

# Science and Medicine

The Science and Medicine Conference was held at the Royal College of Physicians on 8–9 November 1990. This annual conference, organised by Dr Carol Seymour FRCP, Assistant Registrar of the College, is designed to interest and inform physicians of all ages, as well as students of medicine and natural sciences. The next Science and Medicine Conference will be held at the College on 6–7 November 1991; the programme is given elsewhere in the Journal.

## The future of medical research

The conference began with an overview by **Sir James Gowans** entitled 'The future of medical research'. He stressed that research is highly unpredictable and that there is often a long delay between scientific advances and their applications in clinical medicine; for example, polio vaccination, the discovery of the hepatitis B surface antigen (Australia antigen) and monoclonal antibodies were instances where the initial aim of the researcher had not been primarily directed towards achieving their ultimate practical application, thus arguing in favour of a policy of supporting good individual researchers rather than goal orientated research. Basic research and clinical work should ideally be taking place side by side. He looked forward to developments such as autologous stem cell bone marrow transplants in which the stem cell had been rendered resistant, by gene transfer, to chemotherapeutic agents and virus infections.

## Regulatory peptides

**Professor Stephen Bloom** (Royal Postgraduate Medical School, London) gave an overview on regulatory peptides, beginning with a hitch-hiker's guide to the vast array of regulatory peptides controlling the gastrointestinal tract and some of their activities. In different organs the same hormone may have completely different functions, and minor differences in apparently identical hormones may radically alter their effects. For example: endothelin appears in some contexts to be a hormone controlling blood pressure, but different species of endothelin also exist in the hypothalamus and the pituitary where they undoubtedly mediate different effects; neuropeptide Y which stimulates the appetite can trigger experimental animals to eat continuously without any escape mechanism in the form of satiety; islet amyloid polypeptide which is found in type II diabetes mellitus causing amyloid in the pancreatic islets seems to inhibit insulin release and decrease glucose uptake by muscle in experimental situations but its role in diabetes is as yet unclear.

**Dr Mike Waterfield** (Ludwig Institute for Cancer Research, London) gave a talk entitled 'Signal pathways initiated by growth factors: their subversion in

cancer cells'. Tumour initiation could be the result of expressing the correct growth factor in the wrong place and at the wrong time, as was suggested by Dr Waterfield's original finding that a gene product from an oncogenic virus, simian sarcoma virus, was found to be homologous with platelet derived growth factor (PDGF), suggesting that virus infection may initiate expression of this gene causing the infected cells to express PDGF-like activity which gives them an inappropriate and possibly transforming growth stimulus. This is one example of an oncogene, an altered normal gene which may or may not be a growth factor; 40–50 of these have now been described.

**Dr T. M. Dexter** (Christie Hospital and Holt Radium Institute, Manchester) spoke on 'The control of blood cell development by growth factors: biological implications in clinical use'. There is mounting evidence that the stroma cells keep the bone marrow progenitor cells alive by providing growth factors. The bone marrow precursor cells interact with the surface of the stromal cell, where they are bound by intercellular adhesion molecules and can interact with cell-bound growth factors. The different types of stromal cells produce different growth factors, different adhesion molecules and different extracellular matrix-binding molecules, giving rise to the concept of the bone marrow as a series of micro-environments, each concentrating on the production of different committed cell lines. Describing the clinical applications of this knowledge of growth factors, Dr Dexter showed good evidence that G-CSF enhanced neutrophil output and prevented infection in patients whose bone marrow had been depleted by chemotherapy. This makes it possible to use higher doses of toxic drugs such as adriamycin than would otherwise be possible.

**Dr Julia Polak** (Royal Postgraduate Medical School, London) gave an elegant demonstration of the power of the confocal microscope in the study of neural, endocrine and endothelial peptides in the respiratory tract and cardiovascular system. Using immunocytochemistry against a protein gene product, PGP 9.5, which is present throughout the complete length of neurones, she found a dense network of nerve fibres in the respiratory tract. In transplanted (denervated) lung, although the sensory innervation is lost, the intrinsic sympathetic nerves remain unchanged and continue actively to secrete neuropeptides. In asthma, and in atopic individuals, PGP 9.5 and VIP (vasoactive intestinal peptide) are both present, but her finding contrasts with published work from other groups which suggested that VIP was completely lacking in asthmatic lungs. Endothelin, which appears to be a bronchoconstrictor peptide in the lungs, is detectable in greater amounts in asthmatics than in control subjects.

Neuropeptide Y, the appetite peptide, is the most

abundant neuropeptide in the human heart. In a transplanted heart its level decreases dramatically whereas PGP 9.5 is still present 5 months after transplant.

Endothelial derived relaxing factor (EDRF), now known to be nitric oxide, derived from L-arginine, is responsible for relaxation of small blood vessels. Dr Polak demonstrated the presence in endothelium of nitric oxide synthetase, the synthetic enzyme by immunochemical staining.

In Raynaud's phenomenon she demonstrated that the general level of innervation of digital blood vessels is decreased. Calcitonin gene-related peptide (CGRP) which increases the digital blood flow can be detected in some digital nerves however. In both primary and secondary Raynaud's phenomenon the only CGRP nerves that remain visible are in the epidermis whereas those characteristically found round blood vessels in normal skin are absent.

### Glycobiology

**Professor R. A. Dwek** (University of Oxford) gave an overview entitled 'Glycobiology: concepts, principles and the future'. Sugar molecules can attach themselves to proteins by a process known as glycosylation and thereby change the structure and functions of the protein. Professor Dwek described the two main classes of glycosylation linkage: first, the N and O linked glycosylation sites through specific amino acid sequences on to protein molecules; second, a class of glycan anchors involving a link through glucosamine inositol into a diacyl glycerol lipid which inserts into but does not traverse a membrane.

Glycosylation of proteins is achieved by oligosaccharide transferases, following which the transferred oligosaccharides are processed, and branching is altered by terminal transferases. This process may stop at any stage, leading to a variety of glycoforms. Some evidence that the different glycoforms may subserve different functions comes from studies of the thy-1 molecule, in which eight glycoforms are found in the thymocyte and four in brain tissue, all twelve of which are distinct. Tissue plasminogen activator (TPA) also shows some diversity of function in the two different forms of glycosylation. Type 1 has three oligosaccharides and type 2 two oligosaccharides. There are at least eight glycoforms of TPA1 and at least four of TPA2. TPA2, the only form found in menstrual fluid, has a much higher activity than TPA1. In terms of disease pathogenesis, abnormalities of protein glycosylation occur in several specific diseases, including rheumatoid arthritis, Crohn's disease and tuberculosis. In the diagnostic field, variations in glycosylation of serum proteins may be able to distinguish between normal pregnancies and those in which pre-eclampsia is a potential problem.

**Dr Ten Feizi** (Clinical Research Centre, Harrow) spoke on 'Oligosaccharides of glycoproteins as attach-

ment factors in microbe-host cell interactions'. She stressed that protein glycosylation sites are important sites of adhesion for different micro-organisms. *Mycoplasma pneumoniae* triggers the production of autoantibodies to the I antigen on red blood cells. This I antigen is a carbohydrate and is a major part of the host cell receptor for mycoplasmas. The autoantibodies produced by *in vivo* infection are monoclonal, causing a cold haemagglutinin disease. These antibodies can be purified and used to detect mycoplasma receptors on bronchial epithelium.

Bacterial adhesion through carbohydrate is important in some *E. coli* urinary tract infections in which the bacteria bind to oligosaccharides on the bladder epithelium.

In HIV there are interactions between the glycosylated regions of the viral envelope protein gp120 and various cellular ligands, although these are of considerably lower affinity than the interaction between gp120 and its cellular receptor CD4 molecule. The physical magnitude of the glycosyl moiety in some proteins is well illustrated by the HIV gp120 external glycoprotein, in which half the molecular weight consists of carbohydrate, attached to 20 different glycosylation sites. Dr Feizi's group has found a huge diversity of glycosylation structures in different gp120s, adding yet another level of antigenic variability to this virus, which undoubtedly helps it escape the immune response. Gp120 binds to CD4 on lymphocytes, which itself has two quite heterogeneous glycosylation sites. The gp120/CD4 interaction requires both molecules to be glycosylated, although the sugars are not directly involved in the binding.

**Dr Anne Dell** (Imperial College, London) spoke on 'The mass spectrometer: a powerful tool for probing links between abnormal glycosylation and disease'. An example of its use is the identification of parasite-specific toxocara secretory glycoproteins. The advantage of the mass spectrometer is that very tiny amounts of material can be analysed, obviating the problems previously encountered in which toxocara larvae had to be grown in the laboratory and took 8 months to provide the 30 mg of material required for conventional analysis. Another example is the band 3 protein of the red blood cell membrane, an anion transport protein which probably has some cytoskeletal properties as well. This protein is abnormal in congenital dyserythropoietic anaemia type 2, and the red blood cell membrane is disorganised. Mass spectrometry has shown abnormally short carbohydrate side chains in this protein. It is speculated that the band 3 proteins may therefore be clustered in the membrane rather than separated by the carbohydrates and so unable to provide a normal framework for the cytoskeleton. The enzyme abnormality here appears to be a defect in *N*-acetylglucosamine transferase 2.

**Dr M. A. J. Ferguson** (University of Dundee) spoke on 'Glycolipid anchors of membrane proteins' and gave more details about the glycosyl phosphatidyl inos-

itol anchors (GPI anchors) already mentioned by Professor Dwek. These are predominantly extracellular and plug into the lipid bilayer of the cell membrane without passing through it. They are found only in eukaryotic organisms. The surface coat proteins of certain organisms are linked to membranes in this way, as are some hydrolases and adhesion molecules, including neuronal adhesion molecules. The acetylcholine esterase receptor of red blood cells and the thy-1 molecule in mammals are also GPI linked external surface proteins. In trypanosomes this external protein is so heavily glycosylated that the dimensions of the resulting molecule and the density of packing of these molecules on the parasite surface can serve as a diffusion barrier between the internal and external environments. It provides an immunological defence mechanism in that it is the major protein to which antibody responses are made, and the parasite has 1,000 genes coding for variations in this coat protein. The switching on of new versions of this external glycoprotein gives rise to parasites which are antigenically distinct and can avoid the immune response; this process is responsible for the cyclical parasitaemia characteristics of the disease. The space filling capabilities of this protein are due entirely to the glycosyl groups. In *Leishmania*, the major gp63 protein is a surface protein involved in parasite attachment which also provides protection against enzymic degradation. It protects by being a scavenger of toxic oxygen radicals and it is also a protein kinase C inhibitor. Different subspecies of *Leishmania* vary in the number of repeating oligosaccharides in the GPI linked protein. *Leishmania donovani* has a disaccharide only, whereas *Leishmania major* has very complex side chains, and the increasing complexity of these side chains appears to correlate with a lower level of pathogenicity. The suggestion was made that *Leishmania* may have evolved more complex gp63 molecules in order to become better adapted to man, the host, and cause less damage.

**Dr D. Ashford** (University of Oxford) spoke on the 'Analysis of the glycosylation of soluble CD4 expressed in Chinese hamster ovary (CHO) cells'. Dr Ashford began by commenting that in organisms such as *Drosophila* the same number of genes were involved in control of glycosylation as were involved in defining the animals' body plan, and this must reflect the importance of glycosylation. He went on to describe the variations in glycosylation between different species of CD4 molecules produced by transfer of mutant CD4 genes into CHO cells as an expression system and demonstrated that by altering the glycosylation sites one could alter the level of CD4 production. He concluded that soluble CD4 carries oligosaccharides with a terminal  $\alpha$  galactose residue and that, as 1% of our circulating IgG is directed against an epitope of two galactoses joined in a 1:3 linkage, most of us are likely to have circulating antibody which would be expected to interact with the soluble CD4.

## Scientific basis of infection

**Professor H. P. Lambert** (University of London) began his overview on 'The scientific basis of infection' by describing aspects of bacterial pathogenesis. He reminded us that a bacterium has to cope with many different environments and must adapt to deal with each one. Attachment and invasion are two important pathogenic attributes. Some bacteria (such as *Vibrio cholerae* and *Bordetella pertussis*) have fimbriae or pili, to help attachment. Enteropathogenic *E. coli* adhere specifically to localised areas of epithelial damage. By contrast, *Streptococcus pyogenes* uses a host protein, fibronectin, as a ligand between its lipoteichoic acid and the eukaryotic cell to which it adheres. Some organisms, such as cholera and pertussis, are purely surface pathogens and do not invade cells. Although cholera has a well characterised toxin gene, elegant genetic exchange experiments have shown that it can colonise perfectly well without the toxin gene being expressed. By contrast, if the gene for pili is switched off no colonisation will occur. Pertussis attaches to the base of a cilium via its fimbriae and has a variety of toxins, including a classical AB subunit toxin, where the B is the oligomer involved in attachment and the A is the active monomer. Pertussis also has an adenylate cyclase toxin which can penetrate cells and make neutrophils and macrophages less able to kill bacteria. Pertussis has yet another toxin, a tracheal cytotoxin, which can damage ciliated epithelium and mucosa.

Bacteria have several methods of immune evasion. The capsules of pneumococcus, meningococcus and *Haemophilus influenzae* resist phagocytosis, and other organisms show antigenic variation and some secrete immunoglobulin A proteases. The immune response can be responsible for much tissue damage in bacterial infections. In experimental models of meningitis using an anti-adhesion factor antibody which decreases lymphocyte accumulation in the central nervous system, there was less damage than in the natural infection.

The ability of bacteria to adapt to different environments, such as variations in temperature, osmolarity etc, involves regulons consisting of a regulator gene and virulence genes. The regulator gene codes for a sensor protein and a regulator protein, and these lead to expression of a variety of unlinked virulence genes, which allow for adaptation to varying physical, chemical and immunological changes.

Professor Lambert concluded by describing some 'new' infectious diseases: the haemolytic uraemic syndrome, toxic shock syndrome, Chlamydia pneumonia, Lyme disease and some viral illnesses such as HIV, HHV6, the new hepatitis viruses and HTLV1. He picked out Brazilian purpuric/haemorrhagic fever (BHF) as a 'new' disease in which children aged 1-10 present with purulent conjunctivitis followed by a clinical picture of fever, vomiting and abdominal pain, and purpuric shock within 1-2 days. The causative agent has now been identified as a well known organism,

*Haemophilus aegyptius*, first isolated by Koch in 1883, renamed *Haemophilus influenzae* biotype 3. This organism has long been known to cause conjunctivitis, but the purpuric fever appears to be a new problem associated with the presence of a novel 25 megadalton plasmid occurring only in BHF strains. This plasmid must code for virulence factors responsible for the illness.

**Professor H. C. Thomas** (St Mary's Hospital, London) described 'Molecular variants of hepatitis B'. Hepatitis B virus (HBV) is a small partially double stranded DNA virus with four coding regions. The region coding for the envelope protein can produce three different envelope proteins of varying length. The nucleocapsid protein is present in two forms, a longer 'e' protein and a shorter virion-associated 'core' protein. The purpose of the secretion of soluble 'e' antigen is unclear, but it may block cytotoxic T-cell activity. Soluble e antigen may be involved in the neonatal tolerance of hepatitis B acquired perinatally from the mother. In the individual infected at birth, immunological tolerance is broken some time later and the adult does develop an immune response to the core gene; however, by this stage hepatitis B sequences are integrated in the liver cell genome and these individuals remain hepatitis B surface antigen (HBSAg) positive for life.

Some HBSAg negative individuals but with detectable antibody to hepatitis B core were found to remain viraemic and have a rapidly progressive disease. This picture is also commonly associated with fulminant hepatitis and occurs predominantly in the Mediterranean region and the Far East. Such patients are seen to seroconvert from e antigen to e antibody positivity and then to develop rapidly progressive disease leading to cirrhosis over the course of 2-3 years. Molecular analysis has shown an escape mutation in the nucleocapsid gene leading to failure to produce 'e' antigen but normal 'core' protein production. Children born to mothers producing mutant virus, and sexual contacts of these individuals, are more prone to fulminant hepatitis, possibly due to the loss of production of the 'immunosuppressive' e antigen.

The surface antigen also shows escape mutation and a proportion of individuals given anti-HBV vaccine composed only of the shortest of the envelope protein surface antigen molecules subsequently become infected after exposure to the virus, despite an apparently good antibody response. Polymerase chain reaction analysis of the 'a' determinant of the surface antigen of virus infecting these individuals shows a mutation of glycine to arginine which disrupts the region's immunoreactivity and allows the virus to escape neutralisation by antibodies raised against the wild type 'a' determinant. It can be argued therefore that hepatitis B vaccine should also contain the apparently invariant pre-S region of surface antigen, as found in the longer surface antigen molecule produced *in vivo* by the virus.

**Dr J. W. Almond** (University of Reading) spoke on 'Redesigning picornaviruses'. The polio virus has a single messenger RNA molecule which is translated into a polyprotein and is subsequently cleaved into structural and non-structural proteins. The viral structural proteins consist of 60 copies of each of the VP1, 2, 3 and 4 proteins, which assemble to form an icosahedral particle. The three-dimensional structure of the virus shows that all the antigenic sites are surface projections of the VP proteins. Modifications can be made to the cDNA of the polio virus and restriction sites built in around the sequences coding for these protruding areas, into which can be inserted antigenic regions from other viruses, including hepatitis A, papilloma viruses such as HPV16, BPV, HIV envelope, rabies etc. By using this highly attenuated virus as a vehicle to present these proteins, immunological responses have been raised in experimental animals against the envelope glycoprotein of HIV; the antibody produced appears to neutralise a range of HIV isolates. Antibody responses have also been raised against HPV16, foot and mouth disease virus and hepatitis A. Building in of these extra antigenic regions gives a surprisingly high (70%) incidence of viable chimeras, and the antigenicity of the final structure seems to be best when the inserted fragment has already been shown to be antigenic in the free peptide form.

### HLA antigens and T-cell receptors

**Dr J. S. S. Lanchbury** (Guy's Hospital, London) spoke on the 'Structure and function of HLA molecules'. HLA molecules are cell surface proteins, the class I consisting of a three-domain  $\alpha$  chain associated with  $\beta$ -2 microglobulin; the class II consists of a two-domain  $\alpha$  chain and a two-domain  $\beta$  chain. The  $\alpha$ -1 and  $\alpha$ -2 domains of class I are highly polymorphic with a large number of cellular alleles. The  $\alpha$  chain of class II is invariant but the  $\beta$  chain is highly polymorphic. Class I is involved in presenting antigen to CD8 positive cells and class II to CD4 positive cells, both in association with T-cell receptors and various other intercellular adhesion molecules. Many disease associations with HLA have been documented since the well known ankylosing spondylitis/HLA B27 association. Psoriasis is associated with Cw6, coeliac disease and dermatitis herpetiformis with DQw2, insulin dependent diabetes mellitus with DR3 and DR4, rheumatoid arthritis with DR4, pemphigus with DR4 and DRw6, Goodpasture syndrome with DR2, and multiple sclerosis with DR2; interestingly, possibly through a non-immunological mechanism, narcolepsy is strongly associated with DR2. Class I molecules carrying a foreign antigen mark an infected or a transformed cell which then becomes a target for immunopathic killing. In this case the antigen is synthesised within the cell. Class II presents antigen which has been endocytosed and degraded in a low pH endosome and then expressed

on the cell surface. Some endogenous antigens can also assemble on class II molecules.

Taking the specific example of rheumatoid arthritis, individuals who are HLADw13 appear to be protected against the disease and those who are Dw4 are slightly more prone, but most of the susceptibility to rheumatoid arthritis is determined by environmental factors (70%) and only 30% can be attributed to an inherited predisposition. Inherited susceptibility could be secondary to positive selection of a pathogenic T-cell or negative selection of a regulatory T-cell, to the inability to tolerise cells extrathymically, or to hyperpresentation of autoantigens. Defects in tolerance to cross-reactive antigens could also trigger an autoimmune disease.

**Dr T. H. Rabbitts** (MRC Laboratory of Molecular Biology, Cambridge) gave an outstanding presentation on the 'Structure and function of T-cell receptor genes and proteins'. The T-cell receptor (TCR) is the cell surface molecule defining T-cell antigen specificity. It is either a heterodimer of an  $\alpha$  and a  $\beta$  chain, or of a  $\gamma$  and a  $\delta$  chain;  $\alpha/\beta$  T-cells are commonest in the peripheral circulation and  $\gamma/\delta$  cells are much less common but occur more frequently in the skin and the uterine epithelium. Unlike antibody, which simply recognises antigen, T-cell receptors recognise antigen presented by 'self' major histocompatibility complex (MHC) on an antigen presenting cell. Each of the two chains of the TCR consists of a variable and a constant region, the variable chains being selected from one of a number of pre-existing genes encoded on the genome. Connecting the variable to the constant regions are junctional regions composed of diversity (D) and joining (J) segments. After the joining of the J and/or D regions, an enzyme called terminal transferase adds random nucleotides to the junction, which increases the diversity of the region. Diversity is thus produced by selection of a particular V gene with particular D and J genes, the junctions of which are all acted upon by terminal transferase; it does not depend on somatic mutation of the V, D or J genes. When the T-cell receptor on a CD4 or CD8 cell interacts with MHC and antigen, signal transduction occurs via the CD13 and CD4 or CD8 complex to activate the cell.

In the thymus, early T-cells express CD3 and CD8, and if there is no interaction with local antigen in the thymic epithelium these cells are killed. If self MHC reactive T-cell receptors are produced by the diversity mechanisms noted above, then the cell can come out of the thymus. Bringing together the VDJ regions involves chromosomal rearrangement in the thymus. Occasionally, instead of the usual VDJ linkage, one can get joining of two irrelevant chromosomes, such as 11 and 14. Interchromosomal joins are commonly seen in T-cell tumours, probably because cellular proto-oncogenes have been placed into a new chromosomal context. A translocation might be expected to make the T-cell a target for intrathymic destruction. However, in all T-cells only the VDJ genes from a single chromo-

some copy are used and the other chromosome copy is inactivated by a process called allelic exclusion. In the tumours the translocation appears to have occurred in the allelically excluded chromosome and the T-cell makes its normal rearrangements on the other chromosome, leading to a phenotypically normal T-cell. This may then exit from the thymus and, when exposed to antigen and possibly other stimuli, it will be triggered into proliferation. In the situation where a proto-oncogene has already been activated in a cell by the rearranged allelically excluded chromosome, this may lead to a potentially leukaemic cell.

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### Ion transport and disease

The session of the conference reported below included the following papers:

Overview—by **T. J. Rink** (*Amylin Corporation, San Diego*); Alveolar ion and water transport—by **R. E. Olver** (*Ninewells Hospital and Medical School, Dundee*); Abnormal ion transporting activities in CF; relationship to the gene—by **A. W. Cuthbert**, FRS (*University of Cambridge*); Implications of epithelial design for disease—by **C. A. R. Boyd** (*University of Oxford*).

Ion transport is fundamental to cellular existence. The many ion pumps, exchangers and channels in different membranes are vital to the control of the ion and water content of cells and of their intracellular organelles, to energy production in mitochondria and chloroplasts (proton gradients), to cell signalling (action potentials of nerve and muscle), and to mass transfer across secretory epithelia. While the physiological properties of many of the transporters are well known, their structure is only now being revealed by cloning techniques.

Secretory epithelia, which line the airways, intestine, nephron, parts of the genital tract, ependyma and secretory glands and their ducts, control the secretion and absorption of water, ions and certain solutes in to and out of these structures. Ions can pass through or between epithelial cells; the importance of the second, paracellular, pathway and its control by the cell has only recently been realised. There are 'tight' epithelia, such as gastric epithelium or the distal renal tubule, which can develop considerable transepithelial gradients of ions and water, and 'leaky' epithelia, such as small intestine, proximal renal tubule or gall bladder, which allow bulk flow between cells. For example, glucose absorption in the small intestine is both transcellular and paracellular, with an increasing percent-

age absorbed through the paracellular pathway as the luminal glucose concentration rises. The paracellular spaces of villous enterocytes widen and change their transporting properties in glucose-containing media. This was assumed to be secondary to water transport but in fact seems to be under cellular control. The structure of the intercellular junction is being actively investigated and the zonula occludens has recently been found to be linked to the brush border cytoskeleton.

The medical relevance of ion transport across secretory epithelia is demonstrated by alveolar pathophysiology and cystic fibrosis. Alveoli have a complex epithelial structure, containing type 2 cells, which have anatomic characteristics of transporting cells and make up 50% of cell numbers, but only 7% of the surface area. The growth of the fetal lung is regulated by active alveolar secretion of lung fluid, which can reach rates of 45 ml/kg body-weight/hour. Insufficient fluid, due to excess drainage, results in lung hypoplasia and major respiratory problems at birth, while obstruction causes hyperplasia. However, at birth fetal lung fluid is rapidly removed. This is associated with a massive stress-induced rise in levels of catecholamines and arginine-vasopressin, as well as other hormones. If the hormonal rise is reduced, as after elective Caesarean section, or if ion absorption is blocked by amiloride, the fluid is not cleared and respiratory distress occurs. The development of active absorption is influenced by steroid and thyroid hormones. Postnatally, alveolar fluid is again a product of active transport and has to be deep enough for surfactant molecules to orientate vertically. Active absorption is also essential to the clearance of pulmonary oedema. Thus alveolar ion transport affects many aspects of lung function.

Cystic fibrosis (CF) is a disease of epithelial ion transport. In CF, sweat duct and airway show reduced chloride absorption, causing the characteristic sweat electrolyte concentrations, as well as an absent chloride (and water) secretory response to agents such as  $\beta$  agonists which act through intracellular cAMP, in spite of a normal cAMP response. Patch clamp studies, a major technical advance of the last decade which allows study of the action of single membrane macromolecules in real time, have shown that chloride channels are present and open normally when the patch is detached from the cell but not when the patch is part of the cell, which suggests that the CF gene product regulates the channel. The gene is large and codes for a structure suggestive of a transmembrane protein with a highly conserved nucleotide binding fold which is the site of the mutation in the majority of CF patients. Its function is unknown but it is homologous to the multiple drug resistance proteins which transport cytotoxic drugs out of neoplastic cells. Epithelial cell cultures transfected with the mutant gene show abnormal chloride transport while those transfected with normal genes do not. The laboratory correction of defective chloride channel function raises the excit-

ing possibility of gene replacement therapy. However, there is also excessive sodium absorption in the airway and excess sodium channel activity on patch clamping, which may contribute to the dehydration of airway secretions. The effect of gene substitution on this less-emphasised but equally important ion transport abnormality is unknown.

Other major diseases of epithelial ion transport include secretory diarrhoea, with cholera as a classic example, and renal tubular diseases, such as the Fanconi syndrome. Diseases involving abnormal ion transport in other tissues include epilepsy, ventricular fibrillation and myotonic dystrophy.

Many drugs and toxins affect ion transport. Local anaesthetics, some anti-arrhythmics, sulphonylureas and tetrodotoxin block sodium channels, while dantrolene, curare and drugs such as verapamil and nifedipine block calcium channels. Several anti-epileptics block sodium or calcium channels, as well as opening chloride channels at higher concentrations. Vasodilators such as diazoxide and pinacidil open potassium channels. Antibiotics such as nystatin and amphotericin are ionophores. Omeprazole, cardiac glycosides and thapsigargin are pump inhibitors. Diuretics such as amiloride, thiazides and loop diuretics, and phlorizin inhibit exchangers. Finally, in acute diarrhoea oral rehydration using the intestinal sodium glucose transporter has been a major therapeutic advance.

Future developments include the better understanding of known medicines, techniques to screen new drugs, such as blockers of intestinal chloride secretion or of neuronal sodium channels, and designing drugs for new targets, for example to reduce bone reabsorption by blocking osteoclastic intracellular proton pumps, or for specific GABA receptor subtypes. Ion transport is fundamental not only to life but also to much of medicine.

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### Free radicals and disease

President Franklin D. Roosevelt defined a 'radical' as a man with both feet firmly planted—in the air. A straw poll of conference delegates, prior to the session on free radicals and disease, produced a range of similar if less erudite answers to the question 'what is a radical?'. Professor Slater, Professor Willson and Dr Gutteridge bravely attempted to dispel such prejudices and to suggest a fundamental role for both oxygen- and carbon-centred radicals in a variety of human and animal diseases.

## Overview

**Professor T. F. Slater** (Brunel University, Uxbridge) provided a much needed overview, putting forward the questions: what are free radicals?, how can they be produced?, how can they be detected?, and how can their chemical reactivity be measured?

In answer to the first question, he reminded us that electrons shared humanoid (and swan like) characteristics, preferring to couple and, when separated, becoming generally, and perhaps randomly, aggressively reactive. A radical consists of an atom, molecule or ion with one or more unpaired electrons and capable of independent existence (ie 'free'). Such a definition defines oxygen as a biradical since it has two unpaired electrons, each located in a different ( $\pi^*$  antibonding) molecular orbital. Moreover, all transition metals (defined as a group of metals with incompletely filled inner electron orbitals, eg  $3d$  orbitals for the first-row elements of the  $d$  block in the periodic table) and, dependent upon the element concerned, a large proportion of their corresponding complex ions can also be considered as radical species since they contain one or more unpaired electrons in their incompletely filled  $d$  shells (ie  $d$  electron orbitals). Agents that can generate free radical species include ionising radiation, ultraviolet light, ultrasonic radiation and thermal processes—one puff of cigarette smoke is said to contain  $10^{15}$  radical species. In biological systems, radicals are generated by virtue of reduction-oxidation (redox) reactions, catalysed either by transition metal ions or by enzymes which usually contain a transition metal ion at their catalytically active centre. Radical generation is clearly an essential accompaniment of life. The conversion of  $O_2$  to  $H_2O$ , a fundamental process in aerobic metabolism, proceeds in four sequential reduction steps which are conducted by the terminal component of the mitochondrial oxidative pathway, cytochrome oxidase. The chemistry of radicals is, however, open to some essential control, as indeed it is for non-free radical species (ie molecules or ions with all electrons paired), and it is for the most part only as a consequence of disease that control mechanisms are disturbed and radical production may cause damage.

Since unpaired electrons actively seek to pair, radical reactions usually occur extremely rapidly (half-lives  $< 10^{-9}$  second), and this creates difficulties in measuring radical species in biological systems. Professor Slater described the technique of electron spin resonance (ESR) spectroscopy which he employed to detect a peroxy radical ( $ROO^*$ ) present in normal human cervix but absent from neoplastic material. This technique is based on the magnetic behaviour of an unpaired electron which arises from the coupling of its negative electric charge with its spinning motion. When exposed to an external magnetic field, the magnetic moment tends to align itself either with the applied field or against it, giving rise to two separate

energy levels. If energy from the microwave region of the electromagnetic spectrum (at a frequency corresponding to the energy separation between the two permitted energy levels) is then applied to the sample, it is absorbed and utilised to excite the unpaired electron from its lower energy state to the higher one. Absorption spectra obtained in this manner contain signals that are characteristic of the precise chemical nature of the radical investigated. Since free radicals are chemically very reactive (particularly the hydroxyl radical  $\cdot OH$ ), their decay is usually suppressed by allowing them to react with an exogenous component (a spin trap) to form a relatively stable radical adduct with a characteristic and measurable ESR signal. Using a spin trap ( $\alpha$ -(4-pyridyl-1-oxide)- $N$ -t-butylnitron), he was able to define the active radical generated in carbon tetrachloride toxicity ( $\cdot CCl_3$ ). However, this spin trap, although useful for *ex vivo* measurement, proved to be too toxic for *in vivo* use. Professor Slater then progressed to discuss examples of diseases where free radicals had been implicated in either their aetiology or their progression, but he stressed that the detection of free radicals and/or products derived from radical reactions in damaged or diseased tissues is not sufficient to warrant a direct causative association.

Polyunsaturated fatty acids (PUFAs) are one of the most susceptible classes of biomolecules to free radical attack. PUFAs have chemical structures containing sequences of two or more methylene-interrupted unconjugated carbon-carbon double bonds (Fig. 1). These molecules are markedly susceptible to free radical attack by virtue of the low dissociation energy of their methylene C-H bonds. Radicals of sufficient reactivity (eg  $\cdot OH$ ) readily initiate the oxidative degradation of PUFAs by combining with the abstracted hydrogen atom. Subsequently, one major reaction pathway for the carbon-centred radical produced in this way involves its ability to combine with molecular oxygen to form a diene peroxy radical which in turn can abstract a hydrogen atom from a neighbouring PUFA and hence perpetuate an autocatalytic chain reaction. The resulting conjugated hydroperoxydienes (Figs 2 and 3) can then be further degraded to a wide range of so-called lipid peroxidation 'end-products', eg aldehydes such as malondialdehyde and polymeric material. PUFAs are major constituents of cell membranes, and free radical attack can give rise to a reduction in fluidity and a rise in permeability, leading to cell disruption and death when no antioxidants such as vitamin E are available to terminate the chain reaction.

Professor Slater then proceeded to outline the relevance of lipid peroxidation processes to a variety of diseases, including atherosclerosis, porphyria and the malnutrition disease kwashiorkor.

*Free radicals, radiation and cancer: the vital need for broad-visioned basic research.*

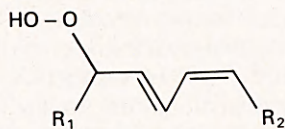
**Professor R. L. Willson** (Brunel University, Uxbridge) began his presentation by stressing the importance of the rates of free radical reactions and their pathways as factors which control the nature and extent of radical-mediated oxidative damage in biological systems. Indeed, the above factors are themselves critically dependent on the precise molecular nature of free radicals, scavenging antioxidants and cell components, together with the polarity of the molecules that constitute the medium or 'solvent system' in which free radical species are generated, eg water.

The relative reactivities of hydroxyl, peroxy and superoxide ( $O_2^{\cdot-}$ ) radicals were respectively specified as being strongly, moderately and weakly oxidising. Professor Willson then gave a visual demonstration of the rapid nature of free radical reactions which involved the oxidation of an iron(II)-cysteinate chelate to a transiently formed blue/violet-coloured iron(III) complex by exhaled oxygen. He also described the precise measurement of the rate of free radical reactions by a technique known as pulse radiolysis. This involves the application of a short 'pulse' of ionising radiation (which generates  $\cdot OH$  and other radical species from water) to an aqueous solution of a 'target' molecule under controlled experimental conditions, giving rise to the production of transient radical species. The decay of intermediates and the corresponding increase in the concentrations of stable products can be monitored by various detection systems, usually those involving differences in the electronic absorption spectra of the radical-scavenging reagent and the various interme-

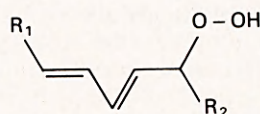
**Fig. 1.** Fatty acid with two double bonds.  $R_1$  and  $R_2$  represent the hydrocarbon backbone of the molecule



**Fig. 2.** cis,trans conjugated hydroperoxydiene



**Fig. 3.** trans,trans conjugated hydroperoxydiene



diates and products derived therefrom. In this manner, specific radical species can be generated and their chemical reactions monitored over extremely short time scales (ca.  $10^{-9}$  second).

The antioxidant activity of vitamins A, C and E and their potential in offering some protection against cancer was also discussed. These antioxidants are particularly good examples of the striking influence of molecular structure and associated physical properties on the endogenous environment and radical-scavenging abilities of these molecules. For example, ascorbate (vitamin C) is water-soluble and has a wide range of antioxidant activities, whereas  $\alpha$ -tocopherol (vitamin E) is lipid-soluble and is primarily a chain-breaking antioxidant that prevents propagation of the lipid peroxidation process. Interestingly, the application of pulse radiolysis was illustrated by a study of the interaction of ascorbate with  $\alpha$ -tocopheryl radicals. Regeneration of  $\alpha$ -tocopherol in this manner at the surface of membranes is believed to be an important antioxidant action of ascorbate *in vivo*.

Professor Willson emphasised that the induction of cancer by exposure to ionising radiation is a process dominated by free radical biochemistry and posed the question of whether these aggressively reactive species could also be responsible for other (non-radiolytically induced) classes of cancer.

In view of the clinical application of gamma irradiation to the treatment of various classes of malignant tumours and leukaemias, Professor Willson then outlined the ability of radiosensitising nitroimidazole drugs such as metronidazole (2-methyl-5-nitroimidazole-1-ethanol) to enhance radiolytic damage to hypoxic tumour cells. Indeed, the hypoxic nature of tumour cells poses a major problem in cancer radiotherapy since the so-called 'oxygen effect' is markedly retarded. The oxygen effect involves the enhancement of radiation injury to living organisms by molecular oxygen and appears to be attributable to the transformation of  $O_2$  to  $O_2^{\cdot-}$  by radiolytically generated aquated electrons ( $e^-_{(aq.)}$ ) and/or the reaction of  $O_2$  with radiolytically generated hydrogen atoms ( $H^{\cdot}$ ) to form the reactive  $HO_2^{\cdot}$  radical species which dissociates at physiological pH to form  $O_2^{\cdot-}$  and  $H^+$ . The selective role of nitroimidazole drugs as radiosensitisers of hypoxic tumour cells is thought to be attributable to the activation of nitroreductase enzyme systems in the absence of oxygen. Indeed, experimental evidence suggests that the metabolic reduction of these agents is responsible for their cytotoxicity. A recent clinical trial (unpublished) conducted in Denmark has apparently demonstrated the efficacy of a related compound, nimorazole, in prolonging the local therapeutic response to radiotherapy.

*Free radicals and atheroma*

**Dr J. Gutteridge** (National Institute for Biological Standards and Control) began by outlining the potentially deleterious effects of the oxidising actions of oxy-



gen, a molecule which he described as an environment pollutant in high abundance. He described the mechanisms for the generation of reactive oxygen radical species *in vivo*, including phagocytosis and the adverse production of  $O_2^{\cdot-}$  via electron 'leakage' from the mitochondrial electron transport chain.

Low molecular mass transition metal ion complexes (specifically those of iron and copper) can readily catalyse the generation of  $\cdot OH$  radicals from hydrogen peroxide ( $H_2O_2$ ) in the presence of endogenous electron donors such as  $O_2^{\cdot-}$  or ascorbate. The purpose of the reductant in these reaction systems is primarily to convert the oxidised form of the metal ion to its lower oxidation state, eg iron(III) to iron(II). In addition, both iron and copper ion complexes can promote the oxidative deterioration of polyunsaturated lipids. In view of these observations, Dr Gutteridge reminded the audience that the average human body contains ca. 4.5 g of iron and 80 mg of copper. The precise chemical nature of iron and copper ions in biological matrices is a critical factor in determining their ability to promote oxygen radical production. An assay system which appears to detect (and quantify) iron ions in a form that is able to catalyse the generation of  $\cdot OH$  radicals, the 'bleomycin assay', has been applied to a range of biological fluids. Such 'catalytic' iron complexes have been shown to be present in blood plasma obtained from subjects with idiopathic haemochromatosis and thalassaemia, and in synovial fluid from rheumatoid and osteoarthritic patients. These observations support the apparent prevalence of oxygen radical mediated oxidative damage in these disease states. Interestingly, caeruloplasmin, a copper-containing protein in plasma, expresses its antioxidant ability by catalysing the conversion of iron(II) to iron(III) (ie it has ferroxidase activity), a reaction which does not release any oxygen radical species.

Dr Gutteridge also discussed the relevance of iron and copper ion-catalysed lipid peroxidation processes to atherosclerosis. In addition to ascorbate,  $\alpha$ -tocopherol and caeruloplasmin, human blood plasma contains a further range of endogenous antioxidants which include albumin, bilirubin, glucose, haemopexin, haptoglobin, the iron-transport protein transferrin and urate. Each of these antioxidants has one or more

protective roles to play. With respect to low density lipoproteins (LDLs),  $\alpha$ -tocopherol is considered to be the most important antioxidant. Peroxidation of the lipid moiety of LDLs can give rise to the production of fluorescent adducts similar to those reported for lipofuscin. Cultured endothelial cells, macrophages, fibroblasts and smooth muscle cells can all chemically modify LDLs. Interestingly, LDLs are particularly susceptible to oxidative damage induced by micromolar concentrations of copper ions. As expected from the autocatalytic nature of the lipid peroxidation process, the cytotoxicity of oxidised LDLs is attributable to the lipid portion of the molecule. Such oxidised species can trigger the release of thrombin from plasma.

Suggested further reading: Halliwell B, Gutteridge JMC. *Free radicals in biology and medicine*, 2nd edn. Oxford: Clarendon Press, 1989.

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### Summary of conference

The conference provided an overview of some of the exciting growing points at the interface of science and medicine. The importance of the long neglected glycosylation of proteins was clearly demonstrated. The molecular and cellular biology of growth factors and their importance in pathogenesis and therapy were also shown. The standard of presentation was very high, but the temptation not to omit anything led a number of presenters to exceed their allocated time; as a consequence it was sad to see the dwindling audiences at the end of the day. However, those who remained were rewarded by some of the best presentations. Many of those attending will not have needed reminding of the importance and growing relevance of basic science to clinical medicine, and it was encouraging to see so many junior doctors and medical students there as guests of the College.

A. M. L. L.