

Opportunities to Replace the Use of Animals in Sepsis Research

The Report and Recommendations of a *Focus on Alternatives* Workshop¹

Chris Langley,² Chris Brock,³ Gerard Brouwer,⁴ Alun Brown,⁵ Lucie Clapp,⁶ Jon Cohen,⁷ Tom Evans,⁸ Carol Newman,⁹ Samantha Orr,¹⁰ Barry Phillips,¹¹ Andy Rhodes,¹² Nigel Webster¹³ and Karl Wooldridge¹⁴

²ScienceSources, Hitchin, UK; ³Lord Dowding Fund for Humane Research, London, UK; ⁴The Home Office, London, UK; ⁵Department of Immunobiology, Guy's, King's and St Thomas' Medical School, Guy's Hospital, London, UK; ⁶Department of Medicine, University College London, London, UK; ⁷Brighton and Sussex Medical School, University of Sussex, Brighton, UK; ⁸Department of Immunology, Glasgow University, Glasgow, UK; ⁹Dr Hadwen Trust for Humane Research, Hitchin, UK; ¹⁰UK Human Tissue Bank, The Innovation Centre, De Montfort University, Leicester, UK; ¹¹RSPCA, Horsham, UK; ¹²Department of Anaesthesia and Intensive Care, St George's Hospital, London, UK; ¹³Department of Anaesthesia and Intensive Care, Institute of Medical Sciences, Aberdeen, UK; ¹⁴Molecular Bacteriology and Immunology Group, University of Nottingham, Nottingham, UK

Summary — Sepsis and multiple organ failure are common causes of death in patients admitted to intensive care units. The incidence of sepsis and associated mortalities has been steadily increasing over the past 20 years. Sepsis is a complex inflammatory condition, the precise causes of which are still poorly understood. Animal models of sepsis have the potential to cause substantial suffering, and many of them have been poorly representative of the human syndrome. However, a number of non-animal approaches, including *in vitro*, *in silico* and clinical studies, show promise for addressing this situation. This report is based on discussions held at an expert workshop convened by *Focus on Alternatives* and held in 2004 at the Wellcome Trust, London. It provides an overview of some non-animal approaches to sepsis research, including their strengths and weaknesses, and argues that they should be prioritised for further development.

Key words: *animal models, animal use alternatives, cell culture, computer simulation, replacement, sepsis, Three Rs.*

Address for correspondence: Carol Newman, Dr Hadwen Trust, 84A Tilehouse Street, Hitchin, Hertfordshire SG5 2DY, UK.
E-mail: carol@drhadwentrust.org.uk

Introduction

Sepsis is a complex clinical syndrome, characterised by an amplified and dysregulated inflammatory response to infection. Despite more than 20 years of extensive research involving both animal models and clinical approaches, the precise causes of the dysfunctional inflammatory response and the subsequent multi-organ failure, elude understanding.

Hospital mortality rates of patients admitted with sepsis to intensive care units (ICUs) in the UK range from 17% for those in the 16–19 age group, to 64% in patients over 85 years of age (1). The incidence of sepsis is also rising at rates between 1.5% and 8% per year (2, 3), due in part to sepsis being a

condition disproportionately affecting the elderly in an ageing population (4). Additionally, individuals show marked differences in susceptibility to infection and progression to organ failure. As well as the human costs, the economic burden of sepsis is substantial.

Sepsis research with animals may cause substantial levels of pain and suffering, as well as death. Many of these models are poorly representative of the human syndrome (5–7), and at best provide proof of principle of the role of various pathways in sepsis (8–10). Certain therapeutic interventions, successful in animal models (for example, antagonising the activities of cytokines, eicosanoids, lipopolysaccharide [LPS] and nitric oxide), have not led to patient benefit (7).

¹Focus on Alternatives comprises British non-profit-making organisations working together to replace animal experiments. Its members include the Dr Hadwen Trust, FRAME, Humane Research Trust, Lord Dowding Fund, RSPCA, St Andrew Animal Fund and UK Human Tissue Bank.

Legislation, both in Britain (11) and in Europe (12), requires the reduction, refinement and replacement of animal experiments wherever feasible, placing a responsibility on researchers, funding bodies and policy-makers to implement these concepts in the planning, design, approval and conduct of research. However, there has been limited discussion of the potential role of various non-animal approaches in sepsis research.

Thus, there is a pressing need for methods which both spare animals and indicate the potential to lead to improvements in patient treatment and prognosis. *Focus on Alternatives* (FoA) held an expert workshop at the Wellcome Trust in 2004, to review the possibilities for replacing animals in sepsis research. Non-animal methods not only hold potential for a better understanding of the clinical syndrome, but also satisfy the aims of UK and European legislation on animal experimentation.

Animals in Sepsis Research: The Issues

Animal experiments in sepsis research worldwide are extensive, and involve rabbits, rodents, dogs, pigs, sheep and non-human primates. Their main applications are: to assess the pathological, pharmacological, biochemical and physiological bases of sepsis; to characterise the genetic and molecular responses to various infective insults; and to test the efficacy of potential therapeutic treatments.

Sepsis is experimentally induced in animals by a variety of methods, including the administration of whole bacteria or cell-wall constituents such as lipopolysaccharides (LPS) into the bloodstream or the peritoneum. In the caecal ligation and puncture (CLP) model, sepsis is produced by facilitating entry into the circulation of bacteria from the gut flora. Predominantly short-term animal models of overwhelming bacteraemia have questionable relevance to sepsis in humans, despite the fact that the animals show evidence of multiple organ failure, which is also found in patients (7).

Animal models may be “observational”, to characterise the model or disease process, or “interventional”, when a putative therapeutic agent is tested for efficacy. In some studies, animals are anaesthetised in order to be instrumented or to induce sepsis, and are conscious for the remainder of the experiment. In non-recovery investigations, the intention is to investigate physiological parameters in addition to the others described above. Genetically modified (for example, “knock-out”) and normal animals have also been used in attempts to understand the roles of specific proteins, enzymes and mediators in sepsis.

The duration of experiments can vary from short-term (hours) to long-term (days to weeks), and their severity ranges from mild inflammation to mortality. Choices of endpoint, use of anaesthesia, level of resus-

citation and experimental duration, influence the degree of pain and distress experienced by the animals.

The research on animals predominantly uses young and initially healthy individuals, unlike the often elderly patients with pre-existing co-morbidities, who typically present with sepsis at ICUs. Furthermore, human populations are not genetically homogeneous, whilst animal studies often make use of in-bred strains. The natural history of severe sepsis in laboratory animals is generally distinct from its clinical manifestation. Animals more often have a rapid onset of hypodynamic circulatory collapse, unlike the hyperdynamic response seen in patients (13). Septic animals also show a more rapid resolution or onset of death, and react differently to equivalent doses of the sepsis-inducing agent.

Many of the failed attempts to transfer the findings from animal studies into the clinical arena have resulted from incorrect assumptions; for example, that LPS is present in high concentrations in the patient’s plasma (5). In addition to the question of extrapolation from animal studies, there have been difficulties in developing appropriate clinical trial designs for these complex conditions.

These significant issues surrounding the use of animals in sepsis research justify a call for an urgent reappraisal of alternative experimental approaches, which could also point the way to improved clinical therapies. This report highlights how certain non-animal methods might be used to progressively replace animal experiments and to improve understanding of sepsis.

Non-Animal Methods: Some Possibilities

The potential advantages of using non-animal methods in sepsis research include: the avoidance of animal suffering; a more economic approach to research questions, since animal experimentation can be costly; and the improved clinical relevance offered by using human material and ethical studies on humans. Table 1 compares the advantages and disadvantages of various animal and non-animal methods of studying sepsis.

Human volunteers: biomarkers, patient monitoring and genomics/proteomics

Biomarkers are characteristics which can be measured objectively and evaluated as indicators of normal biological processes, pathogenic processes or the response to therapeutic intervention (14) in human subjects.

Biomarkers identified during the course of volunteer studies, by using increasingly sophisticated techniques, can also be used to assist the diagnosis

Table 1: Comparison of various animal and non-animal approaches to researching sepsis

Model	Advantages	Disadvantages
Animal models	Whole physiology can be investigated Controlled homogeneous population Well-described model species	Substantial pain and suffering Animals are young and healthy Poor predictors of the clinical picture (species differences) Artificially induced sepsis High cost Genetically homogeneous animals unlike the human population
Human volunteers	Relevant patient population Avoid animal suffering Can identify genetic factors involved in sepsis Avoid species differences	Variety of co-morbidities Limited availability of trained staff for clinical research Difficulties in obtaining informed consent from critically ill patients or next-of-kin Clinical trials complex to organise
Cell and tissue approaches	Avoid animal suffering Avoid species differences, if human tissue used Easily controlled biological variables Economical	Lack systems complexity Extrapolation <i>in vitro</i> to <i>in vivo</i> Problems with human cells, e.g. phenotypic changes Inappropriate culture conditions
Computer and mathematical simulations	Avoid animal suffering Analysis of complex data from several systems — models of inflammation exist Highly controlled and reproducible	Incomplete datasets for sepsis Early stage of sepsis modelling

or prognosis of sepsis in patients (15) or to improve dosage and the timing of therapies. An ideal marker of sepsis should permit an early diagnosis, should potentially help to differentiate infectious from non-infectious causes of systemic inflammation, and should inform about both the course and prognosis of the syndrome.

Recently, it has been shown that procalcitonin fulfils some of these features. Russwurm and Reinhart (16) discuss how procalcitonin is more useful than C-reactive protein and pro-inflammatory cytokines in discriminating between viral and bacterial infections, and between infectious and non-infectious causes of acute respiratory distress syndrome. Its use has markedly improved the sensitivity and specificity of diagnosis of clinical sepsis (17). Nevertheless, these are still early days in the development of reliable biomarkers for understanding the biology of sepsis. The signs are encouraging, but more research is needed.

Potential exists for using a range of *in vivo* techniques to improve the monitoring of the microcirculation, of mitochondrial function, and of the metabolic *milieu* in septic patients. Examples include the use of: orthogonal polarisation spectroscopy, used in real time to follow changes in the microcirculation (18, 19); near infra-red spectroscopy, which monitors tissue oxygenation (20); tissue oxygen electrodes (21); radionuclide tracers; and microdialytic techniques which measure a variety of metabolic indices (22) and antibiotic pharma-

cokinetics at the tissue site of action (23). Techniques such as magnetic resonance spectroscopy, can be applied to obtain hitherto unavailable metabolic data from critically ill patients.

Information from the Human Genome Project and proteomics research will assist in the modelling of sepsis and other infections, especially in view of the technological advances in nucleic acid and protein analysis, coupled with increased computational power. Such approaches provide the ability to characterise, in humans, the determinants of and responses to infection and sepsis on a genome-wide scale (24).

Single nucleotide polymorphisms (SNPs) — common genetic variants associated with disease susceptibility — have been used to tease apart how individuals respond to injury and other inflammatory or infective stimuli (24). SNPs, whilst not disease-causing, are influential in determining the outcome of a disease or condition, including death from sepsis. In addition, other single base substitutions, in which the least common allele is present at a frequency of 1% or less, and also other variations, such as insertion or deletion polymorphisms, may play a role in the septic syndrome.

The strongest evidence suggesting a role for SNPs as markers of sepsis severity and outcome, comes from a report concerning patients with septic shock with a variety of aetiologies, as discussed in Cobb and O'Keefe (24). Lin and Albertson have reviewed the numerous studies that have shown a correlation between specific SNPs and sepsis (25).

In contrast to array-based gene expression experiments that are the mainstay of genomics research, the field of proteomics, which aims to measure and categorise the full array of proteins contained within a single organism, includes details of all post-translational modifications and their localisation within cells. Functional proteomics, which specifically addresses protein–protein interactions and the post-translational modifications that regulate them, and tends to focus on molecular interactions regulating signal transduction events, has been considered as potentially capable of producing significant insight into the molecular basis of sepsis (26).

Selective labels and purification approaches can be applied to look for phospho-proteins, which are either expressed or repressed during stimulation in sepsis. Signalling intermediates regulated by phosphorylation during sepsis, can be followed, so a better understanding of the clinical course of the syndrome can be characterised.

Human tissues and cells *ex vivo*

Several studies have used material such as *ex vivo* cells, tissues and other biological samples from patients, from their relatives or from healthy volunteers. For instance, researchers have investigated the role of polymorphonuclear cells in sepsis, without recourse to the use of animals, providing important insights into the disease process (27, 28).

Tissue samples obtained from patients undergoing surgical procedures such as resection or transplantation, could be incubated with mediators to derive mechanistic data on individual tissue responses, which could provide clues about the septic process. *In vitro* models are being developed to examine the interactions of *in vivo*-stimulated or *ex vivo*-stimulated polymorphonuclear cells on endothelial monolayers (28), and two-chamber models can be used to assess the influence of released mediators on the inflammatory process (29).

Blood samples from septic patients, which are available during normal treatment and investigation, can be analysed for the levels and activities of circulating cytokines, hormones and other factors involved in the development of multiple organ failure. Post-mortem tissue from both previously healthy and septic patients, can provide insight into pathological processes.

Cell culture approaches

By using serum from septic patients, it is possible to assess the effects of factors such as cytokines on primary cultures and human cell lines, and hence to derive valuable mechanistic data (30). Methods are being developed that allow for three-dimensional tissue culture, in which two or more cell types are

grown together and can interact with one another (Figure 1). Several types of flow-based cell culture chambers are commercially available, which facilitate the control of oxygen tension (important in sepsis) and the establishment of dynamic culture conditions, thereby promoting cell polarisation and vectorial transport functions that more closely resemble the conditions in the human body.

Immortalised endothelial cell lines maintain many of the adhesion molecules expressed by freshly isolated human endothelial cells. Such cell lines lend themselves to experimental studies of leucocyte-binding, trans-endothelial migration and cytotoxicity, all of which are important in sepsis. Advances in stem cell research may aid understanding of some of the mechanisms of sepsis.

The immortalisation of human cells can result in phenotypic changes which alter cellular responses to particular stimuli. However, this can be overcome, as illustrated by the use of vectors expressing telomerase, which produce immortalised cells which more closely resemble the primary cells from which they were derived (31). Cells are often grown in non-physiological culture media, at unphysiologically high oxygen tensions, or as artificially static monocultures, but there are also solutions to these potential problems.

Many such cellular approaches could benefit from earmarked funding in order to fully develop their possible utility and potential in the replacement of animal models of sepsis.

Computer simulations

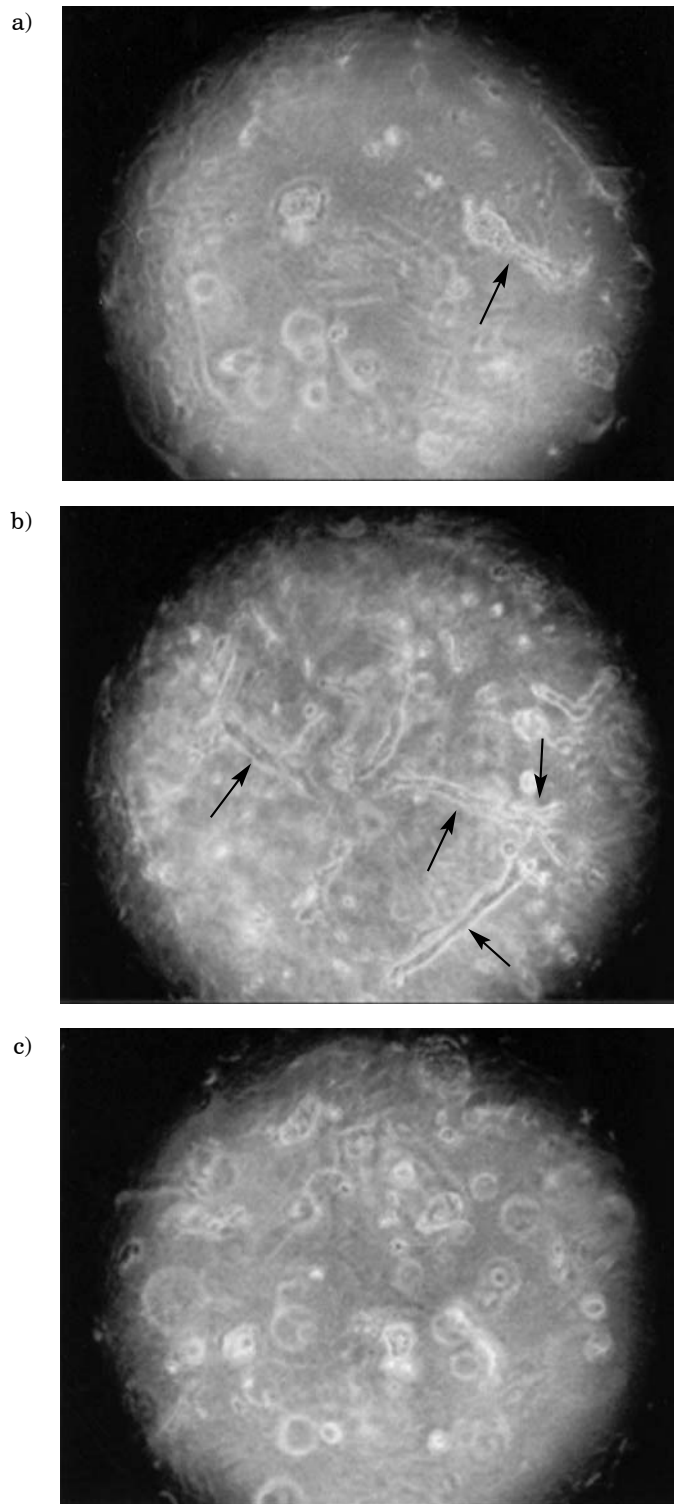
There is increasing scope for the use of well-constructed computer models of physiological systems; of patient populations for an improved understanding of the epidemiology and susceptibility to sepsis; and for elucidation of appropriate therapeutic interventions. In the USA, Immunetrics, with support from the National Institutes of Health, is developing *in silico* software tools to model sepsis and to improve clinical trial design (32; see also, website www.immunetrics.com).

Mathematical and computational models provide a very powerful set of tools for studies on complex phenomena such as inflammation. By using data held by pharmaceutical companies and by ICUs, physiological and biochemical simulations of the body's response to infections (33) and to therapeutic interventions (34) could be derived.

Replacing Animals: Overcoming the Barriers

A variety of non-animal methods could be developed and used more extensively by the sepsis research community, in order to move away from reliance on

Figure 1: Human proximal tubule epithelial cells (PTECs) cast into collagen gels form a novel three-dimensional model for studying the pathophysiology of renal failure in sepsis



The effects of nitric oxide synthase-inhibition on hepatocyte growth factor-induced tubulogenesis.

a) untreated PTECs; b) PTECs treated with hepatocyte growth factor form tubules (arrows) resembling proximal tubules in vivo; c) PTECs treated with hepatocyte growth factor and L-NIL, a nitric oxide synthase-inhibitor, which inhibits tubule formation.

Credit: Professor T.J. Evans, University of Glasgow.

experimental animals and thereby to better characterise and treat sepsis. However, various barriers need to be overcome.

Clinical studies have the potential to replace certain animal-based methods, as described above, but are sometimes limited by the availability of appropriate patients. This is particularly relevant to hospitals with small ICUs, and with few skilled clinical and supportive research staff. For detecting clinically and statistically significant differences in outcome to interventions, large groups of patients could be provided by fostering greater collaborative links between clinical and non-clinical researchers, and by conducting non-industry-funded multi-centre trials, such as those pioneered in Australasia and Canada.

Most critically-ill patients are incapable of giving informed consent to participate in a study. Depending on the country, region, or sometimes the local ethics committee, there will be a requirement to obtain next-of-kin consent or even judicial approval. In England and Wales, the consent of relatives has no legal validity, so patient agreement must be sought. Increasingly, retrospective consent is sought from patients, should they regain mental competency. A number of recent studies have highlighted the problems associated with obtaining consent from next-of-kin sufficiently quickly to initiate innovative therapy in septic shock (35, 36).

The UK *Human Tissue Act* is designed to control the removal of organs and tissues *post-mortem* and during surgical procedures. It may limit the scope for research with human tissue, with a consequent impact on various alternatives to the use of animals. The European Directive on Human Tissues and Cells and the European Directive on Clinical Trials potentially compound these difficulties, by introducing additional regulatory and ethical constraints that would impede clinical and *in vitro* research on sepsis.

The role of the pharmaceutical companies is paramount in the sharing of data from clinical research (not only on sepsis) with the wider research community. Details meticulously collected and validated from thousands of patients enrolled into numerous sepsis trials are currently sitting in secure databases, unavailable for external use. The industry should be strongly encouraged, if not mandated, to release data into the public domain. Patient information from ICUs could also be used to derive mathematical and computer models, leading to a potential reduction in animal use, but also to the improvement of clinical trials on novel agents.

Public attitudes can also be a barrier to the non-therapeutic use of patient material for clinical research. The public are keen on the replacement of animal experiments, and an educational drive could highlight the double advantage — emphasising the rationale for using human cells and tissues in clinical

and pathological investigations, and reducing reliance on the use of experimental animals. Additionally, links with the national transplantation programme, as pioneered by the UK Human Tissue Bank, would facilitate the provision of suitable clinical material.

Funding for the replacement of animal experiments in medical research, including sepsis, has been relatively limited. Many of the approaches discussed above, particularly those showing encouraging signs of clinical relevance, would greatly benefit from specifically directed funding in order for their full potential to be realised. It is encouraging that the Medical Research Council and the Biotechnology and Biological Sciences Research Council now provide funds specifically for developing alternatives to animal experimentation. The new UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) should also make this a priority area.

Sepsis is not only a significant cause of mortality, but is a complex condition which has not been reliably modelled in non-human species. The coming together of a number of novel, non-animal methods could address different aspects of the syndrome and yield clues of direct utility to clinical treatment.

Recommendations

1. More emphasis should be placed on the use of human material obtained from healthy volunteers and patients with sepsis. When combined with cell and tissue culture procedures, especially using co-culture and three-dimensional approaches, these could provide important mechanistic data.
2. There should be a public outreach programme to stress the importance of using human biological material, with full consent, from critically ill patients or *post-mortem*. This use could have a major impact on future patient care and could also reduce animal use — both of which are of widespread public concern.
3. The sepsis research community should continue to inform policy-makers and the public of the value of having secure, ethical access to human biological material for research purposes.
4. Further encouragement should be given to large multi-centre clinical trials on new therapeutic interventions in sepsis that are reported in full. Pharmaceutical companies and ICUs should make data relating to sepsis and other inflammatory conditions widely available, subject to the preservation of patient anonymity.

5. Better collaboration is needed between clinical researchers, cell biologists, pharmaceutical companies and those from the physical sciences, to build powerful and reliable computer models of the course and possible outcomes of sepsis.
 6. Funders supporting sepsis research should earmark sums for the development of non-animal methods to further the understanding and treatment of sepsis.
 7. The new UK National Centre for the Replacement, Refinement and Reduction of Animals in Research should address sepsis research and alternatives to animal use as a priority.
 8. There should be a critical and objective review of the value of animal models of sepsis.
 9. The research community should consider how the replacement of animals might best be undertaken in various areas of sepsis research.
 10. Journal editors should seek, from authors submitting papers for publication, a detailed justification for using animal models, and why appropriate alternative approaches, capable of yielding robust and reliable data, were not used.
 11. Where animal use is currently considered unavoidable, methodologies need to be agreed by the sepsis research community, in order to reduce variations in experimental protocols and hence reduce the need for unnecessary replication. The sharing of experimental data, derived from maximising the use of each animal, providing that this did not entail increasing the severity of the procedures applied, should be encouraged by funders, policy-makers and journal editors.
- Ireland. *Critical Care Medicine* **31**, 2332–2338.
 2. Angus, D.C., Linde-Zwirble, W.T., Lidicker, J., Clermont, G., Carcillo, J. & Pinsky, M.R. (2001). Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Critical Care Medicine* **29**, 1303–1310.
 3. Martin, G.S., Mannino, D.M., Eaton, S. & Moss, M. (2003). The epidemiology of sepsis in the United States from 1979 through 2000. *New England Journal of Medicine* **348**, 1546–1554.
 4. Sessler, C.N. & Shepherd, W. (2002). New concepts in sepsis. *Current Opinion in Critical Care* **8**, 465–472.
 5. Riedemann, N.C., Guo, R.F. & Ward, P.A. (2003). The enigma of sepsis. *Journal of Clinical Investigation* **112**, 460–467.
 6. Redl, H., Schlag, G., Bahrami, S. & Yao, Y.M. (1996). Animal models as the basis of pharmacological intervention in trauma and sepsis patients. *World Journal of Surgery* **20**, 487–492.
 7. Ward, P.A. (2004). The dark side of C5a in sepsis. *Nature Reviews: Immunology* **4**, 133–142.
 8. Hotchkiss, R.S. & Karl, I.E. (2003). The pathophysiology and treatment of sepsis. *New England Journal of Medicine* **348**, 138–150.
 9. Riedemann, N.C., Guo, R.F. & Ward, P.A. (2003). Novel strategies for the treatment of sepsis. *Nature Medicine* **9**, 517–524.
 10. Deitch, E.A. (1998). Animal models of sepsis and shock: a review and lessons learnt. *Shock* **9**, 1–11.
 11. Anon. (1986). *The Animals (Scientific Procedures) Act 1986*. 24pp. London, UK: HMSO.
 12. Anon. (1986). Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Official Journal of the European Communities* **358**, 1–29.
 13. American College of Chest Physicians (1997). Executive summary. From bench to the bedside: the future of sepsis research. National Institute of Allergy and Infectious Disease and National Heart, Lung and Blood Institute Workshop. *Chest* **111**, 744–753.
 14. Lewin, D.A. & Weiner, M.P. (2004). Molecular biomarkers in drug development. *Drug Discovery Today* **9**, 976–983.
 15. Calvano, S.E., Thompson, W.A., Marra, M.N., Coyle, S.M., de Riesthal, H.F., Trousdale, R.K., Barie, P.S., Scott, R.W., Moldawer, L.L. & Lowry, S.F. (1994). Changes in polymorphonuclear leukocyte surface and plasma bactericidal/permeability-increasing protein and plasma lipopolysaccharide-binding protein during endotoxemia or sepsis. *Archives of Surgery* **129**, 220–226.
 16. Russwurm, S. & Reinhart, K. (2004). Procalcitonin mode of action: New pieces in a complex puzzle. *Critical Care Medicine* **32**, 1801–1802.
 17. Harbarth, S., Holeckova, K., Froidevaux, C., Pittet, D., Ricou, B., Grau, G.E., Vadas, L. & Pugin, J. (2001). Diagnostic value of procalcitonin, interleukin-6, and interleukin-8 in critically ill patients admitted with suspected sepsis. Geneva Sepsis Network. *American Journal of Respiratory and Critical Care Medicine* **164**, 396–402.
 18. De Backer, D. (2003). OPS Techniques. *Minerva anesthesiologica* **69**, 388–391.
 19. De Backer, D., Creteur, J., Preiser, J.C., Dubois, M.J. & Vincent, J.L. (2002). Microvascular blood flow is

Acknowledgements

We wish to thank the Wellcome Trust for their generous hospitality and support, and Dr Gill Langley for helping to organise the meeting and for contributing to this report. Professor T.W. Evans of Imperial College School of Medicine, also made helpful comments in advance of the Workshop.

References

1. Padkin, A., Goldfrad, C., Brady, A.R., Young, D., Black, N. & Rowan, K. (2003). Epidemiology of severe sepsis occurring in the first 24 hours in intensive care units in England, Wales and Northern

- altered in patients with sepsis. *American Journal of Respiratory and Critical Care Medicine* **166**, 1–2.
20. Giradis, M., Rinaldi, L., Busani, S., Flore, I., Mauro, S. & Pasetto, A. (2003). Muscle perfusion and oxygen consumption by near-infrared spectroscopy in septic-shock and non-septic-shock patients. *Intensive Care Medicine* **29**, 1173–1176.
 21. Sair, M., Etherington, P.J., Winlove, C. & Evans, T.W. (2001). Tissue oxygenation and perfusion in patients with systemic sepsis. *Critical Care Medicine* **29**, 1343–1349.
 22. Martinez, A., Chioloro, R., Bollman, M., Revelly, J.P., Berger, M., Cayeux, C. & Tappy, L. (2003). Assessment of adipose tissue metabolism by means of subcutaneous microdialysis in patients with sepsis or circulatory failure. *Clinical Physiology and Functional Imaging* **23**, 286–292.
 23. Tomaselli, F., Maier, A. & Smolle-Juttner, F.M. (2003). Pharmacokinetics of antibiotics in inflamed and healthy lung tissue. *Wiener medizinische Wochenschrift* **153**, 342–344.
 24. Cobb, J.P. & O'Keefe, G.E. (2004). Injury research in the genomic era. *The Lancet* **363**, 2076–2083.
 25. Lin, M.T. & Albertson, T.E. (2004). Genomic polymorphisms in sepsis. *Critical Care Medicine* **32**, 569–579.
 26. Nguyen, A. & Yaffe, M.B. (2003). Proteomics and systems biology approaches to signal transduction in sepsis. *Critical Care Medicine* **31**, Suppl. S1–6.
 27. Chen, X. & Christou, N.V. (1998). Protective effect of plasma in polymorphonuclear neutrophil-mediated cytotoxicity of endothelial cells in the systemic inflammatory response syndrome. *Journal of Leukocyte Biology* **63**, 68–74.
 28. Brown, K.A., Lewis, S.M., Hill, T.A., Macey, M.G., McCarthy, D.A., Grant, V.A. & Treacher, D.F. (2001). Leucodepletion and the interaction of polymorphonuclear cells with endothelium in systemic inflammatory response syndrome. *Perfusion* **16**, Suppl. 75–83.
 29. Sendt, W., Wolff-Vorbeck, G., Leipziger, J., von Specht, B.U. & Schoffel, U. (2000). *In vitro* peritonitis: basic inflammatory reactions in a two-chamber co-culture model of human peritoneum. *International Journal of Colorectal Disease* **15**, 229–235.
 30. Boulos, M., Astiz, M.E., Barua, R.S. & Osman, M. (2003). Impaired mitochondrial function induced by serum from septic shock patients is attenuated by inhibition of nitric oxide synthase and poly(ADP-ribose) synthase. *Critical Care Medicine* **31**, 352–358.
 31. Morales, C.P., Gandia, K.G., Ramirez, R.D., Wright, W.E., Shay, J.W. & Spechler, S.J. (2003). Characterisation of telomerase immortalised normal human oesophageal squamous cells. *Gut* **52**, 327–333.
 32. Clermont, G., Bartels, J., Kumar, R., Constantine, G., Vodovotz, Y. & Chow, C. (2004). *In silico* design of clinical trials: a method coming of age. *Critical Care Medicine* **32**, 2061–2070.
 33. An, G. (2001). Agent-based computer simulation and SIRS: building a bridge between basic science and clinical trials. *Shock* **16**, 266–273.
 34. Lamy, B., Roy, P., Carret, G., Flandrois, J.P. & Delignette-Muller, M.L. (2002). What is the relevance of obtaining multiple blood samples for culture? A comprehensive model to optimise the strategy for diagnosing bacteremia. *Clinical Infectious Diseases* **35**, 842–850.
 35. Annane, D., Outin, H., Fisch, C. & Bellissant, E. (2004). The effect of waiving consent on enrolment in a sepsis trial. *Intensive Care Medicine* **30**, 321–324.
 36. Corwin, H.L., Gettinger, A., Pearl, R.G., Fink, M.P., Levy, M.M., Shapiro, M.J., Corwin, M.J. & Colton, T. (2002). Efficacy of recombinant human erythropoietin in critically ill patients. A randomised controlled trial. EPO Critical Care Trials Group. *Journal of the American Medical Association* **288**, 2827–2835.