Fortnightly Review

Biology and clinical relevance of nitric oxide

Patrick Vallance, Joe Collier

Ten years ago the idea that nitric oxide might be a biological mediator would have seemed far fetched. Clinicians might have recalled from schooldays that nitric oxide is a clear, colourless gas which rapidly turns brown on contact with air, anaesthetists might have remembered the series of disasters related to inadvertent contamination of cylinders of nitrous oxide with nitric oxide, the environmentally aware would have been familiar with the gas as a product of car exhaust and cigarette smoke and as a pollutant, and for others it would have conjured up images of primordial swamps and electrochemical storms. However, in 1987 it was shown that mammalian cells synthesise nitric oxide, a year later it was suggested that cells communicate with each other by the synthesis of nitric oxide from the amino acid arginine,[1] and in 1993 there were over 1000 publications on the biology of nitric oxide. This improbable biological mediator has been implicated in the pathogenesis of diseases ranging from hypertension to septic shock and dementia.[1,2]

Summary points

- Nitric oxide is a widespread biological mediator
- Three nitric oxide synthases have been identified
- The cardiovascular system is actively dilated by continuous synthesis of nitric oxide in the endothelium
- Peripheral and central nerves synthesise nitric oxide. Peripheral "nitrergic" nerves relax smooth muscle, and central nerves may be important in memory
- Production of large amounts of nitric oxide contributes to immune function and inflammation
- Nitric oxide is the active moiety of glyceryl trinitrate and other nitrovasodilators
- New drugs based on nitric oxide's actions are emerging

Synthesis and characteristics of nitric oxide

Nitric oxide is synthesised from arginine by nitric oxide synthases. Despite the apparent simplicity of nitric oxide (a 1:1 combination of two abundant elements) nitric oxide synthases are large and complex proteins. They have common features with cytochrome P450 reductase and contain oxidative and reductive domains.

Three isoforms of nitric oxide synthase have been identified: an endothelial type, a neuronal type, and a macrophage (inducible) type. The genes for these enzymes have been localised to chromosome 7 (endothelial type), chromosome 12 (neuronal type), and chromosome 17 (macrophage type).[3] The neuronal isoform is found in some central and peripheral neurones and the endothelial isoform is in vascular endothelium, platelets, and the heart (endocardium and myocardium). These two isoforms are normal constituents of the cells but the macrophage enzyme is not normally found in any healthy, quiescent cell type. Macrophage type nitric oxide synthase is expressed only after activation of cells with products of infection, including bacterial endotoxin or exotoxin, or certain inflammatory mediators, including the cytokines tumour necrosis factor or interleukin-1. It can be expressed in many types of cells including vascular smooth muscle, heart muscle, gut, immune cells, and hepatocytes. This is an inducible enzyme, and produces larger amounts of nitric oxide than the other isoforms.

Nitric oxide is a free radical (it has an unpaired electron) and is therefore highly reactive. It has a half life of a few seconds and readily combines with other free radicals.[4] In biological systems it decomposes rapidly to yield nitrite and nitrate, and this reaction is catalysed by transition metals, including iron. Haemoglobin inactivates nitric oxide by binding it to form nitrosohaemoglobin and by catalysing the degradation of nitric oxide to nitrite and nitrate, resulting in the formation of methaemoglobin. Nitric oxide synthase has a haem moiety and negative feedback of nitric oxide production may occur (fig 1). The very short half life of nitric oxide and its reactivity mean that it is most likely to act as a local messenger molecule transferring messages within and between individual cells.

The main target for nitric oxide synthesised by the

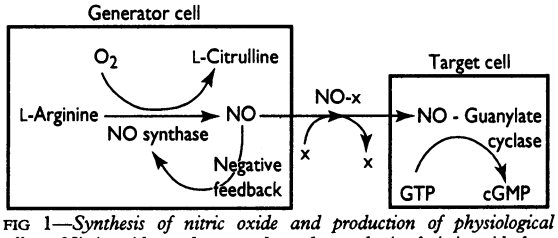

FIG 1—*Synthesis of nitric oxide and production of physiological effects. Nitric oxide synthase catalyses the synthesis of nitric oxide from* L-*arginine and molecular oxygen.* L-*citrulline is the byproduct. Nitric oxide itself might inhibit the activity of nitric oxide synthase by interacting with the haem moiety of this enzyme. Physiological effects are produced after nitric oxide binds to the haem moiety of guanylate cyclase and activates this enzyme to produce cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) in target and generator cells. Carrier molecules (x) that stabilise nitric oxide have been proposed*

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endothelial and neuronal isoforms of nitric oxide synthase seems to be soluble guanylate cyclase. This enzyme catalyses the formation of cyclic guanosine monophosphate (cGMP) (fig 1). Nitric oxide interacts with the haem moiety of this enzyme to activate it, and the rise in cyclic guanosine monophosphate concentrations produces changes in cell function, often by affecting intracellular calcium concentrations.

Nitric oxide synthesised by inducible nitric oxide synthase also activates guanylate cyclase, but the large quantities have additional toxic effects. High concentrations of nitric oxide inactivate enzymes containing tranisition metals, including certain mitochondrial enzymes. Inducible nitric oxide synthase seems to be part of the immune system.[5]

Effects of nitric oxide

Researchers have identified possible roles for nitric oxide by using techniques such as inhibiting the synthesis of nitric oxide with analogues of arginine, identifying nitric oxide synthases with immunohistochemical or biochemical assays, and detecting the messenger RNA for nitric oxide synthases.

BLOOD VESSELS AND HEART

Nitric oxide is released continuously from arterial and arteriolar endothelium. Infusion of a nitric oxide synthase inhibitor (for example, N^G-monomethyl-L-arginine) into the brachial artery of healthy volunteers causes a substantial fall in resting forearm blood flow, indicating that continuous synthesis of nitric oxide is an important determinant of the basal tone of small arteries and arterioles (fig 2). In contrast, veins do not seem to synthesise nitric oxide basally. Systemic injection of the inhibitor increases blood pressure in experimental animals and healthy volunteers but produces little change in venous pressure. Pulmonary vessels also synthesise nitric oxide continuously, and this seems to be important for maintaining blood flow within the lungs and matching ventilation to perfusion. It is now clear that a rise in blood pressure or vasospasm in a vessel does not have to be due to an increase in vasoconstrictors; it could be due to a loss of the basal dilator tone mediated by nitric oxide.[6] Nitric oxide synthesised in the endothelium maintains the vasodilatation of the cardiovascular system and contributes to the thromboresistance of vessel walls (box). It inhibits platelet aggregation and attenuates adhesion of platelets and white cells to the vessel wall. Furthermore, the antiaggregatory effects of nitric oxide are synergistic with those of another endothelium derived mediator, prostacyclin. Platelets also express nitric oxide synthase, and the nitric oxide produced seems to prevent excessive platelet activation in response to aggregating stimuli.[2]

The endocardium is a continuation of endothelium, and it is therefore unsurprising that it synthesises nitric oxide. Myocardial cells have also been shown to possess nitric oxide synthase. Nitric oxide affects cardiac relaxation during diastole and may be negatively inotropic.[7]

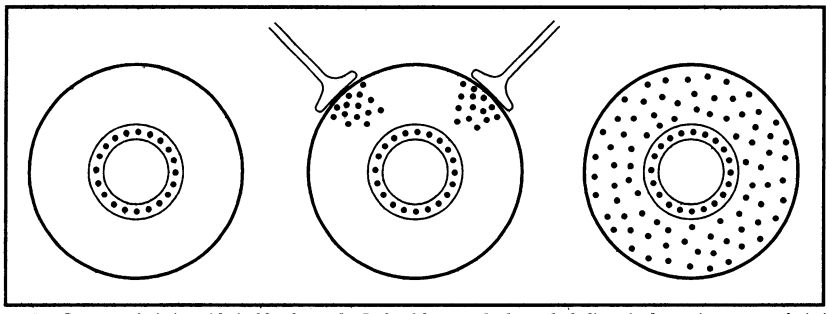

FIG 2—*Sources of nitric oxide in blood vessels. In healthy vessels the endothelium is the major source of nitric oxide (left). Certain vessels (cerebral arteries, corpus cavernosum) have nitrergic nerves that synthesise nitric oxide (middle). Vessels exposed to endotoxin or inflammatory cytokines express an inducible isoform of nitric oxide synthase throughout the vessel wall (right)*

Actions of nitric oxide in the cardiovascular system

In most healthy vessels the endothelium is the main source of nitric oxide

Arterial circulation is continuously dilated by basal synthesis of nitric oxide

Nitric oxide inhibits adhesion and aggregation of platelets and white cells

Some blood vessels are innervated by nitrergic nerves

Bacterial endotoxins or inflammatory cytokines lead to expression of an inducible isoform of nitric oxide synthase throughout the vessel wall

PERIPHERAL AND CENTRAL NERVES

Nerves staining for nitric oxide synthase have been found in the cardiovascular system, bronchial tree, urinary tract, and gut, and a "nitrergic" nervous system has been proposed. Nitric oxide is released from many nerves previously classified as non-adrenergic, non-cholinergic nerves; however, it is probably only one of several transmitter substances released. Nitric oxide relaxes smooth muscle, and nitrergic nerves are thought to play an important part in the dilatation of certain blood vessels (including the corpus cavernosum), adaptive relaxation of the stomach (the process by which the stomach accommodates food), relaxation of sphincters (including the sphincter of Oddi), the relaxant part of the peristaltic cycle, bronchodilatation, and relaxation of smooth muscle in the upper and lower urinary tract.

Mice with a selective mutation in the gene for neuronal nitric oxide synthase have grossly distended stomachs and show hypertrophy of the circular muscle,[8] a pattern similar to that of pyloric stenosis. The potential importance of nitrergic nerves in the vasculature is shown by the finding that inhibitors of nitric oxide synthesis block relaxation of the corpus cavernosum and prevent erection.[9]

Nerves containing nitric oxide synthase are distributed throughout the brain. They are commonest in the cerebellum, superior and inferior colliculi, and the granule cell layer of the olfactory bulb but also occur in the cerebral cortex, hippocampus, posterior pituitary, and autonomic fibres in the retina. Stimulation of the excitatory *N*-methyl-D-aspartate glutamate receptor leads to release of nitric oxide.

Nitric oxide may act as a retrograde messenger, allowing postsynaptic cells to send signals back to the presynaptic neurone. In the central nervous system three major physiological roles for nitric oxide have been proposed: (*a*) a mediator of long term depression and potentiation, the fundamental mechanisms of memory formation by which neurones "remember" the signals they have received previously[10]; (*b*) a mediator of short term electrocortical activation,[11] an alerting response important in control of arousal; and (*c*) a modulator of pain perception.[12] The role of nitric oxide in human brain has not been studied.

IMMUNITY AND INFLAMMATION

Nitric oxide synthesised by inducible nitric oxide synthase in activated murine macrophages is an important host defence mechanism.[5] It kills pathogens including leishmania, *Mycobacterium tuberculosis*, malaria parasites, and certain fungi; mediates "non-specific" immunity; and is toxic to tumour cells. Nitric oxide also regulates lymphocyte function and may have a role in inhibiting certain subsets of T helper cells.[13] Lymphocytes and neutrophils also synthesise and

release nitric oxide, although its role in the normal functioning of these cells is unknown.

Despite the abundance of data from animal studies, the importance of the arginine-nitric oxide pathway for the function of human macrophages is still uncertain. Nitrite and nitrate (stable breakdown products of nitric oxide) are produced by human macrophages, but the amounts are variable and less than those produced by murine cells. No direct evidence exists for production of cytotoxic amounts of nitric oxide by human immune cells or an immunosuppressive effect of nitric oxide synthase inhibitors.

Immune function

Nitric oxide is toxic to pathogens including certain fungi, protozoa, and *Mycobacterium tuberculosis*

Nitric oxide is toxic to tumour cells

Nitric oxide is toxic to certain host cells

Human cells express inducible enzyme after exposure to endotoxin or cytokines, but there is no direct evidence for a role for nitric oxide in host defence in humans

Nitric oxide also contributes to the inflammatory response. Vasodilatation may be mediated by inflammatory mediators stimulating endothelial nitric oxide synthase or by induction of macrophage isoform in endothelium, smooth muscle, and inflammatory cells in the vessel wall (fig 2). The nitric oxide produced might contribute to tissue leakage and damage.[14]

PREGNANCY

Evidence is accumulating that nitric oxide contributes to certain physiological changes that occur during pregnancy. Studies in animals suggest that there is increased expression of nitric oxide synthase during pregnancy, and excretion of nitrite and nitrate is raised.[15] The vasodilatation and fall in blood pressure that occur in pregnancy may be partly due to nitric oxide, and nitric oxide synthesised in the uterus may prevent uterine contraction.[16]

ENDOCRINE FUNCTION

Arginine, the substrate for synthesis of nitric oxide, has been used by endocrinologists for many years to stimulate the release of growth hormone, insulin, pancreatic polypeptide, and other hormones.[17] It is unclear whether these effects are due to nitric oxide. Nitric oxide has also been proposed to have a role in regulating renin production and sodium homoeostasis in the kidney. Endothelial cell nitric oxide synthase is activated by increased shear stress across the cell surface and nitric oxide inhibits release of renin. This might provide a link between renal blood flow and the control of renin and sodium balance, with the endothelial cell acting as a signal transducer.

Diseases

The speed of advance in nitric oxide research and the enthusiasm of investigators has meant that excess or deficiency of nitric oxide has been linked with many conditions. As drugs that interfere with the pathway are developed its precise roles will be more clearly defined. In the cardiovascular system loss of nitric oxide mediated effects has been implicated in virtually every disease associated with increased vascular tone, vasospasm, or enhanced adhesion of platelets and white cells to the vessel wall. Abnormalities of the arginine-nitric oxide pathway have been shown in patients with hypertension, diabetes, hyperlipidaemia, and overt atheroma.[2 6]

Abnormalities of peripheral nitrergic nerves might contribute to altered motility or sphincter function in the gut or urinary tract, or to disordered reactivity of airways or certain blood vessels. Infantile hypertrophic pyloric stenosis and achalasia are associated with loss of nitrergic nerves, and deficient nitrergic relaxation of the corpus cavernosum is associated with impotence.[2 3 6]

In the central nervous system, inhibition of nitric oxide synthesis impairs learning in rats, induces somnolence in sheep, enhances the action of certain anaesthetic agents, protects against epilepsy, and reduces or increases damage caused by stroke depending on the experimental model. Few data exist on humans, but neurones staining for nitric oxide synthase are spared in Huntington's chorea, and vast overproduction of nitric oxide could contribute to programmed or pathological cell death in the central nervous system. Indeed, nitric oxide has been implicated in cerebral damage produced by stroke, Parkinson's disease, and AIDS dementia.[18]

Expression of inducible nitric oxide synthase in response to cytokines or endotoxin seems to be part of the inflammatory response and could contribute to vasodilatation, vascular leakage, and tissue damage in some inflammatory conditions. Nitric oxide synthase is induced in the joints of patients with rheumatoid arthritis, the gut of patients with ulcerative colitis,[19 20] and the ventricles of patients with cardiomyopathy.[21] Nitric oxide synthesis is enhanced in patients with septic shock, and injection of an inhibitor reverses the hypotension, suggesting that overproduction of nitric oxide contributes to the pathophysiology.[22] Nitric oxide has joined the list of mediators involved in local and systemic inflammation in humans. In patients with renal failure, abnormalities of the arginine-nitric oxide pathway might be due to accumulation of arginine analogues, which are known to accumulate as renal function deteriorates.[23]

Clinical uses

Drugs based on nitric oxide have been used for over a century. It is now recognised that nitric oxide is the active moiety of glyceryl trinitrate and related nitrovasodilators, which may be considered nitric oxide donors (box).[24] Glyceryl trinitrate preferentially dilates veins, and this accounts for a large part of its efficacy in angina and heart failure and for the unwanted effect of postural hypotension. The reason for the venoselectivity seems to be that veins have a low basal output of nitric oxide and consequently the guanylate cyclase in venous smooth muscle is upregulated and responds more readily to exogenous nitric oxide. Similarly, loss of nitric oxide synthesis at sites of endothelial damage or dysfunction in arteries or arterioles would make these vessels more sensitive to the dilator actions of nitrovasodilators. The antiplatelet effects of nitric

Established drugs that may influence the arginine-nitric oxide pathway

Nitric oxide is the active moiety of glyceryl trinitrate and other nitrovasodilators

Angiotensin converting enzyme inhibitors inhibit the degradation of bradykinin which stimulates endothelial cells to produce nitric oxide

Glucocorticoids inhibit induction of the macrophage type enzyme

Methotrexate inhibits the synthesis of an essential cofactor for nitric oxide synthesis

Antifungal imidazoles inhibit induction of nitric oxide synthase

Antioxidants protect nitric oxide

oxide released by these drugs may also have therapeutic importance.

New uses for nitric oxide donors are being explored —for example, local application for the treatment of impotence, systemic administration for acute stroke or delaying labour, and inhalation for certain lung diseases. Use of nitric oxide for lung disease is not totally new since inhaled amyl nitrite was used to treat asthma in 1866.[25] Now, however, nitric oxide gas is being used: very low concentrations of nitric oxide (around 100 parts per billion) seem to cause selective dilatation of vessels supplying ventilated alveoli, and this approach might be useful in patients with acute lung injury or other causes of pulmonary hypertension and mismatch of ventilation and perfusion.[26]

New nitrovasodilators are likely to emerge, and there are already hints that it should be possible to deliver nitric oxide to specific tissues.[27] This might lead to nitrovasodilators that act preferentially to inhibit platelet aggregation, affect gastrointestinal smooth muscle, bronchodilate, or relax uterine or urinary tract muscle. Furthermore, with greater understanding of the mechanisms by which nitric oxide donors release nitric oxide the problems of tolerance might be overcome.

Other drugs may increase the production of endogenous nitric oxide. Angiotensin converting enzyme inhibitors block the breakdown of bradykinin, which stimulates release of nitric oxide from the endothelium. Cytokines seem to lead to expression of inducible nitric oxide synthase, and this accounts for the side effect of hypotension and may also contribute to the antitumour efficacy of these drugs. It may be possible to use agonists to stimulate release of nitric oxide in different tissues or "protect" nitric oxide by preventing the production of, or scavenging, other free radicals.

Recent interest has focused on the possibility of enhancing nitric oxide production by providing excess of the substrate, arginine. Although the mechanism by which this might work is far from clear (the amino acid is present in abundance and does not seem to be rate limiting for nitric oxide synthesis), there are reports that arginine prevents the onset of atheroma in experimental models and restores certain aspects of endothelial function in humans.[28] Arginine is present in relatively large amounts in nuts (particularly brazil nuts and almonds), shellfish, and meats including beef, bacon, and game.

DECREASING NITRIC OXIDE

In some situations it may be desirable to inhibit the synthesis or action of nitric oxide—for example, to reverse hypotension, prevent the toxic effects of large quantities of the radical, or inhibit cortical arousal or epileptiform activity. Inhibition of nitric oxide synthases with substrate analogues such as N^G-monomethyl-L-arginine reverses the local vasodilatation associated with inflammation and the profound hypotension seen in experimental models of septic shock and protects against cytotoxic effects of endotoxin and cytokines in vitro. In patients with septic shock N^G-monomethyl-L-arginine restores blood pressure (fig 3), although its effects on tissue damage, morbidity, and mortality are unknown. Selective inhibitors of the inducible isoform of nitric oxide synthase are being developed as possible anti-inflammatory drugs. These would have the theoretical advantage that they would inhibit the pathophysiological production of nitric oxide without affecting normal endothelial, neuronal, or platelet function.

Selective inhibitors of cerebral nitric oxide synthase have been described.[29] These should be useful for studying the physiological role of the pathway in the central nervous system.

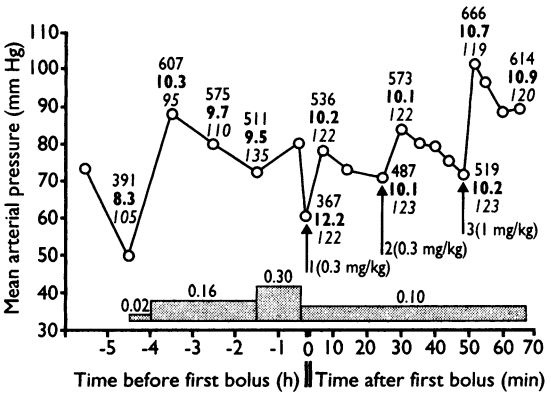

FIG 3—*Effects of N^G-monomethyl-L-arginine on mean arterial blood pressure and other parameters. The numbers above the points represent systemic vascular resistance (dyne s/cm^5), cardiac output (l/min (bold numbers)), and heart rate (beats/min (italic numbers)). Arrows show bolus injections of N^G-monomethyl-L-arginine. Hatched columns indicate noradrenaline infusions (μg/kg/min) (L-NMMA)*[22]

Other common drugs may also work partly by affecting nitric oxide synthesis. Anti-inflammatory glucocorticoids inhibit induction of nitric oxide synthase but are ineffective once the enzyme is expressed. The anti-inflammatory and cytotoxic drug methotrexate has a similar profile but acts by inhibiting the synthesis of tetrahydrobiopterin, an essential cofactor for the induction and activity of nitric oxide synthase.[30] It is not known how these effects relate to the therapeutic efficacy of these drugs, but the findings raise the possibility of developing more specific anti-inflammatory or immunosuppressive drugs based on modification of the inducible nitric oxide pathway.

Conclusions

Within 10 years it has been established that the formation of nitric oxide from arginine is a ubiquitous biochemical pathway that regulates the activity of soluble guanylate cyclase. Constitutive isoforms of nitric oxide synthase seem to have a role in controlling blood vessel tone and blood flow and regulating platelet function, gastrointestinal motility, and reactivity of certain airways. Nitric oxide produced in large amounts by an inducible isoform of nitric oxide synthase contributes to host defence (at least in animals) and pathophysiological changes in inflammation (including sepsis) and might cause tissue damage. As drugs are developed that selectively enhance or inhibit the pathways, the role of the arginine-nitric oxide pathway in health and disease will become clearer. New uses for old drugs are emerging, some new drugs are in the pipeline, and many more treatments based on nitric oxide can be expected.

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Conducting clinical research in the new NHS: the model of cancer

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The United Kingdom Coordinating Committee on Cancer Research represents the major organisations funding cancer research in the United Kingdom. The deliberations of a working party convened by the committee to evaluate recently expressed concerns that the changes in the NHS threaten research, especially clinical trials to evaluate new treatments, are reported. A survey of contributors to trials coordinated by the committee showed that half are now experiencing difficulties in continuing to participate in clinical trials. The two major problems identified were lack of time and of staff, especially for NHS staff in non-teaching hospitals. Recent changes in junior doctors' hours and proposed reductions in the length of time for training will exacerbate this. It is possible to identify the direct and indirect excess costs of conducting research in the NHS, but currently the mechanism does not exist to designate funds specifically for this purpose. Consultation with the regional directors of research and development confirmed that the service increment for teaching and research is not the solution for this. Proposals are made to secure future clinical research in the NHS, including finance, indemnity, the licensing of new drugs, the greater use of nurse counsellors, and the value of cancer registries.

Introduction

The United Kingdom Coordinating Committee on Cancer Research is an independent body representing the major organisations funding cancer research in the United Kingdom. Clinical cancer research can cover many aspects of malignant diseases, and it is important to distinguish research involving NHS organisational and managerial issues from those dealing with the evaluation of (new) treatments. It is the latter that is of particular concern to the committee. We seek to encourage, promote, and facilitate cancer research, particularly through the medium of randomised clinical trials. This has never been easy, and the changes now being introduced in the NHS are further exacerbating the difficulties of conducting clinical research. We examined some of the issues involved and have made proposals for improvement.

Cancer is a major health problem in the United Kingdom and was responsible for 163 940 deaths in 1991. As the population lives longer cancer is likely to become a greater burden to the NHS. Despite improvements in techniques and equipment in surgery and radiotherapy, better imaging and staging, and the development of new cytotoxic drugs, it remains the case that most cancer patients die of cancer. There is, therefore, a compelling need to improve treatment by the introduction of new regimens, new combinations, or better scheduling of existing treatments.

It is now widely recognised that the most accurate and effective way of evaluating treatments is a randomised controlled trial. Randomisation avoids bias in the selection of patients, and analysing results on an intention to treat basis ensures that treatments are tested in a way that reflects the realities of clinical practice. Also, effective assessment of new therapeutic strategies is essential to avoid the inappropriate use of scarce resources. There remains, however, a lack of understanding by many of the clinical community and NHS management about the need for clinical trials and the methods entailed. There is still a widely held view that decisions about which treatment is better can be based on clinical experience rather than randomised comparisons.

To identify small or moderate differences in outcome requires large numbers of patients. Even before the introduction of the current changes in the NHS it was a struggle to recruit adequate numbers of patients for clinical trials. Most clinicians are already hard pressed, and few with NHS contracts have any sessions available for research. For the most common solid tumours less than 5% of patients have the benefit of being entered into clinical trials. If this number declines further the impact of such trials on routine NHS practice and the academic skill which currently exists in the United Kingdom will be lost.

The Department of Health itself has recently drawn attention to the need to evaluate new treatments properly before they are introduced on a wide scale into