RAPID, SIMPLE, AND SENSITIVE BLOOD BIOCOMPATIBILITY TESTS WITH THE LIGHT-EMITTING PROTEIN PHOLASIN®

Jan Knight

jan@knightscientific.com
www.knightscientific.com

Proceedings of the TechMed Medical Device conference, 10-11 June 1999 Frankfurt, Germany

20-1 to 20-17

Proceedings published by Advantar Communications, UK

RAPID, SIMPLE, AND SENSITIVE BLOOD BIOCOMPATIBILITY TESTS WITH THE LIGHT-EMITTING PROTEIN PHOLASIN®

Jan Knight

Knight Scientific Limited, 15 Wolseley Close, Wolseley Business Park, PLYMOUTH, PL2 3BY UK

Background

The many advances in medical procedures have fed the multi-billion dollar, and growing, medical device industry. The industry itself is dependent upon the provision of biocompatible materials for use in its devices. Such materials do not elicit an adverse reaction when they come into contact with biological systems, both within and outside the body. The world-wide market for artificial, biocompatible materials has been estimated at $1.5 billion and is still growing[1].

Materials and the design process

In the normal course of the design process, an engineer will select from a handbook of quantitative information a range of materials that satisfy the specifications. If he is required, however, to design a *medical* device, especially one that comes into contact with blood, he will not find any table of blood-compatible materials. He will instead find a list of types of materials that are currently being viewed as promising with respect to biocompatibility. The list will include materials that are hydrophilic, hydrophobic, carry charges or can become variously charged at different values of pH or are electrically neutral, are either rigid or flexible. Such a list offers very few clues about design principles[2]. The reason for this is that a particular material when in contact with a biological system can elicit anything from no response to a potentially fatal response. And while it is now possible to identify certain materials that more frequently elicit adverse biological reactions and should therefore be avoided, a universally biocompatible material, if ever found, would need to be evaluated and compared to existing materials. This evaluation and subsequent comparison requires appropriate test methods. There is at present a lack of consistent assessment within and between laboratories and as a result potentially good materials may be overlooked or discredited prematurely.

What is biocompatibility?

It used to be accepted that biocompatibility was equated with inertness and if you could achieve complete chemical stability you would have a universally biocompatible material. That view no longer prevails. Interpreting the characteristics of biocompatibility under one set of conditions and projecting those characteristics onto another set of conditions is likely to lead to disaster. The problems derive at least in part from the way in which individual blood cells interact with and respond to foreign material.

While there are of course tests that are used to measure different aspects of biocompatibility, at present there are no really fast, reliable, screening techniques that would allow a new material to be assessed for its suitability in a particular application or

even in a particular individual. A perfectly acceptable material for one individual could be potentially deadly to someone else. There are no good standardised methods for assessing batch to batch variation in relation to individual responses. As a result the market is suffering and the introduction and exploitation of new materials is being thwarted.

There are also certain specific problems with the development of biomaterials suitable for a number of specific clinical fields. Problems have developed due to defects in materials used for the urinary tract and bladder. There are difficulties associated with the much needed development of small diameter, synthetic vascular graft materials for cardiovascular surgery. There are problems associated with extracorporeal circulation of blood during renal dialysis, oxygenation during cardiopulmonary bypass surgery and other procedures in which blood is circulated through or around membranes and other materials. The need for reliable, biocompatible materials will increase with an ever-ageing population. But until the industry has good standardised methods effectively to compare materials for biocompatibility there will be a reluctance to test new materials in difficult clinical settings.

International Standards

Medical devices are regulated in order to protect patients and users from devices that are not safe. The challenge is to define what is safe and what is not.

All medical devices must have a CE mark before they can be sold in Europe. To obtain a CE mark manufacturers must assign a risk class to each device and follow other safety and performance requirements defined by the Medical Device Directives. The finished device and the materials from which it is made must be tested for biocompatibility.

Biocompatible materials do not cause:

- direct injury to living tissues
- adverse immunological responses
- other adverse systemic effects
- delayed adverse effects

In approaching the regulation of medical devices the European Community chose not to follow the regulation of drugs, where many specified detailed testing requirements have led to heavy and inflexible legislation. The European Community's approach, in the recently introduced legislation concerning medical devices, was to have only general requirements within the legislation, the so-called *Essential Requirements*, leaving the mandated standards to provide details.

Particularly in the field of Biological Safety, the work on the ISO standard 10993 was to a large extent motivated by the fears that the specific European standards would create new barriers to trade. In the light of these concerns there was a general conviction that the opportunity should be taken to harmonise the requirements internationally. An underlying objective of ISO/Technical Committee (TC) 194 was to achieve a high level of safety for medical devices internationally.

Biocompatibility Tests

In order to avoid potentially fatal effects of incompatible materials there is an urgent need to have reliable and reproducible test methods to evaluate biocompatibility of materials and devices. There are many commercially available assays which measure particular factors, but deciding which factors are more important to measure than others under different conditions is not that easy. Such considerations always lead to doubt. Will, for example, the elimination of one or more tests lead to inappropriate conclusions?

ISO 10993 (ISO/TC 194) Biological Testing Of Medical Devices provide details of the way tests are to be conducted when long-standing methods, such as those described in the US Pharmacopoeia, are available.

Categories in which detailed methods are provided include:

- cytotoxicity
- haemolysis
- muscle implants
- irritation
- sensitisation tests

However standard methods do not exist to address all categories of biological effects. Those not well covered include:

- haemocompatibility
- genotoxicity
- sub-chronic and chronic toxicity
- sub-chronic and chronic carcinogenicity

ABEL® Blood Biocompatibility Tests

The ABEL®[3] blood biocompatibility tests, based on the light-emitting protein Pholasin®, are directed at the needs of haemocompatibility testing, in particular immunological reactions involving white blood cells (leucocytes). They can also be used for *in vitro* testing of materials to assess the potential of materials used in medical devices to elicit adverse reactions in individuals. The tests may also provide a means of assessing the onset of peritonitis, which can be a side effect of ambulatory peritoneal dialysis, by measuring superoxide production in macrophages[4] from waste dialysis fluid.

The tests which use only 1μL of whole blood or a few thousand isolated cells are being evaluated for:

- rapid screening of new materials
- assessing coated materials for improved biocompatibility
- monitoring patients during surgery
- monitoring patients during dialysis
- determining individual immunological reactions of blood to devices prior to exposure

- monitoring side effects after long-term exposure
- evaluating individual abnormalities as a measure of potential risk

Pholasin® and its Reactions

Pholasin®[5] is the photoprotein of the marine, rock-boring bioluminescent mollusc, *Pholas dactylus*, the Common Piddock[6]. Pholasin® does not glow on its own but needs to be switched on. Free radicals [7] and other reactive oxygen species produced by activated leucocytes (and other types of cell) turn on the light. It is an ultrasensitive detector of activated leucocytes[8].

Pholasin® reacts with the following products of cells[9]:

- the oxygen free radicals:
  - superoxide anion,
  - hydroxyl radical and/or ferryl radical [10],
  - nitric oxide[11]
- and the oxidants:
  - hypochlorous acid: derived from myeloperoxidase
  - n-chlorotaurine: derived from hypochlorous acid
  - hypobromous acid: derived from eosinophil peroxidase
  - bromamine: derived from hyprobromous acid
  - peroxynitrite[12]: derived from reactions between superoxide and nitric oxide

Leucocytes on activation produce free radicals and degranulate, releasing damaging granule enzymes. As Pholasin® also reacts with peroxidases such as myeloperoxidase (from granules in neutrophils) and bromoperoxidase (from eosinophils) an understanding of the nature of these light emitting reactions[13] has led to the use of Pholasin® in quantifying degranulation[14,15], a phenomenon of especial relevance to biocompatibility testing.

Background Science

The types of leucocyte: neutrophils (also called polymorphonuclear leucocytes), eosinophils and monocytes that are capable of producing superoxide (oxygen with an extra electron) and of undergoing the respiratory burst[16], do so only after the NADPH oxidase system has become activated.

The NADPH oxidase is made up of a flavoprotein and a cytochrome (now called a flavocytochrome) in the membrane of either the whole cell or of the granules contained within the cytoplasm of the cell. For the NADPH oxidase to become operational a series of events must occur within the cytosol in which five other proteins become activated in a cascade of events leading eventually to the activation of the membrane-bound flavocytochrome. Superoxide is produced again by a series of steps one of which involves the transfer by the flavoprotein of an electron from NADPH to a cytochrome β subunit and the direct reduction by the cytochrome of oxygen to superoxide[17].

When the NADPH oxidase of the plasma membrane is activated, superoxide is secreted to the outside of the cell. When the oxidase of the membranes of secondary

granules is activated, the superoxide may also be secreted outside the cell if, on degranulation, the granules fuse with the plasma membrane. However, the superoxide derived from the secondary granule NADPH oxidase may also be secreted into vacuoles within the cell where consumption of superoxide by myeloperoxidase is possible. It is assumed that these intracellular vacuoles eventually fuse with the plasma membrane and release their contents.

Stimulation of Cells

The NADPH oxidase system of leucocytes circulating in the peripheral blood is very seldom activated. It is usually when the cells leave the circulation and migrate to organs such as the lungs or kidneys or a site of inflammation (such as the foreign implanted material) that they become activated and cause harm. It is therefore the object of the biocompatibility tests to assess how normal looking leucocytes will behave when activated.

In the ABEL® biocompatibility assays, the leucocytes (neutrophils and monocytes) are artificially stimulated *in vitro*.

Two stimulants: fMLP (n-formyl-methionyl-leucyl-phenylalanine) and PMA (phorbol-12-myristate-13- acetate) are used and tests may be carried out on samples of blood in which either heparin or EDTA is used as anticoagulant.

The substances fMLP and PMA stimulate the initiation within the human neutrophil of a series of events leading to the activation of the NADPH oxidase system. This results in the respiratory burst and concomitant production of superoxide. Superoxide, released extracellularly, is initially detected by Pholasin®; later light producing reactions involve degranulation of myeloperoxidase and possible lactoferrin with possibly secondary production of other free radicals.

Activation with fMLP is through a receptor protein-tyrosine kinase and is calcium dependent. PMA, in contrast to fMLP, is not dependent upon calcium and migrates through the cell membrane to activate protein kinase C directly. This route to the activation of the NADPH oxidase bypasses the receptor interaction and the initial stages of the integrated signal transduction pathway.

There is a lag time between the presentation of the stimulus and the start of the respiratory burst. This lag reflects the complex series of integrated signal transduction events that take place during activation. Both the duration of the lag phase preceding the onset of the respiratory burst and the duration and magnitude of activation, are determined by the particular stimulus, or combination of stimuli, as well as by the pre-activation status of the cell (the degree to which it has been primed).

Assay Protocols for Blood Biocompatibility Tests

Superoxide and other reactive oxygen species (ROS) are measured with the commercially available ABEL® Whole Blood and ABEL® Isolated Cell test kits (Knight Scientific Limited, Plymouth, UK)[18]. These are chemiluminescent assay kits for measuring activation of leucocytes (predominantly neutrophils) from whole blood or leucocytes isolated from blood or grown in culture. The whole blood test kit contains: Pholasin, Adjuvant-P™, phorbol-12-myristate-13-acetate (PMA), assay buffer (HBSS with 20 mmol/L HEPES, pH 7.4) and blood dilution buffer (HBSS without calcium and magnesium but with 20m mol/L HEPES, pH 7.4). Adjuvant-P™, an enhancer of

Pholasin®, is not required when isolated cells are used. The reagents are reconstituted with assay buffer to obtain stock solutions of: Pholasin (10µg/mL) Adjuvant-P™ (10 enhancement units/mL) PMA (8 µmol/L) and fMLP (10 µmol/L)

Procedure

- 1:100 dilutions are made in 1mL of blood dilution buffer; 100µL of diluted blood is used in each test luminometer cuvette
- The cuvettes contain: 100µL diluted blood, 450µL assay buffer, 250µL Pholasin® (2.5µg), 100 µL Adjuvant-P™ (1 enhancement unit);
- They are mixed and then incubated for 5 minutes at 37^{o}C
- Light is then measured every second for 90 seconds. (The light readings are the accumulated light detected over each second and expressed as mV).
- At 90s the stimulants are added
- Light is measured for a further 5-8 minutes

Leucocytes will Respond to PMA After Stimulation with fMLP

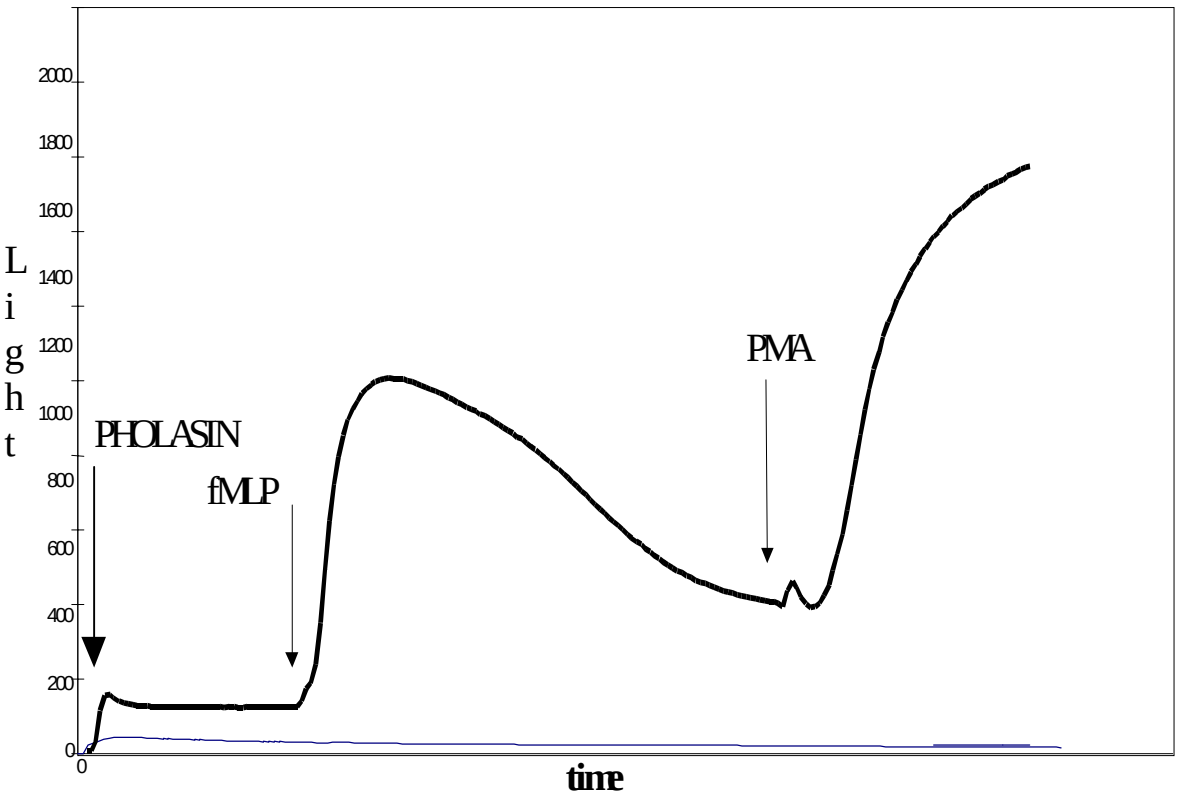
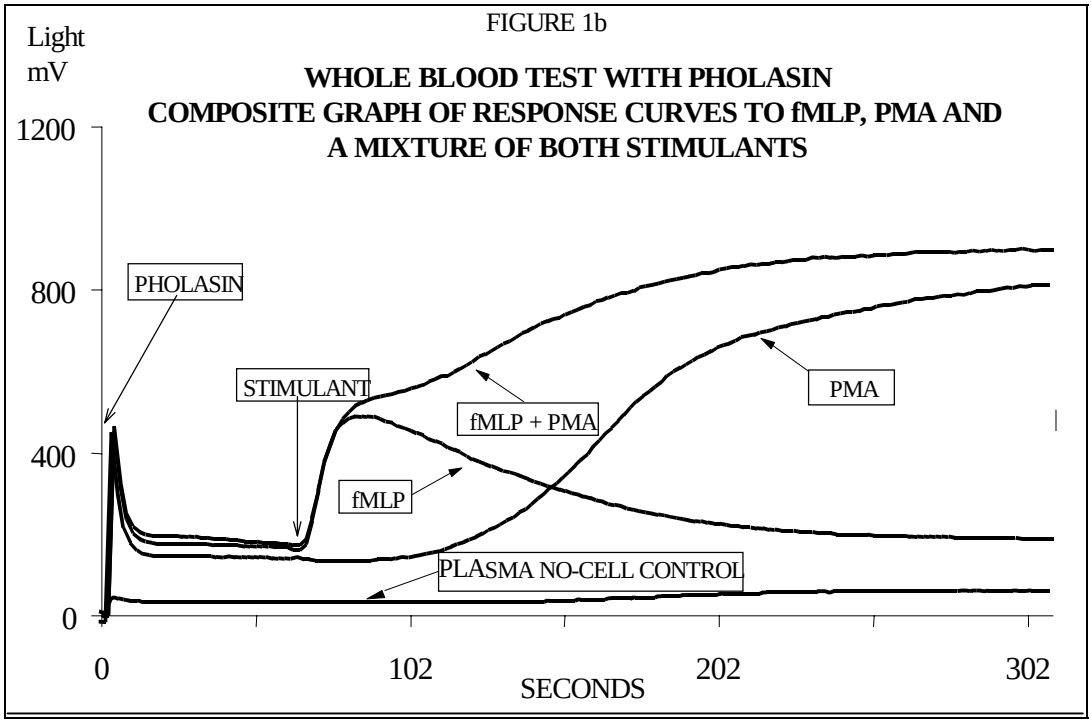


Figure 1a

A shown in Figure 1a, cells activated by fMLP can again respond to stimulation with PMA. It is seen in Figure 1b that responses to fMLP and PMA (in normal cells) are additive and both stimulants can work simultaneously.

FIGURE 1b



Typical Light Response Curve of Normal Blood

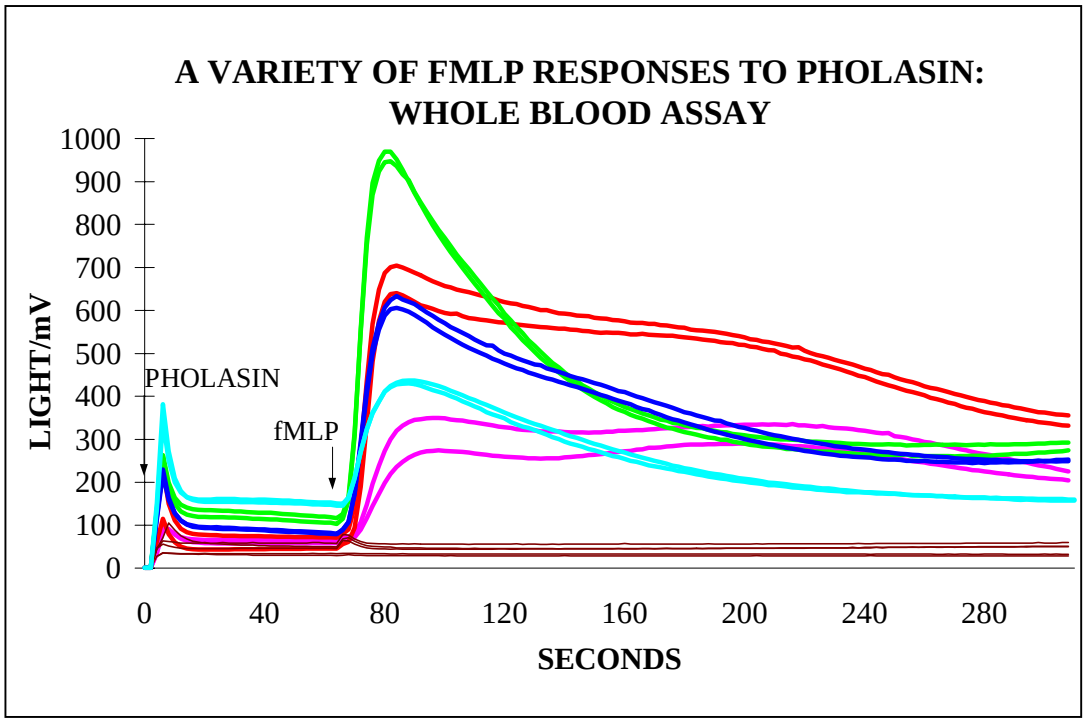
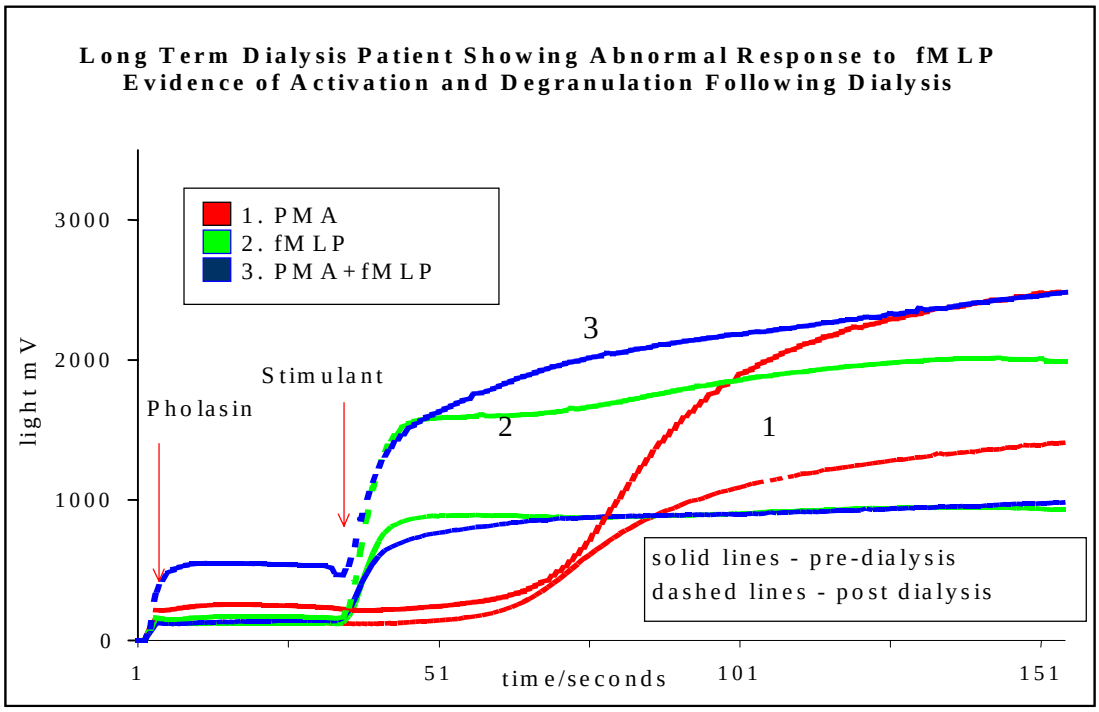


Figure 2

Abnormal Responses to fMLP

A typical normal response to fMLP is for superoxide to be detected within 5 seconds, peak at about 90 seconds and then gradually decay to baseline. However (Figure 2) some individuals show abnormal responses to fMLP and these people may be at greater risk of complications when their blood is exposed to a foreign material .

Figure 3



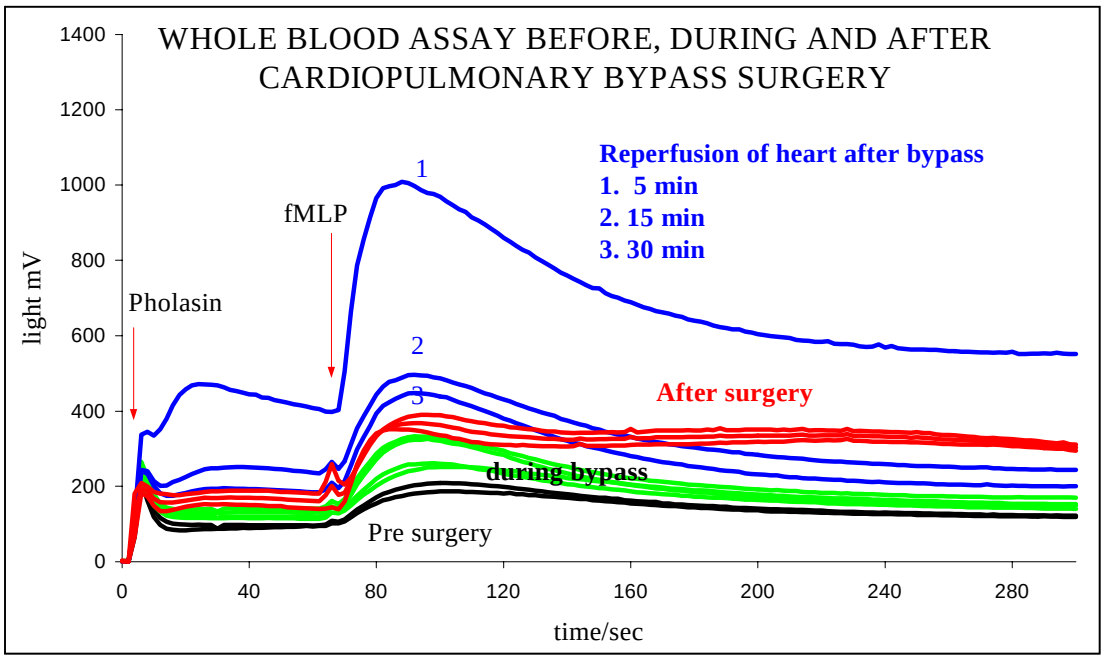
We have found that a number of patients who have been on haemodialysis for many years (>10) have abnormal neutrophils, that is their neutrophils respond to fMLP in a way similar to that seen using a combination of fMLP and PMA. It is hypothesised that PMA may act on the NADPH oxidase of the secondary granules as well as of the plasma membrane and may promote degranulation of myeloperoxidase-containing primary granules[19] whereas fMLP, in normal cells, is more likely to be confined to the NADPH oxidase of the plasma membrane and to a lesser extent secondary granules.

The results from all these experiments can be expressed as the peak value of the luminescence or as the integral of light energy emitted over a specified time.

Exposure of Blood or Isolated Leucocytes to Foreign Material

Exposure of blood to different kinds of foreign material can lead to changes in the way the free radical generating system (the NADPH oxidase) responds to receptor stimuli such as fMLP or agonists such as PMA. The identification of these changed states is the basis of the ABEL® biocompatibility tests with Pholasin®.

Figure 4



In figure 4 above an abnormal response was seen in a patient undergoing cardiopulmonary bypass surgery at the time of reperfusion. This abnormality was manifest in two ways: i) as a pre-stimulus light signal and ii) as a very rapid intense response, suggesting priming of cells. The patient was followed for three further days following surgery. During this recovery stage the cells continued to respond to fMLP in an abnormal way, that is with a continual light emission. This patient developed a number of complications described by the cardiologist as attributable to an auto-immune reaction.

What Are Possible Effect of Exposure of Blood to Foreign Material?

The leucocytes may become activated and produce free radicals and release destructive enzymes.

The newly emerged neutrophils and monocytes can migrate to the lungs, liver, spleen and kidneys where they can continue to produce free radicals and degranulate further, releasing a whole battery of destructive enzymes. These accumulating leucocytes create sites of inflammation to which more and more cells migrate, following gradient trails of chemicals (chemotaxis). The result of this increased inflammatory activity can be to damage or destroy the organs, reduce blood pressure and subsequent cardiac output, and set into motion a series of events leading to adult respiratory distress syndrome and multi-organ failure. And while these occurrences are frequently associated with septicaemia or toxic shock, the same effects can occur in the absence of sepsis as a result of exposure to bio-incompatible material.

Sometimes, however, the effect of exposure is insidious with cells having changed their ability to respond to stimuli but not actually responding at the time (priming). This effect can lead to a variety of types of delayed reactions, sometimes enhanced and sometimes depressed. Both abnormal responses can be potentially damaging if they affect the cells' normal ability to respond to invading micro-organisms. It is therefore desirable to be able to monitor **subtle** changes in the white blood cells which is one of the uses for which the ABEL® whole blood test for biocompatibility monitoring is being evaluated.

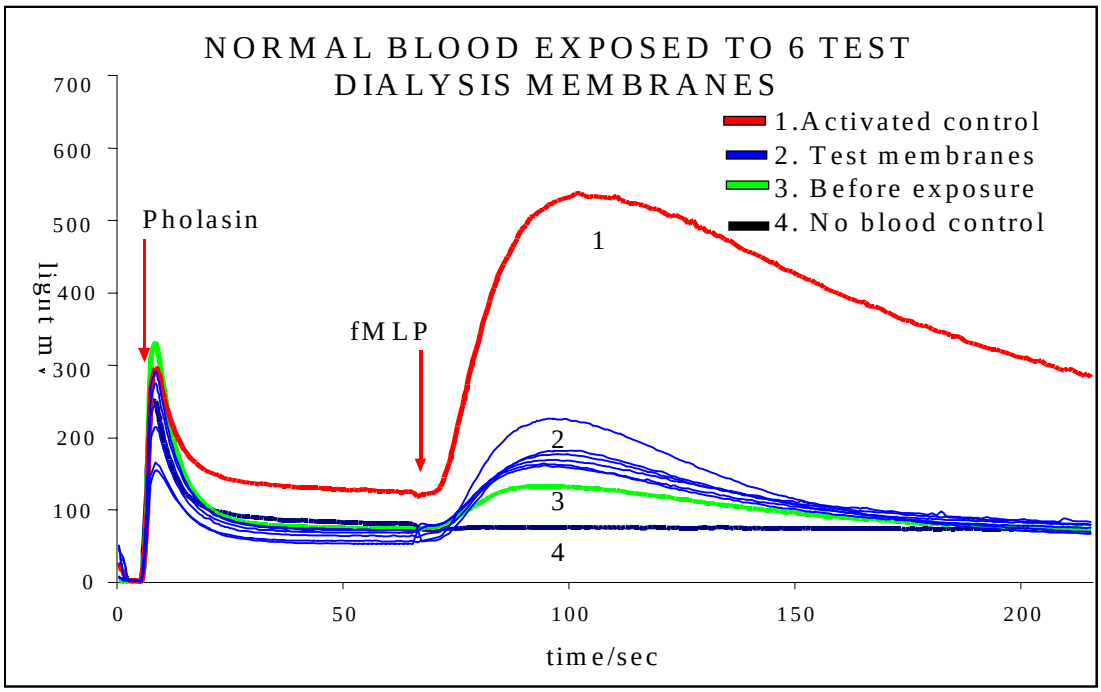
How does the ABEL® test work and what does it measure?

The test is based on the assumption that exposure of blood to bio-incompatible materials can be detected and identified by the way the neutrophils, monocytes and macrophages respond, in vitro, when given appropriate stimuli.

The test relies on the ability of Pholasin® to produce light in the presence of free radicals, some oxidants and certain enzymes. Light is measured, in the presence of Pholasin®, before and after stimulation. The measurement before stimulation indicates whether the cells are already activated to produce free radicals or have degranulated. The measurements following stimulation give information regarding any changes in the way the cells respond as a result of their prior exposure to a biomaterial used, for example, during renal dialysis or cardiopulmonary bypass surgery.

Example: Exposure of Blood to Different Test Dialysis Filters in an *ex vivo* System

The experiment consisted of carrying out dialysis on six test hollow fibre filters, using one donor, but six individual pumps. Results shown here were taken before dialysis and after 60 minutes. In addition two controls were run: i).a negative control in which a sample of blood was not exposed to the material but incubated at 30°C and ii) a positive



control in which a sample of blood was exposed to a complement activating lipopolysaccharide (LPS). The experiment was run on two occasions with identical filters but with different blood donors. The results are presented in Figures 5a and 5b.

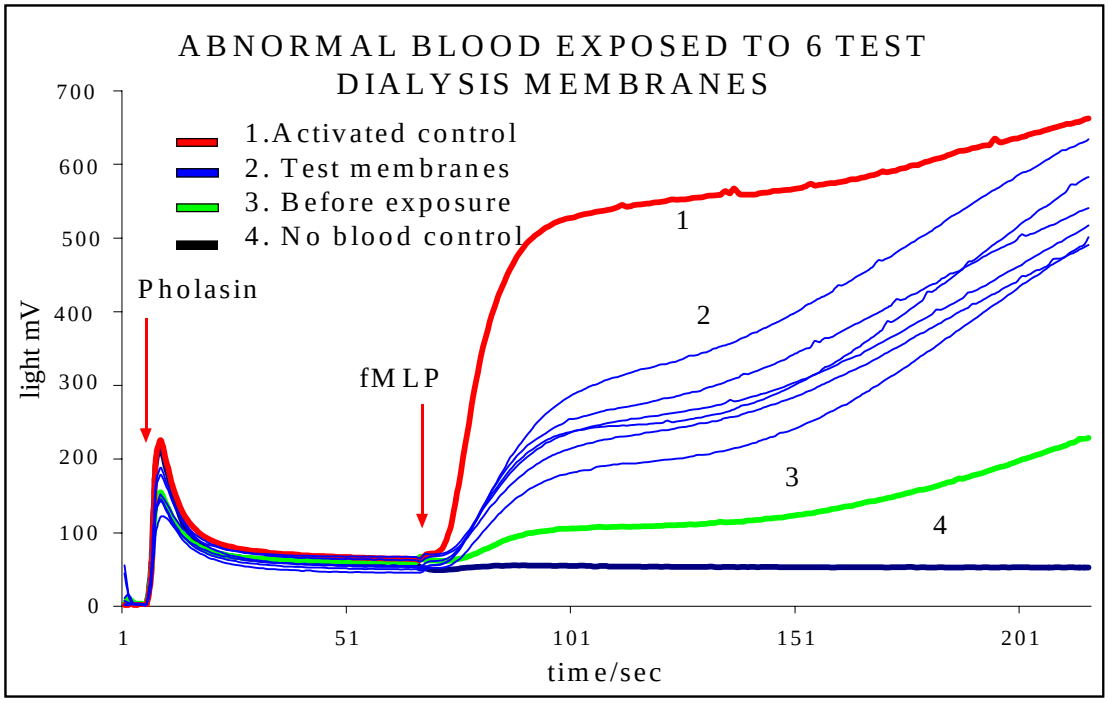


Figure 5a

Evidence of Degranulation in the Absence of Complement Activation

The anticoagulant used, EDTA or heparin, has normally very little bearing on the subsequent response of the sample to fMLP or PMA. However, if the neutrophils in the blood sample have been previously primed to degranulate and release enzymes, then a dependency on the anticoagulant used it seen.

A patient had acute kidney failure following surgery and suffered an anaphylactic reaction during dialysis with a cellulosic type filter. She later was dialysed with a low complement-activating membrane. Samples of blood were taken before and during dialysis and collected in tubes containing either lithium heparin or sodium EDTA. PMA was used as stimulant. The results are illustrated in Figure 6.

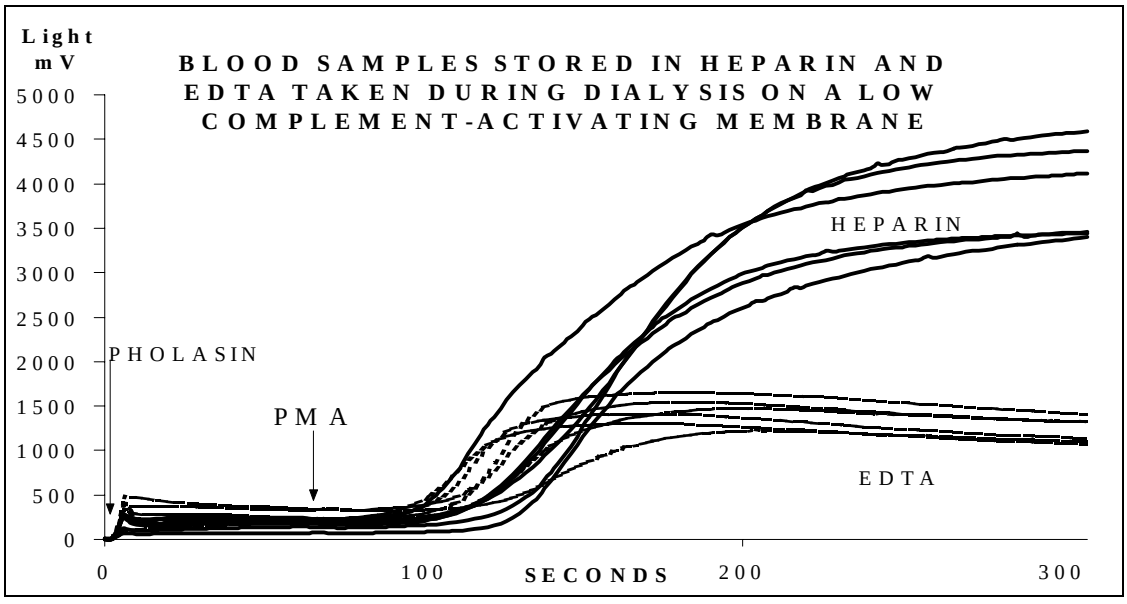


Figure 6

In Figure 6 two groups of responses, one corresponding to heparin blood samples and the other to EDTA, can be seen in which the response to heparin is very much greater. The individual curves, within each group, represent different times (before, during and after dialysis); they are reasonably similar in magnitude, presumably as a result of the use of a low complement-activating filter. However, the very much greater response of the heparinised blood (a way we propose to quantify degranulation[13]) supports the hypothesis that complement activation can be distinct from degranulation and can occur in the presence of low-complement activating materials.

In Figure 7 below the differential is again assumed to be due to degranulation, this time from blood of a patient who had been on dialysis for 15 years.

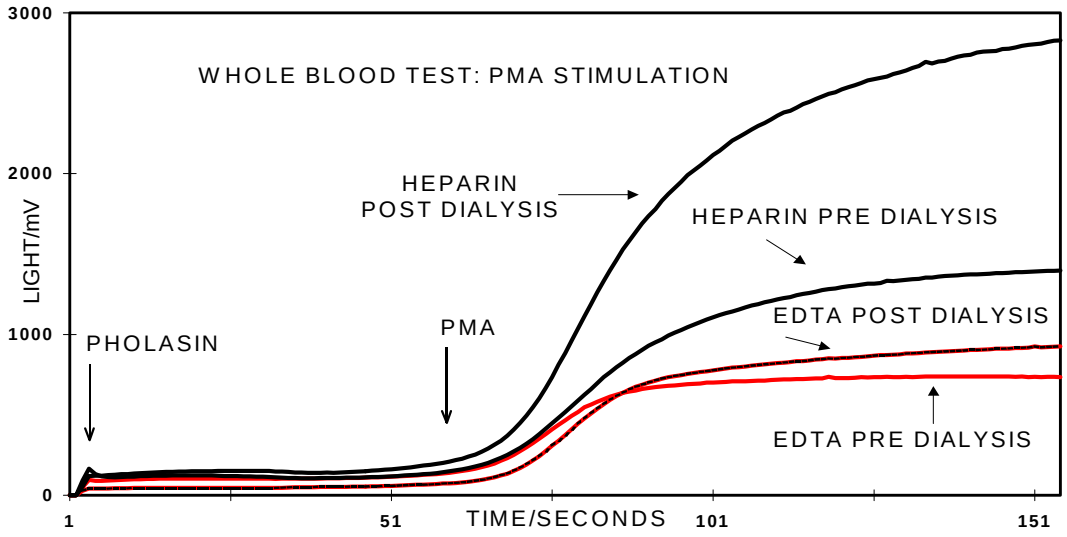


Figure 7

Proposed Screening of New Materials

A convenient, and relevant approach to such testing would be to expose the test material, both in powder form and as a fabrication, to blood and then to assess whether the leucocytes have been affected by this exposure. Exposure could lead directly to activation of the cells or to a priming of the cells.

Activation in a patient could lead to the production of free radicals and the expression of adhesion molecules as well as the production of chemicals (cytokines, chemokines, etc) that would activate other leucocytes and signal the recruitment of vast numbers of leucocytes to the site of inflammation, represented by the foreign material. However exposure might not actually activate the cells but instead prime them to respond later in an abnormal way.

Assessing Bioactivity of Bioceramic Powders

The results shown in Table 1 are from trials being carried out in collaboration with Dr Brian Meenan of the Northern Ireland Bio-Engineering Centre, University of Ulster. The object of the tests was to assess the bioactivity of various calcium phosphate bioceramic materials produced in different ways. The sample powder was exposed to a

1:100 dilution of blood for 6.5 minutes after which the ABEL® whole blood test was carried out with PMA as stimulant. Blood from five volunteers was used, two of whom had rheumatoid arthritis.

The effect of exposure to these bioactive materials was to prime the cells so that their response to PMA was reduced in comparison to the controls (no powder). The cells, in spite of having been primed, showed 100% viability and tests to demonstrate the possibility that the powders quenched the light and/or free radicals produced by the stimulated cells proved negative.

Table 1

BLOOD DONORS	CONTROL PEAK mV	COMMERCIAL TCP PEAK mV	COMMERCIAL TCP % INHIBITION	SINTERED TCP mV	SINTERED TCP % REDUCTION	COMMERCIAL HA mV	COMMERCIAL HA % REDUCTION	SINTERED HA mV	SINTERED HA % REDUCTION	MICROWAVE HA mV	MICROWAVE HA % REDUCTION	SINTERED MICROWAVE HA mV	SINTERED MICROWAVE HA % REDUCTI
A	898	578	36	781	13	563	37	671	25	673	25	762	15
B	940	534	43	845	10	369	61	724	23	617	34	674	28
C	1191	759	36	1059	11	638	46	930	22	842	29	1030	14
D	1002	590	41	914	9	488	51	844	16	722	28	769	23
E	1147	680	41	1022	11	524	54	882	23	787	31	805	30
mean			**39.4**		**10.8**		**49.8**		**21.8**		**29.4**		**22**
average													
deviation			**2.72**		**1.04**		**6.64**		**2.32**		**2.48**		**6**

The reduction in peak luminescence is expressed as a percent reduction compared to the control. The ranking of bioactivity for these six materials can be summarized as: HA (hydroxyapatite)>TCP (tricalcium phosphate)>microwave synthesized TCP>microwave synthesized HA>microwave synthesised and sintered HA>sintered TCP. In general, sintering of the powders reduced bioactivity and perhaps improved biocompatibility. The results, using PMA as stimulant, are independent of differences between individuals, thus making the test suited to fast screening of test materials.

However, using fMLP as stimulant, responses sometimes varied between individuals, possibly indicating degrees of potential risk. Valuable and rapidly achieved laboratory data on the biocompatibility of a material derives from the use of PMA as a stimulant in the ABEL® tests. However more specific data accrues if the blood of an individual potential recipient is tested using fMLP. This latter test could provide the patient (and clinician) with an assessment of possible risk.

Risk Assessment and Predictability

The exposure of a patient's blood to foreign materials, such as those used in medical devices, can sometimes elicit an abnormal (and even lethal) response in one individual

while having no effect on another. In the case of transplanted organs, if the organ has been affected, for example during transport, then the organ is more likely to malfunction or be rejected. In the case of heart and liver transplants, where at present there are no good artificial organs, then the patient is very likely to die while another organ is sought. One object of the present evaluation studies using the ABEL® tests is the prediction of the likely outcome of such exposure in a patient.

The tests should eventually be used for screening and quality assessment by manufacturers, as well as in the clinic prior to use during heart surgery or treatment (renal dialysis, chelation therapy etc) in order to predict complications that might arise after surgery, or during treatment. And because an individuals might respond adversely to a material or device that causes no response in others, the ability to carry out tests in in order more accurately to predict the likely outcome of the use of a particular device, material or possibly even an organ in an individual patient prior to treatment is a long-term aim of this work.

Appendix

What Can Happen When Blood Is Exposed To Bio-Incompatible Material?

- Inactive complement proenzymes circulating in the blood can be activated by the biomaterial[*].
- Cells in the blood, in particular the neutrophils, may bind the complement fragment C3b.
- Neutrophils may also bind, by specific receptors, the active complement fragments C3a and C5a[†] generated by the activation of complement.
- The binding of C3a and C5a frequently leads to the reversible aggregation of neutrophils and the sequestration of these cells to the lungs. This may occur during exposure of blood to complement activating membranes used in renal dialysis and cardiopulmonary bypass surgery.
- However, another more serious possible effect of this binding is the irreversible aggregation of neutrophils leading to clotting and deposition of emboli in the micro-vessels of the lungs here the products of neutrophil degranulation, enzymes and free radicals, may make the vessels leaky resulting in an accumulation of fluid in the alveoli with consequent arterial hypoxaemia.
- An upregulation of the receptor molecules of the leucocytes may occur leading to an increase in the response to receptor stimuli.

[*] Complement can be activated by two distinct pathways, the classical and alternative pathways, each leading to a common point, the cleavage of C5 to C5b and C5a and the formation of the membrane attack complex (MAC). Both pathways produce a C3 convertase and lead to the production of C3b. The classical pathway is initiated by immune complexes whereas the alternative pathway can be activated by non immunogenic means including biomaterials. The binding of C3b to the biomaterial is one means by which complement activation can be initiated with the resultant production of the anaphylatoxins C3a and C5a which are rapidly converted to des Arg derivatives.

[†] Activation of complement in which the anaphylatoxins C5a and C3a are produced usually occurs in response to bacteria and their toxins. The binding of C5a and C3a to phagocytic cells induces these cells to migrate to the site of infection and switch on the mechanisms for destruction of the pathogen.

- Receptor stimuli such as formyl-leucyl-methionyl-phenylalanine (fMLP), platelet activating factor (PAF), tumour necrosis factor α (TNFα), and granulocyte macrophage colony stimulating factor (GM-CSF) lead to an immediate (2-5 seconds) activation of the NADPH oxidase; other responses to these stimuli follow.

- An increase in the magnitude of the response of neutrophils to phorbol myristate acetate (PMA) (which bypasses the receptor-mediated signal transduction pathway by acting directly on intracellular protein kinase C) leads, we believe, to the activation of the NADPH oxidase on both the cell membrane and the secondary granules of neutrophil concomitant with their degranulation.

References

1. Ratner B D (1994) Blood Biocompatible Materials? Medical Device Technology 5(1) 17-18
2. Braybrook, J H (1994) Biocompatibility a fundamental approach, *Medical Device Technology* **5**(8)
3. ABEL, the registered trade mark of Knight Scientific Limited, is an acronym for Analsysis By Emitted Light
4 Humphries, J.E, Corr, B., Alexander, H.D., McConnell, J., Bell, A.L., Markey, G.M., Morris, T.C.M. (1999) Why discard the peritoneal macrophages of patients on CAPD? B J Haematol **105:** 319-320
5. Pholasin is a registered trade mark of Knight Scientific Limited
6. Knight, J (1997) The piddock and the immunologist. *Immunology News* **4**(1), 26-31.
7. Michelson , AM (1978) Purification and properties of Pholas dactylus luciférin and luciférase Meth Enzymol 57: 385-406.
8. Roberts PA, Knight J and Campbell AK (1987) Pholasin: a bioluminescent indicator for detecting activation of single neutrophils. *Anal Biochem* **160**: 139-148
9. Kukreja, RC and Hess, ML (1992) The oxygen free radical system: from equations through membrane-protein interactions to cardiovascular injury and protection. Cardiovasc Res 26 641-655.
10. Knight, J, McCafferty, A, Noon E and Bell A (1996) Indirect evidence to support the production of hydroxyl and/or ferryl radicals at sites of inflammation. Br J Rheum 35 Abstr Suppl 2: 13.
9. Hothersal, J (1999) personal communication
12. Hothersal, J (1999) personal communication
13 .Knight, J (1999) Reactions of Pholasin® with superoxide and peroxidases: applications to cellular luminescence.
14. Knight J, McCafferty A (1997) Pholasin® monitors neutrophil degranulation and activation. In Hastings JW, Kricka, Stanley PE, editors. *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons*. Chichester UK: John Wiley & Son Ltd 334-337.
15. Reichl,S,Bernard, S, Arnhold, J, Schiller, J and Arnold K (1999) Factors influencing the Pholasin® chemiluminescence. In *Bioluminescence and Chemiluminescence Perspectives for the 21st Century*. Chichester UK: John Wiley & Son Ltd 280-283.
16. Non mitochodrial consumption of oxygen and the continuous supply of NADPH generated from glucose via the hexose monophosphate shunt.
17. Segal A.W. (1995).The NADPH oxidase of phagocytic cells is an electron pump that alkalinises the phagocytic vacuole,*Protoplasm* **184,** 86-103.
18. Knight, J and McCafferty, A. Luminescent reactions of Pholasin® with isolated human leucocytes and diluted whole blood. *Eur J Clin Invest* **26** Suppl 1 A37 (1996).
19 Mócsai,A, Jakus,Z, Ligeti, E (1999) Diffferences in the signalling pathways to the different granules of neutrophils. *Eur J Clin Invest* **29** Suppl 1 A174