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Monitoring Bioactivity with the Light-Emitting Protein Pholasin®

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INTRODUCTION

Biocompatibility

Chemical stability was once equated with biocompatibility; it was considered that an inert substance would be compatible with all biological systems. That view can no longer be sustained. Not only must the chemical nature of a substance or product be taken into account but also, *and at the same time*, the individual response of the recipient. To do otherwise is to court disaster.

Problems of bio-*in*compatibility derive, at least in part, from the way in which an individual's blood cells interact with and respond to foreign material. A material that is perfectly acceptable when used for one individual could be potentially lethal to another.

Thus, the ability to predict *an individual's* susceptibility to *a specific product* will have wide reaching benefits.

Problems associated with biocompatibility in specific clinical fields are well known. Defects in materials used for the urinary tract have caused trouble. There are difficulties associated with the much needed development of small diameter, synthetic graft materials for cardiovascular surgery. There are problems associated with materials used in the extracorporeal circulation of blood during renal dialysis; oxygenation during cardiopulmonary bypass surgery; and other procedures in which blood is circulated through tubes or around membranes.

The need for reliable materials will increase with an ever-ageing population. But until the industry has good standardised methods with which to compare materials for biocompatibility there will be a reluctance to test new materials in difficult clinical settings.

Applications of Pholasin® (of which more later) to meet these needs are currently being developed.

Bioactivity

The present goal for materials used in medical devices (including wound healing) is, however, more than just biocompatibility. New materials are being designed to interact in predictable ways with surrounding tissue. For example it may be advantageous to actually generate a small amount of local inflammation in order to remove dead tissue and thus reduce scar formation. This occurs naturally in the body when neutrophils (one of the phagocytic white blood cells) are attracted to a site of injury (such as occurs during a heart attack) where they remove necrotic tissue. The actions of these phagocytic cells are initially beneficial, speeding the healing process; however if too many cells are attracted to the site of inflammation (in the heart for example) then the mild inflammation can lead to a rapidly increasing inflammatory response in which living tissue is attacked.

In the light of this, the term biocompatibility must now include having a designed and desired effect as well as having no effect.

Wound Healing

One of the goals in the development of wound healing preparations is the design of materials that will initially cause mild inflammation leading to the removal of dead tissue and a reduction in scar formation, followed by a turning off of the inflammatory response to

facilitate tissue repair. Novel materials are being developed, such as inert polypropylene non-woven materials incorporating chitosan fibres and controlled release glass (CRG), which dissolves at an appreciable rate. Such materials are designed to deliver a constant stream of substances with a variety of properties including bioactivity and bacteriostasis[1]. And the challenge is especially great in impaired wound healing such as occurs in chronic ulcers associated with diabetes, heart and vascular disease.

Standardised Methods

The provision of simple, but highly sensitive and accurate methods for assessing bioactivity is a key element in the successful development of a material for clinical application. Moreover, simple and effective screening techniques have the potential to make significant savings in the time and expense associated with product development.

This presentation will include a description of novel methods for the assessment of biocompatibility based on the light-emitting protein Pholasin® using diluted whole blood or isolated cells. The methods are currently being assessed for their value in:

1. screening materials used in dialysis and surgery,
2. determining the efficacy of novel coatings of medical devices,
3. screening a range of calcium phosphate bioceramic powders for their bioactivity,
4. determining the bioactivity of substances delivered from controlled release glasses,
5. monitoring patients before, during and after treatment involving medical devices that come into contact with blood.

ABEL® is an acronym for Analysis By Emitted Light and is the registered trade mark of Knight Scientific Limited.

It is the eventual plan for the ABEL® biocompatibility tests with Pholasin® to be listed as recommended tests in the International Standard ISO 10993, Biological Evaluation of Medical Devices, part 4, Blood Biocompatibility Testing.

WHAT IS PHOLASIN® AND WHAT DOES IT DO?

Pholasin®[2] is the photoprotein of the marine, rock-boring bioluminescent mollusc, *Pholas dactylus*, the Common Piddock. Pholasin® does not glow on its own[i], but needs to be switched on. Free radicals and other reactive oxygen species produced by activated leucocytes (and other types of cell) turn on the light.

Pholasin® emits light in the presence of:

- free radicals: superoxide anion, singlet oxygen, hydroxyl radical and/or ferryl radical,
- oxidant: hypochlorous and hypobromous acids, chloramines, bromamines and peroxynitrite
- enzymes: peroxidases and certain oxidases

Pholasin® is isolated and purified by Knight Scientific Limited from molluscs cultivated in land-based systems.

[i] In solution Pholasin® emits low-level light referred to as the resting glow

Before discussing the ways in which we have investigated biocompatibility we will set the theoretical background to the work. This will include a discussion of some of the species involved and their place in the biochemistry of the normal cell.

FREE RADICALS AND REACTIVE OXYGEN SPECIES[3]

Free Radicals

Free radicals are molecules that contain atoms with unpaired electrons. Molecules are most stable if all their electrons are paired; free radicals are very unstable, that is they are very reactive. When reacting with another molecule the second molecule loses an electron to the free radical that now, with a full complement of paired electrons, is more stable. However, in the process, the second molecule itself becomes a reactive free radical and a chain reaction is thus initiated. The chain is broken when two free radicals combine. If the second molecule should be important to the biochemistry of the organism then the whole organism may suffer as a result of the initial free radical attack.

As an example, consider the fate of the all important polyunsaturated lipids in a cell membrane. Attack by a free radical on one of the carbon to carbon double bonds can lead to the formation of the corresponding lipid peroxyl radical and the initiation of a chain reaction in which the final products, lipid peroxides, no longer maintain the integrity of the cell wall. This process of lipid peroxidation may be initiated by the hydroxyl radical $OH^{\bullet}$ in a reaction in which iron, as Fe^{++}, is involved. Hence the use of chelation therapy in the iron overload disease such as that which ensues when children have eaten their parent's iron tablets.

Reactive Oxygen Species:
superoxide, hydroxyl radical and hydrogen peroxide

In biological systems much attention is focussed on the two free radicals, superoxide anion ($O_2^{\bullet -}$) and the hydroxyl radical ($OH^{\bullet}$), which is formed by the reduction of oxygen; and on hydrogen peroxide (which is not a free radical). There is a specific enzyme complex, the NADPH oxidase system, usually associated with phagocytic cells, which when activated releases large quantities of superoxide. Superoxide is also produced by leakage of electrons onto oxygen during the electron transport chain, and as a by-product of the activity of enzymes such as xanthine oxidase and aldehyde oxidase, as well as by the auto-oxidation of hydroquinones, catecholamines and thiols. The reactive species hydrogen peroxide (not a free radical) is formed from superoxide (usually catalysed by superoxide dismutase), as well as, by the enzymes D-amino acid oxidase and amine oxidase. Hydroxyl radicals can be formed by either superoxide anion or from hydrogen peroxide especially in the presence of the transition metals, iron and copper.

halogenated oxidants

The reaction of the neutrophil and monocyte granule enzyme myeloperoxidase with hydrogen peroxide (derived from superoxide) and chlorine leads to the production of hypochlorous acid, whereas eosinophil peroxidase reacts with hydrogen peroxide and bromine to produce hypobromous acid. Both oxidants are highly reactive and participate in the killing of micro-organisms. At sites of inflammation, however, leucocytes may accumulate in vast numbers, and there the release of large amounts of these granule enzymes can cause much damage.

Halogenated oxidants are capable of oxidising many biological molecules, especially those containing –SH groups. Hypochlorous acid can damage cartilage but it also rapidly inactivates α_1-antiprotease, the enzyme that prevents proteolytic enzymes such as elastase from destroying tissue.

The extent to which inactivation of the halogenated oxidants occurs in vivo depends largely upon the environment of the oxidant. In plasma, HOCl is relatively unimportant because it reacts preferentially with albumin (concentration 50-60mg mL^{-1}) rather than with α_1-antiprotease (1-3mg mL^{-1}). However, in many other biological fluids such as cerebrospinal fluid, synovial fluid, and fluid surrounding the alveoli of the lungs, the concentrations of albumin are very much lower. In such environments the inactivation of α_1-antiprotease can be significant. Ascorbic acid is also oxidised by HOCl and its presence as an antioxidant in biological fluids can prevent the inactivation of α_1-antiprotease and thus protect tissue from damage.

It should be noted that HOCl can activate collagenase (which digests native collagen) and gelatinase (which attacks denatured collagen) and so presents a very great threat of damage.

Peroxynitrite

Peroxynitrite anion ($ONOO^-$) is produced by the reaction between nitric oxide and superoxide anion. This reaction is important to the organism when it occurs in inflammatory cells, such as neutrophils and macrophages, as the resultant peroxynitrite kills bacteria and other microorganisms. Excessive production of peroxynitrite, however, can damage normal tissue by oxidation and nitration. For example, its reaction with DNA can lead to breaks in the chain, modification of the bases and mutations. Peroxynitrite can give rise to radical chain reactions, including lipid peroxidation. Nitration occurs mainly on phenols, as in the nitration of tyrosine residues in proteins. It is known to initiate lipid peroxidation of phosphatidylcholine liposomes and low density lipoprotein.

While the damage by peroxynitrite to some molecules is harmful to the organism, the function of others molecules, such as vitamins C and E, includes the harmless removal of excess peroxynitrite.

FREE RADICAL GENERATION AND INTERACTION OF BIOMATERIALS WITH LIVING TISSUE

Exposure of blood or cells to biomaterials can modify free radical generating systems. These modifications may be deliberately planned for in the design of a particular bioactive material, or accidental when an individual person reacts in an adverse manner to a material. The information obtained from monitoring light emitted from Pholasin® in contact with activated cells can be used to identify the nature of the modification.

The mechanisms underlying the emission of light by Pholasin® involve the activation of the NADPH oxidase system (the superoxide generating system), especially in leucocytes, as well as the degranulation of the enzyme myeloperoxidase. A fuller description of the system and the mode of activation is given below, but in essence the exposure of a cell such as a neutrophil to a bioactive material may modify the activation of the oxidase. As discussed below, this activation may be triggered by the binding of molecules to receptors on the surface of the cell or by the direct stimulation of protein kinase C (PKC) in the cytosol.

The effect of exposure to a biomaterial may be to:

- bring large numbers of receptors to the cell surface (upregulation) leading to an enhanced rate of production of superoxide when the cell is later exposed to a receptor stimulant.
- Receptor stimulants include:
- N-formylated methionylpeptides such as fMLP,
- complement fragments such as C5a,

- bioactive lipids such as platelet activating factor (PAF),
- leukotriene B4 (LTB4),
- several neutrophil activating proteins.
- reduce the number (down regulate) or effect the binding of receptor stimulants
- act directly on PKC by either inhibiting or enhancing its activation

SOME DETAILS OF THE FUNDAMENTAL MECHANISMS INVOLVED

Activation of the NADPH Oxidase

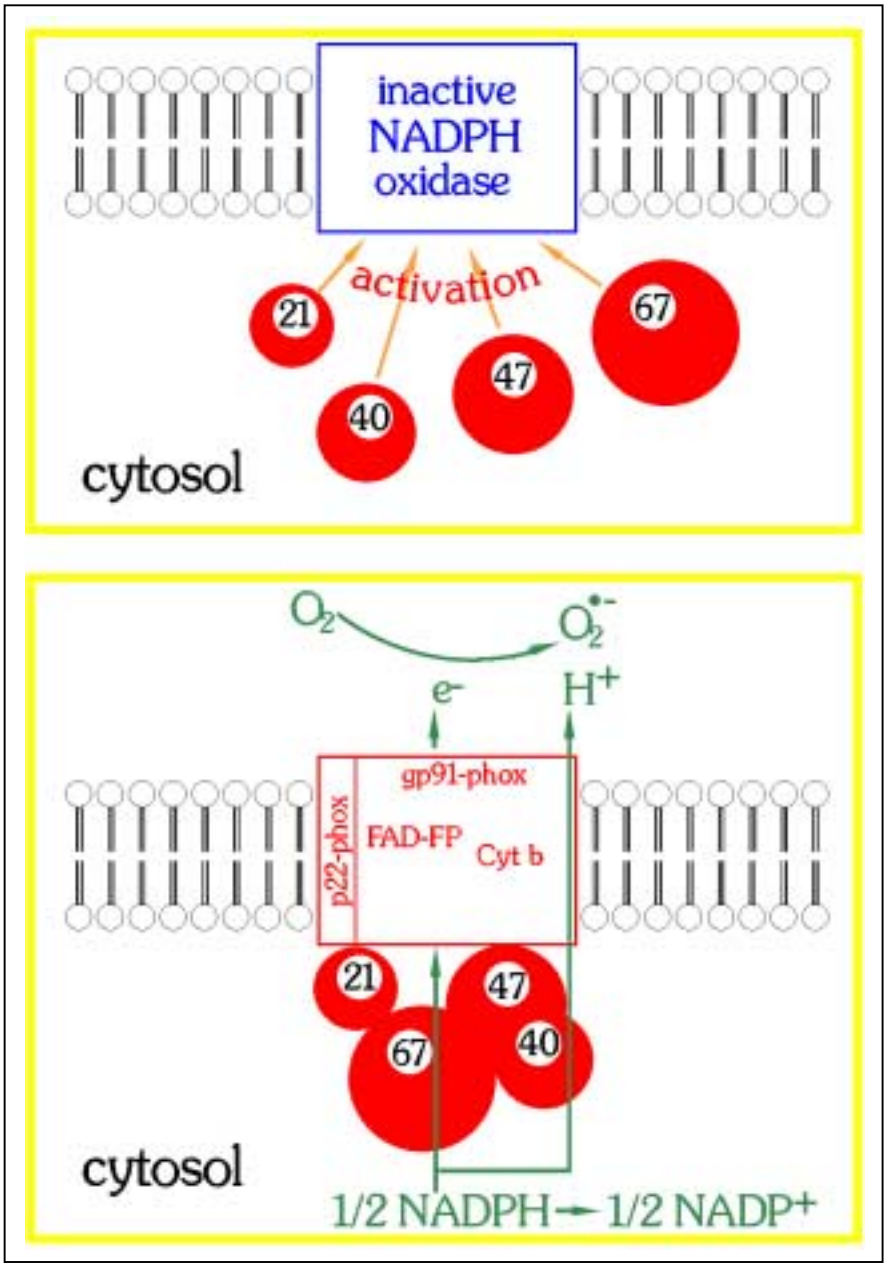

figure 1

The NADPH oxidase (figure 1) consists of a membrane-bound flavoprotein and cytochrome B together with four other proteins in the cytosol[i] . The NADPH oxidase is inactive until stimulated. On activation all the components come together at the cell membrane with the result that the transfer of an electron through the membrane and the direct reduction of oxygen to superoxide is effected.

The fully active NADPH oxidase is a membrane-bound complex consisting of short electron transport chain[4] (flavocytochrome b_{245}) and four cytosolic proteins: $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and $p21^{rac}$; where the superscript "phox" represents phagocyte oxidase. The induction of electron transport is most likely related to translocation of cytosolic proteins to the membrane as that event coincides with oxidase activity[4]. The $p21^{rac}$ is a GTP-binding protein-that occurs in the cytosol as a complex with Rho-GDI, a GDP-dissociation inhibition factor. The function of the oxidase is to accept an electron from NADPH and transfer it to the inside of the cell membrane whence it is passed to oxygen on the outside wall. This one electron reduction of oxygen leads to the direct production of superoxide at the surface of the cell. The dissociation of Rho-GDI from $p21^{rac}$ is the most probable switch that initiates the activation of the oxidase[5]. Upon activation, $p21^{rac}$ dissociates from Rho-GDI and translocates to the cell membrane[6] where an activation complex involving $p21^{rac}$, $p47^{phox}$ and $p67^{phox}$ docks with flavocytochrome b_{245} inducing a conformational change conducive to electron transport. NADPH, the substrate and the source of the electrons for reduction of oxygen, is produced from the oxidation of glucose via the hexose monophosphate shunt. This so-called respiratory burst of phagocytes has been recognised for some time as being distinct from mitochondrial respiration[7].

In the plasma membrane and in the membrane of secondary granules of neutrophils is the membrane bound component of the NADPH oxidase. This component is a flavocytochrome that is in fact a heterodimer. The smaller subunit is approximately 22kDa and is referred to as $p22^{phox}$, while the larger ß subunit is approximately 91 kDa ($gp91\text{-}^{phox}$) but is heavily glycosylated. In unstimulated neutrophils, the oxidase is inactive and no other polypeptide is associated with the membrane. On activation, three further polypeptides are recruited from the cytosol to the membrane. These are: 67 kDa ($p67^{phox}$), 47 kDa ($p47^{phox}$) and 40kDa ($p40\text{-}^{phox}$). It is speculated that these cytosolic components aid the correct conformation of the flavocytochrome and facilitate substrate binding and electron flow through the complex.

The redox activity of the complex (figure 2) is associated with the $gp91\text{-}^{phox}$ subunit. Electrons derived from intracellular NADPH are passed through the membrane complex to extracellular oxygen which is reduced to superoxide.

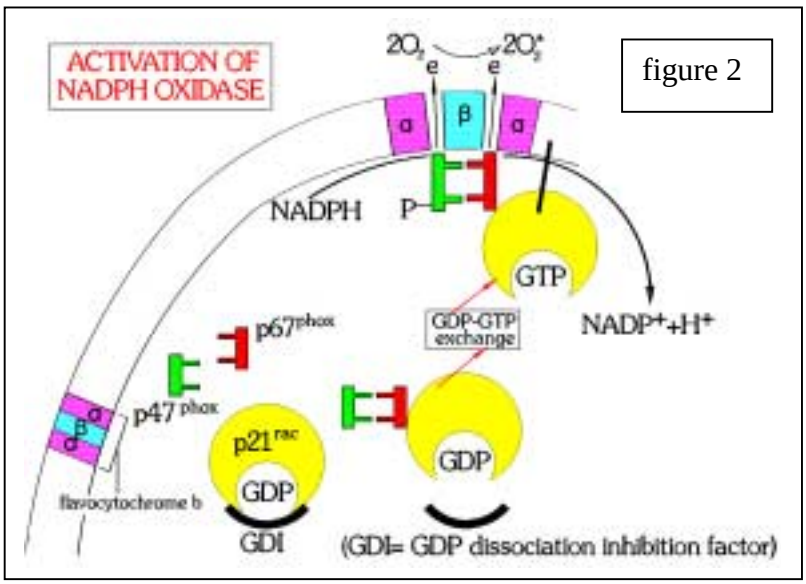

figure 2

Activation of the complex also involves two G proteins (see figure 2). These act as molecular switches, alternating between binding GDP in the inactive state and GDP in the active state. The G protein Rap1A copurifies with the flavocytochrome while the G protein p21rac2 translocates to the membrane like the other cytosolic polypeptides. In the cytosol p21rac2 normally is associated with the guanine nucleotide exchange inhibitor rhoGDI. On activation GDS (a GDP dissociated stimulator protein) catalyses the exchange of GDP/GTP.

Phagocytosis

figure 3

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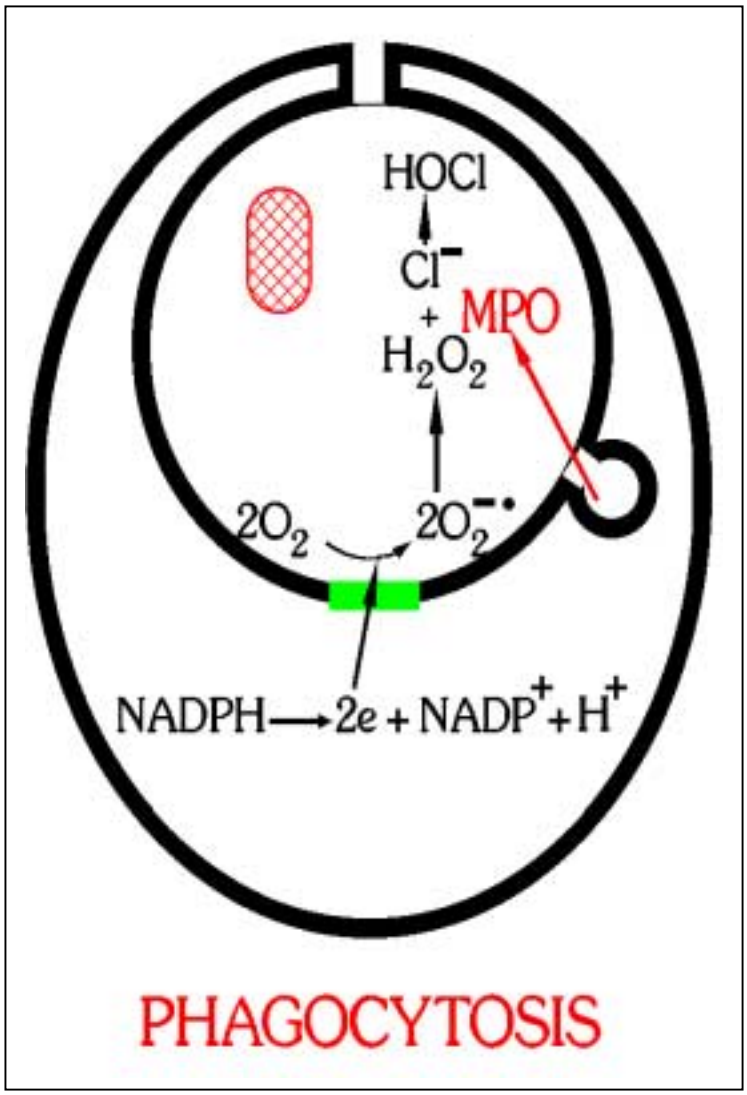


Phagocytic cells, such as neutrophils, eosinophils, monocytes/macrophages recognise the presence of a foreign body such as a bacterium or perhaps a particle derived from a medical device. The response of the phagocytic cells is initially via a receptor which leads to the invagination of the cell membrane to form a vacuole (phagosome), in which the foreign particle is trapped (see figure 3). The NADPH oxidase on the plasma membrane is activated to produce superoxide which is released into the vacuole. Accompanying the formation of the

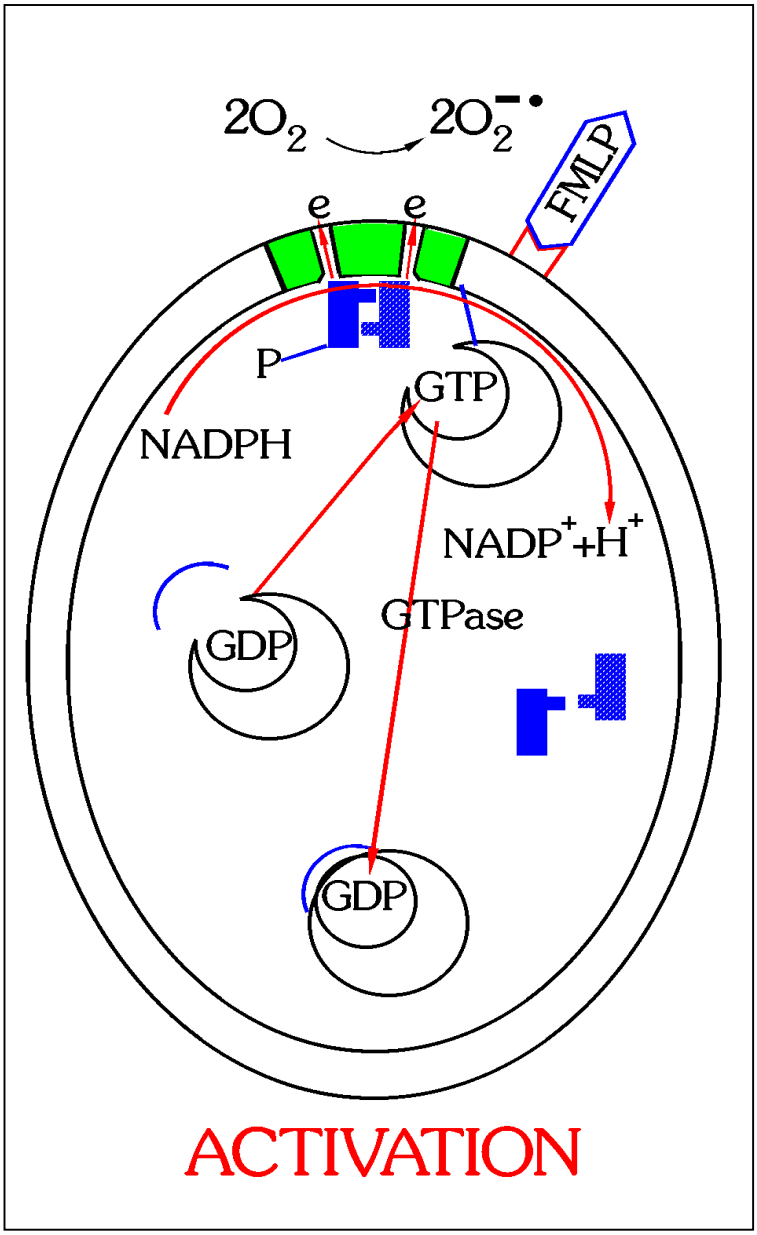
phagosome is degranulation, the fusing of the membrane of a granule within the cell with another membrane, usually the plasma membrane. During phagocytosis the primary granules fuse with the membrane of the vacuole and then release into the vacuole the enzyme

myeloperoxidase, MPO. MPO produces hypochlorous acid from hydrogen peroxide (H_2O_2)[ii] and chlorine.

In phagocytosis, during which foreign bodies such bacteria, spent red blood cells or dead tissue are trapped and usually destroyed (figure 3) oxygen consumption can increase 10-20 fold. The consumption of oxygen in the "respiratory burst" leads to the production of NADPH from glucose via the hexose monophosphate shunt. This oxygen consumption **does not** occur in the mitochondria and is not affected by poisons such as cyanide.

Receptor-Mediated Activation

figure 4



[ii] hydrogen peroxide is formed from the dismutation of superoxide usually catalysed by superoxide dismutase.

Phagocytosis is initiated by receptors at the cell surface which detect chemotactic substances emanating from the foreign body or dead and dying cells. The complete process of phagocytosis can take a number of hours. However, certain soluble substances can activate the NADPH oxidase at the cell membrane without formation of a phagocytic vacuole (see figure 4). These include:

- activated complement fragment C5a,
- the chemotactic peptide derived from the cell wall of a bacterium formyl-leucyl-methionyl-phenylalanine (fMLP),
- bioactive lipids such as platelet activating factor (PAF) and leukotriene B4 (LTB4),
- and several neutrophil activation proteins including IL-8 and granulocyte macrophage colony stimulating factor (GM-CSF).

The time from presentation of stimulus to response (lag time) is usually 2-5 seconds with the maximum rate of production of superoxide attained after about 60 seconds.

The activation of the oxidase by receptor-mediated stimuli

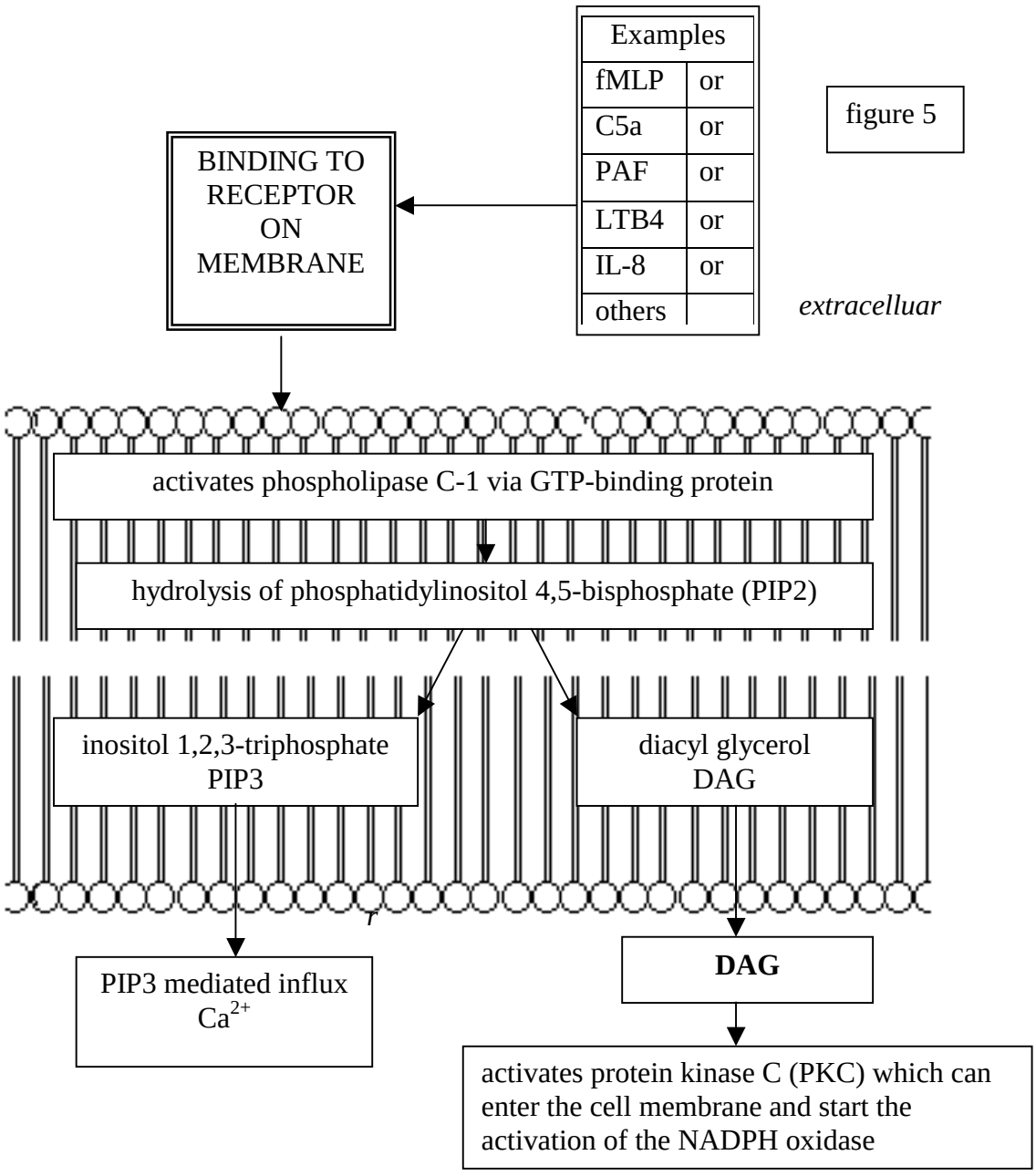


figure 5

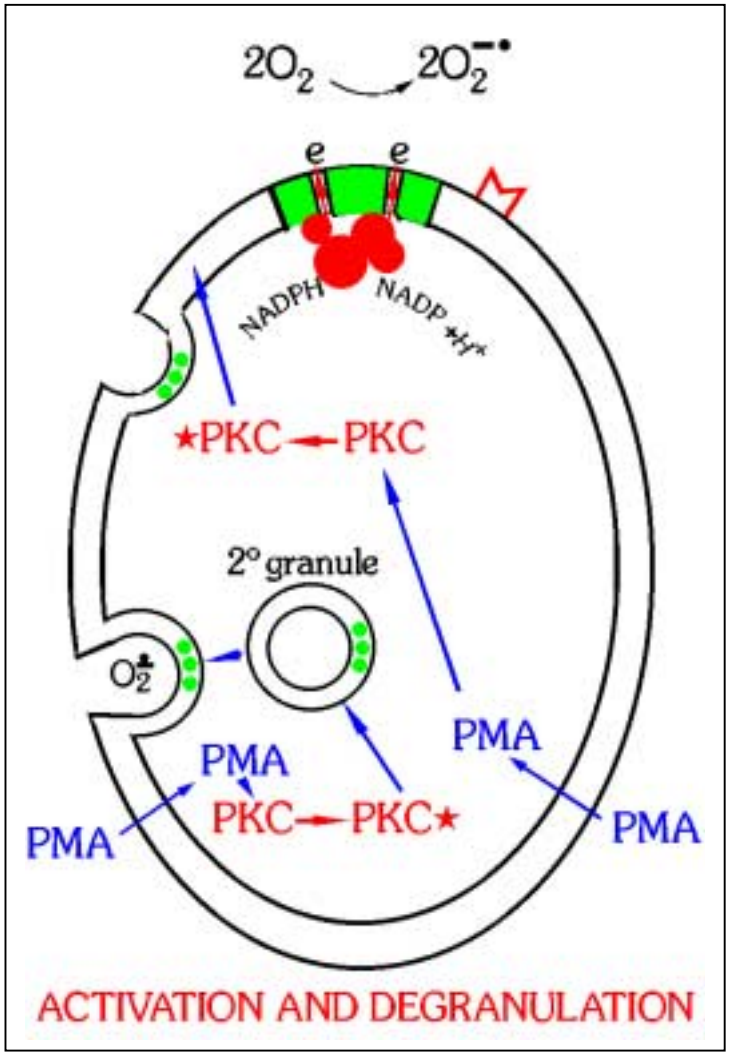
The stimulant must remain in contact with the receptor for activation to continue; removing the stimulant deactivates the oxidase. Phosphorylation and dephosphorylation also appear to be key in the activation and deactivation of the oxidase[8]. Tyrosine kinase inhibitors, such as erbstatin, inhibit receptor mediated activation such as that by fMLP. They do not inhibit activation by phorbol-12-myristate-13-acetate (PMA) which activates protein kinase C directly (see later for details).

Stimulation of the NADPH oxidase via receptors is calcium dependent and takes 2-5 seconds. The maximum rate of production of superoxide is attained after about 60 seconds and usually lasts for less than 5 minutes. Inhibitors of PKC have no effect on receptor activation[9] even though activation of PKC is mediated by diacyl glycerol (see figure 6). The fungal metabolite 17-hydroxywortmannin, an inhibitor of P13 kinase, inhibits the receptor mediated activation[10].but not the receptor-independent activation.

Receptor independent activation of the NADPH oxidase is not calcium dependent and occurs by direct activation of PKC (see figures 6 and 7). Long-chain unsaturated fatty acids and other PKC agonists, such as PMA, migrate into the cell, acting as a diacyl glycerol (DAG) analogue and activating PKC. Activated PKC then migrates into the cell membrane and presumably the membrane of secondary granules, which also contain the membrane components of the NADPH oxidase system, the flavocytochrome b (see figure 2).

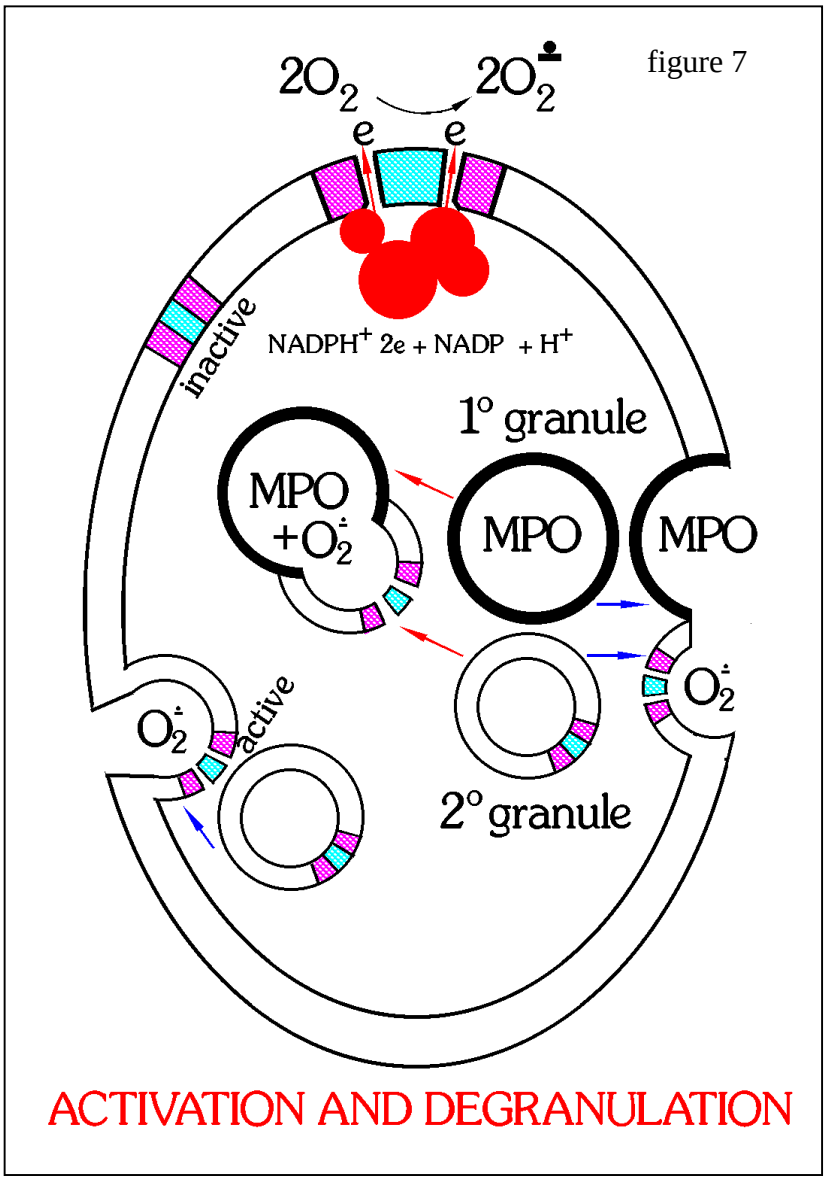
Receptor-Independent Activation

figure 6



The activation of the NADPH oxidase is often linked to degranulation, a phenomenon in which membrane bound intracellular granules containing a range of enzymes fuse with the plasma membrane of the cell and release their enzyme contents to the surrounding tissue. Degranulation of primary as well as secondary granules can be detected by Pholasin®[11,12] which reacts with myeloperoxidase (MPO) in a reaction which is very

figure 7



much enhanced by hydrogen peroxide. However, when degranulation occurs the MPO released from the primary granules can bind to adhesion molecules (CD11b/CD18) which are expressed on the membrane of the secondary granule, which also contains the NADPH oxidase. MPO is therefore bound at the membrane where hydrogen peroxide (derived from the dismutation of superoxide) can participate in luminescent reactions with Pholasin®.

METHODS

One way to monitor bioactivity is to expose a material to be tested, or a complete medical device to blood or isolated cells for varying lengths of time. Small samples of blood or cells are removed on several occasions during the course of an experiment and assayed in one of ABEL® cell activation test kits with Pholasin®.

The tests reveal changes brought about by the material in the way leucocytes respond after stimulation of the NADPH oxidase system. The activation of the NADPH oxidase is monitored by the luminescent response of Pholasin®.

The material under test may be in crude powder form or may be an entire medical device. Alternatively, samples of blood may be taken from a patient (or animal) undergoing treatment involving exposure of their blood to the medical devices.

Procedure with Whole Blood: Tube Luminometer

- 1:100 dilutions are made in 2mL of blood dilution buffer[iii], and 100µL of diluted blood is used in each luminometer cuvette.
- The cuvettes contain: 100µL diluted blood, 450µL assay buffer, 250µL Pholasin® solution (2.5µg), 100µL Adjuvant-K™ solution(1 enhancement unit).
- Note that Pholasin® may be added before or after the cuvette enters the luminometer.
- The mixture is incubated for 1 minute at 37°C
- .Light is then measured every second for 60 seconds. (The light readings are the accumulated light detected over each second and expressed as relative light units or, in some luminometers, as mV).
- After 1 minute the stimulants are added.
- Light is measured for a further 5 minutes with fMLP and 8-10 minutes when PMA or a mixture of fMLP and PMA is used.

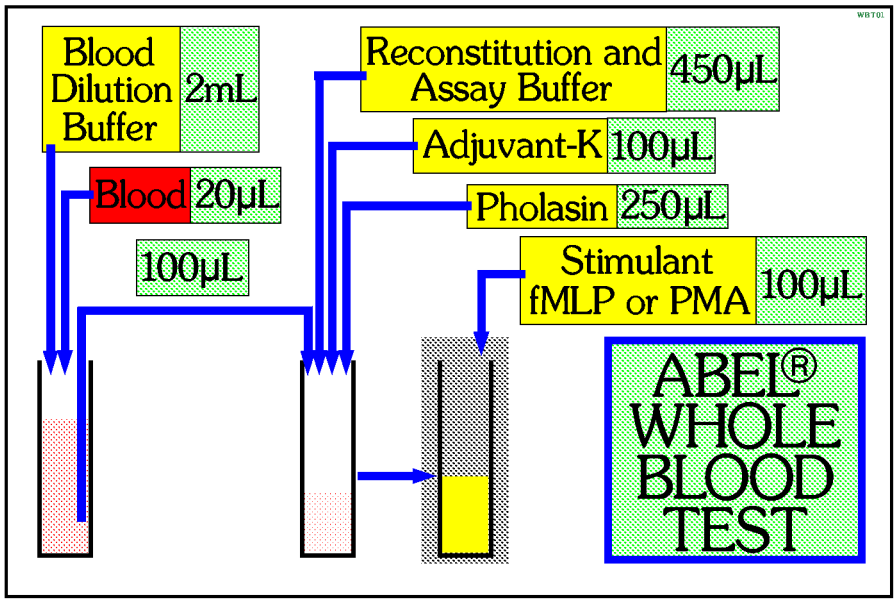


figure 8

[iii] The equivalent of 1µL of blood is used in the test . Because of the imprecision in measuring 1µL it is recommended that 20µL of blood is diluted with 2mL of blood dilution buffer and 100µL (containing 1µL of whole blood) is pipetted into the cuvette. However, if insufficient blood is available then a 1:100 dilution can be made with less than 20µL; if necessary, 1µL of whole blood can also be delivered directly to the cuvette.

Procedure with Whole Blood: Microplate Luminometer

Follow the instructions above but use volumes of reagents shown in the diagram below.

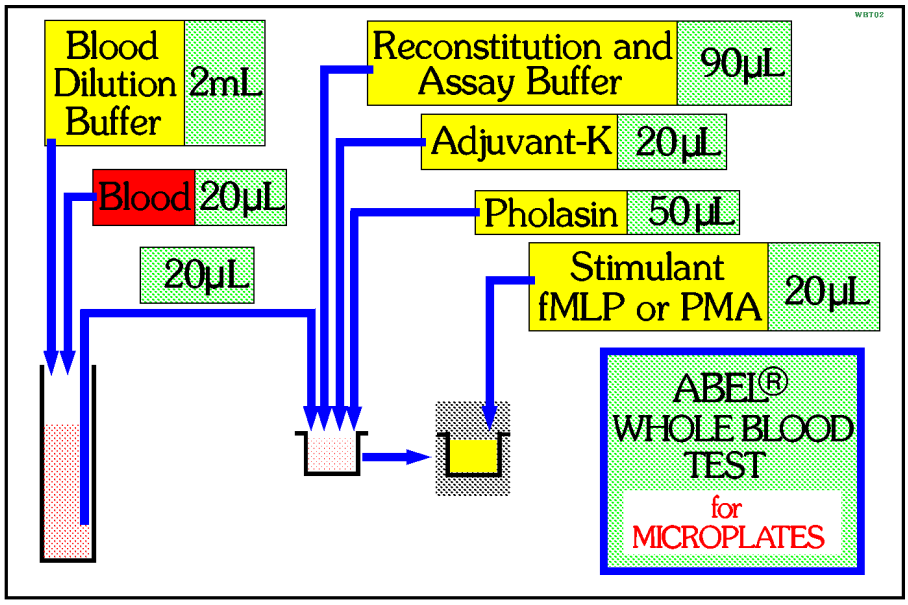


figure 9

Procedure with Isolated Cells

- Isolated cells are transferred to reconstitution and assay buffer[iv] and then 100µL (tube luminometer) or 20µL (microplate luminometer) of cell suspension is used in each test.
- The test can be carried out with or without Adjuvant-K™. A trial with and without Adjuvant-K™ should be carried out first.
- All other procedures are identical to the procedure with whole blood

figure 10

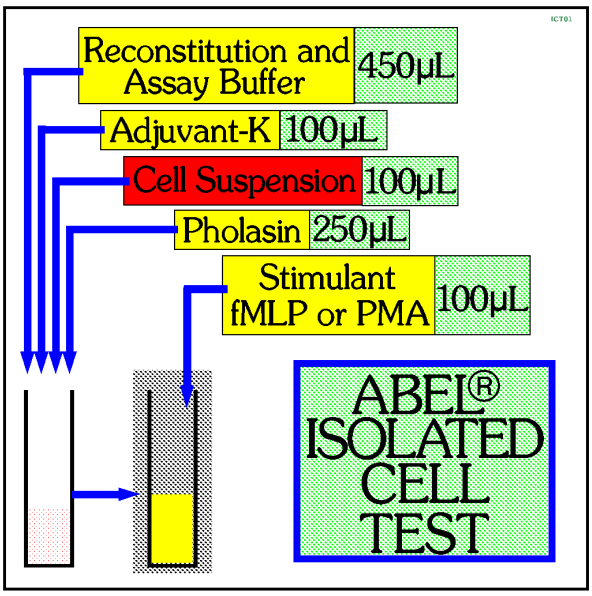
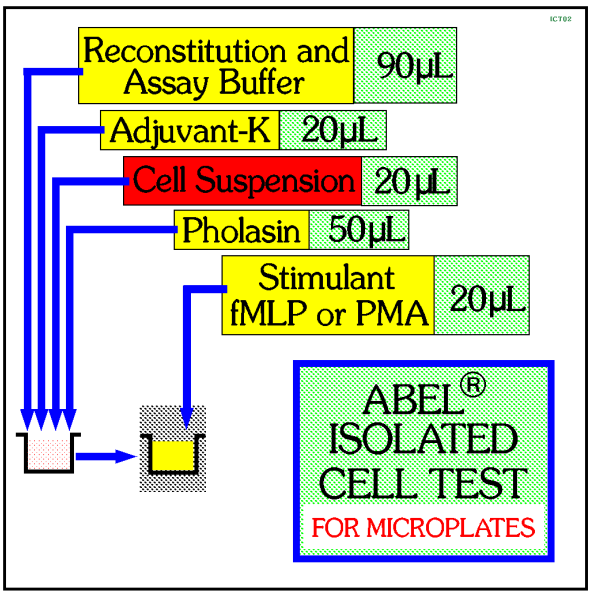


figure 11



Other stimulants

[iv] Tissue culture media and physiological fluids may contain antioxidants that could quench free radicals produced when the cells are activated.

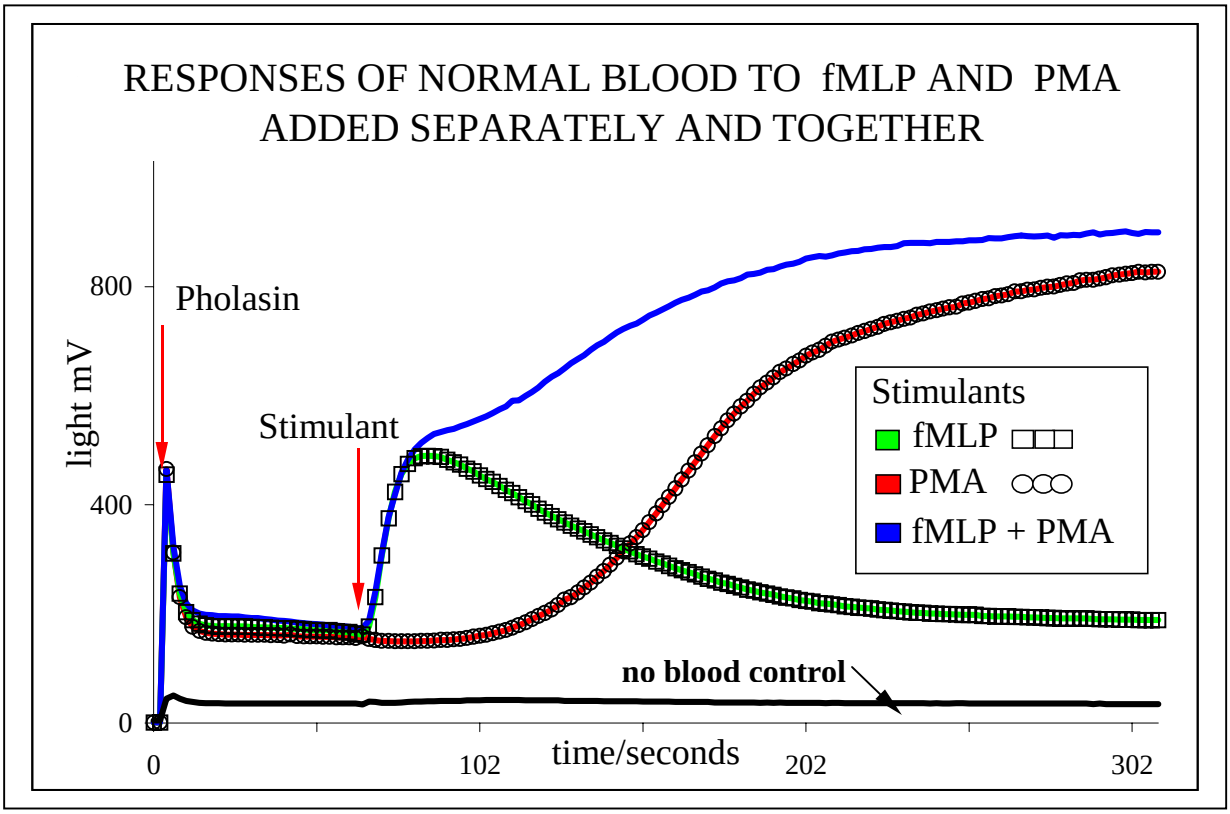
Cells may be stimulated with particles of various sizes, such as controlled release glasses (CRG) or with stimulants such as: platelet activating factor, calcium ionophore, various chemotactic peptides and diacyl glycerol analogues.

RESULTS

Typical Response Curve for Control Samples

A composite of luminescent response curves with Pholasin® after stimulation of the NADPH oxidase via receptor stimulants such as fMLP or by activation of protein kinase C is shown in figure 13.

figure 13

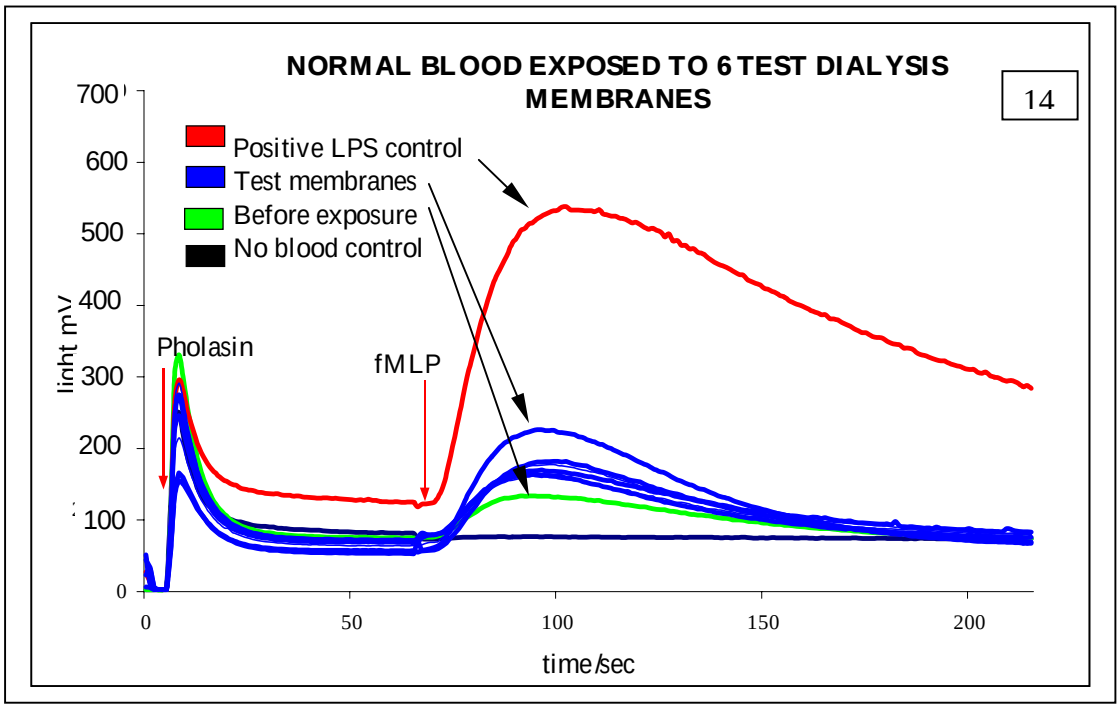


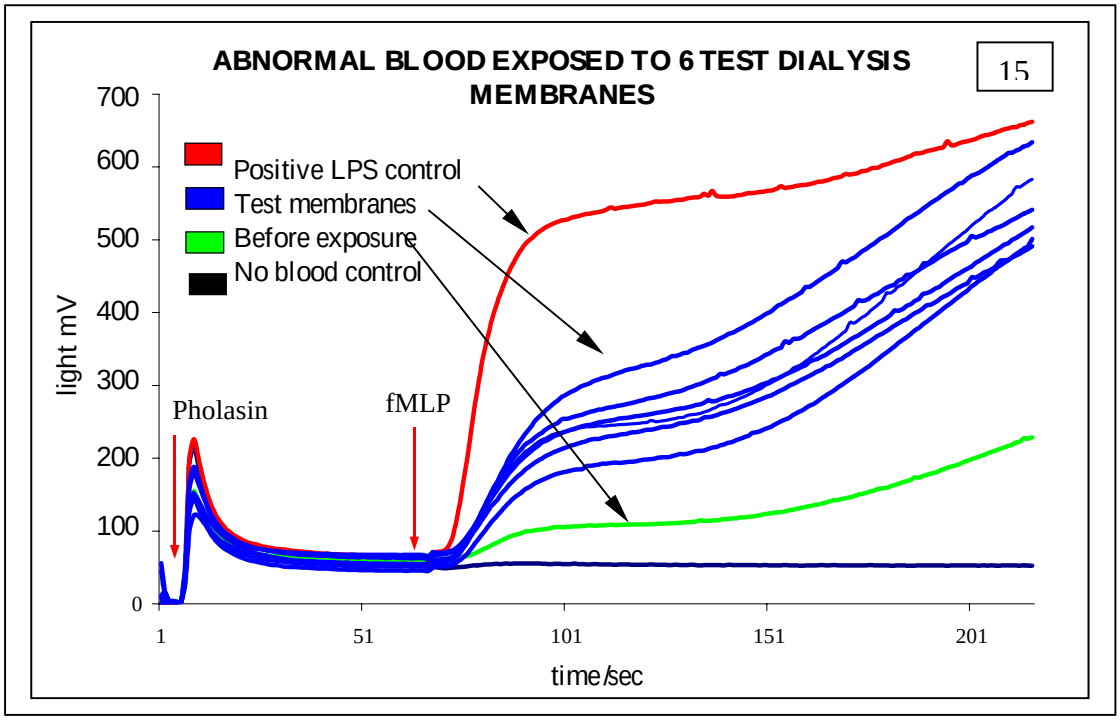
The tests work by stimulating the NADPH oxidase system[v] of cells such as neutrophils, eosinophils, monocytes and macrophages, in the presence of Pholasin®, either previously isolated or in diluted whole blood, and monitoring the resultant luminescent response. The results shown in figure 13 are representative of a typical control blood sample used in the bioactivity/biocompatibility tests. The response from stimulation with receptor stimulant fMLP (1μM) and the diacyl glycerol analogue PMA, which enters the cell and activates protein kinase C, are shown in figure 1. Presentation of fMLP and PMA together enables the activation of the NADPH oxidase on the cell surface to be monitored simultaneously with the activation of the NADPH oxidase on the membrane of the secondary granules. PMA in fact activates the NADPH oxidase throughout the cell, but at a slower rate than fMLP; it also promotes degranulation.

Screening Materials Used in Renal Dialysis

[v] Activation accompanies a 2-20 fold increase in non-mitochondrial consumption of oxygen (the so-called *respiratory burst*) and a continuous supply of NADPH generated via the hexose monophosphate shunt.

Blood from one donor was circulated via 6 separate peristaltic pumps to 6 miniature dialysis cartridges each containing different experimental hollow fibre filter material. Samples of blood (20µL) 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 below in figures 14 and 15.

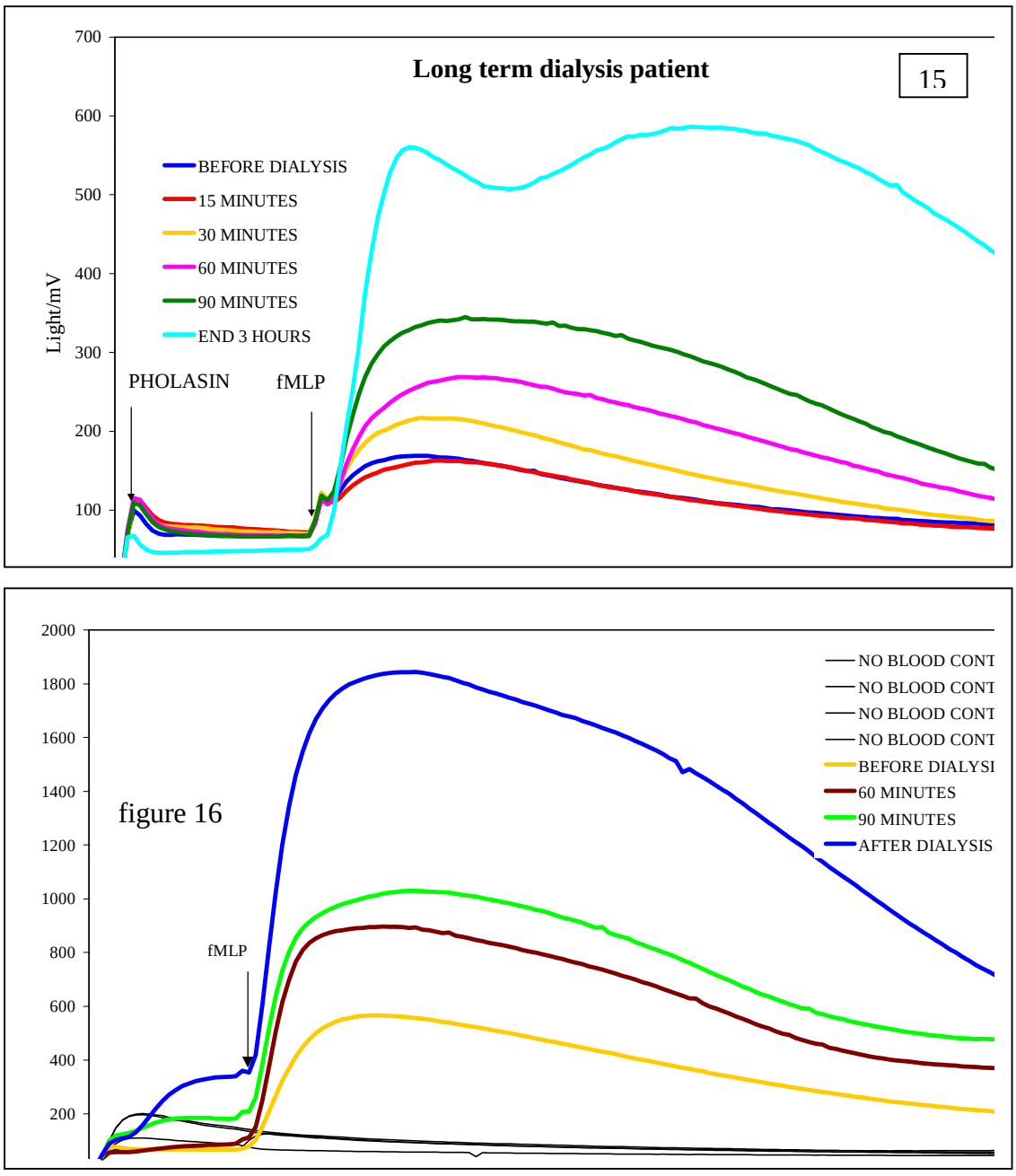




The experiment here illustrates the need when testing new materials to assess the material on a range of different bloods. Results from the blood shown in figure 14 would indicate of

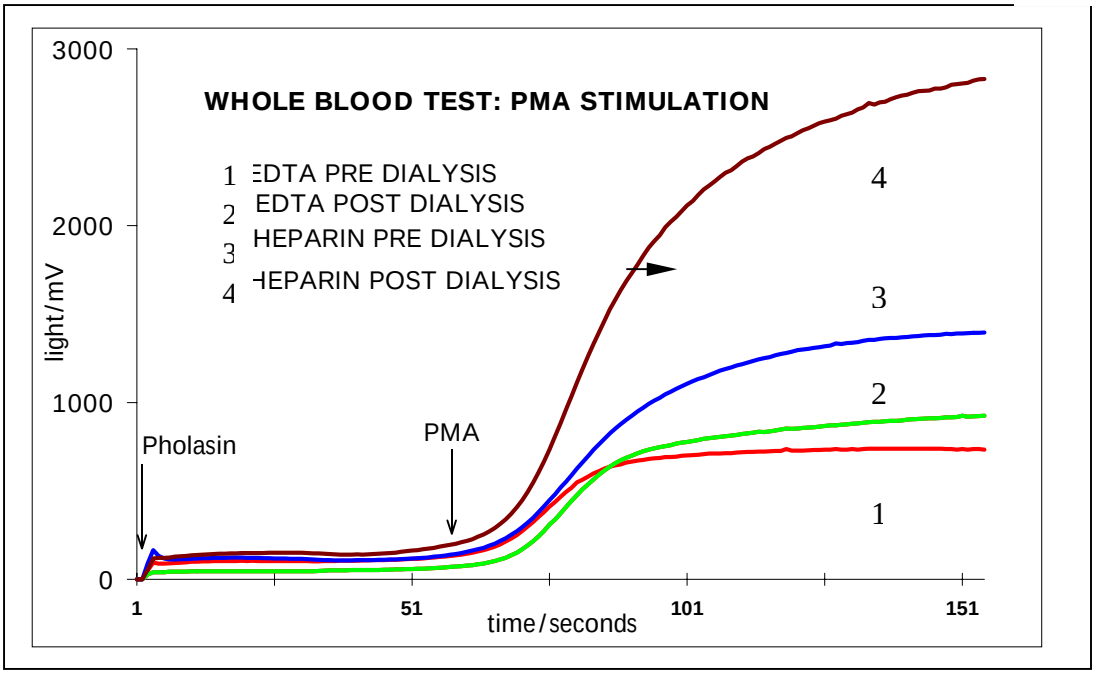
filter material prior to dialysis. While it had been assumed that both donors were normal, further analyses of the donor in figure 15 showed other abnormalities as well. that all the test materials would have no adverse affect on this particular potential patient whereas the results shown in figure 15 could lead to the opposite conclusion. This experiment also illustrates the potential for reducing risk to new patients by testing their blood on various types

Assess*ing *patients while on haemodialysis



Figures 15 and 16 show the results of the whole blood assay performed on blood taken before, during and after renal dialysis from patients who have been on dialysis for over 15 years. These patients show an abnormal response to the receptor stimulus fMLP. The effect of dialysis is shown by these results to prime the cells (perhaps by upregulation of receptors) to respond in an enhanced way to a receptor stimulus. Such patients it is assumed are under potential threat of complications arising from oxidative stress.

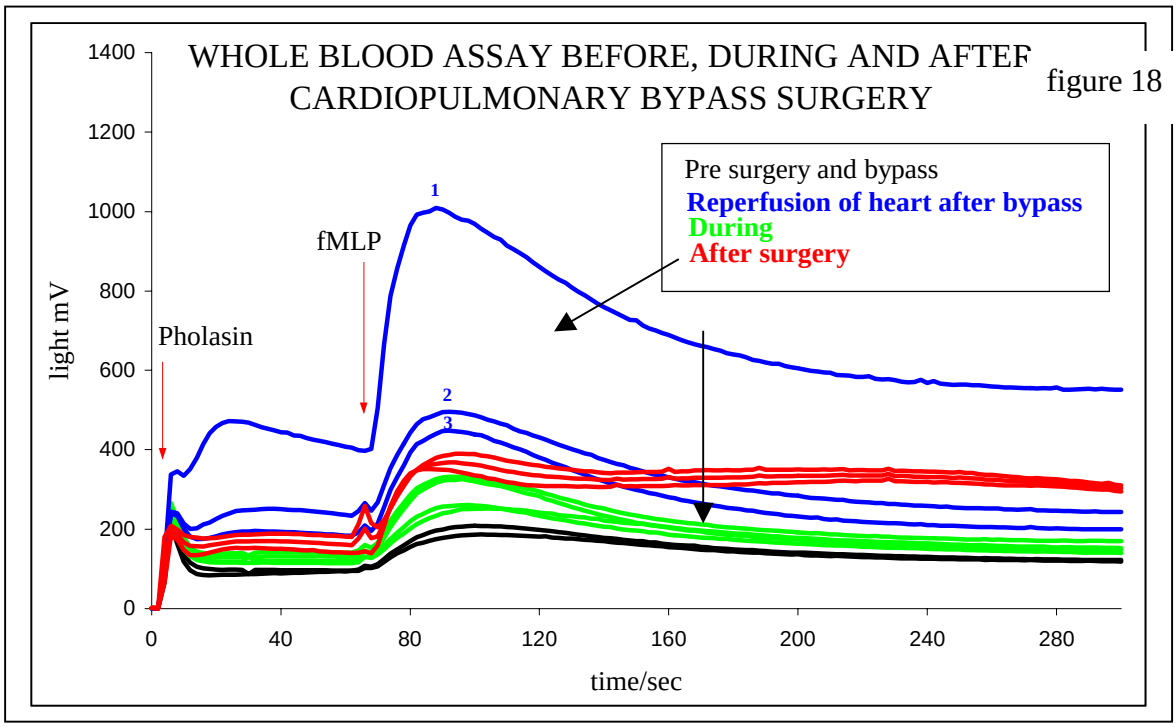
figure 17



In figure 17 samples of blood were taken before and after dialysis from a patient on dialysis for over 15 years. The stimulus used was PMA which as a diacyl glycerol analogue analogue activates the NADPH oxidase by activating PKC directly. PMA can also promote degranulation. If cells become more prone to degranulate as a result of dialysis this is easy to recognize by the difference in peak height of the luminescence of the blood stored in the presence of heparin compared to EDTA. It is seen here that at the end of dialysis there was evidence of degranulation**.**

Monitoring a Patient undergoing Cardiopulmonary Bypass Surgery

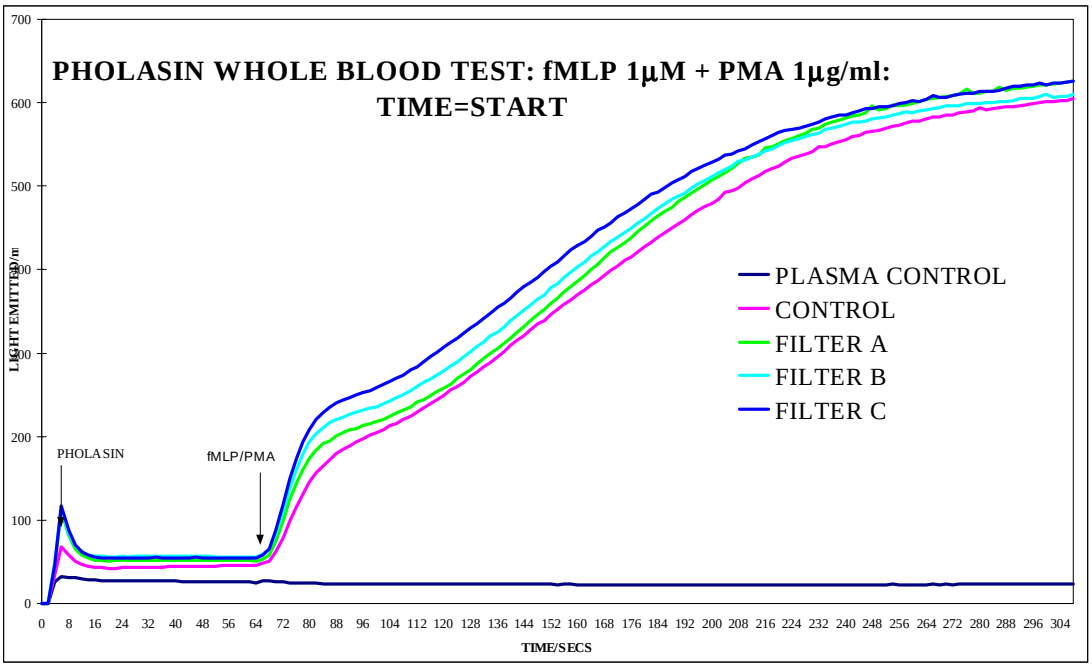
figure 18

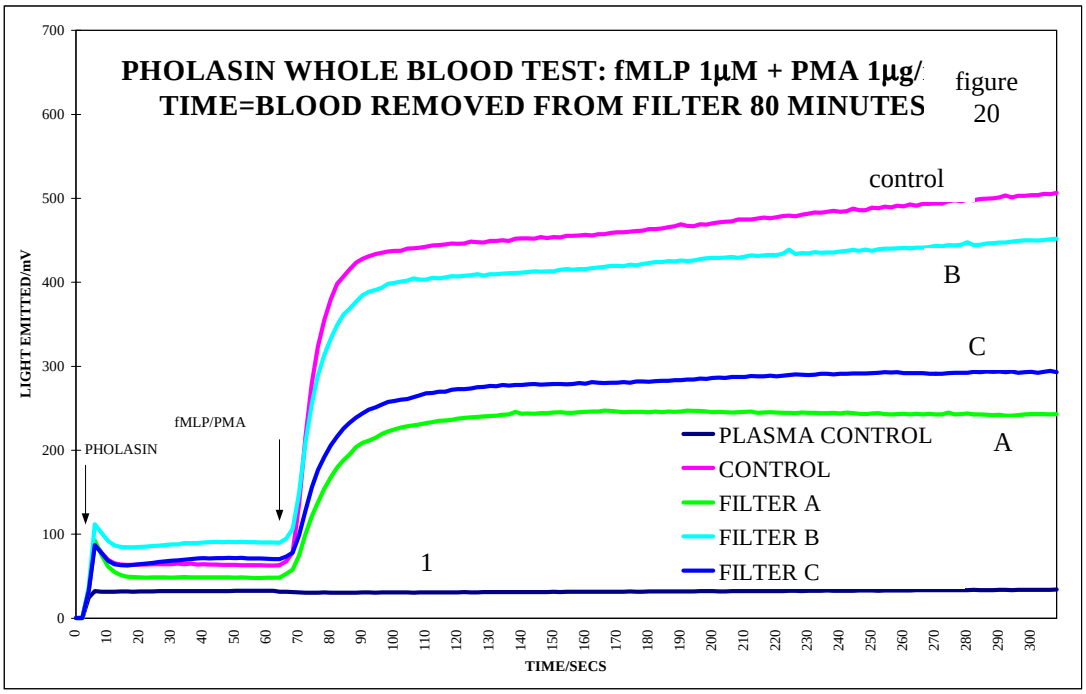


In figure 18 above an abnormal response was seen in a patient undergoing cardiopulmonary bypass surgery at the time of reperfusion. This abnormality was manifest in two ways: i) as a pre-stimulus light signal and ii) as a very rapid intense response, suggesting priming of cells. The patient was followed 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.

Assessing Artificial Coating on Arterial Filter Material

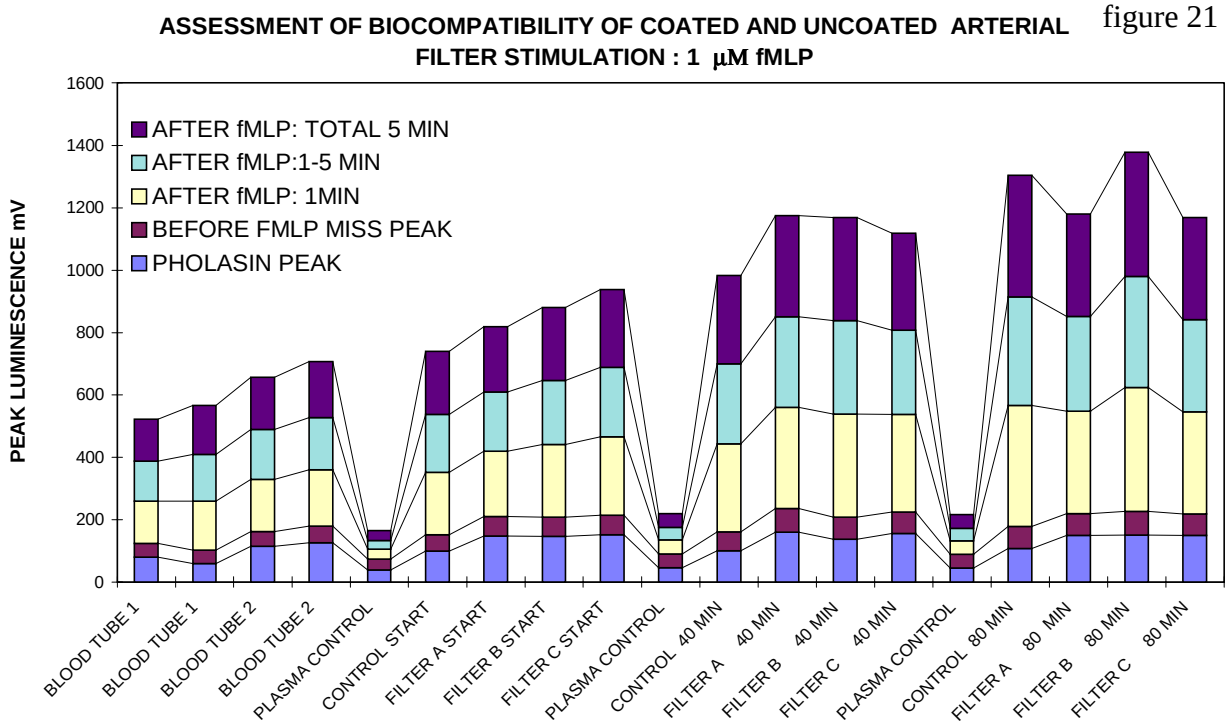
figure 19





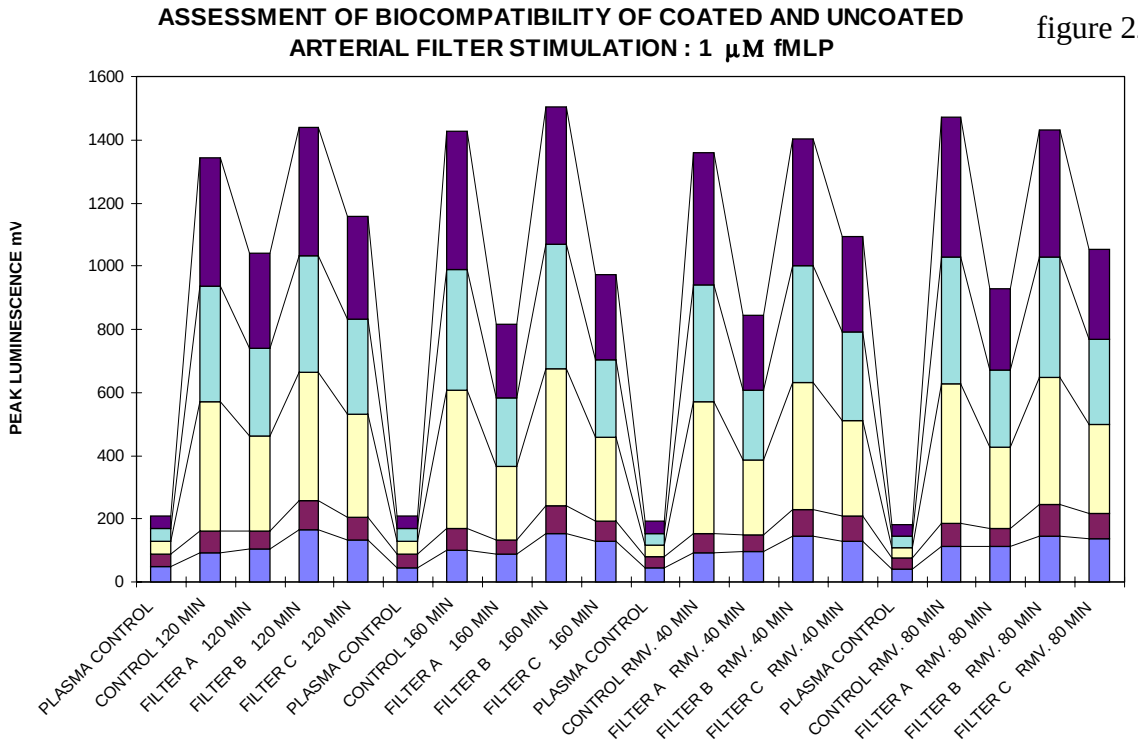
The experiment summarized above in figures 19 and 20 consisted of exposing pieces of arterial filter material to blood contained in a tube and maintained in contact with the filter by contant rolling on a blood roller. The two luminescent response graphs are representative samples of a much more extensive experiment involving longer times and additional sampling; furthermore, the experiment was repeated on more that one donor's blood. Insignificant amounts of blood (20µL from 5mL total) were removed at various times and diluted in accordance with the instructions for the ABEL® whole blood assay (figure 8). Figure 19 shows the response to stimulation with a mixture of fMLP and PMA at the start of the experiment; figure 20 shows the response after 80 minutes. The control sample was blood contained in a tube, but without any filter material and treated in an identical manner to the tubes containing filter material. Filter A was uncoated, filter B was coated with a commercially available proprietary material and filter C was coated with heparin. The results show clearly a marked reduction in the peak height of the response to the uncoated material and to the heparin coated material compared to the control and filter B.

figure 21



The results from the same experiment are presented in figure 20 and 21 as the peak luminescent response after stimulation with the receptor stimulant fMLP. The luminescent response curve (see figure 8) is divided into time segments: when Pholasin is injected, peak after 1 minute stimulation, from 1-5 minutes stimulation for the entire 5 minutes after stimulation. Figure 21 depicts the results at start, 40 minutes and 60 minutes; figures 22 shows the results at 80, 120 and 180 minutes. The results show clearly a reduction in luminescence between the uncoated filter material and the filter coated with heparin.

figure 22



Screening Bioactivity of a Range of Calcium Phosphate bioceramic Powders

figure 23

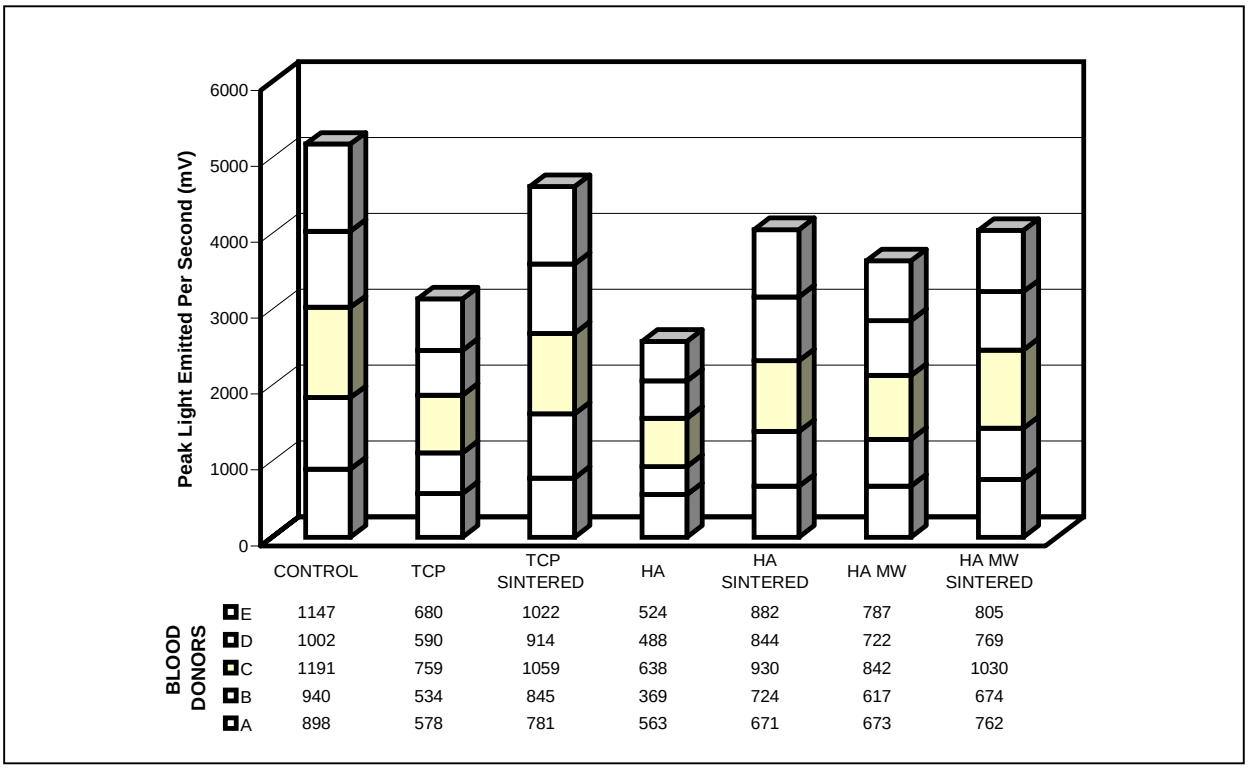
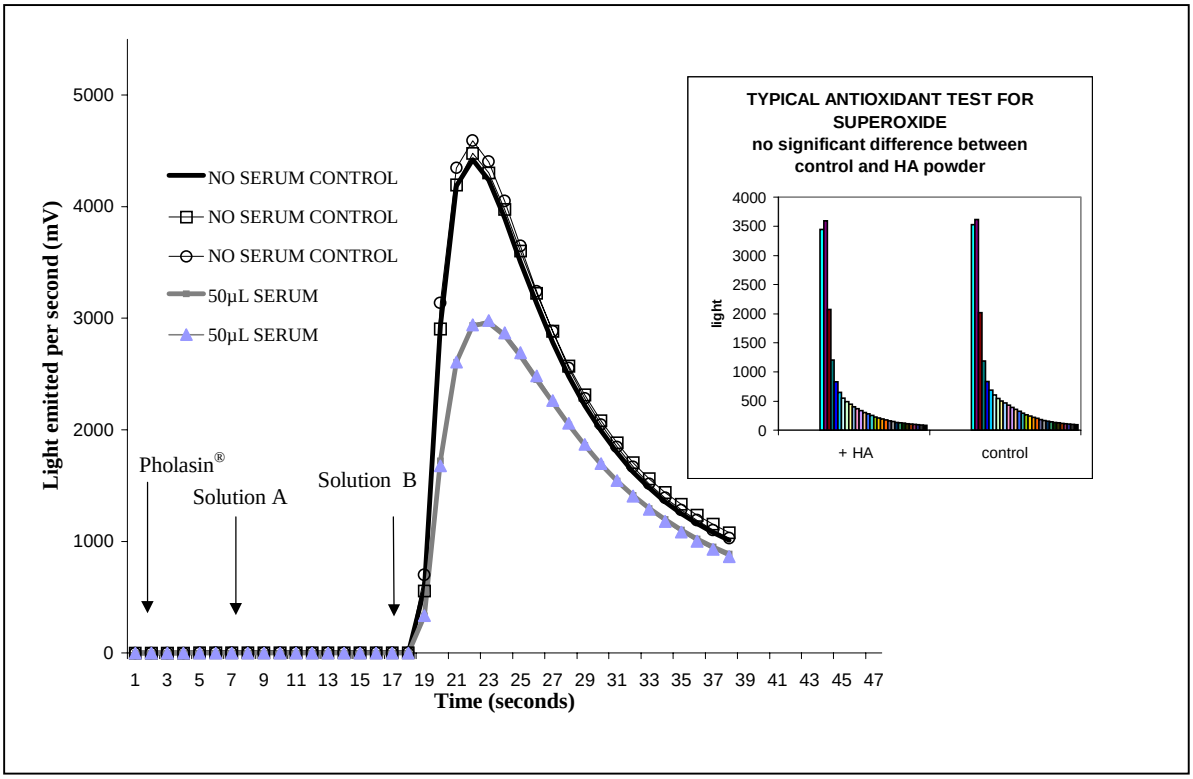


figure 24



Figures 23 and 24 are from a paper[13] submitted for publication in *Biomaterials.*

The calcium phosphate powders used in this study were commercial grade tricalcium phosphate (TCP), sintered tricalcium phosphate, commercial hydroxyapatite (HA), sintered hydroxyapatite, sintered and microwave treated hydroxyapatite. The results shown in figure 23 are from trials 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.

The effect of exposure of diluted blood to these bioactive materials was to lead to a reduction in the response of the cells to stimulation with PMA. The higher the bioactivity of the powders tested in this trial the lower the peak light signal after the NADPH oxidase was stimulated with PMA. The cells showed 100% viability. Tests in which superoxide was generated in the presence of Pholasin, with and without a sample of powder, demonstrated that the effect of reduction in light was neither due to quenching of light by the powder nor to a possible antioxidant effect of the powder (see figure 24). The most likely hypothesis to support the observations seen here was the bioactivity of the powders was indicated by the varying degrees of inhibitory action on protein kinase C.

Assessing Bioactivity of Substances Delivered from Controlled Release Glasses

Figures 25 and 26 are two sets of results from a much larger study[14] carried out by Paul Jackson and John Hunt at Clinical Engineering, University of Liverpool. They have been

using the ABEL® assays with Pholasin® with isolated human neutrophils to assess changes in the response to activation of the NADPH oxidase (the respiratory burst) following exposure

figure 25

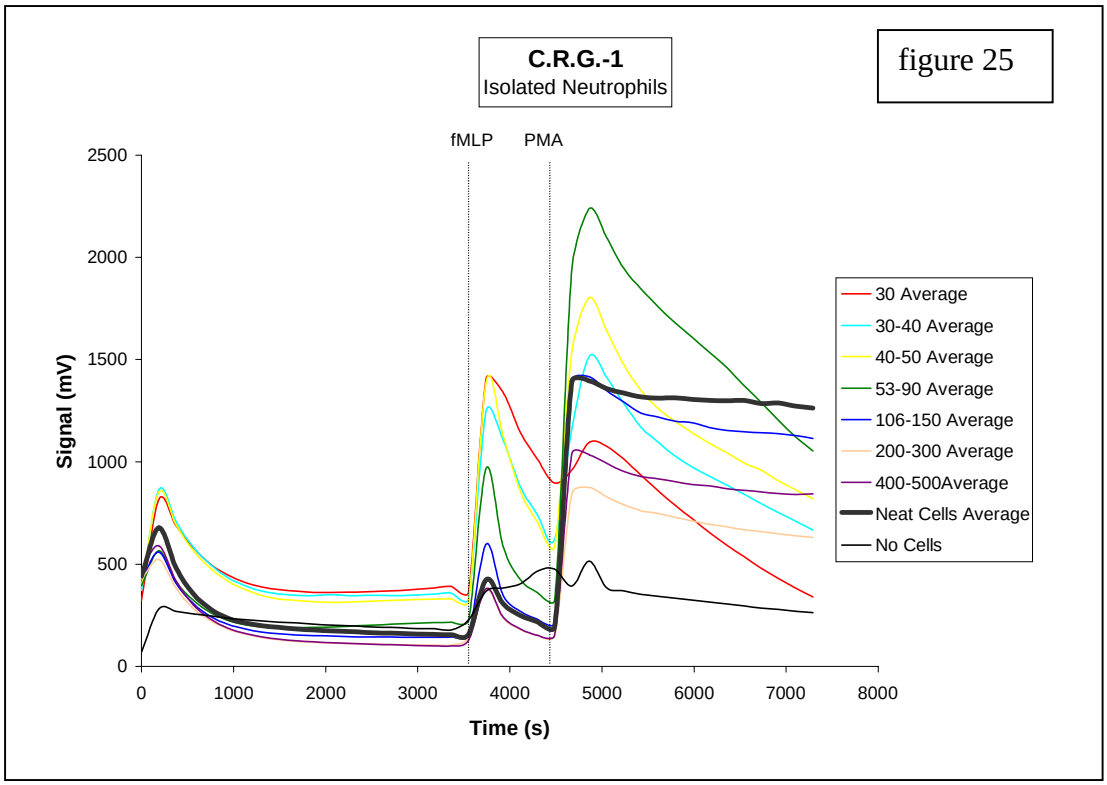
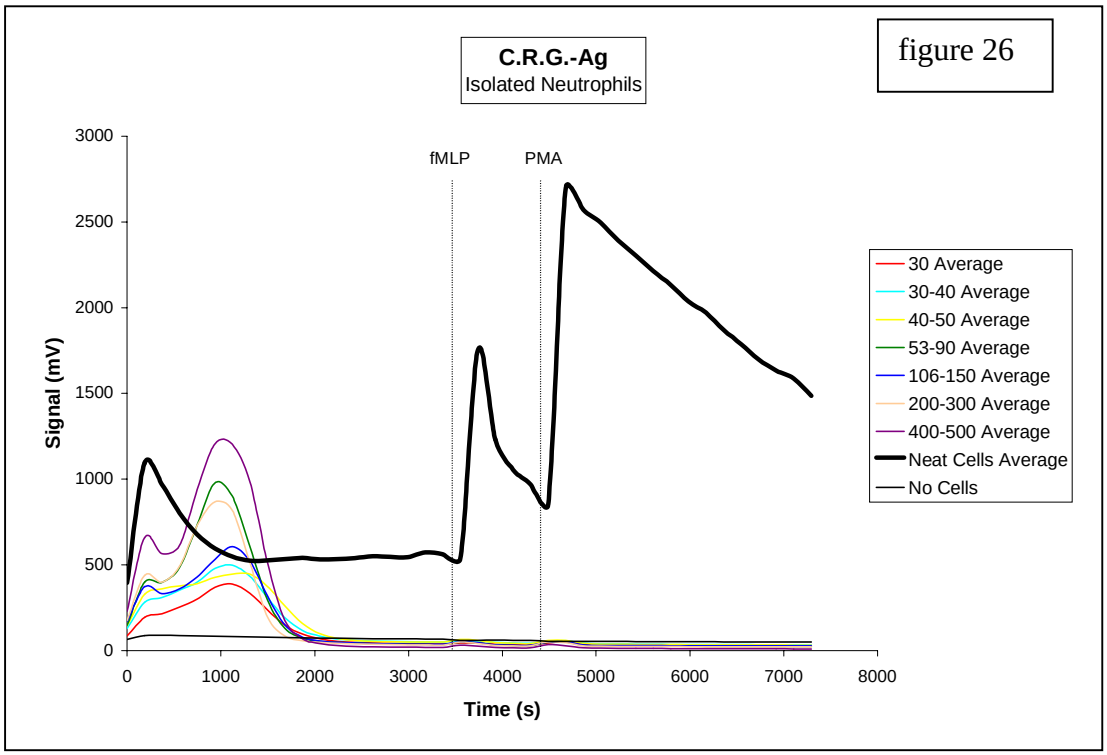


figure 26



of the neutrophils to CRGs of various particle size and containing a variety of metals: Ag, Cu, Mg, Zn. The CRGs dissolve at an appreciable rate in an aqueous environment. A biomedical application is in the wound dressing Arglaes® where Ag^{2+} ions are continually released into the region of the wound to control infection. Their results reproduced here in figures 25 and 26 demonstrate the dramatic effect inclusion of silver ions has on the inhibition of the respiratory burst compared to the effects of the size of the particles alone (figure 26). Effects of Cu, Mg, Zn, Ni ions released from CRG onto isolated neutrophils affected the respiratory response to varying degrees.

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