 1B (PTP1B), a new drug target for type 2 diabetes and obesity.

With the advent of powerful computers with high resolution graphics and molecular modeling software packages, the time scale of drug development and the cost of development of new drugs has been reduced very significantly. Fig. 1 gives and idea of the time scale for the development of a drug by traditional methods. Through the use of computers, the initial process of screening, say 5000 compounds, can be reduced from 8-10 years to 8-10 weeks! Therefore, it is not surprising that computer modeling is now an essential and integral component of the pharmaceutical industry.

THE DRUG DISCOVERY PIPELINE

Let us start with the essential steps involved in the drug development process. Fig. 2 gives the flowchart of the process. The first step is the identification of the target based on the biological and biochemical data for a given medical condition. The target could be a known target, and the aim therefore would be to design new and more efficient drugs for it, or it could be a new target discovered through the genomics initiative, for which the active site if it is an enzyme, or the binding site if it is a receptor, has been characterized.

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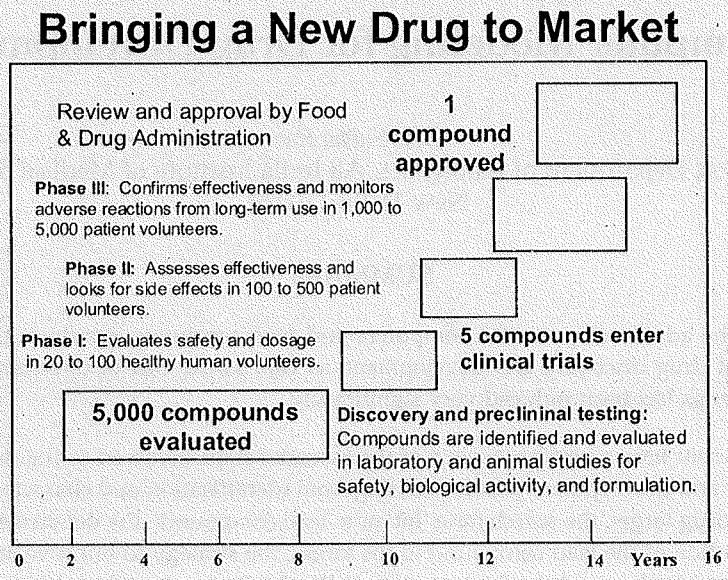

Figure 1

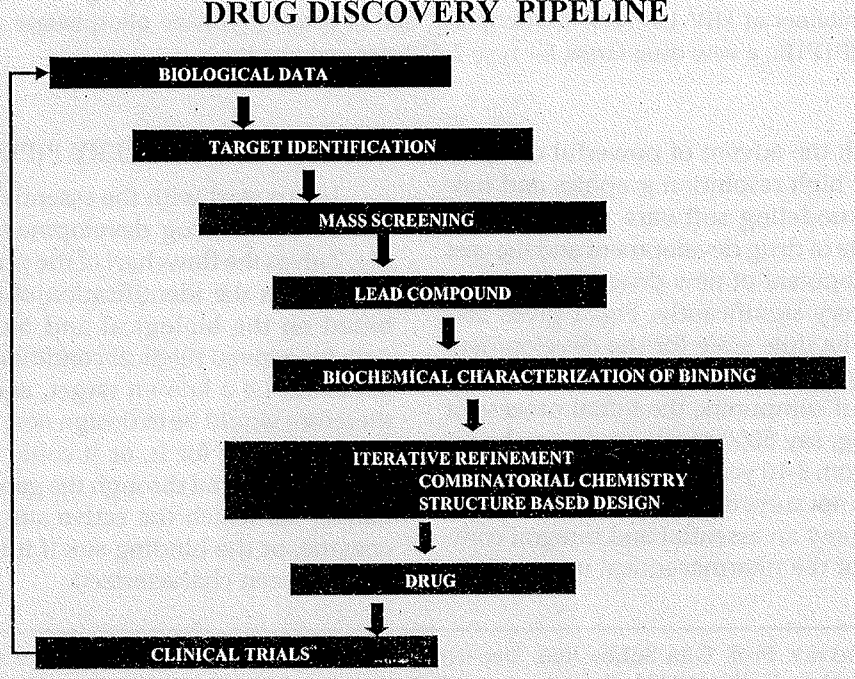

Figure 2

Once the binding region is known, the next challenge is to find suitable small molecule ligands. This is by far the most daunting task of the drug design process and can be achieved through the following stages:

1. **Mass screening**

A database of hundreds of thousand of compounds is screened by determining the activity of each of the compounds and, if a successful match is found, the initial hit is called a 'lead compound'. The lead compound is usually a weakly binding ligand and has minimal receptor activity.

In the next step of the pipeline, the binding of the lead compound to the target binding site is studied in order to determine the steric, electrostatic, hydrogen bonding and hydrophilic interactions between the ligand and the receptor. The most accurate way of doing this is through a determination of the three-dimensional structure of the complex by X-ray crystallography. A strategy can then be developed based upon the interactions to improve and optimize the binding of the lead compound. One then enters a cycle of iterative chemical refinement and testing until a drug is developed which then undergoes clinical trials. The commonly used refinement techniques are combinatorial chemistry and structure based design.

Combinatorial chemistry

This is a synthetic tool that enables chemists to rapidly generate thousands of lead compound derivatives for testing. As shown in Fig. 3, starting from a scaffold which contains a constant part and variable substituent groups, a large number of derivative structures can be generated as a result of the combinatorial process. The combinatorial libraries selected are based on the study of the binding site.

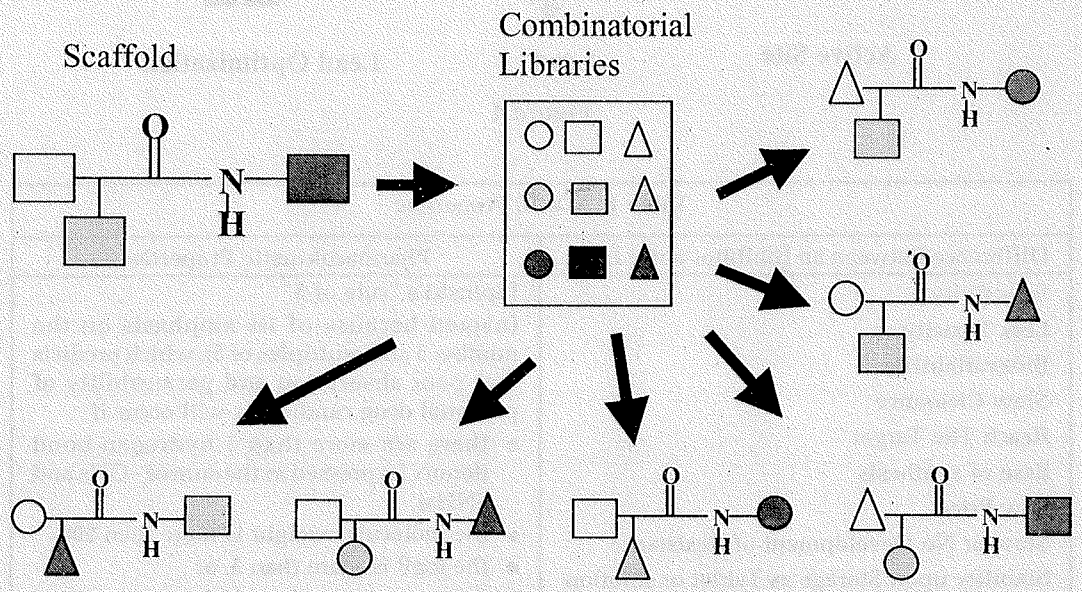

Figure 3

Structure based design

This is often called rational drug design. Based on the crystal structure of the complex, one can target regions of the ligand that fit poorly within the binding site and postulate chemical changes to improve steric and electrostatic complementarity with the receptor, as shown in Fig. 4. It is a much more focused method as compared to combinatorial chemistry.

Use of computers

The process of identifying a lead compound and refinement of the lead compound can be implemented with speed and accuracy with the help of powerful computers and molecular modeling software. Docking and scoring are the two major steps involved in computer-aided drug design. The process of docking is essentially the modeling of the complex of the receptor

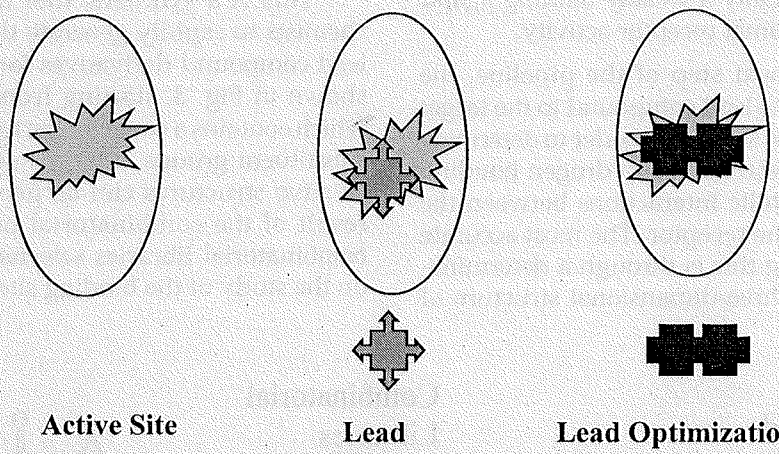

Figure 4

Table 1. ADME Properties	
Difference Between an Inhibitor and a Drug	Pharmacokinetic Properties
Selectivity Less Toxicity Bioavailability Slow Clearance Reach The Target Ease of synthesis Low Price Slow or No Development of Resistance Stability upon Storage as Tablet or solution Pharmacokinetic Parameters No Allergies	Lipinski's "rule of 5" (named because of its emphasis on the number 5 and multiples of 5), which predicts that poor absorption and permeability of potential drug candidates will occur if • there are more than 5 hydrogen-bond donors (expressed as the sum of –OHs and –NHs), • the molecular weight is more than 500, • the logP is more than 5, or • there are more than 10 hydrogen-bond acceptors (expressed as the sum of nitrogens and oxygens).

with the ligand. The latter moves around in the receptor space simultaneously changing its own structure in accordance with an energy minimization algorithm, until the optimum position is reached. Scoring is the method of predicting the affinity of binding and hence the activity of the ligand. Generally docking algorithms are combined with scoring functions to arrive at a prediction of both the ligand binding position and the activity.

Once a suitably active and selective ligand has been identified, the next step is to check whether it would be a suitable drug in terms of its ADME (absorption, distribution, metabolism and excretion) properties (Table 1). Computers play a major role in predicting these pharmacokinetic parameters, using quantitative structure-activity relationship (QSAR) methods. The basic principle of these methods is to build a model using a database of known compounds with known parameters and activities, and then to use the model to predict the parameters for an unknown compound.

Thus, given the high resolution structure of the binding site, the entire drug development process leading up to the stage of animal and clinical trials can be accomplished with the help of computers.

We now present some of our research work on the structure based design of novel inhibitors of HIV-1 reverse transcriptase and of protein tyrosine phosphatase 1B, a new drug target for type 2 diabetes mellitus and obesity.

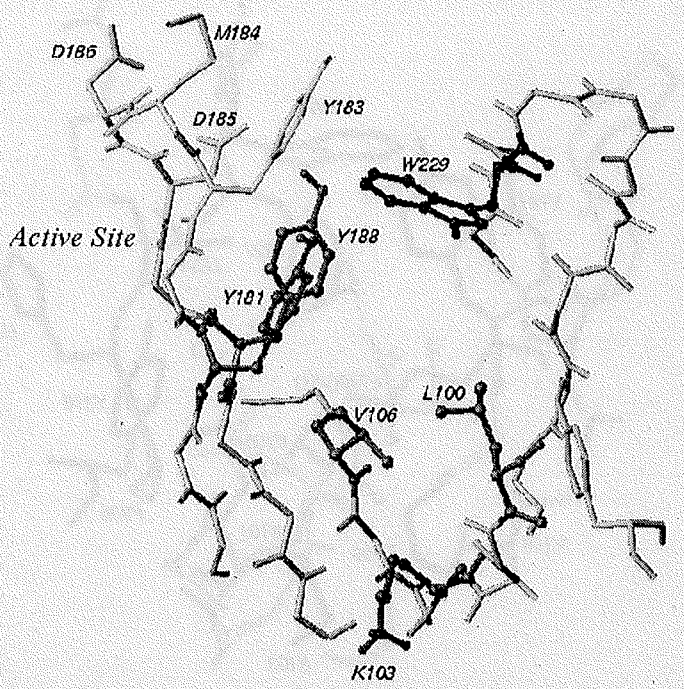

Figure 5. RT Binding pocket. Conserved residue is shown in black, frequently mutating residues in dark gray.

HIV-1 reverse transcriptase

This is a key enzyme in the life cycle of HIV-1, and is one of the targets of HAART. The main problem encountered with anti-HIV drugs is the rapid emergence of drug-resistant mutations and, therefore, the development of new, mutation resilient drugs presents a major challenge in anti-HIV therapy.

The X-ray crystal structure (1) of the complex of HIV-1 reverse transcriptase (RT) with a known inhibitor, nevaripine, indicates a binding pocket which is close to, but not at the active site of RT (Fig.5). This binding pocket has some residues that are conserved and others that mutate in response to binding of the drug. The strategy for new drug development is that the inhibitor should have a large number of interactions with the conserved residues and the backbone of the binding pocket in order to have a more mutation resilient compound. This has given rise to many "second generation" inhibitors, the best amongst them being the compound S-1143. The crystal structure of the complex with S-1143 (2) indeed shows a large number of hydrogen bonds and interactions with the backbone. A second prerequisite for an inhibitor to be potent and mutation resilient is from the analysis of the crystal structures of complexes with mutant RT's, which show that the inhibitor should be able to adapt to a mutated binding pocket.

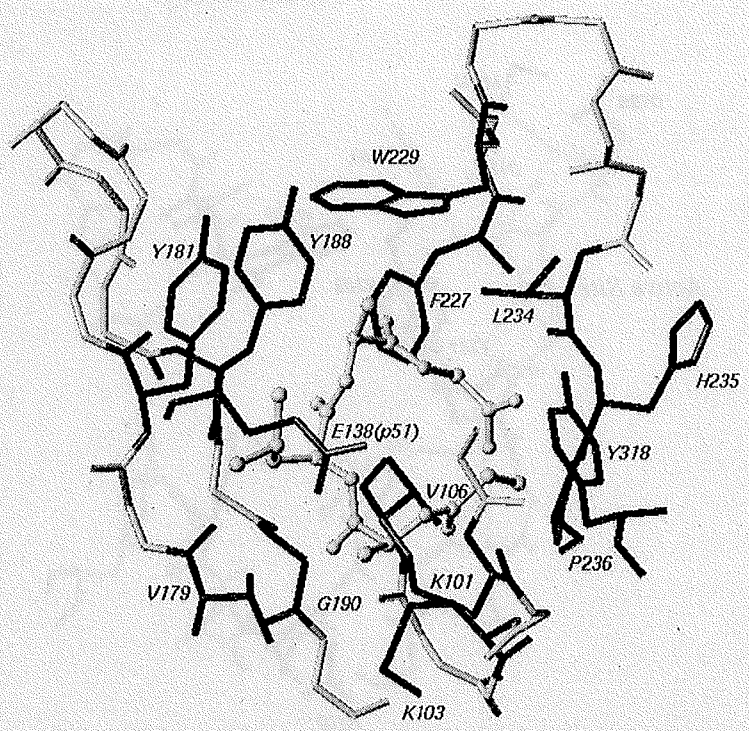

Figure 6. Final docked position of the designed peptide (in ball-and-stick rendering) in the RT binding pocket. The interacting residues are shown in black.

These observations prompted us to design a novel, small peptide inhibitor (3). The energy minimized structure of the peptide was computed, followed by docking studies, and the results are shown in Fig. 6. The peptide is found to have a large number of hydrogen bonds and interactions with the backbone of RT. The peptide also has interactions with the conserved residue W229. In addition, docking with mutant RT's shows that the hydrogen bonds and interactions are retained and, more significantly, the peptide is seen to flip over in order to adapt itself to the mutant RT pocket. Tables 2 and 3 indicate that the designed peptide inhibitor is as potent as the existing inhibitors. Thus, we have identified a new lead compound which is both potent and mutation resilient.

Table2. Measured Activities and molecular weights of known inhibitors

Inhibitor	Activity	Mol. Wt.
Nevirapine	0.08μM	266.3
UC-781	0.009μM	335.0
S-1153	0.01μM	451.4

Table 3. Predicted Activities

Ligand	Docked with	Activity (μM)
Nevirapine	Wild type RT	0.05
Designed inhibitor	Wild type RT	0.01
Designed inhibitor	Y188C mutant	0.06
Designed inhibitor	K103N mutant	0.01

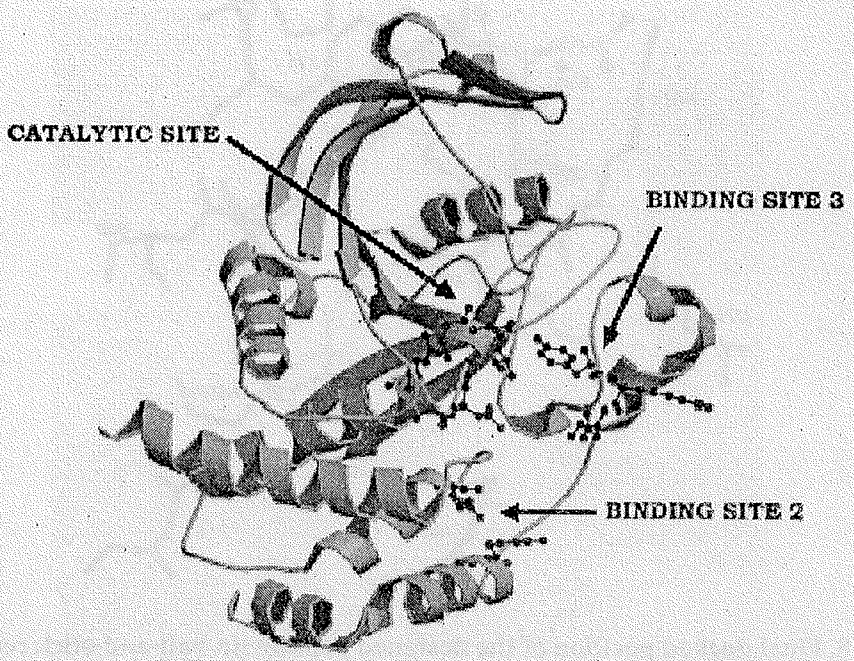

Figure 7. Crystal structure of PTP1B showing the three binding sites.

Protein Tyrosine Phosphatase 1B (PTP1B)

PTP1B has been shown to be a negative regulator of insulin signaling by dephosphorylating key tyrosine residues (4). Recent clinical studies have indicated a correlation between a polymorphism in the PTPN1 gene which encodes PTP1B, and the risk for type 2 diabetes mellitus (5). In addition, recent gene knockout studies in mice have shown the mice to have increased insulin sensitivity and improved glucose tolerance, as well as a resistance to diet-induced obesity (6). Thus there is compelling evidence that selective, small molecule inhibitors of PTP1B may be effective in treating insulin resistance at an early stage thereby leading to a preventive strategy for type 2 diabates and obesity.

The crystal structures of the complexes with known inhibitors reveal that in addition to the phosphotyrosine binding site, there are two other binding sites (Fig. 7).

Inhibitors that bind to these additional binding sites are found to have a high potency and a good selectivity for PTP1B.

Based on the above characteristic features, we have designed a novel, small peptide inhibitor. The modeled complex is shown in Fig.8.

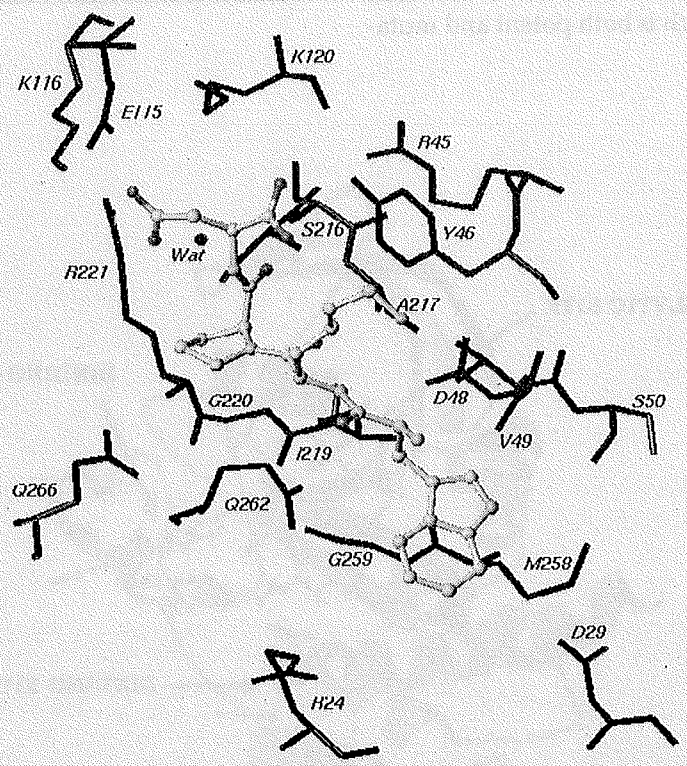

Figure 8. Final docked position of the designed peptide (in ball-and-stick rendering) in the PTP1B binding site. The interacting residues are shown in black.

The peptide is found to have hydrogen bonding with residues in the catalytic site as well as with those in binding site 3, and has interactions with residues in binding site 2. The predicted activity is 1.03 nanomolar, which is comparable to that of the most potent known inhibitor.

The next crucial step is to check whether the designed inhibitor is selective for PTP1B as compared to other closely related protein tyrosine phosphatases such as LAR, calcineurin and the highly homologous T-cell protein tyrosine phosphatase (TCPTP). This was done by modeling the respective complexes and calculating the activities. Table 4 shows that the designed inhibitor is highly selective for PTP1B. Significantly, there is a 800-fold selectivity over TCPTP. This is much higher than the best selectivity of 10-fold achieved so far by the 'best' inhibitor.

Table 4. Predicted Activities

Target	Activity (M)	Selectivity ratio
PTP1B	1.03×10^{-9}	1
TCPTP	8.34×10^{-7}	809
LAR	7.16×10^{-7}	696
Calcineurin	1.64×10^{-7}	159

Thus, the designed small peptide inhibitor is predicted to have a potency comparable to the most potent known inhibitor and a high selectivity over closely related protein tyrosine phosphatases, and is therefore, a suitable lead compound for the development of new drugs for obesity and type 2 diabetes mellitus.

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Stem Cells and their Therapeutic Potential

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SUMMARY

Recent developments on embryonic stem cell lines from human blastocysts have the potential to revolutionize many of our approaches to study human biology and medicine. The current method of treating diseases will be revolutionized in the near future and medicine-based treatment will be replaced by cell-based therapy. Embryonic stem cells appear to have greater potential than adult stem cells because of their flexibility and genetic stability. Combined with therapeutic cloning, embryonic stem cells research has become the focus of intense research across the world.

Embryonic stem cells are pluripotent in nature and extremely valuable because they possess three properties not found together in other cell types. Firstly, they replicate indefinitely without undergoing senescence (aging and death) or mutation in their genetic material. They are thus a large-scale and invaluable source of cells. Secondly, embryonic stem cells are genetically stable. Thirdly, they exhibit marked plasticity i.e., can differentiate into variety of cell types in tissue culture.

There is an urgent need to generate well-characterized embryonic stem cell lines and make them available for further research in our country. Embryonic stem cell lines are derived from surplus cryopreserved embryos generated in IVF labs after taking proper informed consent. Embryonic stem cell programs need to be developed in non- human primates, as this will be essential to carry out pre-clinical evaluation of efficacy and efficiency of embryonic stem cell therapy. Methodologies to derive 'isogenic' embryonic stem cell lines after somatic cell nuclear transfer and therapeutic cloning need to be established since this will take care of problems associated with immune rejection during stem cell therapy to treat various disorders.

Studies have been initiated by us to develop human embryonic stem cell lines in xeno- free environment for future therapeutic purposes. Dedicated efforts, meticulous planning, appropriate ethical guidelines and uninterrupted financial support is required for successful fruition of embryonic stem cell research in our country.

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"Stem cells" are potentially immortal cells capable of self-renewal and also give rise to differentiated cells. No area of research except gene therapy has evoked so much enthusiasm and hope as stem cells. Most medical experts view stem cell research as the new frontier in medicine, a huge breakthrough that could save millions of lives.

Medicines today are based on drug therapy dominated by antibiotics, chemotherapy and other pharmaceuticals. Medicine of future will be based on cell therapies, focused on repair of tissues/ organs by cell transplants i.e. instead of drugs to prevent malfunction or death; diseased cells will be replaced by healthy differentiated stem cells.

Depending on their plasticity i.e. ability to differentiate into various cell types, stem cells may be: (a) **Totipotent** stem cells give rise to a fully functional organism as well as to every cell type of the body; (b) **Pluripotent** stem cells are capable of giving rise to virtually any tissue type, but not a fully formed organism; (c) **Multipotent** stem cells are more differentiated cells and give rise to a limited number of tissues or (d) **Unipotent** stem cells can differentiate into only one kind of cell e.g. germ cells.

Knowledge about stem cells and their potential applications has been known for the last 30 years when mouse embryonic stem cells were discovered. The development of embryonic stem (ES) cells can be traced from mouse teratocarcinomas, tumors in the gonads. It was found that teratocarcinomas produced differentiated embryonic carcinoma (EC) cells that contain a variety of cell types. However, these cells contain genetic mutations and scientists hoped to develop them directly from the blastocyst. This was accomplished in mice

Stem cells have been obtained from various body organs as shown in the following table:

Source	Types of Stem Cells	Potential
Adult	Cord blood stem cells, liver stem cells, epithelial stem cells, neuronal stem cells, pancreatic stem cells, spermatogonial stem cells etc.	Multipotent or unipotent
Embryo	Trophoectodermal stem cells, embryonic stem cells, morula stem cells	Totipotent or pluripotent
Fetus	Fetal germ stem cells, fetal germ carcinoma cells	Pluripotent

in 1981 by Gail Martin and Martin Evans (1, 2) independently. Later, in 1995, Thomson and his group (3) derived ES cells from rhesus monkeys. The major breakthrough came in 1998 when Thomson and his group (4) derived human ES cell lines, which were also shown to be pluripotent in nature. Simultaneously Shamblott and his group (5) derived pluripotent embryonic germ (EG) stem cell lines from primordial gonadal ridge from human abortuses. The potential of EG cells to differentiate into various cell types is relatively limited compared to ES cells, since they occur much further during development (5-9 weeks as compared ES cell derivation on day 5 post fertilization).

In mammals, the fertilized oocyte and the cells of morula stage embryos are totipotent, capable of giving rise to more than 220 cell types. The blastocyst is formed 5- 6 days after fertilization and is about 150 microns in diameter. The outer layer is the trophoblast and gives rise to the placenta whereas the cluster of about 30-50 cells inside the trophoblast is the inner cell mass

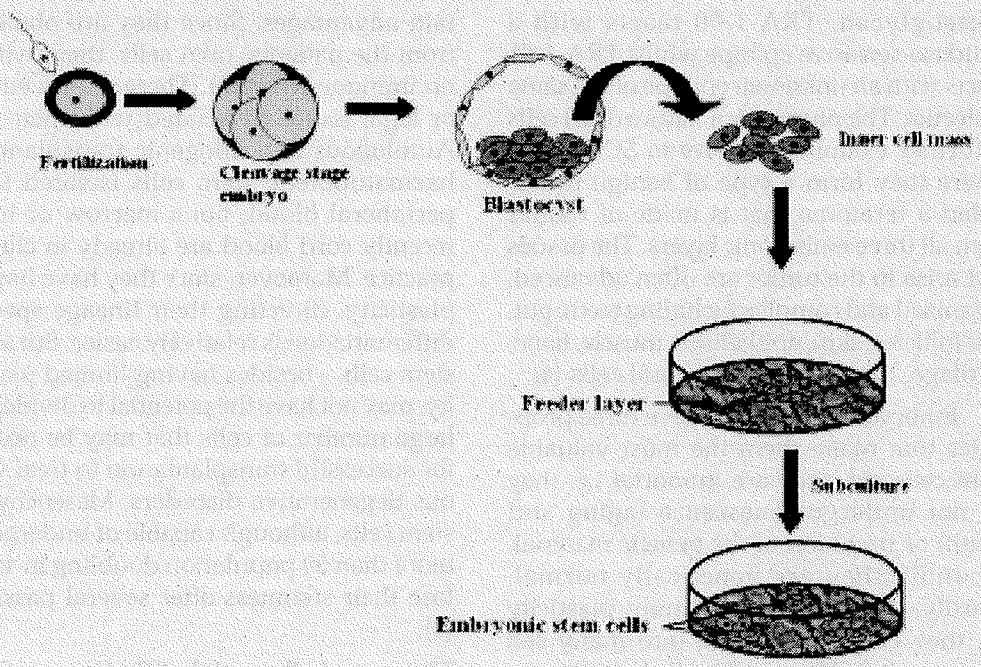

Fig 1. Schematic representation of embryonic stem cell line derivation

which in future will give rise to all cell types of the embryo viz. ectoderm, mesoderm and endoderm. When grown in vitro inner cell mass gives rise to ES cells that are pluripotent. These cells remain undifferentiated with unlimited self-renewal capacity as long as they are grown on feeder layer and spontaneously differentiate into somatic cells when feeder layer is removed (6). In suspension culture these cell aggregate in to balls of differentiated cells called 'embryoid bodies', which contain cells of all the three primary germ cell lineages (7).

Depending on the origin, stem cells express different kind of antigens on their cell surface commonly known as 'markers' which help in characterisation and identification of these cells (8). For example, haematopoetic stem cells express CD34 whereas pluripotent stem cells express stage specific embryonic antigens (SSEA 1, 3 & 4) and tumor recognition antigens (TRA-1-60, TRA-1-81). These antigens were originally identified by monoclonal antibodies recognising defined carbohydrate epitopes associated with lacto- and globo-series glycolipids. SSEA-1 is expressed on pre-implantation eight- cells stage murine embryos and teratocarcinoma stem cells. SSEA-3 and -4 are synthesised during oogenesis and are present in the membranes of oocytes, zygotes and early cleavage-stage embryos. Undifferentiated embryonic stem cells and carcinoma cells of primates and humans express SSEA-3 and SSEA-4, but not SSEA-1. TRA-1-81 and TRA-1-60 monoclonal antibodies (tumor rejection antigen) recognise antigens that are associated with a pericellular matrix

proteoglycan. TRA-1-60 reacts with a sialidase-sensitive epitope whilst TRA-1-81 reacts with an unknown epitope of the same molecule. The pluripotent nature of ES cells is further examined *in vivo* in SCID mice where they form a type of benign tumor called a teratoma that is made of tissues from all three embryonic layers. The tissues that arise in the tumor are often advanced, organized and complex including teeth, gut, hair follicles, skin, epithelium, muscle, bone, cartilage, lung tissue and neural cells (4).

Embryonic stem cells have three properties that make them the most valuable stem cells viz. they are immortal i.e. they do not undergo senescence (aging and death) or mutation of the genetic material. Secondly they are genetically normal. Thirdly, they exhibit maximum plasticity i.e. they can differentiate into many cell types in vitro. Human ES cells hold the potential to revolutionize therapies for many different diseases or disorders caused by cellular degeneration or damage. Only ES cells have the true capacity to develop into all the different cell types of the body and therefore represent the greatest potential for future cell therapies. As ES cells can proliferate indefinitely they represent an inexhaustible supply of cells, which can be cultured in quarantine conditions, free from the risk of other infectious agents.

Stem cells can also be derived from various developed body tissues in post-embryonic life of any organism and are termed as the adult stem cells e.g. hematopoietic stem cells in blood, bone marrow or umbilical cord blood and mesenchymal stem cells in bone, muscle, milk teeth etc. Adult stem cells are multipotent. Stem cell therapies using stem cells from adult organs will complement but cannot replace therapies that may eventually be achieved from ES cells. However adult stem cells have certain advantages. Since they are obtained from the patients' own cells, there will be no immune rejection. There are no ethical or legal issues associated with their use. Autologous and allogenic transplants of haematopoietic stem cells isolated from peripheral blood, bone marrow or more recently cord blood are already in clinical practice. Moreover, since they have limited plasticity, directing their lineage specific differentiation is relatively easier. But adult stem cells – besides having limited plasticity, may not have the potential to divide into large number of cells that may be needed for successful transplantation to treat various degenerative disorders. Mesenchymal stem cells, although capable of undergoing more than 50 population doubling in-vitro, lose their stemness after several passages (9).

Therapeutic Potential of Embryonic Stem Cells

Embryonic stem cells are a source of unlimited supply of specialized cells, which may not be possible with adult stem cells. Most of the age related cellular degenerative diseases cause functional disabilities and can be potentially cured by cell based therapies e.g. diabetes, neurodegenerative disorders like Parkinson's disease, Alzheimer's disease, heart diseases, stroke, spinal cord injuries, burns, immunodeficiency diseases, diseases of bones and cartilage like osteoarthritis, fractures, osteogenesis imperfecta, chondrodysplasias, cancer etc. For treating such type of diseases, large number of cells is required to resolve the function and ES cells are the only source that could generate such a large number of cells.

ES cells can be triggered to differentiate *in vitro* into fat cells, cells of brain and nervous system, insulin- producing cells of

pancreas, bone cells, hematopoietic cells, yolk sac, endothelial cells, endodermal cells, smooth and striated muscle cells including heart muscle cells. (10) Recently mouse ES cells have been reported to differentiate spontaneously into germ cells. (11, 12)

Besides the clinical potential, ES cells are also an ideal tool to understand basic developmental biology i.e. how various cell lineages become committed and differentiate into various lineages. They may also serve as tools to understand the cause of birth defects and may lead to their prevention in future. ES cells may be differentiated into various cell lineages which will serve as *in vitro* models for human diseases that are constrained by animal or culture models at present e.g. HIV and hepatitis C virus grow only in human or chimpanzee cells; only partial representation of Alzheimer's disease is possible in currently existing animal models. Recently human ES cells are also being used to create accurate human Lesch-Nyhan disease model (13), an ideal vehicle to carry the genetic material into diseased tissue that provides a necessary protein for therapeutic effect, as an ideal source of cells for drug screening, testing & toxicity and for functional genomics and proteomics. A new branch of science has emerged due to advances in stem cell research called tissue engineering which will result in making of tissues/ organs using artificial, biodegradable scaffolds for transplantation. Now it is possible to form three -dimensional organs by the use of biodegradable polymer scaffold, which allows proper alignment, and physical disposition of the cells and extracellular matrix to produce a reasonable organ (14) These organs along with stem cell technology allow the creation of biohybrid organs e.g. skin, cartilage, bone etc have been bioengineered *in vitro* for burns patients. Stem cells can also be used to deliver growth factors *in vivo* e.g. in the nervous system in the form of cell based drug delivery.

Availability of Human Embryonic Stem Cell Lines for Research: Till date more than 120 human ES cell line have been reported worldwide. Only a handful is available for research. United States has restricted its researchers to use existing 72 cell lines and no creation of new lines by destroying surplus frozen embryos is permitted. However, existing stem cell lines are gradually degrading and will soon be useless for research. Most of the lines have become useless; some of the lines are genetically identical to others; only 11 remain available for research. Research using embryonic stem cell lines has been authorised in Britain, U.S. private labs and in both government and private labs in the U.K., Japan, France, Australia and other countries. Till date no established human embryonic stem cell line has been reported in India.

Therapeutic Cloning: The embryonic stem cell lines to be used in future for therapeutic purposes are bound to have immunological differences. The impact of this difference will vary with different conditions – while using 'differentiated' stem cell transplantation to cure various degenerative disorders. In the brain, where immune rejection is less effective it may be possible that no immunosuppressive drugs would be required, or perhaps, only a low dose. When cells are transplanted to other sites, the patient would have to choose between the disadvantages of the initial condition and a lifetime of taking immunosuppressive drugs (and the resulting greater vulnerability to infections and cancer)

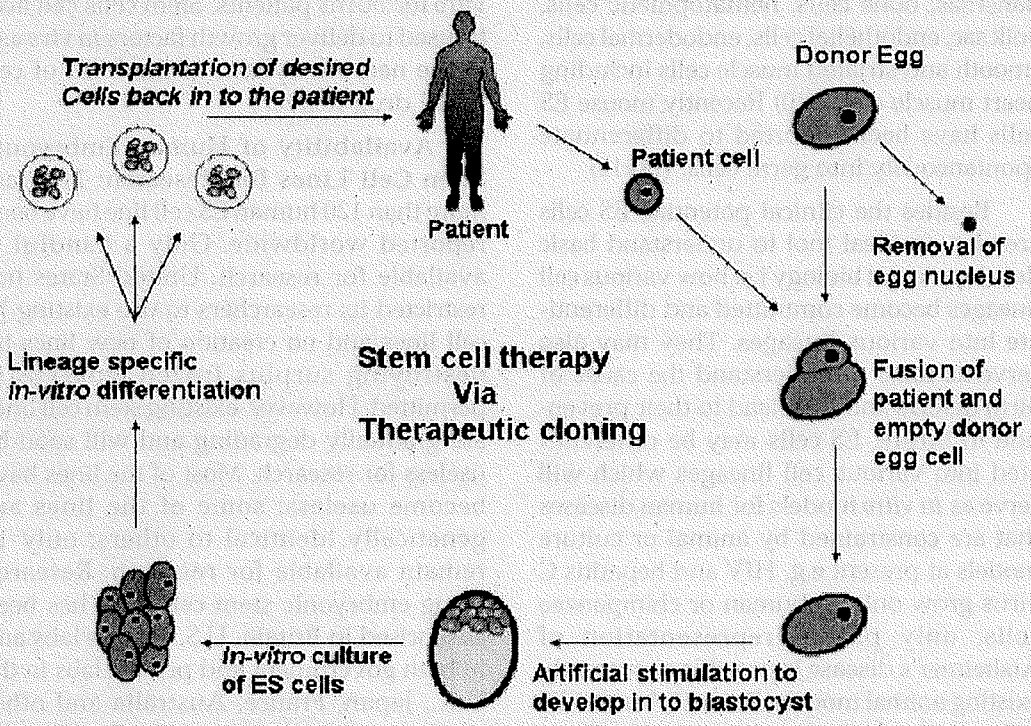

Fig 2. Schematic representation of derivation of stem cell line after "Somatic Cell Nuclear Transfer".

Matching embryonic stem cells to potential recipients may not be ethical, since it will require destruction of a large number of in vitro fertilized embryos. The immunogenicity of a cell depends on its expression of Class I major histocompatability antigens (MHC), which allows the body to distinguish its own cells from foreign. The immunological rejection of human ES-derived cells might be avoided by genetically engineering the ES cells to express the MHC antigens of the transplant recipient or by using somatic nuclear transfer technology.

During therapeutic cloning, somatic cell nuclear transplantation (SCNT) is carried out, with no goal of attempting to implant the resulting blastocyst in a uterus. An egg has its own nucleus removed and replaced by a nucleus taken from a somatic (e.g. skin) cell. The oocyte, thus "fertilized," could be cultured *in vitro* to the blastocyst stage. ES cells could then be derived from its inner cell mass, and directed to lineage specific differentiation. The result would be differentiated (or partly differentiated) ES-derived cells that match exactly the immunological profile of the person who donated the somatic cell nucleus, and who is also the intended recipient of the transplant-a labor intensive, but truly personalized therapy.

Clones have been produced from somatic cells of various animal species viz. sheep, cattle, goats, rabbit, pigs and recently dogs (15-20) However, despite considerable efforts by experienced labs, the same

method has failed to work in non- human primates and humans. Simerly and co-workers reported defective mitotic spindles after SCNT in nonhuman primate embryos and reported that therapeutic cloning may not be possible in primates (21, 22). They hypothesized that this may perhaps be due to the depletion of microtubule motor and centrosome proteins lost to the meiotic spindle after enucleation.

Ethical Issues Involved in ES Cells Research

Embryonic stem cell research along with therapeutic cloning is a highly sensitive area for research as it is associated with several ethical issues. There are three main issues viz. availability of embryos for stem cell research, creation of embryos specifically for research and source of eggs for therapeutic cloning.

A. It is known that very few cryopreserved embryos are available for research and their quality is compromised as the best ones are always implanted. In general, freeze thaw rate of cryopreserved embryos is about 65%, only about 25% of thawed embryos develop to blastocyst stage and the chances to derive successful stem cell lines is only 15%. This means that about 50 cryopreserved embryos are needed to hopefully derive one good cell line.

B. Use of embryos for research is a sensitive issue but it is a condition of "no harms and many benefits". If our society accepts abortions, then embryo research should not be a controversial issue. It needs to be decided the stage up to which embryonic development should be permitted for research and whether we could create embryos specifically for research. In general, it is believed that embryos up to 14 days have no relevant physical properties neither a stable identity since twinning and reabsorption of twins is still possible. Thus embryonic research, which involves the use of blastocyst cultured only up to Day 5, as such is not an issue but the issue is whether embryo could be created specifically for research as a part of therapeutic cloning protocol.

By definition an embryo results from fusion of male and female pronuclei i.e. fertilization of egg by sperm. However, during somatic cell nuclear transfer, there is no fusion of gametes – it is just an adult nucleus transferred into an enucleated egg. The embryo is cultured only up to day 5 to obtain blastocyst from which 'patient specific' cell lines are derived.

C. The other major issue that is extremely sensitive is the source of eggs for somatic cell nuclear transfer. Risks involved in egg donation are minimal but do exist such as bleeding, scarring, pelvic swelling, unintentional pregnancy etc. Donors may experience premenstrual syndrome - like symptoms, could get pregnant since due to hormonal treatment they become highly fertile, ovaries may get hyper-stimulated, could become infertile or might get ovarian cancer. But these risks exist even for patients who undergo IVF cycles. In a typical IVF cycle, the female partner always undergoes hyperstimulation of ovaries to collect eggs for *in vitro* fertilization with the sperm.

Will it be ethical to give financial incentive to egg donors and how much? Most egg donors are fertile young women

in 20s or early 30s and usually earn about $ 3000 to 5000 in USA. The other alternative is to request couples who attend IVF clinics to donate few of their eggs for research or thirdly the scientists need to establish methods to *in vitro* mature human follicles from ovaries removed due to various clinical indications. In the near future it may be also possible to differentiate ES cells into germ cells, which could be used as a source of eggs for therapeutic cloning.

Therapeutic cloning is permitted in our country. However, creation of embryos is not yet permitted by the policy makers. Stem cell research and therapeutic cloning warrants focussed research efforts rather than legal restrictions.

Paradigm Shifts that have occurred due to Stem Cell Research

Cancer Stem Cells: Recently it has been shown that all kinds of cancers are stem cell diseases. Even after extensive research, it appears that current approaches to cure cancer are targeting wrong cells. New treatment modalities need to target cancer stem cells, which form a very small fraction of cells within the tumor. This has resulted in a conceptual paradigm shift of how tumors are formed, spread and are treated. Recurrence of cancer is now understood to be because conventional cancer therapies target majority of cells in the tumor and the cancer stem cells escape the treatment. These immature stem cells are more resistant to chemotherapy and radiotherapy than the mature cancer cells and result in recurrence.

Studies have shown that cancer stem cells are the only cells present in many different kind of cancers, including solid tumours (breast cancer, brain tumours), that have the capacity to keep the tumours growing e.g. in leukaemia cases it is only one in a million cells that has the ability to sustain the disease. To cure cancer it will be ideal to devise therapies that will target cancer stem cells (23).

Stem Cells in Ovaries: An underlying principle of female reproductive biology appears to have been challenged by the compelling evidence reported by Johnson and co-workers (24, 25) that proliferative germ cells exist in the surface epithelial cell layer of mice ovary which keep replenishing the oocytes pool in the postnatal life. He demonstrated the presence of cells in the surface epithelium which stained positive for 5- bromodeoxyuridine and mouse vasa homologue (MVH), a gene expressed exclusively in germ cells. Cells in the surface epithelium are also positive for synaptonemal complex protein 3 in juvenile and adult ovaries. When the wild-type ovaries were grafted into transgenic mice expressing green fluorescent protein (GFP) showed appearance of preantral follicles with GFP negative granulosa cells and GFP positive oocytes. Finally, the germ cell toxicant busulphan is found to eliminate primordial follicle reserves by early adulthood without inducing follicular atresia, indicating the presence of proliferative germ cells between postnatal days 25 and 40. Similar evidence has also been generated in human ovaries. These results have significant clinical implications related to the therapeutic expansion of the follicular reserve as a means to postpone normal and premature ovarian failure.

Future Outlook of Stem Cells

Stem cells have come a long way and are with us to stay. But lot of hurdles need to be overcome and methodologies need to be refined before they can be incorporated in clinical settings. We have to develop technologies to control their differentiation into

pure cell populations in large numbers. The availability of a homogenous cell population will enable stem cell transplantation to replace diseased cells, possible in future. Secondly we need to achieve 100% differentiation of these cells since even a single undifferentiated cell may lead to teratoma formation. Therapeutic cloning protocols need to be developed with fair success rate to avoid immune rejection at the time of cell therapy. Finally we need to produce large number of embryonic stem cell lines and make them available for research purposes immediately. Extensive research needs to be carried out to elucidate the mechanisms of stem cell lineage specific differentiation. They are like a wild horse, which has to be tamed before being used to its full potential.

In future, it might be possible to "reprogram" an adult cell - even one as specialised as a skin cell, for example - to become any other cell type in the body. Extensive work on embryos will help to understand some of the secrets of how early cells are controlled. What makes a cell committed or how they get out of control to form tumor are some basic questions that will be answered in near future. Success to realize full potential of stem cells and to develop effective and safe therapies requires time, money, perseverance and an urgent need to lay down appropriate national ethical guidelines

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The Occurrence Of *Rhinosporidium seeberi* in a fresh water pond at Piranmalai (Sivaganga District, Tamil Nadu, India

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SUMMARY

Rhinosporidium seeberi the casual agent of Rhinosporidiosis is known for about hundred years and its association with stagnant water bodies has been known for as many years. Several reports of its aquatic associations have been published but so far there is no report of its occurrence in any of the water bodies that are associated with Rhinosporidiosis (6). We report for the first time the presence of *Rhinosporidium seeberi* in a pond, which is known to be associated with the disease. The sporangia isolated from the water samples showed typical structure of those isolated from the infected tissue. The pond water samples also yielded several developmental stages for the sporangia comparable to those obtained from the infected tissue. Since the organism cannot be cultured and there is no possibility of verifying Koch's postulates, an identical RAPD profile of the DNA from sporangia occurring in water and from those of infected tissue was considered confirmatory.

Key words: *Rhinosporidium seeberi;* Water Source; Developmental stages; RAPD Profile

INTRODUCTION

The disease Rhinosporidiosis has been known from 1900, when Seeber (1) first described it in his thesis. Comprehensive description of the manifestations of the disease in humans and animals and the developmental stages of the causal organism *Rhinosporidium seeberi* have been reported over the years by several investigators (2-9). Karunaratanae (5)

Correspondence: Prof. C. Rajamanickam, Tamil Nadu; Dr. Pran Nath Chuttani Oration delivered by Dr. S. Kameswaran at Chandigarh - 2005.

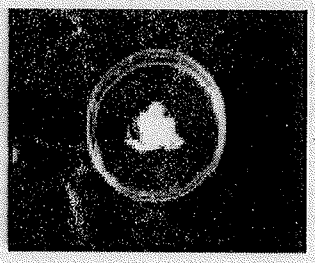
observed in his monograph that the disease was associated with agricultural workers and those who had occupational association with muddy water. In general, persons suffering from Rhinosporidiosis had a history of taking bath in a stagnant pool of water such as pools, ponds, lakes etc. Kameswaran (6) reported that patients of Rhinosporidiosis coming from a hyper endemic area (Rajapalayam-Tamil Nadu) had a history of taking bath in a specific pond. This association of Rhinosporidiosis with a stagnant pool of water has also been reported by Vukovic *et al* (10) who found that the only experience that the Yugoslavian patients had in common was that they all bathed in the same stagnant water called the "silver lake". The occurrence of Rhinosporidiosis in swans (9) also supported an aquatic environment as the reservoir for Rhinosporidiosis seeberi. However the presence of the pathogen Rhinosporidiosis seeberi in aquatic environment has not so far been reported. We reported here for the first time the occurrence of Rhinosporidiosis seeberi in a pond in Piranamlai (Sivaganga District-Tamil Nadu) which has been known to be associated with Rhinosporidiosis (6). Several Rhinosporidiosis patients coming from this area have a history of having taken bath in the specific pond. The samples of water collected from this tank at different time intervals over a year have been examined for the presence of Rhinosporidium and the observations are reported. It has not been possible hitherto either to culture the organism or to verify Koch's postulates in Rhinosporidiosis. The identity of the organism occurring in the pond as Rhinoporidium seeberi was suggested by the morphology and the RAPD profile of the sporangia obtained from water body, which was comparable to that of the sporangia, obtained from the human infected tissue.

MATERIALS AND METHODS

Separation of Rhinosporidium from infected tissue:

The infected tissue was cut into small pieces, teased in phosphate buffered saline (PBS) and treated with collagenase type I enzyme (0.9mg/ml) for 2 hours at 37°C with continuous stirring (see plate in Appendix 1). The sample was diluted ten fold with cold PBS to stop the reaction and centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellet was suspended in 1ml of PBS, the number of sporangia counted and loaded on to a discontinuous (step) density

Infected Rhino Tissue



Collgenase treated

Appendix I

Sporangia at various stage

gradient made of fetal calf serum, ficoll, percoll and sucrose as given in Table I. The 10ml gradient was centrifuged at 1,000 rpm for 30 minutes at 4°C in a swing out bucket. After centrifugation, each fraction was isolated using a Pasteur pipette, washed by centrifugation and sporangia counted in a Neubaur Haemocytometer (Table I). Sporangia of different sizes were found to be distributed in different fractions/layers. There was also some contamination of host tissue debris. Hence sporangia from different fractions were pooled and subjected to a sucrose gradient centrifugation (2M, 1.5M, 1M, 0.5M and 0.25M) at 2500 rpm for 30minutes. Majority of the sporangia were recovered without host cells in the 1-1.5 M gradient interphase. These sporangia were washed and used for DNA extraction.

Table 1 : Density Gradient Separation of *Rhinosporidium* sporangia from infected human tissue in Ficoll-Percol gradiant

	Number of Sporangia				
	Debris Particles	Small (Juvenile) <100µ	Medium (Intermediate) 100µ–250µ	Large (Mature) >250µ	Total
Digested whole sample count in 50µl	Large nos	200	230	30	460
In total sample loaded		48000	55200	7200	110400
Yield on Gradient Fractions Fetal Calf Serum	Present	900	7200	300	8400
Interphase 1	90	7500	19500	3000	30000
Ficoll 1.07 g/ml	40	26250	6900	1200	34350
Interphase 2	20	8800	7000	1800	17600
Percol 1.13 g/ml	10	900	2230	330	3460
Interphase 3	0	0	0	0	0
Sucrose 2.5 M	0	0	0	0	0
Yield		44350	42830	6630	93810
Yield in percentage		94.4	77.59	92.08	84.97

Note : The sporangia from each fraction were separately collected and suspended in appopriate volume of PBS.

Collection of *Rhinosporidium* from pond water

Water samples were collected from the Piranmalai tank (78.2°E, 97°N) during May, August, September and December, 1999. Sporangia of different sizes were observed in every sample collected. The sporangia were collected by passing plankton net several times through the pond water and the samples transported to the laboratory in cold box. Samples were further concentrated by centrifugation and resuspended in 1ml of PBS. One drop of this suspension was placed on a small piece of filter paper (Whatman No.1, 2 cm x 1 cm) placed on a slide and viewed under a microscope. Sporangia were picked up with a sharp needle and transferred to phosphate buffered saline (50µl) in an eppendorf tube. Five hundred such sporangia picked up were washed thrice by centrifugation, and used for extraction of DNA.

Isolation of Genomic DNA

Total genomic DNA of sporangia from water sample and tissue sample was isolated employing "Lysis" method. The sample was treated with lysis buffer containing 0.32M Sucrose 10mM Tris pH 7.5, 5mM MgCl2, 1% Triton 100x in 1:1 ratio.

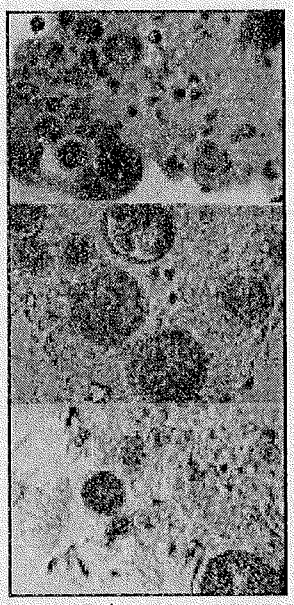


Plate I

Fig 1. Juvenile, intermediate and mature sporangia in the infected tissue. x120.

Fig 2. Infected tissue mass treated with collagenase showing sporangia in different stages of development. x120 (phase).

Fig 3. Collagenase treated infected tissue in advanced stage of digestion showing several juvenile sporangia. Some of them are equal to or smaller than the host cell nucleus. x120 (phase).

The sample and lysis buffer were mixed well using a vortex mixer and then spun at 10,000 rpm for 20 seconds to obtain the pellet. The pellet was suspended in 1ml of lysis buffer and the same step was repeated thrice. The pellet was finally resuspended in 0.5ml of sterile PCR buffer (5mM KCl, 10mM Tris-HCl pH 8.3, 2.5mM MgCl2, 0.1 mg/ml gelatin, 0.45% NP40, 0.45% T20) and stored frozen. The sample was thawed and digested with proteinase-K (0.6 µl of 10 mg/ml per 100µl) at 60°C for one hour and the

reaction terminated by incubating at 97°C for 10 minutes. This was spun at 10,000 rpm for 20 seconds and the pellet was resuspended in 10µl of PCR buffer.

Random Amplified Polymorphic DNA (RAPD) Analysis:

RAPD analysis is a technique employed to detect genomic polymorphism, utilizing oligonucleotide primer for an arbitrary sequence in a Polymerase Chain Reaction (PCR). 50ng of genomic DNA isolated from the sporangia from the water or from tissue was added to the tube containing the RAPD analysis bead (25 pmol of RAPD primer IV) and made up to 25µl with sterile distilled water. PCR was performed in a thermocycler (Perkin Elmer, Gene Amp 2400 USA) with the following cycle profile; 1 cycle at 95°C for 5 min followed by 45 cycles consisting of denaturation at 95°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min.

Results:

Almost all of developmental stages of R.seeberi similar to those obtained in the infected tissue were observed in the pond water samples (Plate I, Figs 1, 2 and 3). These are summarized below.

Several sporangia in juvenile stages of development in a spore mass (Plate II, Figs 1 and 8). However there were also individual occurring very small sporangia of 12 µm diameter (Plate II, Fig 3), which was probably the earliest stage in the

Different Stages of *Rhinosporidium seeberi*

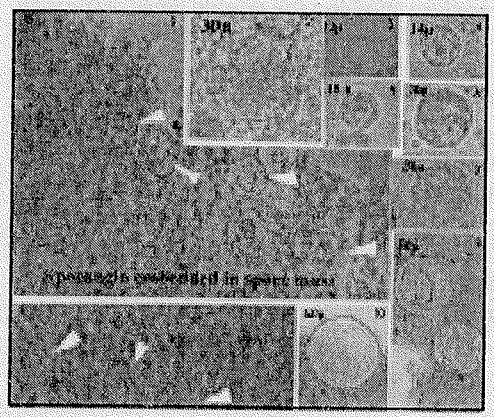


Plate II

Figs 1 and 8. Several sporangia embedded in spore mass. x120.
Fig 2. 30µ sporangium, outer sporangial wall more clearly visible than in the earlier stages as also the inner translucent layer, several nuclei are seen. x760.
Fig 3. 12µ spore probably showing the earliest stage in development nucleus not yet divided. x760.
Fig 4. 14µ sporangium nucleus enlarged, probably undergoing division x760.
Fig 5. 18µ sporangium enclosing a few nuclei. x760.
Fig 6. 20µ sporangium showing further nuclear division having a prominent translucent layer and not so prominent outer wall. x760.
Fig 7. 22µ sporangium showing the beginning of the cleavage of cytoplasmic. The outer wall is not prominent.
Fig 9. 50µ and 50 micro sporangia clearly showing nuclei and cytoplasmic cleavage the thick dark brown, outer wall is clearly visible. x120.
Fig 10. 60µ sporangium (x304) showing a thick dark outer wall and well defined, translucent inner wall exhibiting the initial stages of development of the pore and the annulus. Several nuclei, each surrounded by a small amount of cytoplasm are present inside the sporangium.

development to the sporangia where the nucleus had not yet divided. This was further enlarged to 14 µm (Plate II, Fig 4) where the nucleus was large and was probably undergoing division. The next stage of development was seen in the 18 µm diameter sporangia with a few nuclei inside the sporangium (Plate II, Fig 5). The nuclei underwent further division along with the enlargement of the sporangium to 20µm exhibiting a prominent translucent layer of sporangial wall and not so prominent outer wall (Plate II, Fig 6). The next stage of development namely the 22 µm sporangium showed the prominent nuclei and the beginning of cleavage of the cytoplasm. At this stage also the outer thick wall was not prominent and the translucent inner wall was clearly seen (Plate II, Fig 7). In the next developmental stage observed as 30 µm diameter sporangium (Plate II, Fig 2) the outer sporangial wall was more clearly visible than in the earlier stages as also the inner translucent layer. The sporangial content showed several nuclei. This pattern was more clear in the 50 µm diameter sporangium (Plate II, Fig 9), which clearly showed the nuclei and the

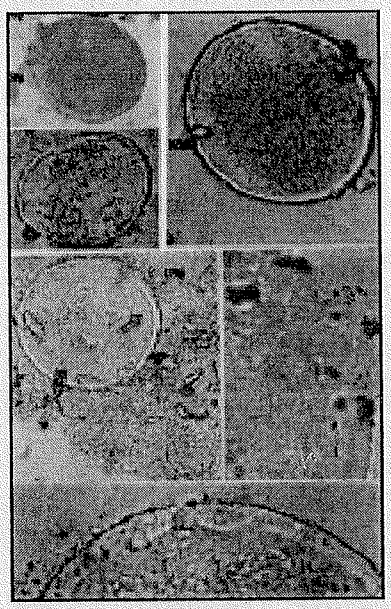


Plate III

Fig 1. 250µ sporangium showing bilamellar wall and a prominent pore large number of spores about to be released.

Fig 2. 200µ sporangium showing prominent dark outer wall and a translucent inner wall containing numerous developing spores (x304).

Fig 3. 135µ sporangium showing prominent wall layers. A few developing spores are clearly seen (x120).

Fig 4. 140µ sporangium showing the double layer of the wall and the formation of the annulus and the pore 35µ sporangium is present at the bottom and 60 micro sporangium is seen at the lower right corner (x760).

Fig 5. The pore and the annulus of a 165µ sporangium enlarged. Some spores are seen between the annulus and the pore region (x760).

Fig 6. 170µ sporangium showing the dark outer wall and a prominent and a prominent trunslucent innter wall forming the thick annulus near the pore region. Several developing spores are clearly visible. (x760).

cytoplasmic cleavage. Further development of the sporangium (60 µm diameter) showed a prominent, dark outer layer of the sporangial wall clearly defined translucent inner wall, which also exhibited initial stage of development on the pore and annulus, which is the thickening of the translucent wall layer surrounding the pore. Inside the sporangium several nuclei, each surrounded by a small amount of cytoplasm could be observed (Plate II, Fig 10). It may be mentioned here that the sporangia described above can be grouped under the category of "Juvenile" sporangia according

to the ontogenic nomenclature proposed by Kennedy *et al.* (9).

This juvenile stage is followed by further development of the sporangium to the intermediate stage where the sporangia attain 100 µm - 200 µm diameter exhibiting a prominent dark outer wall, a well developed, distinct and diffuse inner wall enclosing spores in different stages of development and maturity. In addition, we could also observe the gradual

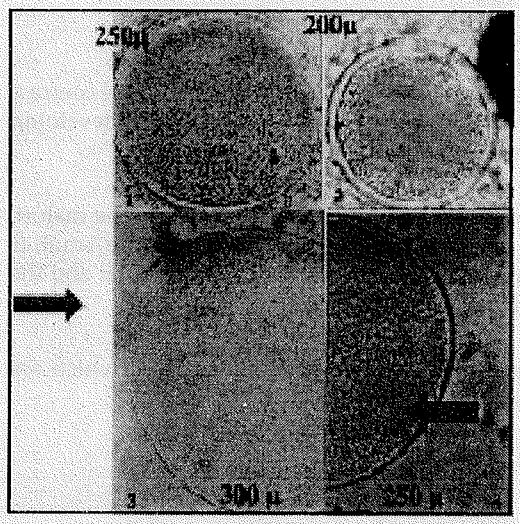


Fig 1. 250µ sporangium showing clearly the thick annulus and the pore, fully packed with numerous spores. (x304).

Fig 2. 200µ sporangium from infected tissue placed here to compare with figure 1.

Fig 3. 300µ sporangium with a thick outer wall but a thin translucent inner wall releasing the spore. The mature spore, are towards center of the sporangium and the small developing spore are towards a periphery. (x304).

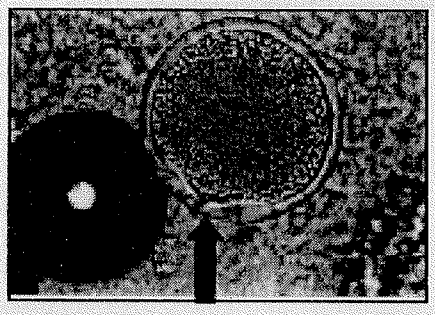
Fig 4. 250µ sporangium showing similar features as figure 3 except the pore is also clearly seen. (x304).

The arrow indicates the annulus opening and release of spores from it.

Plate IV

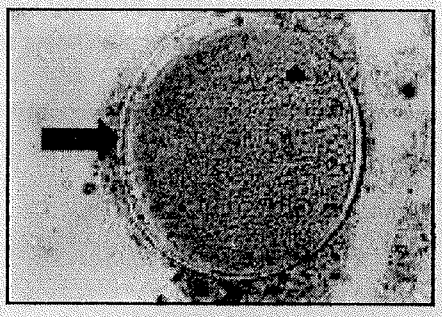
Sporangia isolated from

Infected Tissue



Mature sporangia 200µ thinner bilamellar wall. The pore is also visible in several sporangia. Mature sporangium contains about 16,000 endospores which are released usually through the pore.

Pond Water



Mature sporangia 250µ diameter show comparatively thinner wall of sporangium. The sporangium contains several thousands of spores, which are released through the pore.

Plate IV A

development of the pore and annulus in tissue intermediate stages (Plate III, Figs. 2, 3, 4, 5 and 6).

The intermediate sporangia gradually developed into mature sporangia enclosing progressively maturing process (Plate IV, Fig 1 and 2), which were released through the pore at the appropriate time (Plate III, Fig 1; Plate IV, Fig 3 and 4; Plate V, Fig 3). The morphological similarity of the sporangia during development in the infected tissue and in the water of the Piranmalai tank was remarkable (Table II).

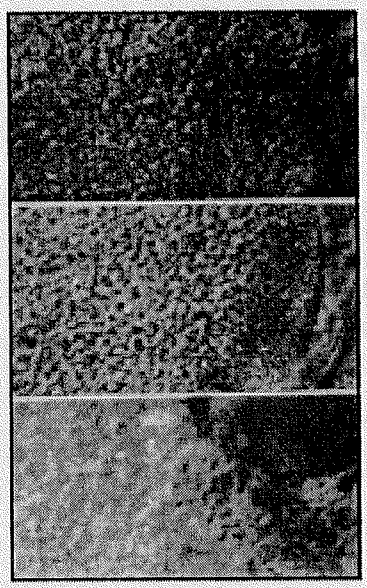


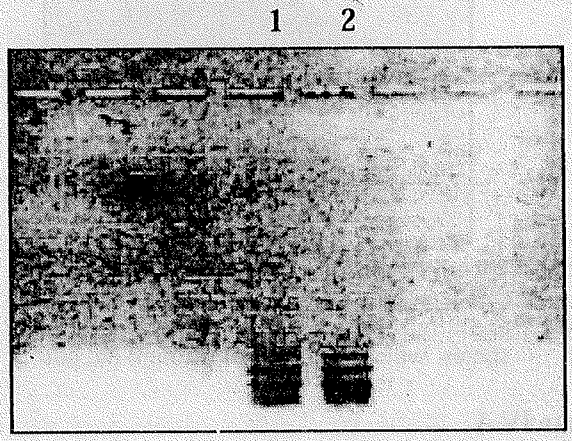
Plate V

Fig 1. A part of 200μ sporangium enlarged under phase contrast to show the mature spores in the center and the develoing spores towards the periphery (x760).

Fig 2. A part of 250μ sporangium showing clearly the mature spores in the center of the sporangium and the smaller developing spores towards the thick translucent wall and the dark outer wall are also clearly seen. (x760).

Fig 3. 250μ sporangium showing mature spores which are about to release.

Fig 1. Comparison of RAPD profile of Rhinosporidium sporangia from human tissue and from using primer 1V



Lane 1. Sporangia from tissue;
Lane 2. Sporangia from water

Almost all the stages that were observed in the surgically removed mass could be observed in the water samples also (compare figures Plate I and II). There was also striking similarity in the development of pore and annulus between the sporangia from water and the one obtained from the infected tissue (Plate IV, Fig 1 and 2; enlarged in Plate IV A).

The results of RAPD analysis of *R.seeberi* obtained from pond water and human infected tissues are presented in Figure I. The two profiles were markedly similar.

DISCUSSION

Ajello (13) attributes an aquatic habitat for *Rhinosporidium* and has given a compendious review of available literature

Table 2 : Comparison of principal morphological characters of Rhinosporidial sporangia occurring in the infected tissue and sporangia occurring in pond water

Sporangia from infected tissue	Sporangia from pond water
Juvenile (9) 12-100 μm diameter also termed "trophocyte" with variable intracellular appearances, chromatin either organized into a single nucleus (with nucleoli) (11) or may be fragmented and diffuse (12). At about 50-60μm diameter nuclear division results in the appearance of chromatin within vesicles.	Several juvenile sporangia with variable intracellular appearances. Chromatin organized into a single nucleus at about 12μ stage, starts dividing very early and appears fragmented. At about 50μ cytoplasmic cleavage is noticeable and at 60μ diameter nuclear division results in the appearance of chromatin with in vesicles.
Intermediate (9), immature sporangium 100-200μ diameter, spherical thick bilamellar wall, outer dark (Chitinous) and inner translucent, contains immature endospores in cytoplasm that is granular or fibrillar. Several thousand endospores in immature sporangium of about 200μ diameter.	Immature, intermediate sporangia 140-170μ diameter stage shows clearly thick bilamellar wall, outer dark and inner translucent and contains immature spores.
The annulus is prominent.	The annulus is prominent
Mature sporangia 250-400μ thinner bilamellar wall (4,13). The annulus (15μ) is prominent. The pore is also visible in several sporangia. Mature sporangium contains about 16,000 endospores (4), which are released usually through the pore.	Mature sporangia 200-300μ diameter show comparatively thinner wall of sporangium. The sporangium contains several thousands of spores, which are released through the pore.

in support of his consideration. However, none of the papers so far published have given evidence for the presence of Rhinosporidium in an aquatic habitat that is associated with the disease. For the first time in the history of Rhinosporidiosis we have identified Rhinosporidium in an aquatic habitat which is implicated in the disease. The spore appears to be the starting point of the life cycle since we have not observed any structure (trophocyte) smaller than 10-12μ in diameter in any of our samples collected. The developmental stages and the mature sporangia obtained from the water sample were markedly similar in structure to the sporangia obtained from the infected tissue. Further, the RAPD profile of DNA from the sporangia occurring in the pond and that obtained from the sporangia isolated from the infected tissue were identical (Fig. I) indicating that the organism occurring in the aquatic habitat is *Rhinosporidium seeberi.*

The absence of evidence hitherto on the occurrence of *Rhinosporidium seeberi* in nature, particularly in ponds or other stagnant water associated with the disease had led to various speculations (14,15). *Microcystis aeruginosa* a unicellar, colonial cyanobacterium was considered as a causal agent of Rhinosporidiosis (15). However this does not appear to be the case since the structure (16) and the ultra structure (17) of M *aeruginosa* are entirely at variance with the structure and the ultra structure of *R. seeberi* (18). *M. aeruginosa* is known to occur in the blooms in several ponds of Tamil Nadu including Piranmalai tank (16), nonetheless not at all these water bodies have been implicated with Rhinosporidiosis. Further the DNA sequence of an RAPD fragment of *R. seeberi* did not show any homology with the published sequences of M *aeruginosa* (unpublished observation). The identification of an organism identical to R *seeberi* in the Piranmalai tank implicated in the disease (6) and its confirmation by RAPD analysis establishes the transmission of Rhinosporidiosis through water bodies. Developing an animal model and culturing of *R. seeberi* will lead to further understanding of the development and transmission.

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Pathogenesis of Infective Endocarditis

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SUMMARY

In vitro studies were conducted to study the early steps of pathogenesis of infective endocarditis caused by *Staphylococcus epidermidis*, which is a significant pathogen in prosthetic valve endocarditis and nosocomial endocarditis.

Staphylococcus epidermidis binds to host tissues (pharyngeal epithelial cells, fibrin-platelet clots, cardiac cell lines, surgical sutures and tissue matrix proteins) by lipoteichoic acid present on the bacterial surface. The active component is lipid as deaceylation of LTA removes the adherence properties. The adherence is unrelated to surface charge, hydrophobicity, slime production, encapsulation, biotype and phage type of the strains used. The receptor on the host cells is a glycoprotein.

Key words: *S. epidermidis*, pathogenesis, endocarditis, bacterial adherence.

Introduction

Infective endocarditis (IE) was first described by William Osler in 1885 in his Gustonian Lecture and stated that "The etiological, clinical and anatomical characters of the disease have been fairly well ascertained" (1). However, this statement was an overreach and the disease has been the subject of continuous study since then. There have been advances in the understanding of various aspects of IE. In 1910 Libman and Celler reported in their experience with over 3000 blood cultures, and stated that for IE "The absolute diagnosis must, for the present, rest on the culture of the blood" (2). Nearly a century later blood culture retains a pre-eminent role in the diagnosis of IE. There have been development of new histologic and molecular techniques for detection of intracellular and difficult-to-culture pathogens that cause the disease (3, 4). In 1940s, the basis of successful antibiotic therapy for IE had been established and mortality had decreased from 100% to 30%. The introduction of intracardiac surgery in the 1960s has assumed a central importance in the management of cases where medical therapy fails. Despite rapid medical progress the mortality remains high, approaching 40% at one year.

Correspondence: Dr. T.D. Chugh, D-702, Som Vihar, R.K. Puram, New Delhi-110022. Gen. Amir Chand Oration delivered at Annual Meeting of the NAMS (India) at Ahmedabad, 2005.

The concepts in pathogenesis of IE based upon experimental findings in animal models of IE have been the subject of intense research in 1970s (5, 6). Most patients with IE have pre-existing valve abnormalities. The normal endothelium is resistant to colonization and infection by circulating bacteria. The primary event of IE is bacterial adherence to target tissues and involves both host tissue and bacterial factors. This is followed by establishment, persistence and microbial growth with local tissue damage and extension to adjacent tissues. Any endothelial lesion results in exposure of the underlying extracellular matrix proteins, production of tissue factors, deposition of fibrin and platelets and non-bacterial thrombotic endocarditis (NBTE) which acts as a nidus for bacterial adherence and colonization during transient bacteremia by even less virulent bacteria (e.g. streptococci). This is also possible on physically intact endothelium when virulent, invasive pathogens (e.g. *S. aureus*) can initiate similar process.

The epidemiology of IE is changing due to increase in lifespan, new predisposing factors and increase in number of nosocomial cases. The incidence of community - acquired native valve endocarditis is 1.7 - 6.2 cases per 10^5 person-years in the developed countries. The risk for prosthetic valve endocarditis is 1 per cent at 12 month and 2-3 per cent at 60 months. Nosocomial endocarditis accounts for 7-29 per cent of all cases of IE seen at tertiary care hospitals (7).

This presentation is a study of various aspects of early events of IE: the physico-chemical basis of adherence of *S. epidermidis*, which has become a significant pathogen in cases of prosthetic valve endocarditis and nosocomial endocarditis (8).

Results

A prospective study of 60 consecutive cases of IE was carried out during the period of May 1985 to December 1988[9]. There were 40 males and 20 females with a mean age of 28 years. Thirty patients were found to have an evidence of chronic rheumatic heart disease, 8 congenital heart disease, 5 mitral valve pro-lapse and 4 had prosthetic aortic valve. Thirteen patients had IE in a previously normal heart. Positive blood and or tissue cultures were obtained in 44 patients (73%). The commonest infecting organism was *Streptococcus viridans*. Early surgery was performed in 31 patients (21 had severe heart failure, 10 embolisation, 15 failure of medical therapy and 19 had large vegetations (more than 10 mm).

For studies on adherence, 8 clinically significant isolates of *S. epidermidis* obtained from blood cultures of the same number of patients of IE were included in the study. These strains were identified by Staph API System (Table 1). All were non-encapsulated, non-slime producers and did

Table 1 : Characteristics of 8 strains of *S. epidermidis*

S. epidermidis strain	Biotype	Phage type
CH1	6706113	Untypable
CH2	6300113	63/138/245/336
CH3	6300113	63/138/245/336
CH4	6306113	63/138/245
CH5	6706113	Untypable
CH6	6300113	Untypable
CH7	6300113	63/138/245
CH8	6700113	Untypable

not agglutinate human, guinea pig, rabbit or sheep erythrocytes. These were stored in skim milk at -70°C in aliquots and used fresh for each experiment. Adherence assays were done to pharyngeal epithelial cells (PEC) with and without pre-treatment of bacteria and PEC with various enzymes. The results are shown in Tables 2, 3. Binding was mannose resistant and was not related to surface hydrophobicity and surface charge of bacteria. Adherence to epithelial cells was reduced four to tenfold ($P < 0.01$) on pretreatment of bacteria with lipase while neuraminidase, phospholipase C, trypsin, and sodium periodate did not alter their binding. The surface carbohydrate profile of bacteria was studied by monitoring adherence to Lectin-Sepharoses. The bacteria did not conform to any pattern, and there was no relation to strain variation or adherence property. The pretreatment of PEC with trypsin and sodium metaperiodate produced a marked reduction in bacterial binding, 3 to 25 fold, ($P < 0.01$), but neuraminidase, phospholipase C, and lipase did not have any such

Table 2 : Effect of lipase pretreatment of *Staphylococcus epidermidis* adherence to pharyngeal epithelial cells

S. epidermidis strains	No. of bacteria adhering to each PEC		
	Control bacteria	Lipase-treated bacteria	% Control adherence
1	21.8 + 3.6	3.0 + 1.1	13.8
2	37.9 + 4.3	3.9 + 1.3	10.3
3	20.9 + 3.0	4.1 + 1.4	19.6
4	8.0 + 2.6	2.1 + 1.3	26.2
5	11.1 + 2.0	2.6 + 1.0	23.4
6	21.7 + 3.0	3.7 + 2.0	17.0
7	20.8 + 3.1	3.9 + 2.1	18.7
8	24.0 + 3.1	2.7 + 1.2	11.2

Table 3 : Effect of periodate and trypsin treatment of human pharyngeal epithelial cells on *Staphylococcus epidermidis* adherence

S. epidermidis strains	No. of bacteria adhering to		
	Control cells	Periodate-treated cells	Trypsinized cells
1	21.1 ± 3.4	2.1 ± 1.7 (9.9)	2.2 ± 1.4 (11.6)
2	38.6 ± 4.7	1.5 ± 1.3 (3.9)	1.7 ± 1.2 (4.5)
3	21.6 ± 2.3	3.1 ± 1.4 (14.3)	2.0 ± 1.3 (9.9)
4	8.4 ± 2.7	2.9 ± 2.0 (34.5)	2.4 ± 1.8 (30.4)
5	11.3 ± 1.8	3.4 ± 1.6 (30.1)	2.6 ± 1.4 (22.4)
6	20.4 ± 3.5	3.2 ± 1.2 (15.7)	3.1 ± 1.0 (14.5)
7	21.3 ± 4.3	4.1 ± 1.4 (19.2)	3.2 ± 1.6 (15.5)
8	24.2 ± 3.9	4.0 ± 1.6 (16.5)	4.2 ± 1.9 (17.9)

Values represent the average number of adherent bacteria per epithelial cell ± standard error of the mean. Figures in parentheses are percentage of control adherence.

Table 4 : Adherence of homologous and two heterologous strains of *Staphylococcus epidermidis* to PEC pretreated with LTA

Treatment (μg/ml)	Strains of *S. epidermidis*		
	CH2	CH4	CH8
Hemoglobin 100	0.2 ± 0.1[a]	0.5 ± 0.2	0.3 ± 0.1
Dl-α-glycerol-phosphate 100	0.5 ± 0.5	0.4 ± 0.4	0.6 ± 0.3
LTA			
3	9.2 ± 1.0	8.3 ± 0.8	3.2 ± 0.9
6	13.8 ± 1.0	21.4 ± 1.4	11.2 ± 1.3
12	25.4 ± 1.3	30.1 ± 1.6	20.3 ± 1.2
25	48.2 ± 1.1	51.3 ± 1.7	46.6 ± 0.9
50	84.4 ± 1.2	68.2 ± 0.9	73.2 ± 1.7
100	87.9 ± 0.5	81.3 ± 1.3	84.8 ± 1.4
200	84.7 ± 1.3	80.2 ± 1.0	78.6 ± 1.9

[a] All figures are percentage inhibition of adherence ± SD after treatment of epithelial cells with LTA derived from *S. epidermidis* CH2 strain.

effect. These findings provide evidence that the receptors on the surface of PEC are glycoprotein in nature, while the bacterial adhesin is a lipase-sensitive material (10).

Table 5 : Inhibition of adherence of *Staphylococcus epidermidis* CH2 to PEC pretreated with LTA of *S. epidermidis, S. aureus* and *S. pyogenes*

Treatment (μg/ml)	Prior treatment of PEC with LTA of		
	S. epidermidis	*S. aureus*	*S. pyogenes*
Hemoglobin 100	0.2 ± 0.1[a]	0.8 ± 0.4	0.8 ± 0.5
Dl-α-glycerol-phosphate 100	0.5 ± 0.5	0.2 ± 0.2	0.6 ± 0.2
LTA			
3	9.2 ± 1.0	4.8 ± 0.1	4.3 ± 0.1
6	13.8 ± 1.0	8.8 ± 0.3	6.8 ± 0.3
12	25.4 ± 1.3	16.1 ± 1.0	1.0 ± 1.9
25	48.1 ± 1.1	32.1 ± 1.0	20.5 ± 1.8
50	84.4 ± 1.2	70.0 ± 1.5	51.2 ± 1.0
100	87.9 ± 0.5	71.7 ± 1.5	61.7 ± 1.5
200	84.7 ± 1.3	69.9 ± 1.9	56.8 ± 1.3

[a] All figures are percentage inhibition of adherence ± SD after treatment of epithelial cells with LTA derived from one of the three bacteria.

Further experiments were done to characterize the adhesin on the surface of bacteria. Lipoteichoic acid (LTA) was extracted, purified, characterized and then used for various blocking experiments. The results are shown in Tables, 4, 5, 6. These experiments show that prior treatment of pharyngeal epithelial cells with lipoteichoic acid derived from *Staphylococcus epidermidis* produced a marked inhibition of adherence of the homologous strain and two heterologous strains. The inhibition was dose-dependent and saturable with 100 μg/ml of LTA. However, pretreatment of PEC with deacylated LTA did not block the adherence of the three strains tested. A similar but less marked blocking effect on the adherence of *S. epidermidis* to PEC was also observed with LTAs derived from *S. aureus* and *Streptococcus pyogenes*. On treatment of bacteria with substances capable of binding to LTA, such as polyclonal mouse anti-LTA antibodies or with human albumin, a marked inhibition of bacterial adherence was observed. Immunofluorescence studies showed that anti-LTA antiserum bound readily to the surface of bacterial cells. These findings provide clear evidence that the lipid component of LTA located on the bacterial surface is centrally involved in the adherence of *S. epidermidis* to human mucosal cells (11).

Table 6 : Adherence of pretreated *Staphylococcus epidermidis* to PEC

Treatment	% Inhibition of adherence of *S. epidermidis*		
	CH2	CH4	CH8
Human albumin (μg/ml)			
1	15.0 ± 1.0	18.3 ± 0.9	12.4 ± 0.7
10	32.1 ± 1.2	28.4 ± 1.3	36.4 ± 1.6
100	81.2 ± 0.8	76.3 ± 1.4	68.4 ± 0.9
1000	87.5 ± 0.5	83.4 ± 1.1	84.3 ± 1.3
Anti-LTA serum (Titer 1/12,800)			
1:5	68.9 ± 1.2	58.1 ± 2.0	56.3 ± 1.7
1:10	56.6 ± 1.1	43.4 ± 1.8	40.6 ± 1.2
1:40	40.3 ± 1.5	30.3 ± 1.1	31.3 ± 1.4
Anti-heat-killed serum (Titer 1/3200)			
1:5	38.8 ± 1.5	30.3 ± 1.3	31.4 ± 0.9
1:10	28.9 ± 1.2	20.4 ± 1.0	20.3 ± 0.9
1:40	3.0 ± 1.0	5.3 ± 0.8	6.4 ± 0.9

Haemoglobin, dla-glycerophosphate and normal mouse serum were negative controls

The next set of similar experiments were done using fibrin-platelet clot as the

substrate. The results are shown in Tables 7, 8. The conclusions from these experiments were that adherence was

Table 7 : Effect of pretreatment of *S. epidermidis* on adherence to platelet-rich clots

Pretreatment with	% Inhibition of adherence of *S. epidermidis*	
	CH2	CH5
Lipase (10 mg/ml)	85.7 ± 0.9	78.5 ± 1.2
Human albumin (µg/ml)		
0.1	6.9 ± 0.3	9.1 ± 0.7
1	10.7 ± 0.7	15.9 ± 1.0
10	28.6 ± 1.0	20.6 ± 1.2
100	68.7 ± 1.2	59.7 ± 1.3
1000	78.8 ± 1.0	75.5 ± 1.0
Mouse anti-LTA serum		
1.5	73.6 ± 1.1	80.6 ± 1.3
1.10	50.1 ± 0.8	42.6 ± 0.9
1.40	30.6 ± 1.0	31.3 ± 1.4
Normal mouse serum 1:5	7.6 ± 0.4	10.9 ± 0.7

reduced four-to six fold ($P < 0.001$) on pretreatment of bacteria with lipase, while neuraminidase, trypsin, phospholipase C, and sodium periodate did not alter their

Table 8 : Adherence of *S. epidermidis* to platelet-rich clots pretreated with LTA and deacylated LTD

Pretreatment with (µg/ml)		% Inhibition of adherence of *S. epidermidis*	
		CH2	CH5
LTA	3	6.4 ± 1.3	4.0 ± 0.3
	6	11.1 ± 1.6	7.1 ± 1.2
	12	20.3 ± 1.9	11.3 ± 1.2
	25	34.2 ± 3.7	29.2 ± 1.9
	50	60.4± 3.2	51.2± 2.1
	100	73.4 ± 2.9	66.7± 3.2
	200	76.3± 3.9	70.2± 3.6
DLTA	100	10.8± 1.0	6.6 ± 1.1

binding. Pretreatment of bacteria with substances known to bind lipoteichoic acid (LTA), such as human albumin and anti-LTA antibodies, also resulted in a four-fold ($P < 0.001$) reduction in adherence. Prior incubation of clots with free LTA, but not with deacylated LTA, produced a fourfold ($P< 0.001$) decrease in the adherence of homologous and heterologous strains of *S.*

Table 9 : Adhesion of two strains of *S. epidermidis* to three cardiac cell lines

Cell Line	Number of bacteria adherent per well	
	CH2	CH5
Girardi	3,712.8 ± 40.4	2,310.4 ± 47.9
HR 9	192.7 ± 18.3	182.6 ± 11.7
CPAE	154.3 ± 10.7	110.1 ± 14.4

epidermidis. A similar reduction was also observed when LTAs derived from *Staphylococcus aureus* and *Streptococcus pyogenes* were used. These data provide evidence that the lipid moiety of LTA has a central role in the adherence of *S. epidermidis* to fibrin-platelet clots in vitro (12).

Table 10. Adherence of bacteria to surgical sutures

Bacterial Strains	Adherent bacteria $X10^8$ per cm^2 (mean ±SD)			
	Poly-propylene	Polyester	Polyester coated with polybutylate	
			White	Green
S. epidermidis				
1	4.6 ± 0.7	11.6 ± 1.3	46.3 ± 4.4	45.1 ± 4.2
2	4.1 ± 0.5	12.5 ± 1.5	40.3 ± 3.4	41.2 ± 3.5
3	4.4 ± 0.5	14.3 ± 0.9	40.6 ± 3.1	41.4 ± 3.3
4	5.0 ± 0.4	10.4 ± 1.0	41.5 ± 3.6	40.7 ± 3.5
S. aureus				
1	3.8 ± 0.4	11.3 ± 0.9	39.8 ± 3.3	40.4 ± 3.3
2	4.3 ± 0.4	12.0 ± 0.9	37.9 ± 3.5	37.4 ± 3.3
Strep. sanguis				
1	3.9 ± 0.3	4.2 ± 0.6	5.1 ± 0.6	5.0 ± 3.3
2	3.6 ± 0.3	4.6 ± 0.5	5.3 ± 0.5	5.4 ± 0.6

The studies were extended to 3 cardiac cell lines (Tables 9, 10, 11). These clearly showed that LTA is an important though not the sole adhesin for bacterium-host cell receptor interaction in this experimental model (13).

Table 11 : Adherence of 2 antibiotic-treated strains of *S. epidermidis* to pharyngeal pathelial cells

	Strain 2	Strain 5
Cephalothin		
¼ MIC	83.3 (< 0.05)	98.7 (NS)
½ MIC	54.7 (< 0.001)	56.8 (0.001)
Cloxacillin		
¼ MIC	141.7 (<0.01)	64.3 (<0.001)
½ MIC	97.6 (NS)	77.2 (<0.001)
Vancomycin		
¼ MIC	81.0 (<0.05)	55.1 (< 0.001)
½ MIC	21.8 (<0.001)	15.1 (<0.001)
Rifampicin		
¼ MIC	57.7 (<0.001)	82.9 (< 0.05)
½ MIC	48.5 (<0.001)	49.9 (<0.001)

All values are expressed as the adhesion index with their p. values in brackets
NS= Difference not significant.

In-vitro bacterial binding to extracellular matrix proteins was studied and the results are shown in Table 12. *S. epidermidis* strains bind well to immobilized fibronectin, laminin, and vitronectin but not to fibrinogen and collagen IV (Author's unpublished data). The adherence of bacteria to sutures used in cardiac surgery was studied by in vitro quantitative determination with [^{3}H]-leucine-labeled *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Streptococcus sanguis* (14). The adherence per unit area for staphylococci was least for monofilament polypropylene (prolene), 3 times higher ($p< 0.05$) for braided polyester (Mersiline) and greatest (10 times, $p< 0.005$) for braided polyester sutures coated with polybutylate (ethibond). Mean values for the adherence of streptococci were low for all the sutures. Sutures pretreated with human plasma showed a 12-37% increase in bacterial adherence. In view of these observations, it is suggested that: (a) the preferential adherence of staphylococci to intra-cardiac sutures may be one of the explanations for its being the commonest cause of early prosthetic valve endocarditis, (b) there is a need for a careful selection of sutures used in cardiac surgery and (c) the described *in vitro* assay for bacterial adherence may be used for monitoring the development of better designed sutures and the effect of incorporation of antibiotics in the sutures (14, 15).

Table 12 : In vitro adhesion of 2 strains of *S. epidermidis* to extracellular matrix proteins

Immobilised	No. of adherent bacteria	
	CH_2	CH_5
Fibronectin	28.6 ± 3.2	6.3 ± 1.2
Laminin	21.4 ± 3.1	9.6 ± 1.7
Vitronectin	16.2 ± 4.1	10.2 ± 1.1
Fibrinogen	3.0 ± 2.1	2.1 ± 0.3
Collagen IV	5.1 ± 1.3	4.0 ± 1.0

Conclusions

The primary event in the pathogenesis of IE is bacterial adherence to host tissues. The present studies clearly show that *Staphylococcus epidermidis* is capable of adhering to pharyngeal epithelial cells, fibrin-platelet clots, tissue proteins, surgical sutures used in cardiac surgery and human cardiac cell line. The slime production, bacterial capsule, hydrophobicity, surface charge, mannose resistance and adherence

to lectin-sepharoses has no effect on the adherence property. The adhesin on the surface of staphylococci was identified to be lipoteichoic acid, the lipid being an essential component as deacylation caused loss of adherence. The receptor on the surface of host cells was found to be glycoprotein.

Lipoteichoic acid has been reported to be the principle adhesin of group A & B streptococci & *S. aureus* as well. LTA is complexed to proteins on bacterial surface in such a way that fatty acid ends of LTA molecules are exposed at the outer ends to interact with specific receptors on host tissues. LTA appears to be the principal adhesin of various pathogens that frequently cause IE.

Immunization against IE is impractical because of a variety of causative pathogens. However, common adherence factors seem to exist between the pathogens. LTA is a common adhesin, shared by streptococci and staphylococci, the most common cause of IE. Impregnation of LTA analogues in the prosthetic valves may be able to prevent prosthetic valve endocarditis. Anti-LTA vaccine is another alternative.

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