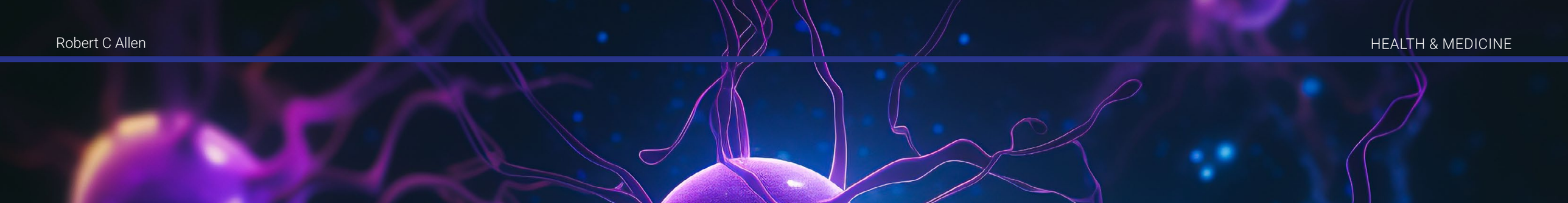


Inflammation measured as blood neutrophil luminescence

- Professor Robert C Allen has investigated how neutrophil respiratory burst metabolism transforms oxygen to a microbe-killing agent. The light emission associated with neutrophil microbicidal action proves the combustive nature of such action.
- Over the past four decades, Allen has invented sensitive luminol and lucigenin luminescence techniques for quantifying and differentiating the oxidase and haloperoxidase activities of phagocytic leukocytes and macrophages.
- Discriminant analysis of luminescence measurements is applicable to gauging the poise of innate immunity. The state of blood neutrophil responsiveness reflects the in vivo state of immune activation and inflammation.

Oxygen is essential to the survival of many organisms on Earth, including humans. In our bodies, however, oxygen also acts as a powerful defence weapon against microbial invaders. In the blood, red cells known as erythrocytes carry oxygen molecules throughout the body as required for metabolism, and the white cells, known as neutrophils, respond to microbe infections by migrating to the site of infection, contacting and phagocytosing the microbe, and converting oxygen to reactants responsible for combustive microbicidal oxygenations. Neutrophil oxygenating activity is fast and focused on killing microbial pathogens.

Combustion and chemiluminescence

Through a series of elegant studies, Robert C Allen has shown that the key to understanding the role of oxygen within the complex biochemical machinery of our immune system is linked to the unique electronic structure of the oxygen molecule. Neutrophils change the spin multiplicity state of the oxygen molecule by modifying the distribution of the frontier orbitals electrons. In neutrophils, the chemically inert triplet multiplicity oxygen molecule ($^3O_2$) from the atmosphere is converted to the metastable electronically excited singlet multiplicity oxygen molecule ($^1O_2$*) which readily reacts with the singlet multiplicity biomolecules of the microbe, resulting in the combustive oxygenation reactions responsible for microbial killing. Allen's central premise is that reactions of $^1O_2$* with the singlet multiplicity molecules of microbes are spin allowed and highly exergonic. Such oxygenation reactions can yield electronically excited singlet multiplicity carbonyl products that relax to ground state by light emission, ie, chemiluminescence.

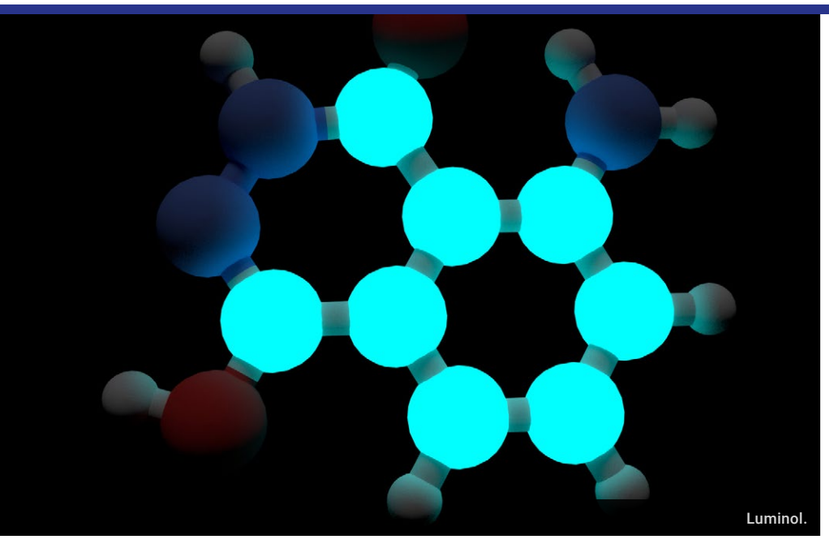
In the conventional chemical process of burning, energy is applied to a singlet multiplicity fuel molecule (^{1}molecule) causing homolytic bond cleavage yielding two radical doublet (^{2} molecule) products. The doublet radicals produced can participate in doublet-triplet reactions with ground state triple oxygen ($^3O_2$) yielding a doublet product capable of reacting with additional $^3O_2$ in a process of radical propagation.

Instead of radicalizing the microbial substrate molecule to facilitate reactivity doublet-triplet reaction with propagation, neutrophils de-radicalize paramagnetic $^3O_2$ to create diamagnetic $^1O_2$*, thus allowing its participation in singlet-singlet reaction with the singlet molecules of microbes. Spin must be conserved for combustion of any type, either burning or neutrophil dioxygenation. Combustions have exergonicities sufficient for light emission.

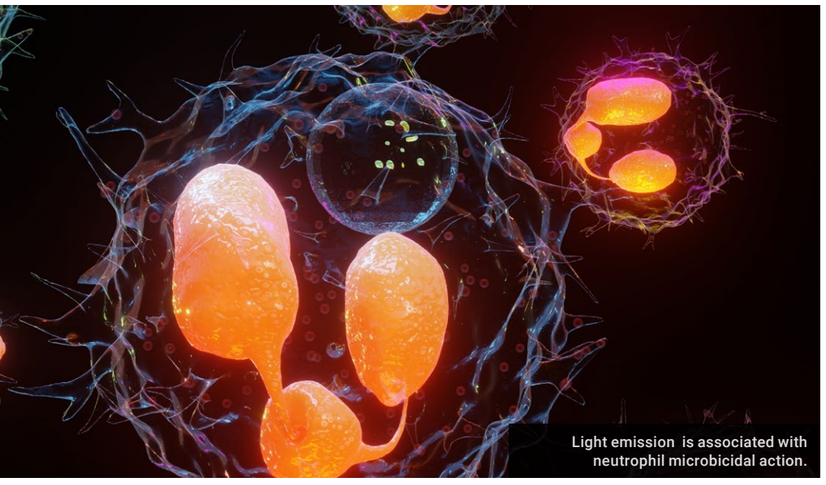
Chemiluminigenic probes enable sensitive measurements of phagocyte respiratory burst combustive actions.

Tracking the path of oxygen in phagocyte microbicidal action

Chemiluminescence allows non-destructive measurement of phagocyte microbicidal oxygenation reactions. The sensitivity and specificity of luminescence measurements of phagocyte redox metabolism and haloperoxidase activity are greatly increased by using chemiluminigenic probes, ie, organic molecules susceptible to dioxygenations yielding electronically excited singlet carbonyl



Luminol.



Light emission is associated with neutrophil microbicidal action.

functions and photon emission. In such regard, lucigenin luminescence requires reductive dioxygenation and allows high sensitivity and specific measurement of phagocyte NADPH oxidase activity. Luminol luminescence requires simple dioxygenation. Luminol permits high-sensitivity luminescence measurement of neutrophil haloperoxidase action, but it is not haloperoxidase specific. Luminol can measure oxidase activities in macrophages lacking haloperoxidase and in haloperoxidase-deficient heterophile leukocytes (Merrill et al, 1996). In an influential paper published in 1986 in *Methods in Enzymology*, Allen demonstrated the use of the chemiluminigenic probes lucigenin and luminol for high sensitivity and differential quantification of phagocyte oxidase and myeloperoxidase activities, respectively.

Microgravity adaptation

Allen's method provides information on immune system dynamics that can be applied to investigating how the body responds to pathogens under a variety of conditions and

Artificial intelligence and the ability to measure cellular luminescence are helping to develop novel diagnostic tools for immune system monitoring.

environments. Intriguingly, these are not limited to life on Earth. In recent work carried out on the International Space Station (ISS), Allen's approach has been used to monitor the reactions occurring in respiratory burst conditions in mammalian macrophages, a type of tissue phagocyte that play an important role in the immune system by engulfing and digesting pathogens such as microbes, cancer cells, and foreign substances. This study has shown that macrophages adapt very quickly, literally within seconds, to microgravity after an initial inhibitory phase of the respiration burst reactions. This is an important finding, which sheds light onto the long-standing question of why many astronauts can spend protracted periods of time in space with no apparent adverse effects on their health, despite the severe stress mammalian cells experience in microgravity conditions.

Applying classification statistical analysis to the immune system

The human immune system is an intricately complex network, characterized by the dynamic interplay of genes, proteins, cells, and tissues. It is a vast and sophisticated architecture, billions of times larger than the human genome. This complexity is further accentuated by individual variations among people and by the continual modulation influence of factors such as age, genetics, and environmental conditions. This intricate system serves as the foundation for crucial health interventions, encompassing vaccines and state-of-the-art immunotherapies.

Advanced computing capabilities combined with the classification statistic method of discriminant analysis facilitate investigation of immune system functions. Chemiluminigenic probing allows measurement and differentiation of resting and stimulated neutrophil oxidase and haloperoxidase functions and permits estimation of the ratio of circulating (COR) to maximal primed neutrophil opsonin receptor (MOR) expression. The COR:MOR ratio gauges *in vivo* state of inflammation. Metric acquisitions require only sub-microliter quantities of blood or tissue fluids. Discriminant analysis of temporally acquired data provides information applicable to research and infectious disease clinical management. Such temporal analysis is also relevant to monitoring of bone marrow myelopoiesis.

A novel point of care diagnostic tool

Allen and collaborators demonstrated the utility of composite chemiluminigenic probing with discriminant analysis. Recent efforts have been directed to procedural and technical simplification regarding minimal blood specimen collection, rapid dilution and direct luminescence measurement using a 96-well microplate format. Blood or fluid undergoes a thousand-fold dilution with buffered medium that reconstitutes divalent cations and obviates the effect of anticoagulant. Less than a microliter of specimen is directly contacted with appropriate chemiluminigenic probes, with or without immune primers, and with or without chemical or phagocytic stimuli. Light emission is measured by an automated Berthold microplate luminometer and the composite data is collected and used for discriminant analysis. Testing is robust, yields reproducible results, and can be adapted to point-of-care testing using a hand-held or portable luminometer.

Personal response

What are the principles on which chemiluminigenic probing is based and what were the steps that led you to extend this approach to create a tool for monitoring the immune system response in real time?

The light emitted, ie, native luminescence, from phagocytosing neutrophils is an energy product of combustive microbicidal dioxygenation activity and is easily measured using the photon-counting capability of a scintillation counter operated in the out-of-coincidence mode. However, the native luminescence activity of phagocytosing macrophages was not sufficiently above instrument background noise. My reasoning was that adding a high-luminescence quantum yield molecule susceptible to reaction with oxygenating agents generated by phagocytosing macrophages would increase light yield, allowing luminescence detection. Luminol, the first chemiluminigenic probe tested, greatly increased the luminescence, allowing sensitive detection of the oxygenation activities of activated macrophages. When applied to the study of phagocytosing neutrophils, luminol increased light emission by several orders of magnitude relative to natural light emission (Allen and Loose, 1976).

A chemiluminigenic probe is defined as a molecular substrate susceptible to dioxygenations and producing endoperoxides or dioxetane intermediates ultimately yielding electronically excited carbonyls that relax by photon emission. Selecting chemiluminigenic probes with different reactive potential can allow differential measurement of dioxygenation activities. Lucigenin luminescence requires reductive dioxygenation, ie, the incorporation of two electrons plus an oxygen molecule. This reductive dioxygenation requirement can be achieved by phagocyte production of one singlet hydrogen peroxide (H_2O_2) or by univalent reduction of lucigenin to a doublet radical plus reaction with doublet multiplicity superoxide anion ($^2O_2^-$). Note that all the possible reactants are products of activated NADPH oxidase (Allen, 1981).

In an alkaline milieu, luminol can participate in complex radical (doublet) reactions ultimately yielding endoperoxide formation with luminescence, but in a mildly acid milieu, luminol luminescence is the product of non-radical haloperoxidase-catalyzed dioxygenation (Allen, R, 2022). Use of lucigenin and luminol allows sensitive and differential luminescence measurements of phagocytosing blood neutrophils. Measuring the luminescence ratio of un-primed- and maximally primed-phagocytosing neutrophils gauges the *in vivo* capacity of neutrophils to respond to phagocytic challenge. This circulating (COR) to maximal receptor (MOR) ratio reflects the *in vivo* state of inflammatory poise at the time of blood sampling. As *in vivo* inflammation approaches maximum, the COR:MOR ratio approximates unity.

What is the role of classification statistical analysis play in the development of new medical tools for measuring the functionality of the immune system?

Relatively large luminescence data bases can be generated by differential chemiluminigenic probing of oxidase and haloperoxidase activities plus COR:MOR ratio determination on each sub-microliter blood or body fluid specimen tested. By the 1980s, the data generated by combined measurements suggested the need for advanced data handling and analysis. Professor AD Mason of the USAISR (US Army Institute of Surgical Research) suggested that a statistical classification or clustering approach might be applicable.

Discriminate analysis is a statistical classification method applicable to relating a categorical (ie, non-metric or descriptive) dependent variable to a metric independent variable or variables. If three or more classifications are involved, the technique is a multiple discriminate analysis (MDA). Linear combination of two or more independent (luminescence metric) variables can facilitate discrimination between defined groups, ie, healthy human host and infected human hosts. Statistically, the objective is to maximise between-group variance relative to the within-group variance (Allen et al, 2000). MDA has been successfully applied to luminescence analysis of human patients under treatment for various infectious diseases, healthy young and older human volunteers treated with the myelopoietic stimulant rG-CSF (recombinant human granulocyte colony stimulating factor), and healthy human volunteers treated with endotoxin. For all studies the collective metric measurements were evaluated over the temporal course of disease (Allen and Stevens, 1992; Stevens et al, 1994), the temporal course following rG-CSF (Chatta et al, 1994; Allen et al, 1997) and the time course following endotoxin infusion (Taylor et al, 2000), respectively.

What will allow creation of a point-of-care test tool for the immune system and make it available for general use?

Presently, neutrophil number and function can be rapidly assessed in real-time using a sub-microliter volume of blood or body fluids by dilution and luminometry measurement. Such measurements are applicable to the clinical laboratory environment or any venue where a point-of-care luminescence detection device can operate (Allen et al, US Patent Appl Pub 2022/0308046 A1).

Details

e: robertallen@creighton.edu
w: exoxemis.com

Bio

Professor Robert C Allen's published research started in 1972, with the report that neutrophils engaged in microbe killing emit light in the visible range of the spectrum. In a seminal paper in 1986, while serving as a military medical officer at the US Army Institute of Surgical Research (aka Burn Center), FSH TX, he demonstrated that neutrophil oxidase and haloperoxidase dependent oxygenation activities could be differentially quantified in real time and with high sensitivity using chemiluminigenic probes (ie, luminol and lucigenin). In recent decades, Allen has applied luminescence measurement techniques and discriminant statistical analysis to evaluation of host systemic inflammation and diagnosis of infectious states.

Further reading

- Allen, R, (2022) Haloperoxidase-catalyzed luminol luminescence, *Antioxidants*, 11, 518.
- Taylor, Jr, F, et al, (2000) Luminescence studies of the phagocyte response to endotoxin infusion into normal human subjects: multiple discriminant analysis of luminescence response and correlation with phagocyte morphologic changes and release of elastase, *J Endotoxin Res,* 6(1), 3–15.
- Allen, RC, Dale, D, Taylor Jr, F, (2000) Blood phagocyte luminescence: Gauging systemic immune activation, *Method Enzymol*, 305, 591–629.
- Thiel, C, et al, (2017) Rapid adaptation to microgravity in mammalian macrophage cells, *Sci Rep,* 7, 43.
- Allen, R, et al, (1997) In vivo effects of recombinant human granulocyte colony-stimulating factor on neutrophil oxidative functions in normal human volunteers, *J Infect Dis*, 175, 1184–119.
- Merrill, G, et al, (1996) Oxygenation activities of chicken polymorphonuclear leukocytes investigated by selective chemiluminigenic probes, *Lab Animal Sci*, 46(5), 503–508.
- Stevens, D, et al, (1994) Analysis of circulating phagocyte activity measured by whole blood luminescence: Correlations with clinical status, *J Infect Dis*, 170, 1463–1472.
- Chatta, GS, et al, (1994) Effects of in vivo recombinant methionyl human granulocyte colony-stimulating factor on the neutrophil response and peripheral blood colony-forming cells in healthy young and elderly adult volunteer, *Blood*, 184(9), 2923–2929.
- Allen, R, Stevens, D, (1992) The circulating phagocyte reflects the in vivo state of immune defense, *Curr Opin Infectious Dis*, 4, 289–298.
- Allen, R, (1986) Phagocytic leukocyte oxygenation activities and chemiluminescence: a kinetic approach to analysis, *Method Enzymol*, 133, 449–493.
- Allen, R, (1981) Lucigenin chemiluminescence: A new approach to the study of polymorphonuclear leukocyte redox activity. In: *Bioluminescence and Chemiluminescence Basic Chemistry and Analytical Applications.* New York: Academic Press, 63–73.
- Allen, RC, Loose, L, (1976) Phagocytic activation of a luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophages, *Biochem Biophys Res Comm*, 69(1), 245–252.