Molecular medicine: from laboratory to clinical practice

The Science and Medicine conference held at the College on November 14/15 1996 illustrated how research into molecular genetics helps us to understand the mechanisms of clinical diseases.

Making genes work for us

Transient gene expression (TGE) systems – Dr J Sinclair (University of Cambridge School of Clinical Medicine) explained how TGE systems are used to analyse genes of interest. Once a gene has been identified and cloned it is important to analyse that gene by reintroducing it into cells of interest. TGE systems introduce cloned DNA into cells and use the cell’s transcription machinery to express that DNA, after which mRNA needs to be transported onto ribosomes and translated into protein. Gene expression is relatively transient (a few days) because DNA is not segregated correctly but that may be enough time to conduct experiments. The cell type chosen for expression depends on whether one is interested in expressing a gene of interest at a high level, in which case any cell type will often suffice, or whether one wants to analyse the control of expression of the gene, in which case it is vital to use a biologically relevant cell type for that gene.

There are two major ways of delivering DNA to cells: mechanically or by viral mechanisms (Table 1). It is unclear how the different mechanical delivery systems work; in essence, DNA must be delivered into the cell cytoplasm, taken up into the nucleus and expressed by the cell. TGE systems can be used to study a wide variety of gene regions, eg 3'UTR sequences (which confer stability upon mRNA). Promoter regions of DNA control expression of genes within cells. It is relatively easy to enforce expression of proteins that are not normally expressed in a cell by splicing these sequences to a promoter region, eg firefly luciferase, chloramphenicol acetyl transferase and betagalactosidase, all of which are good reporter constructs. Enhancers increase activity of any promoter, possibly by changing its structure and so allowing RNA polymerase enzymes access to it. Expression can be increased further by including into expression vectors an intrinsic sequence or polyadenylation signal found at the end of most RNA polymerase-driven genes. Intrinsic sequences and poly-A sites stabilise the mRNA, and hence are always found in laboratory-made expression vectors. Dr Sinclair warned that if virus vectors are used (Table 1) guidelines in handling them must be followed. For safety, virus defective mutants which only infect the first cell can be created.

Transgenics – Dr J Mullins (Centre for Genome Research, University of Edinburgh) defined transgenic animals as animals that contain exogenous DNA that has been introduced by means other than traditional breeding, eg by microinjection or transfection. A wide variety of transgenic animal species has been transfected including mice, pigs, goats and sheep, particularly for generating pharmaceutically important proteins. There are two main areas of transgenics: gene addition and gene targeting. The problem with gene targeting is that gene targeting is its cost, complexity and the fact that it is, at present, limited to mice. The remainder of Dr Mullins’ presentation concentrated on the much cheaper method of gene addition, ie introducing DNA by fine micropipette directly into the pronucleus of fertilised eggs. These embryos go on to produce viable offspring, although many embryos will die because of intolerance to this insult. Because gene incorporation (including the number of copies) is a random phenomenon, problems may occur due to position effects, eg expression of the same transgene array may vary depending on where it has been physically incorporated. Further, genes may integrate into a functionally important gene and disrupt that gene’s function, leading to phenotypes that are not related to the gene being introduced. One way around this problem is to study multiple transgenic lines.

Despite only one gene being introduced into an animal model, phenotypic interpretation is still complicated because gene expression may take place in many different tissues. Smithies et al have managed to use transgenes in a most elegant manner to study blood pressure. His group conducted gene duplication experiments using knockout animal models for the angiotensinogen (AGT) gene and to which they then

| Table 1. Some methods for delivering DNA into cells |
|-----------------------------------------------------|
| Mechanical delivery                                  |
| Calcium phosphate precipitation                      |
| DEAE-dextran complex                                 |
| Lipofection                                          |
| Electroporation                                      |
| Biolistics (firing DNA on gold-plated balls)         |
| Direct DNA injection and micro-manipulation          |
| Viral delivery                                       |
| Retrovirus (especially for primary cells)            |
| Adenovirus                                           |
| Vaccinia virus                                       |

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added extra copies, resulting in animals with zero to four copies of the gene. Plasma AGT levels rose with the number of copies of the gene, as did the animals’ blood pressure.

Unstable genes

**Dr D Rubinsztein** (Addenbrooke’s Hospital, Cambridge) gave an overview of the current state of knowledge in human diseases characterised by trinucleotide repeat (TNR) sequences, concentrating mainly on Huntington’s disease (HD). TNR diseases may be divided into four main types. (1) Myotonic dystrophy is the sole member of this category and results from CTG repeats in the 3’UTR of a protein kinase gene on chromosome 19. Normally there are between 5 and 27 successive repeats, but numbers from 50 to 1,600 result in a decrease in expression of mature mRNA. (2) Fragile X syndrome (the commonest cause of inherited mental retardation) is the most important of the diseases resulting from chromosomal fragile sites. More than 200 CGG repeat sequences at the 5’UTR of the gene cause loss of expression of the transcript. (3) CAG repeats in the coding portion of genes cause expansions of polyglutamine tracts resulting in a mutant extended protein which is thought to cause a toxic gain of function. These diseases are illustrated by Kennedy’s syndrome (spinal bulbar muscular atrophy), the spinal cerebellar ataxias and HD. (4) CAG repeats in intronic regions cause autosomal recessive diseases such as Friedreich’s ataxia with corresponding reduction in mRNA expression.

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder characterised by abnormal choreiform movements and cognitive deterioration. Neostriatal neuronal loss is characteristic, but evidence of pathology can also be found in the frontal cortex. The HD gene is one of the largest genes known and was one of the first to be mapped in 1983 on chromosome 4. Although the HD gene is now considered to be the definitive abnormality in the diagnosis of HD, Dr Rubinsztein cautioned the audience that because of incomplete penetrance some normal individuals may have CAG repeats in the HD range (≥40). This was illustrated by a case report from Johns Hopkins Hospital, USA, where a man was shown to have a large number of CAG repeats but a normal brain on autopsy at the age of 95.

Age of onset of HD is directly proportional to the number of TNRs. Indeed, (CAG)n accounts for around 70% of the variance of age of onset of HD. With each succeeding generation HD presents at a younger age, but it is unclear how this process of ‘anticipation’ occurs although slippage of the TNR during meiosis may be one reason. Work from the USA has shown that sperm of affected males show expanding CAG repeats (spermatic mosaicism). It is not clear how these TNRs cause HD, but what can be said is that localisation of TNRs within certain areas of the brains of affected individuals does not seem to relate to the severity of HD. During discussion it was noted that all TNR diseases seem to affect the central nervous system, and that although they cause no apparent problem in early life, patients usually died within 10 years of clinical onset.

Identifying genes involved in common diseases

Unlike the single gene disorders such as HD, unraveling the genetics of the common diseases is proving much more complex. Even answering the basic question of how many genes are involved in a common disease is difficult enough, let alone trying to identify the actual disease-causing genes. Two main methods were illustrated at this conference.

(i) Association studies. These are case–control studies within a given population comparing the frequency of a variant allele within a candidate gene between unrelated affected and unaffected groups. **Dr R J Bellamy** (Wellcome Trust Centre for Human Genetics, Oxford) in collaboration with the MRC Laboratories in The Gambia, presented work on the genetics of tuberculosis (TB). Worldwide 1.7 billion people are infected (3 million dying per year) and yet only 1 in 10 will go on to get active TB. Why only those 10%? The genetic component for susceptibility to premature death from infectious disease is thought to be greater than for cardiovascular disease or cancer. Dr Bellamy reminded the audience of the evidence for the genetic basis of TB. Blacks are at twice the risk from TB compared with Caucasians, and data suggest that this is more related to heritability than to social conditions. Although HLA DR2 is known to affect disease onset it accounts for only a small percentage of TB susceptibility. Noting that vegetarians are 8.5 times more likely to suffer from TB than those who eat fish (a food high in vitamin D), as well as other evidence suggesting that vitamin D is involved in immunity to TB, Dr Bellamy and co-workers investigated the role of the vitamin D receptor (VDR) as a protective ‘candidate gene’ for TB. They genotyped approximately 400 patients with TB and an equal number of controls for a variant allele of the VDR gene, and found that significantly fewer TB patients than controls were homozygous for the VDR polymorphism ($p < 0.009$). The odds ratio for those with this genotype developing TB, compared to those with other genotypes, was 0.52 (95% confidence interval (CI): 0.32–0.86) which tended to support the hypothesis that vitamin D is protective against TB. Dr Bellamy speculated that vitamin D could be used as a chemoprophylactic agent against TB.

(ii) Linkage studies. **Dr M Parkes** (Nuffield Department of Medicine, Oxford) presented linkage data in Crohn’s disease (CD). Linkage relies on the principle of cosegregation of disease markers within pedigrees with the disease. Epidemiological (including twin and adoption) studies suggest that shared genes play a more important role in the aetiology of inflammatory
bowl disease than shared environment. CD has a three times greater heritability ratio than ulcerative colitis (UC). Dr Parkes and colleagues recruited 186 affected sibling pairs (ASP) (CD = 81, UC = 64, mixed = 41) to see whether more marker alleles were shared by ASPs than expected by Mendelian inheritance. Using five microsatellite markers they confirmed a previously identified putative locus for CD on chromosome 16 with a maximum lod score of 2.3, although no obvious candidates lie in this region. Although Dr Parkes suggested that replication of such genome-wide studies identifying susceptibility loci is the ‘gold standard’, readers should be aware that computer models have managed to ‘replicate’ fictitious (ie computer generated) data sets.

Aspects of infections

Prion diseases – Professor J Collinge (Imperial College, London) reflected the current excitement of working in the field of prion diseases. Molecular biological techniques have unravelled many underlying biochemical mechanisms and the public interest in human prion diseases made this a very topical lecture. An increasing number of transmissible spongiform encephalopathies are recognised and they are united by similar neuropathology (Table 2). Mammals produce the protein PrPc, a glycoprotein expressed on the surface of neuronal cells and encoded by a two-exon gene. PrPc knockout mice are resistant to infection with prions, but in later life develop ataxia associated with loss of Purkinje cells in the cerebellum, implying that PrP has an important physiological function. The prion protein PrPsc (the putative transmissible agent of spongiform encephalopathies) has the same amino acid sequence as PrPc, but different biochemical and functional properties. Whilst PrPc is mainly alpha helical, PrPsc has a beta pleated sheet structure. It is thought that PrPsc is able to induce a change in host PrPc from an alpha helical to a beta sheet structure, thus disrupting normal function and so causing disease.

Human prion diseases – Different types of human prion diseases occur (Table 2), and four variants of Creutzfeldt–Jakob disease (CJD) are now recognised. It is estimated that about 85% of cases of CJD are ‘sporadic’; 15% are inherited forms; rare iatrogenic cases occur (mainly related to human cadaveric growth hormone therapy) whilst the true prevalence of new variant CJD is unknown. Molecular analysis of familial cases has identified point mutations or insertions in the PrP gene. The human PrP gene has a common amino acid polymorphism (129Val/Met) which occurs more commonly in cases of sporadic CJD, implying a genetic susceptibility to this disease.

Species barriers to prion diseases appear to be determined by the PrP gene. Prions from human CJD cases cannot infect normal mice, but can infect transgenic mice expressing the human PrP gene rather than the mouse PrP gene. The effect of bovine spongiform encephalopathy (BSE) prions on transgenic mice expressing human PrP is currently being investigated, but as yet the answers to the important scientific question of whether BSE prions can infect human is unclear.

Modelling of epidemics – Professor R Anderson (Centre for the Epidemiology of Infectious Disease, Oxford) gave a lucid account of the usefulness of mathematical modelling to predict the effects of interventions on the incidence and prevalence of infectious diseases. It is clear that some predicted effects may be counterintuitive, but this is often because our intuitions are based on wrong assumptions. Practical uses of mathematical modelling include predicting the best use of multi-drug regimens to prevent HIV drug resistance, calculating the cost-benefits of vaccinating against chicken pox and assessing the impact of different cattle culling policies on the BSE epidemic.

Inherited disorders of immunity

Professor A Segal (University College, London) talked about the biochemical disorders underlying the chronic granulomatous diseases (CGD). These are characterised clinically by severe recurrent bacterial and fungal infections and inflammatory granulomata. Biochemically, leukocytes from CGD patients are unable to generate superoxide free radicals required to kill phagocytosed bacteria. CGD is rare, affecting 1 in 250,000 of the population, and is biochemically and genetically heterogeneous – but the complexities of the various forms are now fairly well understood. Superoxide free radicals are generated by an assembly of phagocyte membrane-bound cytochrome b, cytosolic protein factors known as phox (phagocyte oxidase) and a small G protein from the Rac family. Two thirds of CGD patients are males heterozygous for mutations in the X-linked gene for the large subunit of

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**Table 2. Prion diseases in various species**

| Prion disease                          | Species affected          |
|----------------------------------------|---------------------------|
| Scrapie                                | Sheep, goats              |
| Transmissible mink encephalopathy      | Mink                      |
| Chronic wasting disease                | Male deer, elk            |
| Bovine spongiform encephalopathy (BSE)| Cattle                    |
| Feline spongiform encephalopathy       | Domestic cats             |
| Kuru                                   | Humans                    |
| CJD (Creutzfeldt–Jakob disease)        | Humans                    |
| Gerstmann–Sträussler–Scheinker (GSS)   | syndrome Humans          |

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cytochrome b. A rare form of autosomal recessive CGD results from defects in the small subunit of cytochrome b, whilst most autosomal recessive forms are associated with mutations in the genes for the \( p_{ox} \) cytosolic proteins \( p_{47}^{\text{phox}} \) and \( p_{67}^{\text{phox}} \). Although the different forms of CGD have now been extensively characterised genetically, this knowledge has yet to provide new treatments for those affected. It is hoped this will change now that the genetic insights have enabled the production of animal models by generating knockout mice that are being used to test new therapies, including various gene transfer protocols – an attractive therapy for a haematological disorder.

Thrombosis – mechanisms and prevention or treatment

**Thrombophilia – Dr M Laffan** (Royal Postgraduate Medical School, London) reviewed the role of inherited thrombophilias in the aetiology of thrombotic episodes. Factor V Leiden is emerging as an important player in thrombophilia in the general population. This polymorphism denotes an amino acid change (Arg 506 Glu) in factor V which alters an important proteolytic cleavage site making factor V Leiden resistant to cleavage by activated protein C, hence prolonging its procoagulant effect. The factor V Leiden polymorphism is common, being found in approximately 5% of Caucasians. It was found in 20% of a small group of patients with stroke before the age of 42, suggesting that it may have a significant role in human disease. This interesting observation needs to be tested prospectively. Factor V Leiden appears to interact with other inherited thrombophilias such as protein S deficiency further to increase the risk of thrombosis in these individuals. The search for other candidates having population-wide effects on thrombosis suggests that raised factor VIII levels may be important. Evidence from the Leiden Thrombophilia Study showed that in a group of 300 subjects with first episode deep vein thrombosis (DVT), 25% had a raised factor VIII:C level compared to age- and sex-matched controls.

**Fibrinogen – Professor G Lowe** (University of Glasgow) presented evidence for a role for fibrinogen in human disease. Fibrinogen accounts for 50% of plasma viscosity, is raised by smoking and lowered by fibrates. High-fibrinogen levels are correlated with an increased risk of ischaemic heart disease (IHD). Professor Lowe postulated that raised fibrinogen levels may be as important a risk factor in IHD as raised low density lipoprotein (LDL) cholesterol. In clinical trials the effect of bezafibrate on IHD event rates is similar to that of statins, but it has only a small effect in reducing LDL cholesterol. Evidence from the Edinburgh Artery Study of 1,500 middle-aged subjects suggests that raised fibrinogen may be a more significant independent risk factor for stroke and fatal myocardial infarction (MI) than either smoking or LDL cholesterol.

When treating acute MI, those with higher pretreatment fibrinogen levels are more likely to have non-patent coronary arteries after thrombolytic therapy and, interestingly, thrombolytic drugs deplete plasma fibrinogen. Professor Lowe advocated population screening and intervention strategies to reduce fibrinogen levels in a fashion similar to that now accepted for cholesterol. This might have an impact not only on IHD but also on the incidence of stroke.

**Low molecular weight heparins (LMW heparins) – Dr T Barrowcliffe** (National Institute for Biological Standards & Control, London) reviewed the genesis, mechanism of action and uses of LMW heparins. Although a number of these preparations is now available for clinical use, all are essentially made by depolymerisation of unfractionated heparin (UFH). These preparations contain a range of compounds with molecular weights of 4,000–5,000 compared to the parent UFH of 13,000. At least two distinct modes of action for LMW heparins are known – first, their pentasaccharide groups bind anti-thrombin III giving them anti-Xa activity; secondly, larger molecules bind thrombin, giving them anti-IIa activity.

*In vivo,* LMW heparins given subcutaneously have longer anti-Xa activity than UFH. This is explained partly by their increased bioavailability (UFH 30%, LMW heparins 90–100%) and partly by the stronger interaction of plasma heparin-binding proteins with UFH than with LMW heparins. In clinical use for general surgical prophylaxis of DVT, LMW heparins have been shown to be at least as efficacious as UFH, with no difference in bleeding events. However, for orthopaedic DVT prophylaxis, LMW heparins appear to be more efficacious. Further, their longer half-life means that they only need to be administered once daily. Although there is less experience in their use for treating established DVT, current evidence suggests that they may be more effective. No LMW heparins are at present licensed for use in the treatment of pulmonary embolism, but clinical trials are assessing this key therapeutic area.

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