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Research activities of the Centre for Genetic Engineering

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Abstract

The Centre for Genetic Engineering was created in 1988 to foster basic research using recombinant DNA techniques. Several research programmes are operative in the areas of transcription, translation, molecular basis of viral and bacterial pathogenesis and tumorigenesis. In addition, molecular and immunological aspects of tuberculosis are being actively pursued. This profile describes in a nutshell the research programme of the Centre.

Key words: Interferon, Japanese encephalitis, transcription activation, translation, *M. tuberculosis*.

Introduction

Genetic engineering can be essentially considered as a method of approach to study biological phenomena at the genetic level. The philosophy of this approach lies in the manipulation of genes by way of altering their structure and studying the consequence of such alterations in terms of biological function. A gene can be mutated and the consequence of its functional absence can be studied or its function can be analysed in isolation in an *in vitro* system. The genetic engineering methodology which facilitates innumerable ways of gene manipulations has gained tremendous importance and technical advantage in studying biological phenomena at the molecular level. The technology has also influenced several applications both in agriculture and health-related areas. When this area of research was fast developing in the western countries, a group of scientists in the biology division of the Indian Institute of Science, mainly from the Biochemistry and Microbiology/Cell Biology Departments, formed a viable team to bring in, adopt, and foster genetic engineering methodology on the Institute campus. This attempt marked the genesis of the Genetic Engineering Unit on campus and from 1982 it started receiving formal funding from the Department of Science and Technology. Later, in order to build sound infrastructure, to expand and foster expertise on recombinant DNA research, the Centre for Genetic Engineering was established in place of the Unit in 1988 with financial support from the Department of Biotechnology, Government of India.

The following objectives were formulated as part of the Centre's academic activity: (i) to foster basic research using genetic engineering approach on the campus and in the country; (ii) to carry out basic research in the area of gene structure and organisation and control of gene expression; (iii) to undertake goal-oriented research in the areas of health and agriculture; (iv) training pre- and post-doctoral research workers and short-term workers from the Institute and from elsewhere in the country; and (v) organising laboratory workshops and symposia in the frontier areas of biology.

The major areas of research pursued by the faculty of the Centre include structure, organisation and regulation of expression of genes and their products involved in the transcriptional activation, cell proliferation, viral and bacterial virulence/pathogenesis, initiation of protein synthesis, meiosis, drug resistance and recombination. In addition to these major areas of research, aspects relating to molecular biology/molecular immunology of *Mycobacterium tuberculosis* were chosen as a common theme for group research activity by the faculty of the Centre. Tuberculosis is still a major health problem in India and only very little is known about the biological peculiarities of *M. tuberculosis* and how it brings about the onset of the disease by overcoming the immunological response of the host. Some of the projects have been initiated in this area involving studies on molecular mechanisms underlying pathogenesis, virulence, drug resistance, and immune suppression. With more research projects getting initiated in this field, it is hoped that the concerted effort put in might bring about some valuable information which might be useful in the management of the organism and of the disease.

The various areas of research being pursued by the Faculty of the Centre are summarized below:

1. Arrest of cell proliferation by gamma interferon

Gamma interferon (IFN–γ) is a biological response modifier synthesized by lymphocytes in response to mitogenic and antigenic stimuli[1]. The IFN–γ molecules are internalised through specific cell-surface receptor-mediated endocytosis and are transported to the cell nucleus and into the areas of dense chromatin[2]. They have been found to induce synthesis of messenger RNAs (mRNAs) from a large number of different genes in the target cells[3]. These include mRNAs for tumour necrosis factor, major histocompatibility molecules, complement C3, the guanylate-binding protein (GBP), Fc receptor, (2′–5′) oligo (A) synthetase, P1/eIF-2α protein kinase, C-myc and many other protein products whose functions have not yet been identified. Gamma interferon also elicits suppression of the synthesis of mRNAs for polypeptides such as Type I and Type II collagen. IFN–γ has been shown to induce significant synthesis of at least 12 distinct polypeptides in fibroblasts, six of which are not induced by either IFN–α (produced by leukocytes) or IFN–β (synthesized by fibroblasts)[4].

Both transcriptional and translational events seem to be playing roles in determining the level of induced mRNA. At least in the case of IFN–α and IFN–β it has

been found that transcriptional activation of interferon-stimulated (responsive) genes (ISGs) is associated with the rapid induction of DNA-binding proteins[5–8]. These proteins bind to the interferon-stimulation response element ISRE, TTCN (G/C) NACC TCNGCAGTTTCTC (C/T) TCT–CT, which is present 5′ to the ISGs′ sequence and brings about transcriptional activation of ISGs. The induction of some of the ISG-specific mRNAs by IFN–γ has been found to require ongoing protein synthesis. This suggests either that a newly synthesized protein regulates the level of inducible mRNAs or that mRNA turnover may be autoregulated by the amount of translation product present in the cell.

The biological effects of IFNs include antiviral action, anti-cellproliferative activity against normal and malignant cells, stimulation of cytotoxic activities of lymphocytes and macrophages and of natural killer cell activity[9]. Several laboratories have tried to exploit the anti-cellproliferative property of IFN in its clinical use against cancer cells. However, it was found that many types of both solid as well as disseminated tumour types such as carcinomas, lymphomas, lymphoblastoid tumours, leukemias and so on are nonresponsive to the anti-growth action of IFNs. The sensitivity of tumour cells to the anti-cellproliferative effect of IFNs depends on the differentiation status of the cells. For instance, malignant embryonal carcinoma cells can neither produce nor respond to IFNs, whereas differentiated cells obtained from embryonal carcinoma cell lines behave normally in both respects. Whereas the proliferation of Hela cells and U-amnion cells are effectively inhibited by IFN-γ, lymphoblastoid cell lines (Daudi and Raji) are relatively insensitive to the anti-proliferation effect of the IFN. Expression of IFN-γ receptors on both sensitive and insensitive cell lines clearly indicates that specific binding is a prerequisite, but not sufficient to confer sensitivity to the IFN[10]. In fact, data showed that binding sites of equally high affinity were present in both IFN-γ-sensitive (for instance, Hela) and IFN-γ-insensitive (for instance, Daudi) cell lines, with the number of receptors per cell being 2·5 fold greater in Daudi than in Hela cells. Thus, sensitivity of a given cell to IFN-γ-mediated inhibition of proliferation appears to be determined at a post-receptor level. These results suggested the existence of critical factors/cellular components which mediate sensitivity to the anti-proliferation effect of IFN-γ. Unlike in Daudi cells, sensitivity of Hela cell growth inhibition was accompanied by inhibition of protein synthesis[11]. Thus, in the case of Hela cells the situation responsible for the sensitivity to growth inhibition by IFN-γ seems to be related to protein synthesis, but not to receptor down-regulation which may be the possibility in the case of Daudi cells.

Two biochemical mechanisms have been proposed for the IFN-induced, double-stranded RNA (viral or synthetic)-dependent growth-inhibitory action of IFN. These are the (2′-5′) oligo (A) pathway and the P1/eIF-2α pathway[12]. However, these mechanisms cannot be considered as explanation for the anti-cellproliferative mechanism of IFN-γ against normal malignant cells since these cells lack double-stranded RNA of viral or synthetic origin. Recently, a process by which IFN-γ might be mediating inhibition of proliferation of KB oral carcinoma cells or WIDR colon carcinoma cells has been found[13]. In these systems, IFN-γ seemed to induce the

synthesis of the enzyme indoleamine 2,3-dioxygenase which leads to increased L-tryptophan metabolism and subsequent depletion of the amino acid in the culture medium, resulting in the growth inhibition of these cells. The authors suggest that this might be one of the several mechanisms by which cell proliferation gets arrested during exposure to IFN-γ.

One of the research projects at the Centre is aimed at the detection and characterisation of specific interferon-responsive genes and gene products in the interferon-responsive and interferon-nonresponsive tumour cell lines. Elucidation of the molecular basis for interferon-nonresponsiveness/responsiveness in tumour cells might throw light on the fundamental processes of cell proliferation *per se*. The experimental strategy involves cloning of specific tumour cell lines into the interferon-responsive and interferon-nonresponsive clones. Then the response of these two types of clones to gamma interferon will be compared at the gene expression level. Such an analysis might reveal the expression defects/product defects of interferon-specific genes in the nonresponsive cell clones.

2. Structure–function relationship studies with transcription activator protein C

The bacteriophage Mu *mom* operon codes for a unique DNA modification protein which protects the phage DNA against a wide variety of host restriction enzymes[14]. In the pentanucleotide recognition sequence 5'-GAGNPy-3' found in Mu DNA, adenine is converted to acetamido adenine by a reaction pathway yet to be elucidated. The host DNA methyl transferase (DAM) and two-phage gene products, C and Com, are involved in positive regulation of *mom* gene expression[14–16]. Earlier studies have shown that C gene product is required for *mom* transactivation[15]. The lambda–Mu hybrid phages carrying the C gene region transactivate the *mom* promoter in a *mom*-lac Z fusion plasmid. The C gene product also activates some other late genes. At least four promoters are specifically activated by C[17]. The C gene has been cloned and sequenced[18,19]. The open reading frame can encode a protein of 140 amino acids with the size of 16·5 kDa. This observation is further confirmed by cloning the gene into expression vectors and subsequent purification of over-produced protein[20]. Gel mobility shift experiments showed that C protein binds specifically to DNA fragments containing the Mu *mom* promoter[21]. The protein primarily binds to two sites in the *mom* gene regulatory region and this binding is implicated in transcription activation[22]. To gain further insight into the mechanism of *mom* gene transactivation by C, additional footprinting experiments have been carried out with *E. Coli* RNA polymerase and C protein on Mu *mom* DNA. In the absence of C, RNA polymerase binds to a site upstream from the functional *mom* promoter; this promoter-like sequence overlaps with one of the primary C-binding sites. In the presence of C protein, RNA polymerase binds to the downstream functional promoter. Thus, C protein seems to transactivate *mom* expression by displacing RNA polymerase from the upstream non-functional site and by positioning it at the downstream functional promoter[22]. *In vivo* footprining experiments confirm these results[23].

Examination of the derived amino acid sequence of the C protein revealed the

presence of 'leucine zipper'-like motif and an adjacent basic amino acid region[22]. Such an organisation has been identified in DNA-binding regulatory proteins from a variety of eukaryotic systems. The term 'leucine zipper' is referred to a stretch of about 30 amino acids containing 4–5 leucine residues separated from each other by six amino acids. This structure was first proposed by McKnight and his colleagues based on the sequence similarity of enhancer-binding protein C/EBP with oncoproteins myc, fos, jun, and yeast transcriptional activator GCN4[24]. They all contain this novel motif and hence can be classified as new class of DNA-binding proteins. The leucines from two chains interdigitate to provide the zipper-like structural framework resulting in dimer formation. Mu C protein represents the first example of a prokaryotic regulatory protein containing this motif. Adjacent and amino terminal to the zipper region is a basic amino acid region similar to the domainal organisation of eukaryotic transcriptional regulatory proteins. A typical example is fos/jun family of transcriptional activators[25–29]. In this group, the role of leucine zipper is in protein–protein interaction to form dimers, either with an identical protein as in the case of GCN4 or with a different protein as exemplified by fos/jun heterodimer. These conclusions are based on elegant studies involving site-directed mutagenesis and domain-swapping experiments. Such experiments have precisely defined the dimerization and DNA-binding motifs of these proteins. When the leucines in the zipper region are changed, the proteins neither dimerise nor bind DNA. On the other hand, mutations in the basic region prevent DNA binding but do not interfere with dimerization[25–29]. It is important to ascertain whether the two observed domains in the C protein are functionally distinct and play roles analogous to those in the eukaryotic proteins. If so, it would suggest the functional organisation of these transcriptional regulatory proteins to be the same. The project being carried out attempts to correlate the structural basis of the C protein to its function.

3. Japanese encephalitis virus—basis for pathogenesis and neurovirulence

Neurotropic viruses are among the most severe pathogens of man and animals. Included in this category are poliovirus, rabies virus, reovirus and many of the flaviviruses. Japanese encephalitis virus is a flavivirus, which as the name implies, causes encephalitis in man. Gaining entry into the central nervous system is the first major step in the pathogenesis brought about by the above-mentioned agents. The mechanisms adopted by these microbes to reach the CNS may vary. An attractive possibility is to breach the blood–brain barrier to directly access the brain. Both rabies virus and reovirus initially infect the peripheral nervous system and travel along the nerves to the brain. Neurotropism may also be mediated through specific receptors on the surface of cells in the CNS that endocytose the infective agent which may subsequently establish a foothold within these cells. Activation of viral regulatory elements by neuron-specific factors is yet another molecular event that can lead to nerve cell-specific expression of viral genes and thereby, neurotropism.

Much of the pathological and degenerative lesions commonly seen in an infected individual may be brought about by immune attack of infected cells rather than directly by the infecting agent itself. This is true of many bacterial, viral and parasitic infections. Complement-mediated lysis of infected cells, antibody-dependent

cytotoxicity, destruction of infected cells by a class I or class II restricted cytotoxic T cells are some of the mechanisms by which the immune system attempts to clear an infecting agent from the host system, with attendant damage to host tissues. In the course of infection by JEV, extensive damage is caused to the host tissues, mainly the CNS, that is of a permanent and irreversible nature. This is seen as speech disturbances, lack of coordination and various other abnormalities[30]. It is this aspect of the virus that makes it a dreaded pathogen where individuals who recover from an infection may still suffer long-lasting neurological disorders.

At present, the molecular basis of pathogenesis by JEV is not understood. Since the damage caused by the virus is only to brain tissue, preventing entry of the virus into the brain can potentially and dramatically reduce the risk of infection by this virus. However, to date, no mutants of JEV are known which have lost the capacity for neurotropism. Generating and screening a number of mutants of this virus for their ability to access the CNS and/or survive within the brain tissues would be a very worthwhile task. Such studies would also help to map the viral gene(s) involved in neurotropism.

In developing vaccines against this virus, it is necessary to first understand the nature of viral antigens that mediate the immunopathology seen during infection. For this, one has to study the nature of the immune response to individual viral antigens in a given population. For these studies, the genes of JEV need to be expressed in specialized vectors which can then be used as tools to study the B and T cell responses to the viral antigens. It is then possible to avoid those antigens that mediate tissue damage in a potential vaccine.

Although JEV has seven nonstructural proteins[31], their functions have yet to be precisely identified. Based on sequence homology, one of the open reading frames is believed to encode a protease[32] while another a polymerase[33]. It is essential to understand the functions of the viral proteins in the viral life cycle which would then give one a handle to attenuate its virulence by functionally inactivating one or more non-essential viral genes. These are the various approaches that are being pursued in the study of Japanese encephalitis virus in this laboratory.

4. Structure–function relationship of initiator tRNA and study of initiation of protein synthesis in prokaryotes

All organisms possess two classes of methionine tRNAs—the initiators and the elongators. The elongators insert methionine internally in the polypeptide. To study the structure–function relationship of *E. coli*-initiator $tRNA_{II}^{fMet}$, we have generated a large number of mutants using site-directed mutagenesis and analysed the mutant tRNAs *in vitro*. Recently, we described an *in vivo* initiation assay where initiation of protein synthesis occurs from UAG codon using formyl glutamine as initiating amino acid[34]. A mutant chloramphenicol acetyl transferase gene (CATam1·2·5) in which the initiation codon AUG has been mutated to UAG is used as a reporter gene along

with an initiator tRNA with a change in its anticodon from CAU to CUA such that it can base pair with the UAG initiation codon of the CATam1·2·5. Production of CAT from CATam1·2·5 is detected by (i) phenotypic resistance of the bacteria to chloramphenicol, (ii) Immunoblots, and (iii) CAT assays in the cellular extracts. To ensure aminoacylation of tRNAs that are poor substrates for GInRS we have also introduced GInRS gene on a second compatible plasmid in these transformants[35]. This assay allowed us to couple the anticodon sequence change to virtually any other mutation in the tRNA body and assess the effect of the mutation on its initiation activity[36].

Using both *in vitro* and *in vivo* assay systems we have recently identified two important structural elements in the initiators that are located in the anticodon and the amino acid acceptor stem regions of the tRNA. We also showed that when these elements are introduced into elongators ($tRNA^{met}$ and $tRNA^{gln,\ 2}$) they function as initiators[37].

Our *in vivo* analysis showed that some of the mutants of the initiator tRNA function both as initiators and elongators (dual function tRNAs), whereas others are inactive in initiation but function as elongators.

Our current research objectives are focussed in two major areas:

1. We are interested to further study the mutants of the initiators that are inactive in initiation. We want to know where in the initiation pathway the defect lies and thus use these mutants to elaborate on the molecular mechanism initiation of protein synthesis in prokaryotes. In particular, we are seeking to isolate and characterize factors that will rescue the initiation activity of these mutants. A general approach is to make genomic libraries of *E. coli* DNA (W.T. or following its treatment with chemical mutagens) in the same vector that harbors the initiation-defective mutant tRNA and the CATam1·2·5 genes, and screen for the transformants that are chloramphenicol resistant as a result of production of chloramphenicol transferase from the CATam1·2·5 gene. We can then characterize the genomic insert(s).

2. Inititation from UAG is quite efficient[38]. We are therefore also exploring the possibilities of using initiation from termination codon as an alternative expression system to overproduce proteins that are of biotechnological significance. This system will also provide us with an opportunity to initiate protein synthesis with a desired amino acid which may be of importance to regulate the stability of a protein *in vivo*.

5. Plasmids from mycobacteria

Plasmids are known to carry genes which code for properties that directly contribute to pathogenicity, antibiotic resistance as well as some metabolic functions of microorganisms. Although research work on plasmids from mycobacterial species had begun only during the late seventies, some of those studies have established a correlation between the presence of plasmid and virulence of the bacterium. Presence of covalently

closed circular (CCC) plasmid DNA molecules was first shown in the lysates of nine strains of *Mycobacterium avium intracellulare* complex[39,40]. One of these strains, LR25, was found to carry three plasmids of molecular weights $11{\cdot}2 \times 10^6$, $18{\cdot}3 \times 10^6$ and 107×10^6 as determined by electron microscopic studies[41]. This *M. avium* complex strain was shown to be of high virulence in beige mice as judged by high mortality and a progressive increase in the bacterial population in the spleen and lungs as determined by counting the number of colony-forming units[42]. However, the *M. avium* LR163 strain, which is the 'cured' derivative of LR25 strain and lacks all the three plasmids, showed low virulence. This suggests that virulence character might be associated with the presence of plasmids in *M. avium*. In contrast to the loss of virulence, no significant change was seen in drug susceptibility with the lack of plasmids. Presence of a restriction modification (R–M) system has also been demonstrated in LR25 strain but not in LR163[41]. Similarly, about 26 AIDS-associated *M. avium* strains of serotype 4 and 8 were found to carry small plasmids of 9 to 15 megadalton size. Ten of the strains also carried large plasmids of 100 megadalton size, and one strain carried a 60 megadalton plasmid. Each of the 26 strains was found to carry a plasmid closely related to a 15·3 megadalton plasmid pLR7 from *M. intracellulare*[43]. Considering the fact that *M. avium* complex (MAC) (*M. avium* and *M. intracellulare*) cause pulmonary disease in humans, which is indistinguishable from tuberculosis, and that these organisms are the opportunistic pathogens encountered in AIDS patients, it is important to analyse the biological role of these plasmids.

Although several species of mycobacteria such as *M. avium*, *M. scrofulaceum*[44], and *M. fortuitum*[45] have been analysed for plasmids, a similar study has not been carried out in *M. tuberculosis*. One and perhaps the only striking report in these lines is that of Ramakrishnan and coworkers who detected a plasmid of molecular weight $1{\cdot}2 \times 10^6$ dalton in the virulent *M. tuberculosis* $H37R_v$ strain by electron microscopic methods [46,47]. No such plasmid could be detected in the avirulent *M. tuberculosis* $H37R_a$ strain. This observation has not been followed up with a systematic analysis of the characteristics and biological role of the plasmid. Although another research group had detected the presence of a plasmid in the virulent strain by agarose gel electrophoresis, no characterization even to the extent of size determination was mentioned in the report[39].

It has become increasingly evident that genes for bacterial virulence are often present in plasmids[48,49]. They carry genes for a number of different virulence factors, sometimes in association with antibiotic-resistance genes[50]. Although many a gene have been characterized as coding for virulence factors in a variety of microorganisms, no such factor could be detected in *M. tuberculosis* so far. The observation on the exclusive presence of a $1{\cdot}2 \times 10^6$ dalton plasmid in the virulent strain but not in the avirulent one, brings in the possibility that this plasmid function might have a role in the virulence character of the bacterium. If it is found to be associated with virulence character then it opens up the scope for developing molecular diagnostic probes based on this plasmid. Moreover, in this context, the study on the molecular basis for virulence of *M. tuberculosis* itself attains utmost medical relevance. The study envisaged in one of the laboratories at the Centre involves the structural and functional

characterization of plasmids from mycobacterial species, specifically from *M. tuberculosis*, if any, using molecular biology techniques. The virulence characteristics of plasmid-bearing strains will be tested in animal models for correlation. These strains will also be analysed for drug resistance pattern which could be plasmid based.

6. Cloning, expression and purification of topoisomerases from mycobacteria

DNA topoisomerases catalyse changes in the supercoiling of DNA. Other topological reactions catalysed by these enzymes are formation and resolution of knotted or catenated structures[51]. The topological change, either negative or positive supercoiling, is brought about by breaking and resealing of DNA strands. All topoisomerases carry out this reaction either by single-strand break as in the case of type I, while the enzymes whose reaction proceeds *via* double-strand breaks are classified as type II. Cells of all organisms examined so far have been found to contain one or the other kind of DNA topoisomerases. They are shown to be essential for cell growth[52-54].

DNA gyrase is a type II topoisomerase and is the only enzyme so far shown to introduce negative supercoils into DNA. Negative supercoiling of the DNA is essential for replication and transcription. DNA gyrases from *E. coli*, Bacillus, Micrococcus and several other bacteria have been isolated and studied in great detail[51,52]. *E. coli* DNA gyrase comprises 2 subunits, gyr A and gyr B; two different groups of antibiotics bind to these subunits. The inhibition of replication by nalidixic acid and oxolonic acid is by interaction with gyr A subunit while novoboicin and coumermycin AI block DNA and RNA synthesis by binding to gyr B subunit[51]. *E. coli* topoisomerases I, DNA gyrase and phage T4 topoisomerase have been extensively studied with respect to their structure, function and enzymatic mechanistics of the reaction[54]. The properties of gyrase and its interaction with DNA substrate have been studied by methods like nuclease protection, filter binding, sedimentation analysis, electron microscopy and a variety of other standard methods[55]. Substantial information is available on DNA binding, DNA cleavage, cleavage site specificity, ATPase activity and drug binding of this enzyme. DNA gyrase[56] and topoisomerases I[57] of *E.coli* have been cloned and overproduced. This has enabled the structural studies of these enzymes and their complexes with DNA. Such experimentations not only yield insight into reaction mechanics but also serve as a means to determine the regions of the protein which can be exploited for the binding of new drugs.

From the foregoing discussion, it is clear that isolation and characterization of such enzymes from mycobacteria could ultimately lead to the discovery of new antitubercular drugs. In general, knowing the gene structure and organisation will add to the information on mycobacteria at molecular level. Though significant research has been carried out on various aspects of mycobacteria, there is still dearth of information in many areas. Study of genetics and molecular biology of this genus attains great importance considering the pathogenic nature of several species and the increased development of drug resistance by the pathogenic organisms. Analysis of DNA gyrase gene and the protein could be one such step to know more about these bacteria. The

cloning and expression of the gyrase genes should facilitate the isolation of the protein in large quantities to carry out a wide variety of *in vitro* experiments. We have detected DNA gyrase activity in Mycobacteria earlier[58]. Experiments are in progress to purify the protein and to clone the genes.

7. Identification of antigens of *Mycobacterium tuberculosis* preferentially expressed in lung alveolar macrophages

The antigens of mycobacteria are some of the most potent stimulators of the immune system, so much so that immunopotentiation using BCG has been resorted to against a wide range of infections including parasitic diseases on the one hand and even malignancies on the other[59,60]. Against this backdrop, the profound immunosuppression seen during active infection by mycobacteria presents a striking paradox[61].

Mycobacterium tuberculosis causes disease in millions worldwide every year, of which pulmonary tuberculosis is the most common and most contagious. Forty per cent of patients with newly diagnosed active pulmonary tuberculosis show suppression of the cell-mediated immune response to tuberculin-purified protein derivative (PPD)[62]. This is manifested in most cases as a lack of skin test reactivity. Prolonged drug therapy reactivates the skin response, showing that reducing the bacterial load in some manner relieves the anergy. It is therefore important to identify the bacterial components important for immunosuppression.

The other striking aspect of pulmonary tuberculosis is the ability of the bacilli to thrive in the harsh phagocytic environment of the lung macrophages which normally engulf and destroy all invading pathogens. This ability to survive in the macrophage must be mediated by one or more mycobacterial antigens expressed in the infected macrophage.

Up to the present time, various antigens of *M.tb* have been identified using the lambda gtll genomic expression library of *M.tb*[63]. These have been isolated using mono- or polyclonal sera raised to culture-grown antigen extracts of *M.tb*. These antigens turned out to be predominantly stress proteins. Subsequently, when the importance of T cell immunity in tuberculosis influenced the identification of antigens, the same B cell reactive antigens were screened for the presence of T cell epitopes. Alternately, T cell clones were elicited from PPD-immunised individuals. These approaches do not have the capacity to identify macrophage-specific antigens of *M.tb*.

In order to understand the basis for pathogenicity of the tubercle bacillus, it is proposed to identify those antigens (protein or otherwise) which are uniquely expressed by *M. tuberculosis* within the infected macrophages.

It is proposed to construct a genomic expression library of the standard virulent strain $H37R_v$ as well as field isolates that score as high virulent in the guinea pig model system. The vector used will be the versatile lambda ZAP vector which permits generation of a plasmid from each recombinant phage by excision as well as generation of strand-specific RNA probes.

Macrophages obtained from human lung by bronchoscopy will be infected in culture using virulent isolates of tubercle bacilli. Antigens expressed by these bacilli will be fractionated into (i) secreted, (ii) cytoplasmic, and (iii) bacilli-associated species. Sera and T cells obtained from healthy contacts will be screened against these antigen fractions using the Western blot technique. The serum samples will be preadsorbed with total extract of culture-grown *M. tuberculosis* to remove the antibodies reacting to antigens common to both culture- and macrophage-grown bacilli. Similarly, T cells reactive to antigens present in culture-grown bacilli will be removed using standard panning techniques following stimulation with culture-grown bacillary extracts.

Once such antigens are identified, their genes will be isolated from the library and their role in pathogenicity/immune suppression will be elucidated.

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