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NEUTROPHIL DEFECT IN THE CHEDIAK-HIGASHI
SYNDROME MINK USING A CHEMILUMINESCENCE ASSAY
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CHARACTERIZATION OF THE
NEUTROPHIL DEFECT IN THE CHEDIAK-HIGASHI
SYNDROME MINK USING A CHEMILUMINESCENCE
ASSAY

By

Wm. Ellis Carter

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ABSTRACT

CHARACTERIZATION OF THE NEUTROPHIL DEFECT IN THE CHEDIAK-HIGASHI SYNDROME MINK USING A CHEMILUMINESCENCE ASSAY

By

William Ellis Carter

A luminol enhanced chemiluminescence (CL) assay, which rapidly, nonsubjectively, measures the high energy antimicrobial oxygen species formed during the initial phagocytic event was adapted to CHS mink to characterize that animal's neutrophil defect.

The CHS mink latex induced, luminol enhanced ($0.5 \times 10^{-5} \text{M}$) neutrophil CL measured in a liquid scintillation counter "in-coincidence" produced $15,345 \pm 6,471$ counts per minute (CPM). This was significantly less ($p < 0.05$) than the $196,440 \pm 66,980$ CPM observed with normal mink neutrophils.

The CHS neutrophil defect could be further amplified in vitro by using a higher concentration of luminol ($8 \times 10^{-6} \text{M}$). A similar significant deficit ($p < 0.05$) of the CHS neutrophil CL response was observed using phorbol myristate acetate as the CL inducer in the $8 \times 10^{-6} \text{M}$ luminol enhanced assay compared to normal mink. Using the peak CPM of normal mink neutrophil CL as an index of phagocytic function it was also shown that the latex induced CL response of CHS mink neutrophils was not enhanced by mixing normal with CHS neutrophils in ratios of 80:20, 50:50, and 20:80.

The time of onset of the peak CL response was documented by transmission electron microscopic examination of gluteraldehyde fixed CHS and normal mink neutrophils two minutes after the addition of latex

particles. The subjective evaluation of the electron micrographs was that both groups of neutrophils were capable of phagocytizing similar numbers of latex particles, and that the time of onset of the peak CL response obtained during the CL assay was valid.

By using enzyme inhibitors of oxidative metabolism, superoxide dismutase and catalase, it was shown that the CHS CL response, even though significantly less than the normal response, consisted of the same reactive oxygen species as the normal CL response because of the similar manner of CL inhibition.

These results demonstrate that the dual "in-coincidence" photomultiplier CL assay, is quick, reproducible, and effective in detection of the CHS neutrophil defect. The CHS neutrophil is not defective in its ability to phagocytize but is defective in its ability to generate the high energy antimicrobial oxygen species assessed by the luminol enhanced CL assay. This defective CL response partially explains the compromised host defense of CHS mink.

To my parents, William, Sr. and June Carter,
for their continual love and support.

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LIST OF SYMBOLS AND ABBREVIATIONS

CHS	The Chediak-Higashi Syndrome
CL	Chemiluminescence
Cl^-	Chloride ion
CPM	Counts Per Minute
EDTA	Ethelenediaminetetraacetic Acid
EPS	EDTA-Phosphate Buffered Saline
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
HBSS	Hanks Balanced Salt Solution
HOCL	Hypochlorous Acid
MPO	Myeloperoxidase
NADP	Oxidized Nicotinamide Adenine Dinucleotide Phospate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
O_2	Molecular Oxygen
O_2^-	Superoxide Anion
OCl^-	Hypochlorite Acid
PBS	Phosphate Buffered Saline
PMA	Phorbol Myristate Acetate
SOD	Superoxide Dismutase

INTRODUCTION

Since Metchnikoff's discovery in 1887 that phagocytes protect the body against infection, the importance of the neutrophil in host defense has been of great research interest (1). The research of the neutrophil has been facilitated by improved neutrophil isolation techniques such as the separation of total leukocytes by sedimenting agents (2) and density gradient centrifugation (3), and has resulted in several assays to evaluate neutrophil competency (4,5).

A great deal of knowledge about neutrophil function, metabolism, and biochemical pathways was generated by the study of families with inherited neutrophil defects (6). The most notable of these are chronic granulomatous disease, myeloperoxidase deficiency, and the Chediak-Higashi syndrome (4,6,7).

Research on the Chediak-Higashi syndrome has been facilitated by documented animal models (8). Aleutian mink, Mustela vison, are one of six species of animals that have this neutrophil defect. As this is an unusual research animal a few comments are necessary.

In 1941 an Oregon mink rancher saved an "off color" mink to use as a breeder. The offspring were back-crossed to reproduce the same phenotype. The coat color was called "Aleutian" after the fox which had a similarly colored pelt. These mutant mink known collectively as "blues", "sapphires" and "Aleutian type" commanded higher market prices during pelting season than the "dark" or "Pastel" mink. Unfortunately, ranchers that took advantage of this economic potential began to report losses due to a condition that appeared to affect only Aleutian type mink. The condition was named "Aleutian disease" and has subsequently been determined to be a chronic viral infection that can affect all mink (9). The viral infection

results in a plasma cell proliferation with an associated polyclonal hypergammaglobulinemia and fibrinoid degeneration of small muscular arteries and arterioles. The disease is transmitted vertically and horizontally as the virus can be shed through most body excretions and secretions (10).

As veterinary researchers determined Aleutian mink were more susceptible to Aleutian disease and bacterial infections such as staphylococcal and corynebacterial abscesses, pastuerellosis and tuberculosis (11,12,13), it became evident that homozygosity for the recessive gene affected more than just coat color. The melanocyte defect, exhibited as partial albinism, and a platelet defect were genetically related to abnormal granulation in all lysosome-containing cells (14,15,16), including one most important in host-defense, the neutrophil.

The present study on the neutrophil defect in CHS mink was undertaken after a pilot study was done in 1981 to assess the CHS genotypic effect on the generalized Schwartzman reaction (GSR) induced by endotoxin. Twenty mink, half normal and half CHS, were administered 55:B5 Escherichia coli endotoxin or saline intraperitoneally followed by a second identical injection 21 hours later. The dual dosage ranged from 200 ug/kg to 26.6 mg/kg. Normal mink were symptomatic at 800 ug/kg and 26.6 mg/kg was lethal while CHS animals succumbed at 12.2 mg/kg. Forty-eight hours later or at the time of death all animals were necropsied and sampled for histopathologic examination. Spleno-hepatomegaly with extravasation was present at a dose of 1.6 mg/kg in CHS while it was not a lesion of normal or control mink. Renal tubular necrosis was also present in CHS mink. The results revealed that CHS mink were more susceptible to the lethal effects of endotoxin and had different signs and lesions (17). With the

information that neutrophils play a role in the inflammatory events subsequent to endothelial damage (18,19) as evidenced in the generalized Schwartzman reaction, and that CHS animals are more susceptible to those inflammatory events (17), it was apparent that a study designed to compare normal and CHS neutrophils should be undertaken.

These studies, outlined in the text of this dissertation, used CHS and normal mink neutrophils isolated by density gradient centrifugation. The isolated cells were assayed in a liquid scintillation counter to assess their function by chemiluminescence. CHS mink neutrophils and normal mink neutrophils were studied as individual groups and in combinations. Two different stimuli, latex particles and phorbol myristate acetate, were used to initiate chemiluminescence. The cells were also examined for post-phagocytic differences in morphology with the aid of transmission electron microscopy. To document the source of neutrophil chemiluminescence two enzymes, superoxide dismutase and catalase, were added to the assay to inhibit the response. The latter studies were carried out to determine to what extent the oxidative mechanisms involved in normal neutrophil defense systems were altered in the CHS neutrophil and to determine if these alterations were part of the CHS neutrophil defect and the compromised host defense in CHS animals.

A severe defect in CHS neutrophil function was revealed by the marked reduction in chemiluminescence in response to latex particle phagocytosis.

LITERATURE REVIEW

A. General Considerations

In the human, the Chediak-Higashi syndrome (CHS) is a rare disorder inherited as an autosomal recessive trait (8). Synonyms for CHS include Bequez-Cesar-Steinbrinck-Chediak-Higashi syndrome, congenital giantism of peroxidase granules, granulation anomaly of leukocytes, and hereditary gigantism of cytoplasmic organelles (20,21,22,23,24). This fatal disease of childhood is characterized by imperfect oculocutaneous albinism with giant melanosomes, giant peroxidase positive lysosomal granules in leukocytes, and giant granules in Schwann cells and other tissues (25). A prominent clinical feature in humans is increased susceptibility to recurrent, severe and often fatal pyogenic infections. Few affected humans have survived to twenty years of age and those that do survive frequently develop a lymphoreticular infiltrate of various organs.

Following the description of CHS in humans in 1943 (20), the disease was described in Aleutian mink (26) and cattle (27) in the early 1960's and mice in 1967 (28). In the 1970's CHS was reported to occur in Persian cats (29) and a killer whale (30). The mode of inheritance in all these animals, except the latter, like man, is known to be autosomal recessive.

The Aleutian (CHS) mink is known to have many characteristics similar to other species with CHS and is considered to have CHS. Increased susceptibility to bacterial infection has also been noted in CHS mink and has been attributed to the abnormally enlarged granules of lysosome-containing cells, the polymorphonuclear neutrophil being the cell of greatest interest to this discussion. The CHS neutrophil has impaired chemotactic and microbicidal activity marked by a decreased fusion of enlarged granules with phagocytic vacuoles (31,32).

Clawson and White (33) have clarified the defective bactericidal function of CHS neutrophils in humans by using electron microscopy to study morphology and ultrastructural cytochemistry. The abnormally enlarged organelles in CHS neutrophils originate by fusion of small azurophilic, peroxidase positive granules in promyelocytes and myelocytes. This fusion process, that leads to the formation of the large granules, continues in mature neutrophils and involves both azurophilic lysosomes and specific granules with entrapment of other cytoplasmic elements. The end result is a reduction in primary granules and formation of a large granule that lacks reactivity because the content of hydrolytic enzymes has been diluted. These large granules, technically lysosomes, continue to react with primary lysosomes, but seldom fuse with each other. Therefore, when CHS neutrophils ingest particles, a phagosome is formed, but since the number of primary granules is reduced, there is inefficient phagolysosome formation. The casual and delayed interplay involving the enlarged myeloperoxidase-containing granules results in a phagolysosome with ineffective killing power.

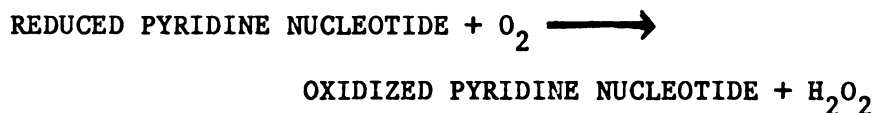
B. Normal Oxygen-Dependent Neutrophil Defense Mechanisms

The killing power or microbicidal capacity of the neutrophil involves a complex interplay of metabolic pathways during phagocytosis which are, in part, dependent upon reactions involving oxygen (34).

Baldrige and Gerard made the initial observations relating oxygen to microbial killing by phagocytes in 1933 by studying the metabolism of the canine neutrophil (35). They reported that oxygen uptake by these cells rose by a factor of 2 to 4 when they were engaged in the phagocytosis of Sarcina lutea. This increase in oxygen uptake was attributed to the

additional substrate oxidation required to provide energy for phagocytosis. In 1959 the real meaning of Baldrige's and Gerard's experiments was elucidated by Karnovsky (36). His monumental finding that the increase in oxygen uptake associated with phagocytosis was not blocked by cyanide meant that the profound change in oxygen uptake was not connected with energy production. Oxygen consumption related to ATP formation would have been abolished by cyanide under the conditions used in Karnovsky's experiments; therefore, oxygen was being used by the phagocyte for some other purpose.

Quastel is credited with elucidating the function of oxygen in phagocytosis (37). He not only confirmed Karnovsky's findings but also reported that phagocytosing neutrophils liberated hydrogen peroxide (H_2O_2) into the surrounding medium. Quastel then postulated that H_2O_2 was generated from the oxygen taken up by phagocytes in response to particle stimulation and that it functioned as an antimicrobial agent. Karnovsky and his associates had previously discovered that phagocytes engaged in particle uptake augmented their rate of glucose oxidation via the hexose monophosphate shunt (38). This led to the formulation of the metabolic event now known as the "respiratory burst".



The above reaction along with the conversion of the respiratory burst products to potential microbicidal oxidants and the destruction of those respiratory burst products which leak into the cytoplasm of the phagocyte by the antioxidant systems of the cell constitute the mechanism of oxygen dependent killing.

The biochemical basis for the respiratory burst is the activation of an enzyme, NADPH oxidase, which is dormant in unstimulated cells and

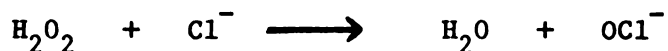
catalyzes the reduction of oxygen to O_2^- (superoxide) at the expense of a reduced pyridine nucleotide NADPH (39,40). The O_2^- forming reaction may therefore be written:



Hexose monophosphate shunt activation results from an increase in the rate of oxidation of NADPH during the respiratory burst. The increase in NADPH oxidation has been documented by measurements of pyridine nucleotide levels in resting and activated neutrophils (41).

Of the two respiratory burst products H_2O_2 is weakly microbicidal (42), and O_2^- (superoxide anion) appears to have little if any microbicidal activity whatsoever (43,44). The major function of these compounds is to serve as precursors of the very powerful oxidants actually employed by phagocytes for killing. These lethal oxidants are formed from the respiratory burst products by two distinct pathways. One of the pathways requires myeloperoxidase, while another less well defined pathway does not.

Myeloperoxidase, a heme-containing enzyme, is found in abundant quantities in azurophilic granules of neutrophils (45,46,47). The enzyme contains two iron porphyrin groups attached to the protein by covalent links (48). The reactions catalyzed by myeloperoxidase are generally typical of those catalyzed by other peroxidases. The most relevant substrate in biologic interactions of myeloperoxidase is the chloride ion. This halide ion is readily oxidized to OCl^- by H_2O_2 :



Myeloperoxidase (MPO) together with H_2O_2 and a halide ion (Cl^-) constitutes an unusually powerful microbicidal system (42,49,50), amplifying the antimicrobial potency of hydrogen peroxide by a factor of 50 (42).

100

100

When discussing the oxygen-dependent killing mechanisms of phagocytes, a second less well defined pathway labeled by the term, oxidizing radicals, refers to a group of compounds produced during the respiratory burst. These compounds are essential components in a myeloperoxidase-independent but oxygen-requiring microbicidal system (34,51,52) and are characterized by exceptionally high reactivity and the presence of an unpaired electron. The information available about the cellular damage caused by the biochemical mechanism of oxidizing radicals is limited (53,54). Lipid peroxidations, membrane damage, and damage to nucleic acids are the probable cellular alterations (55,56,57).

C. Neutrophil Anti-oxidant Systems

The endogenous oxidants produced during the respiratory burst may damage host cells as well as the neutrophils themselves. However, a series of effective antioxidant systems equip the neutrophil to trap or destroy oxidants before they do harm. This category includes superoxide dimutase, catalase, glutathione-dependent enzymes, and low molecular weight antioxidants.

Superoxide dismutases are enzymes that catalyze the conversion of O_2^- (superoxide anion) to oxygen and H_2O_2 (58).



This reaction also occurs spontaneously, but the reaction is bimolecular implying that even at low rates of production, tissue superoxide concentrations could rise to unacceptable levels before the rate of spontaneous dismutation (59,60).

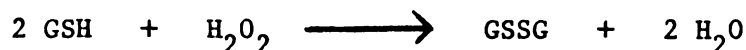
Catalase is a large tetrameric heme enzyme containing one prosthetic group per subunit. The molecular weight is 263,000 and the enzyme is found

in several tissues including neutrophils, erythrocytes, and liver (61,62). Catalase catalyzes the conversion of hydrogen peroxide to oxygen and water:



At high concentrations, H_2O_2 is rapidly destroyed by catalase. Because the reaction rate is strictly proportional to H_2O_2 concentration, catalase mediated destruction of H_2O_2 is inefficient at low peroxide levels (63).

Most of the H_2O_2 that neutrophils are exposed to, including that which leaks into the cytoplasm during the respiratory burst, is destroyed by a glutathione-requiring coupled enzyme system whose ultimate effect is to reduce H_2O_2 to water at the expense of NADPH (64,65). The reactions are as follows:



catalyzed by glutathione peroxidase, and



catalyzed by glutathione reductase.

In this pathway, glutathione cycles between its oxidized and reduced states, serving as a catalyst for H_2O_2 reduction. The NADP is reduced by the hexose monophosphate shunt, just as the NADP generated in the superoxide forming reaction. A substantial fraction of the hexose monophosphate shunt activity expressed during the respiratory burst is attributed to this glutathione-dependent H_2O_2 consuming pathway (66).

D. Chemiluminescence of Neutrophils and Oxidative Mechanisms

The respiratory burst and other subsequent oxygen dependent reactions can be quantitated by chemiluminescence (63). Chemiluminescence, an emission of light during the course of a chemical reaction, implies the presence of energy-rich molecular states in which electrons occupy orbitals

←

←

←

of higher than ground state energy (68). The excess energy is dissipated by thermal decay, increased chemical reactivity (69), or in the case of the activated neutrophil, by light emission (67,68,79,70),

During this metabolic activation of neutrophils electronically excited substances are formed. These substances, theorized to involve the reactive species of oxygen (71,72,73), emit light when they relax to the ground state. Both particulate stimuli (43,74,75), including latex particles, which are consequently phagocytized and soluble stimuli (76,77,78), such as phorbol myristate acetate that binds receptors on the surface of neutrophils can cause membrane activation which initiates the metabolic pathways involved in chemiluminescence. This emission can be inhibited by superoxide dismutase and catalase (68,69,73).

Allen et al. in 1972 (70) and later others (43,72) made the observation that neutrophils involved in phagocytosis of opsonized bacteria, latex particles, or zymosan generate light. Gerbner et al., who demonstrated parallel relationships between phagocytosis and chemiluminescence by altering the opsonization of bacteria (79) and Stevens and Young (80), who earlier reported a correlation between resistance of certain strains of E. coli to opsonization and decreased in vitro killing, oxygen consumption and chemiluminescence, led to a proposal that the biochemical processes that control phagocytosis and chemiluminescence were closely related and interdependent.

In further studies Allen et al. (72) demonstrated a 30% reduction in light emission when superoxide dismutase was added to the medium in which neutrophils were involved in phagocytosis of opsonized bacteria. Webb et al. (73) were able to show a 70% inhibition of chemiluminescence with superoxide dismutase, thus supporting the role of the superoxide anion in

the light emitting process. They also used catalase to explore the possible role of H_2O_2 in neutrophil chemiluminescence and found a slight, consistent inhibition of light emission. Klebanoff and Rosen later inhibited myeloperoxidase with azide and concluded that myeloperoxidase was another possible contributor to chemiluminescence (81).

One technique used to measure small amounts of light emitted by phagocytic cells involves the sensitive photomultiplier tubes available in liquid scintillation counters. Photomultiplier tubes are able to amplify the electrons emitted from a single photon reaching the light-sensitive cathode to a measurable signal. This is counted in the associated circuits (69). However, one of the major drawbacks of this system is the insensitivity to detect the low-level of light output.

The detection of luminescence can be enhanced and the sensitivity can be increased by a factor of 10^4 by adding a substance with a high chemiluminescence quantum yield upon oxidation by oxygen-derived radicals. Luminol (5 amino-2,3 dihydro-1,4 phthalazinedione) can be used in situations where the natural chemiluminescence is too weak for detection (82,83) or in situations where the number of granulocytes for study is limited. Luminol emits light upon oxidation and is converted to an excited aminophthalate ion (69). The light emitted by luminol maybe be related directly to O_2 (84) but the precise mechanism is still not clear (85).

The importance of these biochemical processes known as the respiratory burst in the chemiluminescence phenomenon has been exemplified by reports of significantly depressed chemiluminescence responses by neutrophils from human patients with neutrophil defects such as chronic granulomatous disease (86,87) or myeloperoxidase deficiency (81). The former disease is characterized by a failure of the patient's neutrophils

to generate O_2^- (superoxide anion) and H_2O_2 in significant amounts (87,88). Myeloperoxidase-deficient neutrophils exhibit normal generation of superoxide and H_2O_2 but the absence of the enzyme precludes the formation of O_2 via the $MPO-H_2O_2$ -halide reaction (89,90).

E. Hypothesis

There is definite compromised host defense in CHS mink. This compromised host defense is theorized to also involve and be reflective of the morphological and cytochemical differences between normal mink and CHS mink neutrophils. The neutrophil defect in the Chediak-Higashi syndrome has never been characterized by chemiluminescence; however, some metabolic pathways which are involved in neutrophil chemiluminescence were examined in humans with the Chediak-Higashi Syndrome and the CHS granulocyte oxygen consumption was normal after phagocytosis but reported to be above normal in resting, pre-phagocytic cells (7). In the bovine homologue these same metabolic pathways were suspected to be hypofunctional (91). There are no other defects reported involving CHS neutrophil oxidative systems or anti-oxidant systems. The mink homologue of CHS has not been rigorously tested for a neutrophil metabolic defect involving the hexose monophosphate shunt or activation of the respiratory burst. This study will compare the chemiluminescence response in both normal and CHS mink neutrophils. The abnormality in CHS might be further defined with regard to mechanisms of oxidative metabolism. Since the CHS neutrophil dysfunction involves membrane fusion abnormalities, chemiluminescence may be distorted. It is hypothesized that abnormal membrane functions may be reflected in altered chemiluminescence in CHS and that isolation of this distortion by use of inhibitors of the chemiluminescence response may be possible.

MATERIALS AND METHODS

A. Animals

The mink were housed on the campus of Michigan State University either outside at the Poultry-Fur Bearing Animal Farm or inside at the Laboratory Animal Care Service Center. They were purchased from farms with no recent history of Aleutian Disease and kept for a period known to be far beyond the incubation period for Aleutian disease. Forty adult males, half CHS with the genotype (aa) homozygous recessive and half matched phenotypically normal mink with genotype (aA or AA) for the Aleutian gene were used for the experiments. All animals were at least 1 year of age.

B. Blood Sampling

The blood samples were obtained from the jugular veins of the animals under ether anesthesia. The volume of blood taken was arbitrarily limited to 10 ml per animal per week.

C. Granulocyte Isolation

Venous blood was drawn with 1.5 inch 21-inch guage needles into 3 ml lavender top (EDTA) vacutainer tubes (Becton-Dickinson Co., Rutherford, N.J.) or drawn into disposable plastic syringes containing K_3 EDTA through 21-gauge needles (Beckton-Dickinson Co., Rutherford, N.J.). Blood was transferred to Falcon tubes (Falcon 2057 tubes, Becton, Dickinson and Co., Oxnard, CA) and mixed with 9 ml of Ethelenediaminetetraacetic Acid-Phosphate buffered saline (EPS) solution. This mixture was gently layered over a Ficoll-diatrizoate solution at room temperature and then centrifuged at $675 \times g$ for 15 minutes. Following centrifugation the EDTA plasma layer

and the lymphocyte-platelet layer was removed, saving only the granulocyte-red blood cell layer. This layer was washed in 8 ml of double distilled water for 15 seconds to lyse red blood cells. The 8 ml of double strength EPS solution was added to reconstitute tonicity. Centrifugation of this suspension for 8 minutes at 200 x g pelleted the granulocytes. The wash-lysis-centrifugation procedure was repeated at 110 x g for 8 minutes until a red blood cell-free pellet was obtained. The granulocyte pellet was resuspended in RPMI (RPMI 1640 without L-Glutamine M.A. Bioproducts, Walkersville MD) or Hanks Balanced Salt Solution (HBSS without Phenol red and without Sodium bicarbonate, Gibco Laboratories, Grand Island, NY) and counted with a hemocytometer. The granulocyte preparations were adjusted to 1×10^6 cells/ml of RPMI or HBSS. The isolated cells were routinely stained with filtered 0.5% New Methylene Blue, (Harelico, Gibbstown, NJ) before and with an automatic Wright-giemsa slide stainer (Hema-tek slide stainer, Hema-tek stain pak, Ames Co. Elkhart, Ind.,) after the assays were done. Granulocyte purity was at least 95% with 90% being neutrophils. Granulocyte viability was determined by 0.1% trypan blue dye exclusion and was greater than 95% in all preparations. All neutrophil preparations were assayed within four hours of initial venipuncture. Occasionally, after washing and counting cell clumping was observed. These preparations were discarded. This phenomenon was observed exclusively with CHS mink and often during cold weather.

D. Preparation of Reagents

1. General reagents and latex particles

Phosphate buffered saline (PBS) was prepared with 0.127 M NaCl, 2.7 mM KCL, and 8.33 mM Na_2HPO_4 . EPS was prepared with 0.154 M NaCl, 9.8

M Na_3 EDTA, and 14.6 mM KH_2PO_4 . Double strength EPS was prepared by adding twice the amounts of the above salts to the same amount of distilled water used in the preparation of EPS. Ethelenediaminetetraacetic Acid (K_3 EDTA, Eastman Kodak Co., Rochester, N.Y.) was prepared in PBS to obtain a concentration of 15 mg/ml and was used at 1.5 mg/ml of blood. Polystyrene latex particles were 0.797 microns in diameter and were in a solution containing 10% solids (Dow Diagnostics Co., Midland, MI.). Trypan Blue (Sigma Chemical Co., St. Louis, MO) was prepared and used in a 0.1% solution with 0.154 mM NaCl.

2. Preparation of Luminol

Luminol was prepared in two different molar concentrations and solubilized by two different methods. Luminol $\times 10^{-6}$ M with 0.1% Bovine serum albumin (BSA) was prepared from a stock solution of 10% weight/volume BSA- 10^{-4} M Luminol by dissolving Bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) in PBS and adding Luminol (Luminol, 5-amino-2,3, dihydrol, 4-phtalazinedione, Sigma Chemical Co., St. Louis, MO) and stirring for two hours in the dark. The stock solution was sterile filtered and stored at 4°C. The working solution was prepared by a 1:100 dilution of the stock solution in PBS and stored in the dark at 4°C. This reaction concentration of luminol was $.5 \times 10^{-6}$ M. Luminol without BSA was dissolved in sodium hydroxide and the diluted to 2×10^{-5} M concentration with PBS. The reaction concentration of luminol was 0.8×10^{-5} M.

All above reagents except Trypan Blue were used at pH 7.4.

3. Preparation of Ficoll-Hypaque

Ficoll-hypaque (ficoll-diatrizoate) was prepared by adding a solution containing 27 g of ficoll (Sigma Chemical Co., St. Louis, MO.) dissolved in 240 ml of distilled water to a solution of 75% diatrizoate

(Hypaque-M, Winthrop Laboratories, New York, N.Y.) dissolved in 100 ml of distilled water. The refractive index was adjusted to between 1.3665 - 1.3570 and then sterile filtered.

4. Preparation of Phorbol Myristate Acetate (PMA)

Phorbol 12-Myristate 13-Acetate (Sigma Chemical Co., St. Louis, MO)(PMA) was dissolved in dimethylsulfoxide (DMSO) to give a stock solution of 1 mg/ml. The stock solution was aliquoted and frozen at -70 degrees C. An aliquot was then thawed and diluted with phosphate buffered saline (PBS) immediately prior to use. A similar quantity of DMSO alone in PBS was added in control vials to insure that any observed effect was not due to DMSO (92).

5. Preparation of enzymes

Superoxide dismutase (SOD) from bovine erythrocytes (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS to a concentration of 8 mg/ml. Catalase from bovine liver suspended in water with an activity 40,000 Sigma units per milligram of protein. (Sigma Chemical Co., St. Louis, MO) was used undiluted.

E. Experimental Procedures

1. Experiment #1: Latex Particle Induced Chemiluminescence

In this first procedure the reaction mixture consisted of 0.5×10^6 isolated granulocytes in 0.5 ml of RPMI and 0.5 ml of 1×10^{-6} M luminol in 0.1% BSA. To the cell luminol mixture 25 ul of latex particles were added (93). The reaction took place in Beckman biovials (Beckman Product #566353, Beckman Co., Palo Alto, CA). Luminescence measurements were made with a scintillation counter (Beckman LS-3133 P ambient-temperature liquid scintillation counter, Beckman Co., Palo Alto, CA) in the tritium region of the spectrum. The coincidence circuit remained on

with both photomultiplier tubes activated. The reaction mixtures, prior to latex addition, were counted twice for background readings. Only reaction mixtures with background readings of fifty counts per minute (CPM) or less were assayed. Latex particles were then added, the reaction vial mixed and the scintillation counter placed on sample repeat, 2% error and 0.1 minute count interval.

2. Experiment #2: Ultrastructure of latex particle phagocytosis in normal and CHS mink neutrophils

Isolated neutrophils examined for unstimulated morphology using transmission electron microscopy (TEM) were pipeted into Beckman microfuge tubes and pelleted by centrifugation in a Beckman micro-centrifuge for 30 seconds (Beckman microfuge tubes, Beckman Microfuge-B, Beckman Co., Palo Alto, CA). The supernatant was gently pipeted off so as to not disrupt the neutrophil pellet and 0.1% glutaraldehyde in 0.1 M cacodylate buffer was then carefully pipetted on top of the pellet as a prefixative.

Neutrophils for TEM examination after phagocytosis of latex particles were prepared as followed: isolated neutrophils were counted, adjusted to 1×10^6 ml, and then centrifuged at $110 \times g$ for 5 minutes. The pellet was then resuspended in 1 ml of RPMI to yield a concentration of 4×10^6 cells/ml. 100ul of latex particles were then added to the cell suspensions. After 1.5 minutes the suspension was pipeted into the micro-centrifuge tubes and pelleted in the micro-centrifuge for 30 seconds. Prefixation in 0.1% glutaraldehyde in 0.1 M cacodylate buffer was the same as in the above procedure.

After prefixation of the pellets for ten minutes three percent glutaraldehyde in 0.1 M cacodylate buffer, at room temperature, was carefully layered over the neutrophil pellet and the pellet allowed to fix

for 2 hours. The pellet was then cut from the tip of the microfuge tube with a clean razor blade. Next the pellet was transferred to a glass petri dish containing additional 3.0% glutaraldehyde and processed for TEM as described for platelet pellets by Mastson et al. (94). Briefly, the pellets were cut into 1 mm blocks, postfixed with osmium tetroxide, dehydrated in graded ethanols, and embedded in Epon-Araldite resin. The thin sections were double stained with uranyl acetate and lead citrate and examined on a Phillips 201 Electron Microscope.

3. Experiment #3: Phorbol vs Latex Induced Chemiluminescence

In this procedure 0.5×10^6 cells in a volume of 0.5 ml RPMI were mixed with 0.4 ml of 2×10^{-5} M luminol. Background counts were done as described above. Admixed with the reaction vials was 25 ul latex particles and 75 ul PBS or 1 ug of PMA in 0.1 ml PBS. As described above the reaction was counted in the scintillation counter at least until the peak chemiluminescence had dropped 20%.

4. Experiment #4: Cell Mixing and Latex Induced Chemiluminescence

The reaction vials contained 0.5×10^6 cells in RPMI. The cell ratios were as follows: (a) 100% normal neutrophils: 0% CHS neutrophils, (b) 80% normal neutrophils: 20% CHS neutrophils, (c) 50% normal neutrophils: 40% CHS neutrophils, (d) 20% normal neutrophils: 80% CHS neutrophils (e) 0% normal neutrophils: 100% CHS neutrophils. The cells were counted for background as previously described and admixed with 25 ul of latex particles in 75 ul of PBS and counted until at least a 20% drop in the peak chemiluminescence was reached.

5. Experiment #5: Inhibition of Latex and PMA Induced Chemiluminescence with Superoxide Dismutase and Catalase

The reaction mixture consisted of 0.5×10^6 cells in a 0.5 ml volume of HBSS and 0.4 ml of 2×10^{-5} M luminol. The reaction vials were counted as previously described for background chemiluminescence. To each reaction mixture assayed was added: (a) 25 ul latex, 75 ul PBS, (b) 25 ul latex, 50 ul SOD, 25 ul PBS, (c) 25 ul latex, 25 ul Catalase 50 ul PBS, (d) 25 ul latex, 25 ul catalase, 50 ul SOD, (e) 25 ul PMA at a concentration of 4 ug/ml (f) 25 ul PMA, 50 ul SOD, 25 ul PBS, (g) 25 ul PMA, 25 ul Catalase, 50 ul PBS, or (h) 25 ul PMA, 25 ul Catalase, and 50 ul SOD. The luminescence measurements were made as described above. The degree of inhibition was reported as the percentage by which the chemiluminescence at its highest count per minute was reduced by the test enzyme in comparison with chemiluminescence of the sample without test enzyme added.

F. Data Analysis

All data and comparisons are represented by the use of the group mean followed by the standard deviation. In experiments #1,3, and 5, the differences in group responses were compared by the use of Student's "t" test whenever applicable. When the variances were found to be so unequal as to invalidate the use of the test, a "t'" test was used with an adjusted tabled "t" value and an adjusted value for the degrees of freedom.

In experiment #4, the Chi-square formula for observed versus expected values of peak chemiluminescence was used.

In experiment #5, in addition to the applicable "t" test, a 2 x 4 mixed block analysis of variance design was used to determine the "inducer effect" of PMA versus latex induced chemiluminescence.

The applicable complete block analysis of variance design was used next to compare the "treatment effects" of the enzyme inhibitors when compared to the uninhibited peak chemiluminescence "control" responses.

These responses were then compared by the "Student-Newman Kuel's" test to determine with-in group inhibition effect.

The "p value" or probability level for significance in all analyses is $p < 0.05$ (95).

RESULTS

A. Observation of Animals

During the course of the experiemnts the CHS mink were observed to acquire abscesses usually in the head and neck region. Five CHS mink were excluded from the assays because of unresolved absecesses. These animals were treated with antibiotic and also had their abscesses lanced. In addition to the abscesses the CHS mink blood samples were observed to have a high incidence of clotting regardless of the anticoagulant. This was common when the needle used for venipuncture required unusual force to puncture the skin, when there was a readjustment of a needle after initial venipuncture, and when vacutainer tubes were used to obtain blood. Approximately 50 samples had to be discarded because of clotting. Also blood sampled from normal mink had a brighter oxyhemoglobin appearance than that obtained from the CHS mink.

B. Exp. #1: Latex Particle Induced Chemiluminescence (CL)

Neutrophils were assayed in the liquid scintillation counter "incoincidence" in dark adapted vials. The final concentration of luminol in the reaction mixture was 0.5×10^{-6} M. The mean peak latex particle induced chemiluminescence of neutrophils from normal mink was $196,441 \text{ CPM} \pm 66,981$ (Table 1). The mean peak chemiluminescence for those assayed from CHS mink was $15,345 \text{ CPM} \pm 6,471$ (Table 1). This showed a mean deficit of 181,096 CPM at the peak chemiluminescence in the CHS animals. This difference was significant at $p < .05$.

C. Expt. #2: Ultrastructure of Latex Particle Phagocytosis in Normal and CHS Mink Neutrophils

TABLE 1

LATEX PARTICLE INDUCED CHEMILUMINESCENCE OF NORMAL AND CHS MINK NEUTROPHILS^a

EXPERIMENT #1

Normal Mink (N=10)	Peak Count CPM	CHS Mink (N=9)	Peak Count CPM
K2251	182,970	CHS1	10,825
K2231	168,330	CHS2	b
K1033	236,750	CHS3	14,285
K1023	66,065	CHS4	17,080
J1241	249,670	CHS5	14,090
J1791	208,900	CHS6	20,640
J741	188,690	CHS7	13,670
K141	117,990	CHS8	7,380
K1221	274,170	CHS9	29,285
K2413	270,860	CHS10	10,850
Mean $\pm$ SD*	196,440 $\pm$ 66,980	Mean $\pm$ SD*	15,345 $\pm$ 6,471

a. luminol concentration 0.5×10^{-6} M 0.1% BSA

b. died 5/24/83

* The difference in the mean peak Chemiluminescence (CL) of normal mink when compared to the mean peak CL of the CHS mink was significant at $P < 0.05$.

Transmission electron microscopoc examination of CHS and normal mink neutrophils showed the classically reported large granules present in CHS mink neutrophils when compared to normal mink neutrophils (Figures 1 & 2). Transmission electron microscopoc examination of CHS and normal mink neutrophils two minutes after addition of latex particles revealed that cells from both groups were capable of phagocytizing similar number of the latex particles. There was less phagolysosomal formation in the CHS neutrophils than in the normal neutrophils. This difference was detected by the presence of multiple latex paticles in vacuoles of normal cells compared to CHS cells (Figures 3 & 4,)

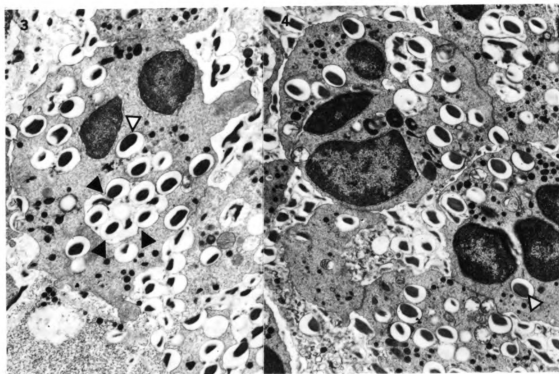
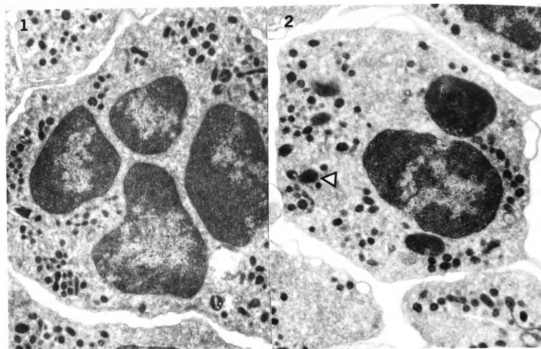
D. Expt. #3: Phorbol Myristate Acetate Induced CL and Latex Particle Induced CL in CHS and Normal Mink Neutrophils

Addition of 0.1 ug/ml of PMA (92) to the reaction mixture containing 8×10^{-6} M luminol resulted in a mean peak response of $541,000 \pm 11,000$ CPM with normal neutrophils and a mean peak response of $409,000 \pm 69,000$ CPM with CHS neutrophils. This was a significant difference at the $p < .05$ level indicating that the CHS mink were less responsive to PMA than the normal mink. The mean response of normal mink neutrophils by latex induced chemiluminescence, $518,000 \pm 50,000$ CPM, when compared to the $541,000 \pm 11,000$ PMA induced chemiluminescence response was not significantly different for the normal mink neutrophils. However the latex induced chemiluminescence response of CHS mink neutrophils, $48,000 \pm 11,000$, was significantly less at the $p < .05$ level than normal mink neutrophil latex or PMA induced chemiluminescence and CHS mink neutrophil PMA induced chemiluminescence (Table 2).

Figures 1, 2, 3 and 4, Experiment #2

THE ULTRASTRUCTURE OF NORMAL AND CHS MINK NEUTROPHILS BEFORE AND AFTER LATEX PARTICLE PHAGOCYTOSIS

The CHS neutrophil before latex particle ingestion (Figure 2) revealed the classically described enlarged primary granule (open arrow head) but was otherwise indistinguishable from the normal mink neutrophil (Figure 1). Both normal (Figure 3) and CHS (Figure 4) neutrophils 2 minutes after mixing with latex particles had ingested similar numbers of particles (open arrow heads). There was a greater tendency for particles to coalesce (solid arrow heads) into groups within phagolysosomes in normal neutrophils (Figure 3). Magnification (4,500 x 2.5), Figures 1 and 2; magnification (3,000 x 2.5), Figures 3 and 4.



E. Expt. #4: The Influence of Mixing of Normal and CHS Mink Neutrophils on Latex Particle Induced CL

Normal and CHS mink neutrophils were mixed in ratios of 100:0, 80:20, 50:50, 20:80, and 0:100. They were then admixed with latex and a 8×10^{-6} M Luminol. Expected values for peak chemiluminescence and for the time that the peak chemiluminescence would occur in the 80:20, 50:50, and 20:80 ratios were extrapolated from the actual values and actual times observed in the 100:0 and 0:100 ratio experiments. The extrapolated values were then compared to the actual values to establish whether there was any difference in the observed latex induced chemiluminescence and the estimated expected chemiluminescence and if this difference was significant.

There was no significant evidence of a positive influence of the normal cell to ameliorate the response of the CHS cell when the data was evaluated by Chi-square observed versus expected statistics. Likewise, there was no significant evidence to show that the two populations of cells acted in the mixtures totally independent of each other. However there was suggestive evidence that the CHS cells had a negative influence on the normal cells. This apparent negative influence was greatest at the 80:20 normal to CHS neutrophil ratio in that the observed peak chemiluminescence was much less than the expected. These 3 cell mixing experiments are shown in Table 3 and graphically summarized in Figure 5. The graphed data of the trends for the 20:80, 50:50, normal to CHS ratios fall within the expected range. The observed 80:20 ratios are at the lower end of the estimated range (Figure 5).

F. Expt. #5: Inhibition of Neutrophil CL with SOD and Catalase

TABLE 3

THE INFLUENCE OF MIXING OF NORMAL AND CHS MINK NEUTROPHILS ON LATEX
PARTICLE INDUCED CHEMILUMINESCENCE (CL)

EXPERIMENT #4
(n=3)^a

TRIAL NUMBER	RATIO NORMAL:CHS	OBSERVED RESPONSE CPM	EXPECTED ^b RESPONSE CPM
1	100:0	422,980	- - -
2	100:0	536,700	- - -
3	100:0	542,450	- - -
Mean	- -	500,710	- - -
1	80:20	264,312	339,230
2	80:20	344,866	444,116
3	80:20	393,533	464,708
Mean	- -	334,237	416,018
1	50:50	200,610	209,830
2	50:50	309,200	306,920
Mean ^c	- -	254,905	258,375
1	20:80	66,740	88,840
2	20:80	134,300	169,739
3	20:80	114,930	140,183
Mean	- -	105,323	132,921
1	0:100	11,060	- - -
2	0:100	80,380	- - -
3	0:100	74,440	- - -
Mean	- -	55,293	- - -

^aEach trial represents CL from 0.5×10^6 pooled neutrophils in RPMI.

^bExpected responses were calculated from the mathematical ratio derived from normal (100:0) and from the CHS (0:100) observed responses of the respective trial number (1,2 or 3).

^cInsufficient cells were available for trial 3.

Figure 5, Experiment #4

THE INFLUENCE OF MIXING NORMAL AND CHS NEUTROPHILS ON LATEX INDUCED CHEMILUMINESCENCE (CL).

Normal and CHS cells were assayed in ratios of 100:0, 80:20, 50:50, 20:80, and 0:100. This figure shows the peak counts per minute ($\text{CPM} \times 10^{-3}$) obtained from the control observed CL responses ($\bullet$, 100:0, and 0:100 ratios). These responses represent the upper and lower limits from which the expected responses ($\blacksquare$, 80:20, 50:50, and 20:80 ratios) were extrapolated. The observed mixed responses ($\circ$, 80:20, 50:50, and 20:80 ratios) are the actual peak CL values obtained from the mixing of normal and CHS cells. The broken lines (- - -) represent the estimated range that all of the values were expected to lie between. See Table 3 for the numerical designation of the data points.

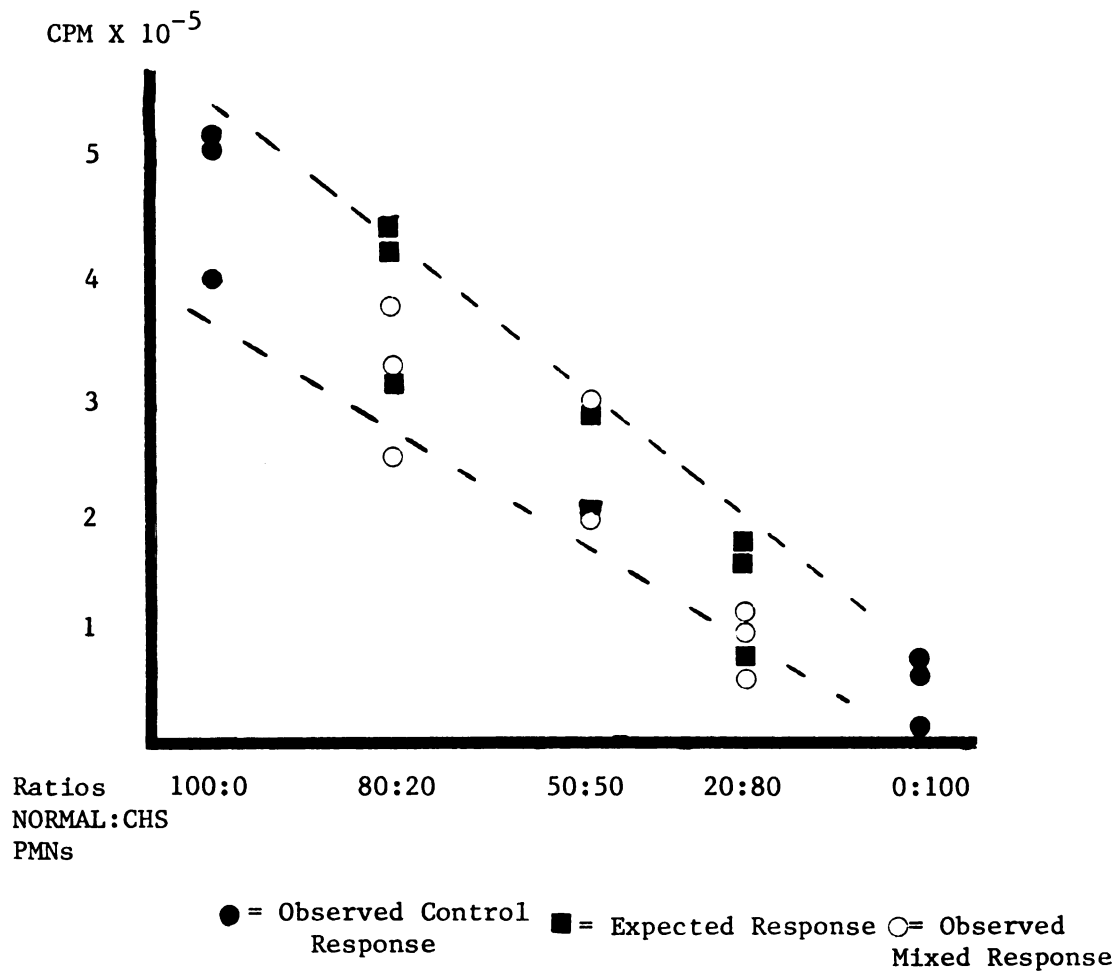


Figure 5.

The peak chemiluminescence values used for the control response of latex and PMA induced chemiluminescence are listed in Table 4 for normal mink neutrophils and Table 5 for CHS mink neutrophils along with the time that peak chemiluminescence occurred during the assay. Enzyme inhibition of the control responses for normal and CHS mink neutrophils are shown in Tables 6 & 7.

The mean peak CPM response of latex induced chemiluminescence of normal mink neutrophils, $557,167 \pm 4,511$ and that achieved with PMA, $511,724 \pm 84,332$ were not significantly different and were used as the control responses for this study which evaluated the effect of catalase and SOD inhibition of the normal mink neutrophil chemiluminescence responses. Figure 6 depicts the normal and CHS response to latex and Figure 7 the responses to PMA induced chemiluminescence.

A mean of 57.0% inhibition of normal mink neutrophil latex induced chemiluminescence was achieved with catalase while the mean inhibition by SOD was 91.7%. The combination of SOD and catalase resulted in a mean inhibition of 88.4%

A mean of 57.5% inhibition of normal mink PMA induced chemiluminescence was achieved with catalase while the mean inhibition achieved with SOD was 77.7%. The combination of SOD and catalase resulted in a mean inhibition of 92.0%.

These observed inhibitions of peak chemiluminescence were significant at $p < .05$ for each enzyme alone or the enzymes in combination. The inhibition achieved with SOD and the combination of SOD and Catalase was significantly greater than that achieved with catalase alone. There was no significant difference in the amount of inhibition achieved with SOD when compared to the inhibition achieved with the combination of SOD and

TABLE 4
CONTROL RESPONSES OF PMA AND LATEX INDUCED CHEMILUMINESCENCE IN NORMAL MINK

EXPERIMENT #5

ANIMAL NUMBER (n=5)	0.1 ug/ml PMA		25ul Latex	
	TIME OF PEAK CPM IN MINUTES	PEAK CPM	TIME OF PEAK CPM IN MINUTES	PEAK CPM
B-2	0.7	557120	3.2	549790
B-11	1.2	559230	2.8	566530
B-9	1.8	557910	3.0	508550
B-14	1.0	549750	2.8	366970
B-12	2.2	561810	3.2	566780
Mean $\pm$ SD*	1.4 $\pm$ 1.4	557164 $\pm$ 4511	3.0 $\pm$ 0.2	511724 $\pm$ 84332

* (see statement in text, pages 31 and 49 for "statistical significance")

TABLE 5

CONTROL RESPONSES OF PMA AND LATEX INDUCED CHEMILUMINESCENCE IN CHS MINK

EXPERIMENT #5

ANIMAL NUMBER (n=5)	0.1 ug/ml PMA		25ul Latex	
	TIME OF PEAK CPM IN MINUTES	PEAK CPM	TIME OF PEAK CPM IN MINUTES	PEAK CPM
C-19	0.8	27550	4.0	43750
C-24	0.9	102700	4.9	84780
C-28	0.9	95600	4.2	53620
C-14	1.1	11440	4.5	85130
C-10	0.8	55370	4.5	103270
Mean $\pm$ SD*	0.9 $\pm$ 0.12	58532 $\pm$ 40350	4.42 $\pm$ 0.34	74110 $\pm$ 24633

* (see statement in text, pages 40 and 49 for "statistical significance")

TABLE 6

INHIBITION OF LATEX AND PMA INDUCED CL IN NORMAL MINK NEUTROPHILS

EXPERIMENT #5

ANIMAL NUMBER (n=5)	CONTROL RESPONSE CPM WITH LATEX	CATALASE INHIBITION CPM (%)	SOD INHIBITION (CPM) (%)	CATALASE+SOD INHIBITION CPM (%)
B-2	557120	365470 (34.4%)	3340 (99.4%)	99170 (82.2%)
B-11	559230	290240 (48.1%)	133650 (76.1%)	45860 (91.8%)
B-9	557910	159000 (71.5%)	41290 (92.6%)	88150 (84.2%)
B-14	549750	112590 (77.7%)	6050 (98.9%)	10995 (98.0%)
B-12	561810	270790 (51.8%)	46070 (91.8%)	79770 (85.8%)
Mean $\pm$ SD *	557164 $\pm$ 4511	239618 $\pm$ 102479 (57.0%)	46082 $\pm$ 52731 (91.7%)	64789 $\pm$ 36070 (88.4%)

ANIMAL NUMBER (n=5)	CONTROL RESPONSE CPM WITH PMA	CATALASE INHIBITION CPM (%)	SOD INHIBITION CPM (%)	CATALASE+SOD INHIBITION CPM (%)
B-2	549790	293590 (46.6%)	183630 (66.6%)	63230 (88.5%)
B-11	566530	146165 (74.2%)	62885 (88.9%)	17560 (96.9%)
B-9	508550	151550 (70.2%)	110860 (78.2%)	34070 (93.3%)
B-14	366970	122935 (66.5%)	28260 (92.3%)	110864 (97.0%)
B-12	566780	397880 (67.4%)	184770 (67.4%)	79350 (86.0%)
Mean $\pm$ SD *	511724 $\pm$ 84332	222424 $\pm$ 118939 (57.5%)	114081 $\pm$ 70412 (77.7%)	41044 $\pm$ 29418 (92.0%)

* (see statement in text, pages 31 and 40 for "statistical significance")

TABLE 7

INHIBITION OF LATEX AND PMA INDUCED CL IN CHS MINK NEUTROPHILS

EXPERIMENT #5

ANIMAL NUMBER (n=5)	CONTROL RESPONSE CPM WITH LATEX	CATALASE INHIBITION CPM (%)	SOD INHIBITION CPM (%)	CATALASE+SOD INHIBITION CPM (%)
C-19	27550	1520	140	*50
C-24	102700	14070	720	210
C-28	95600	14050	760	190
C-14	11440	680	520	310
C-10	55370	280	170	*50
Mean $\pm$ SD *	58532 $\pm$ 40350	6120 $\pm$ 7262	462 $\pm$ 295	161 $\pm$ 112

* Inhibited response indistinguishable from background

ANIMAL NUMBER (n=5)	CONTROL RESPONSE CPM WITH PMA	CATALASE INHIBITION CPM (%)	SOD INHIBITION CPM (%)	CATALASE+SOD INHIBITION CPM (%)
C-19	43750	18070	1140	740
C-24	84780	10770	3560	2460
C-28	53620	19200	2150	860
C-14	85130	33460	4510	2130
C-10	103270	18070	720	520
Mean $\pm$ SD *	74110 $\pm$ 24633	19914 $\pm$ 8283	2416 $\pm$ 1602	1342 $\pm$ 886

* (see statement in text, page 40 for "statistical significance")

Figure 6, Experiment #5

Time trace of CL emitted from normal (N=5) and CHS (N=5) mink neutrophils after Latex particle stimulation. The averaged responses of individual CL assays indicate a significant ($p < 0.05$) deficit of the CHS mink (C = CHS genotype) neutrophils when compared to the normal mink (B = black phenotype).

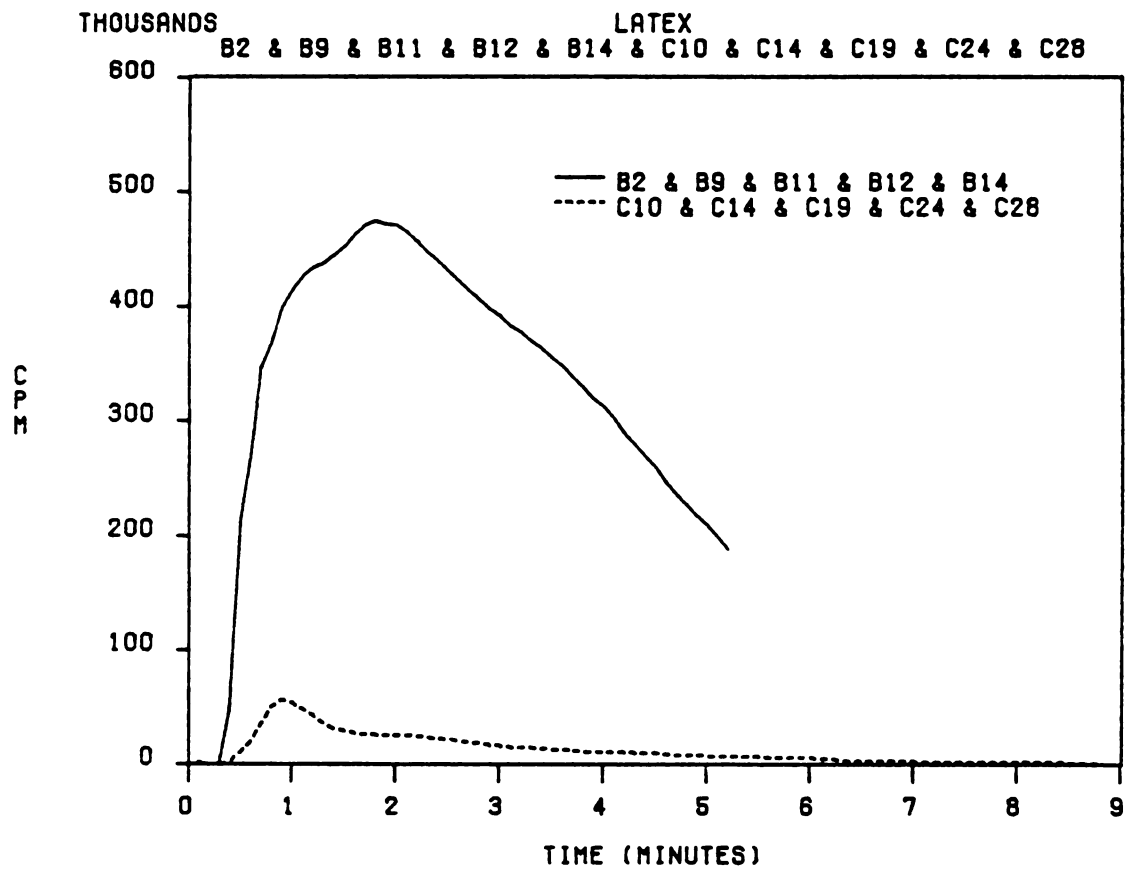


Figure 6.

Figure 7, Experiment #5

Time trace of CL emitted from normal (N=5) and CHS (N=5) mink neutrophils after PMA stimulation. The averaged responses of individual CL assays indicate a significant ($p < 0.05$) deficit of the CHS mink (C = CHS genotype) neutrophils when compared to the normal mink (B = normal, black phenotype).

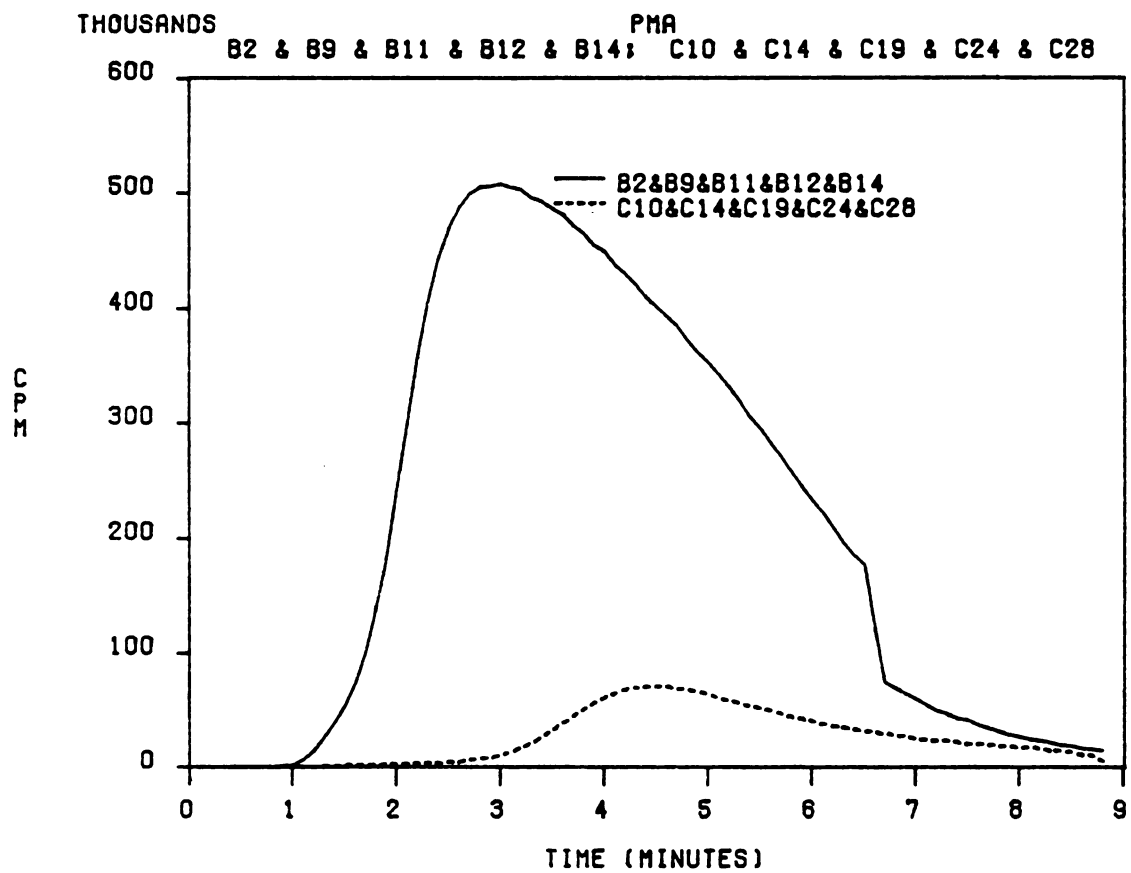


Figure 7.

catalase. In addition to the control peak chemiluminescence values for the enzyme inhibition of the response, Figure 8 & 9 graphically demonstrate the inhibition of chemiluminescence in normal mink.

The mean peak CPM response of latex induced chemiluminescence with CHS mink neutrophils was $58,532 \pm 40,350$ while that achieved with PMA was $74,110 \pm 24,633$. These peak responses were not significantly different and likewise were used as the control group for enzyme inhibition of CHS neutrophil chemiluminescence.

A mean of 89.5% inhibition of CHS mink neutrophil latex induced chemiluminescence was achieved with catalase while the mean inhibition by SOD was 99.2%. The combination of SOD and catalase resulted in a mean inhibition of 99.7%

A mean of 73.1% inhibition of CHS mink neutrophil PMA induced chemiluminescence was achieved with catalase while the mean inhibition by SOD was 96.7%. The combination of SOD and catalase resulted in a mean inhibition of 98.1%.

There was significant inhibitions at the $p < 0.05$ level of the CHS control peak chemiluminescence response by catalase, SOD, and the combination of SOD and catalase. There was no significant difference in the amount of inhibition achieved with catalase versus SOD or the combination of SOD and catalase inhibition when compared to either enzyme's inhibition. In addition to the control peak chemiluminescence values for the enzyme inhibition of the responses, Figures 10 and 11 graphically demonstrate the inhibition of chemiluminescence in CHS mink.

Whereas in Experiment #3, the design only determined differences in peak chemiluminescence, induced by latex or PMA, in Experiment #5 the rate at which the peak chemiluminescence responses occurred was also studied.

Figure 8, Experiment #5

Time trace of CL emitted from normal (N=5) mink neutrophils (B = normal, black phenotype) after Latex particle stimulation with and without the addition of enzyme inhibitors. The averaged responses of individual CL assays indicate significant ($p < 0.05$) inhibition of the Latex induced CL response by the addition of SOD (L + S), catalase (L + C), or the combination of SOD + catalase (L + S + C). Due to the graphic program resolution (L + S) is not discernable from the abscissa. Please refer to table 6 for actual normal mink values.

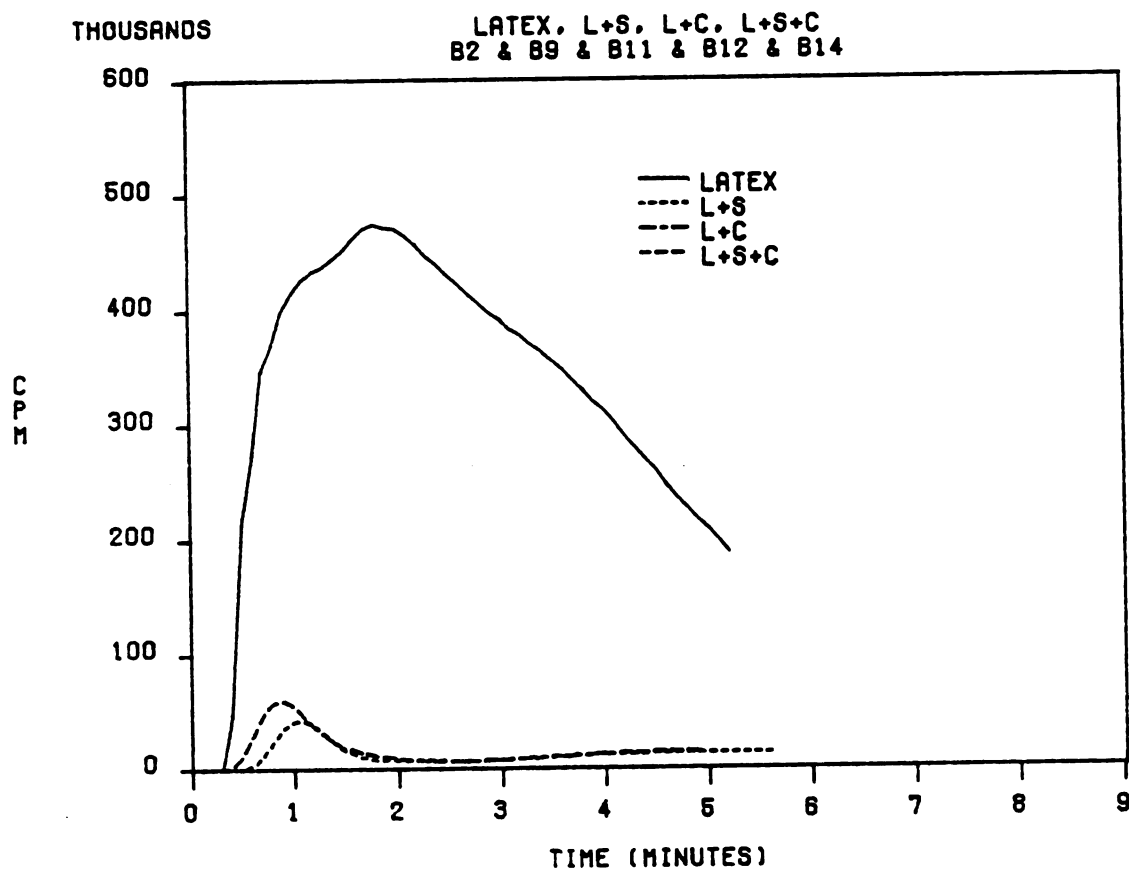


Figure 8.

Figure 9, Experiment #5

Time trace of CL emitted from normal (N=5) mink neutrophils (B = normal, black phenotype) after PMA stimulation with and without the addition of enzyme inhibitors. The averaged responses of individual CL assays indicate significant ($p < 0.05$) inhibition of the PMA induced CL response by the addition of SOD (P + S), catalase (P + C), or the combination of SOD + catalase (P + S + C).

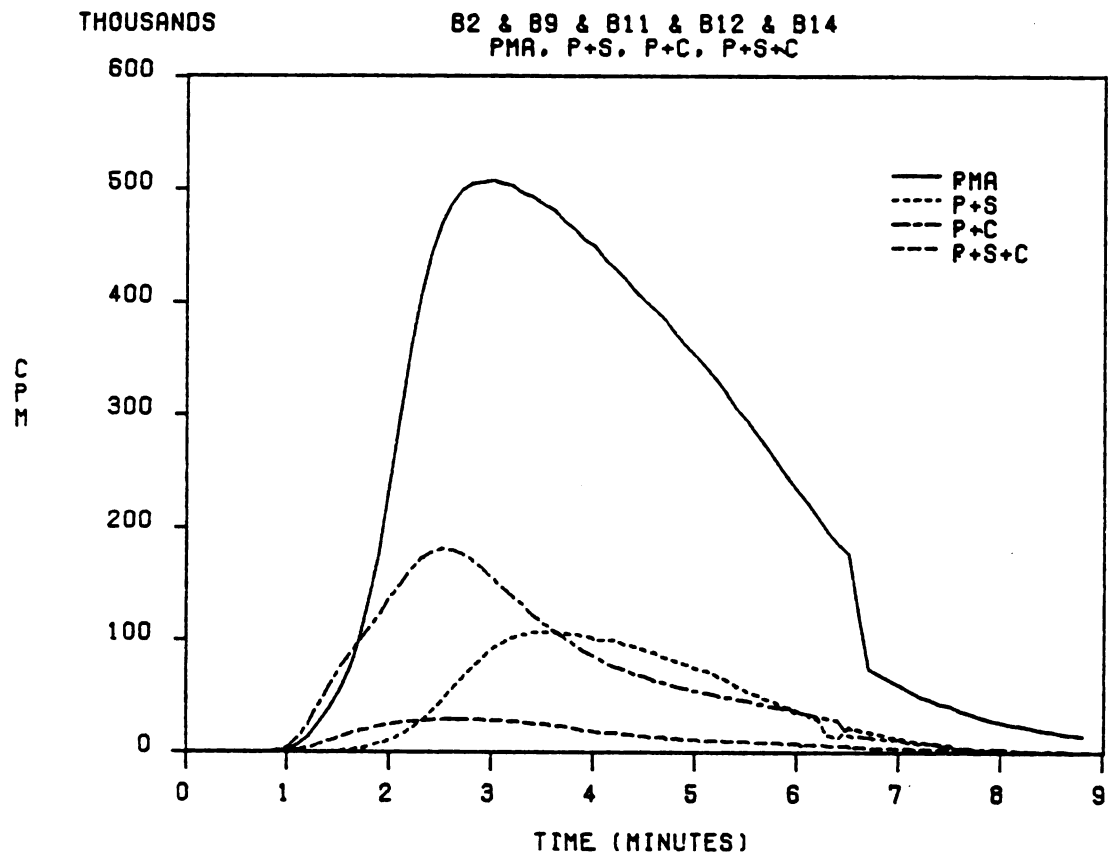


Figure 9.

Figure 10, Experiment #5

Time trace of CL emitted from CHS (N=5) mink neutrophils (C = CHS genotype) after Latex particle stimulation with and without the addition of enzyme inhibitors. The averaged responses of individual CL assays indicate significant ($p < 0.05$) inhibition of the Latex induced CL response by the addition of SOD (L + S), catalase (L + C), or the combination of SOD + catalase (L + S + C). Due to the graphic program resolution (L + S + C) is not discernable from the abscissa. Please refer to table 7 for actual CHS values.

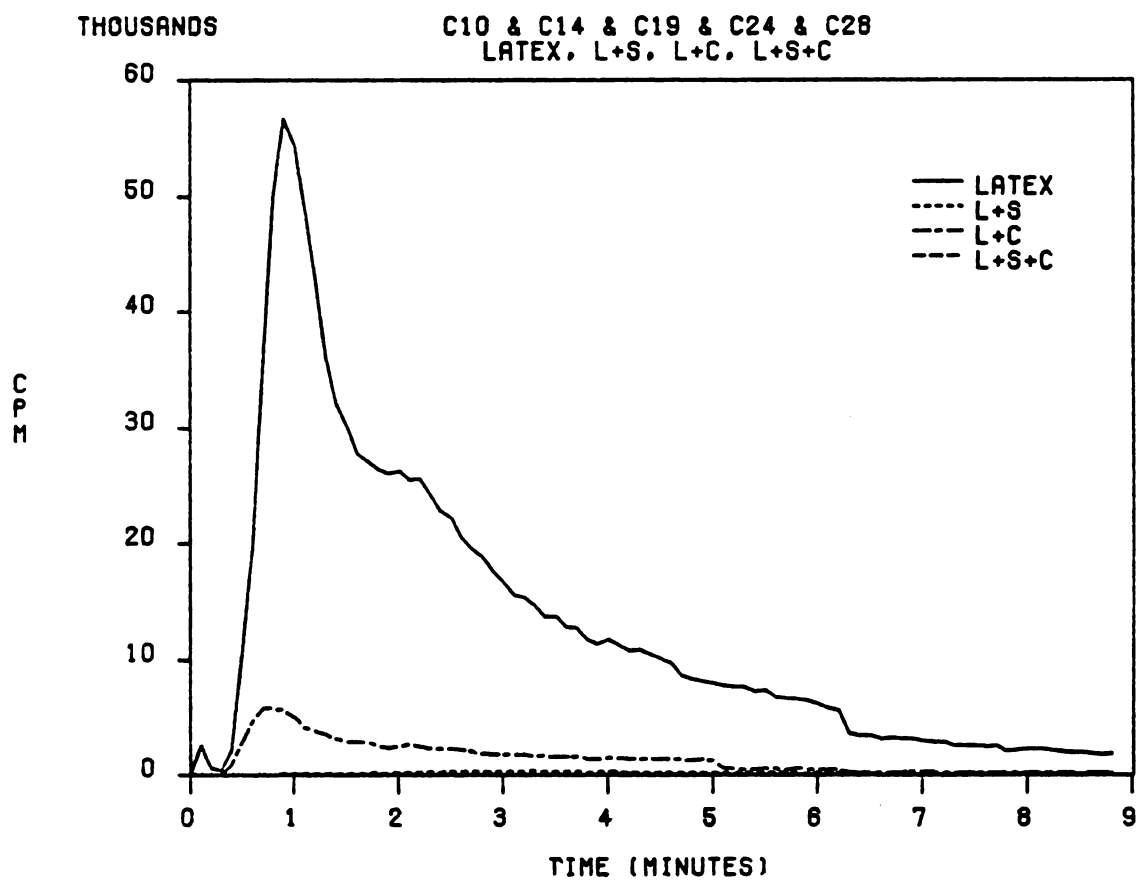


Figure 10.

Figure 11, Experiment #5

Time trace of CL emitted from CHS (N=5) mink neutrophils (C = CHS genotype) after PMA stimulation with and without the addition of enzyme inhibitors. The averaged responses of individual CL assays indicate significant ($p < 0.05$) inhibition of the PMA induced CL response by the addition of SOD (P + S), catalase (P + C), or the combination of SOD + catalase (P + S + C).

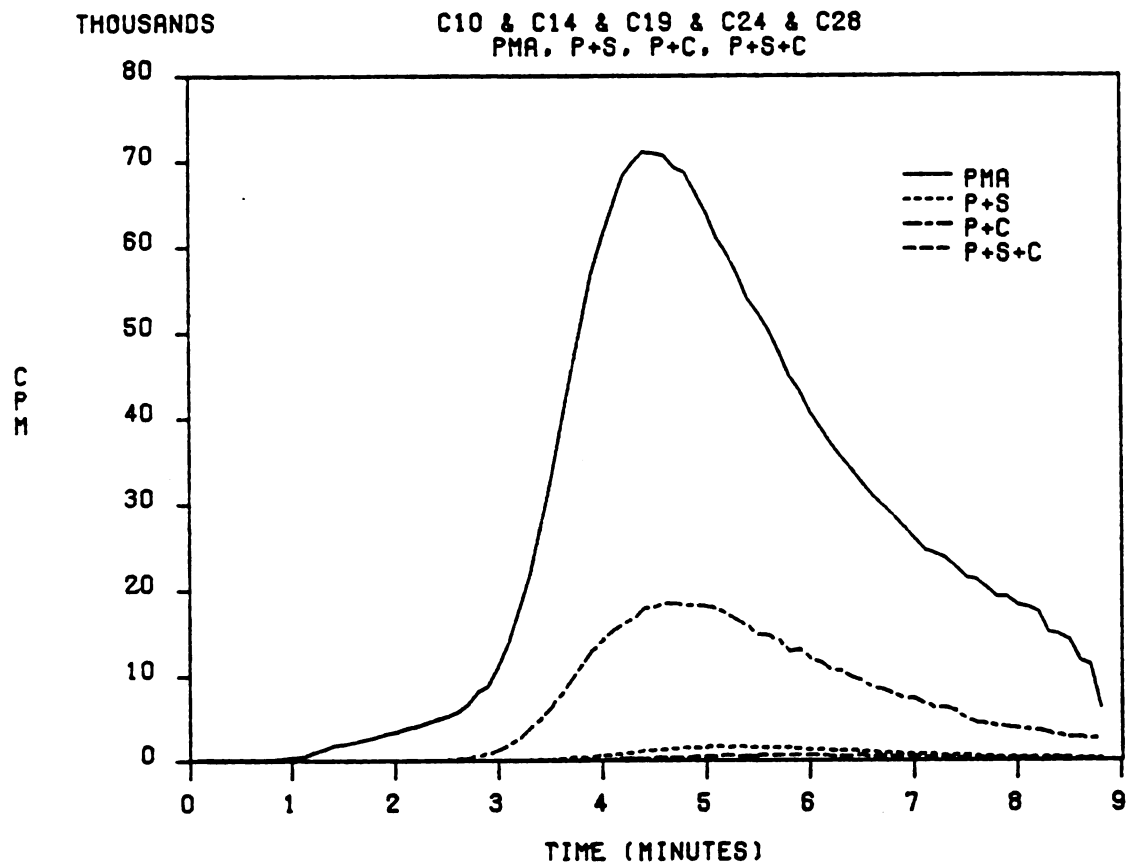


Figure 11.

The onset of peak chemiluminescence induced by latex was 1.4 ± 1.4 minutes which was significantly quicker than the time of onset, 3.0 ± 0.2 minutes, for the peak CPM of PMA induced chemiluminescence of normal mink neutrophils (Figure 12).

The time of onset of peak latex induced chemiluminescence in CHS neutrophils was 0.9 ± 0.12 minutes. This was not significantly different than the onset of peak latex (1.4 ± 1.4 minutes) induced chemiluminescence with normal mink neutrophils (Figure 13).

There was a significantly greater time required, 4.42 ± 0.34 minutes, for the onset of peak PMA induced chemiluminescence of CHS mink neutrophils when compared to onset times of latex induced chemiluminescence with CHS (0.9 ± 0.12 minutes). Likewise, for normal mink neutrophils, peak latex induced chemiluminescence occurred at a significantly quicker time when compared to the PMA induced chemiluminescence peak time (3.0 ± 0.2 minutes).

Figure 12, Experiment #5

Time trace of CL emitted from normal (N=5) mink neutrophils (B = normal, black phenotype) after Latex particle stimulation and PMA stimulation. The averaged responses of individual CL assays indicate the significantly ($p < 0.05$) slower time for peak CL to occur with PMA in comparison to Latex.

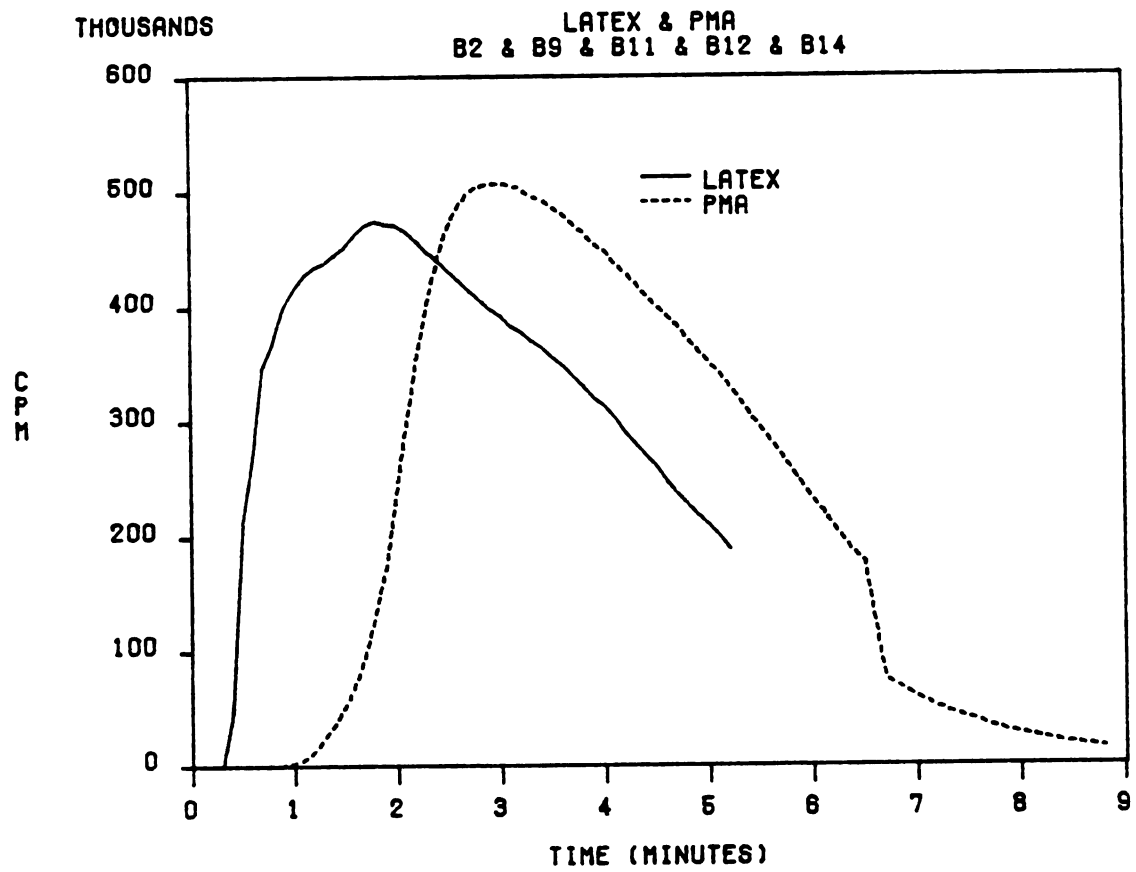


Figure 12.

Figure 13, Experiment #5

Time trace of CL emitted from CHS (N=5) mink neutrophils (C = CHS genotype) after Latex particle stimulation and PMA stimulation. The averaged responses of individual CL assays indicate the significantly ($p < 0.05$) slower time for peak CL to occur with PMA in comparison to Latex.

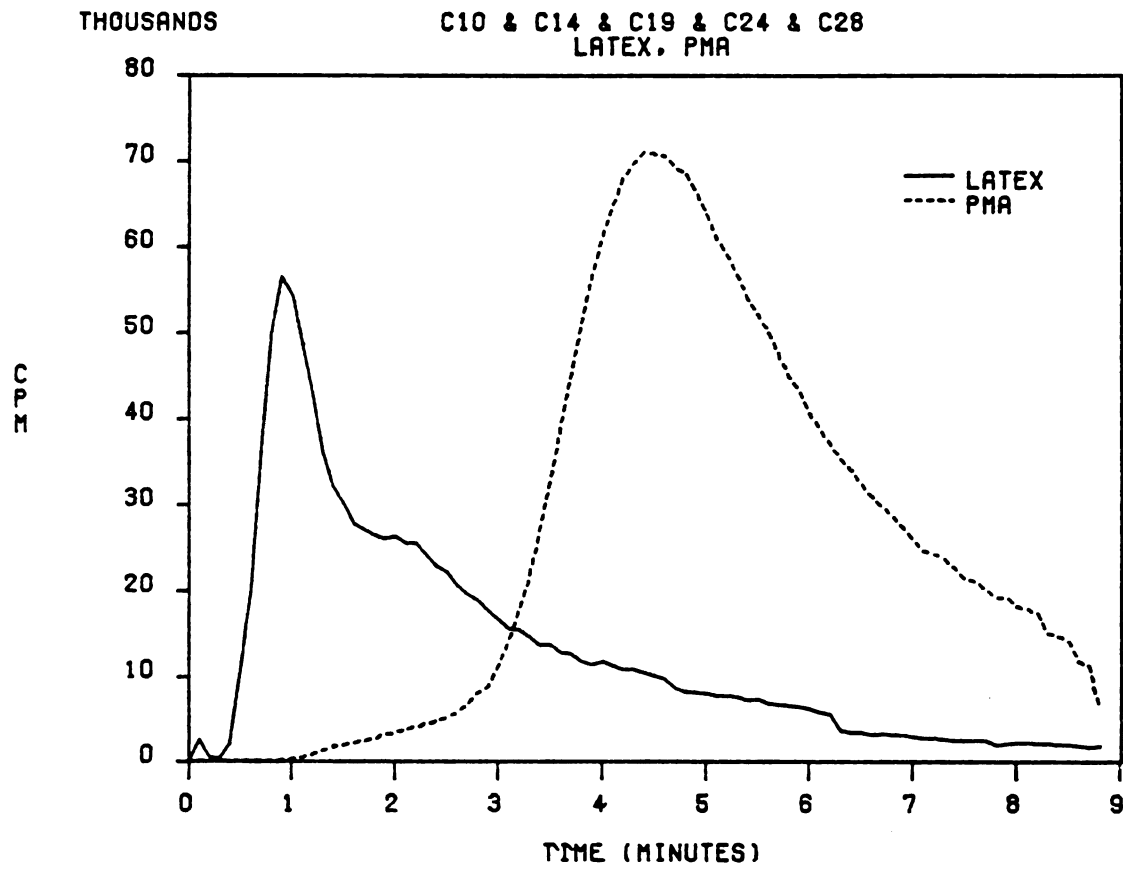


Figure 13.

DISCUSSION AND SUMMARY

The importance of the production of reactive oxygen species such as O_2^- , H_2O_2 , and OH to the microbicidal function of the neutrophil has been well documented (6,34,43), though the exact mechanisms involved in the generation of the reactive species is poorly defined. Previous investigators have used the chemiluminescence assay to study the phenomenon of neutrophil metabolic activation and to diagnose patients with inherent neutrophil defects (81, 83, 87). Therefore, it was hypothesized that it may be possible to isolate the defect in CHS mink neutrophils using inhibitors of the chemiluminescence response. It was the aim of this study to develop a standardized "in-coincidence" enhanced chemiluminescence assay and to characterize, by using this assay, the neutrophil defect in CHS mink with the use of normal mink as controls.

CHS neutrophils in Experiment #1 had a mean CPM of 15,345 which represented a mean deficit of 181,046 CPM when compared with the mean value of 196,441 CPM obtained from normal mink neutrophils. In this first study done with luminol at a concentration of 0.5×10^{-6} M the "within group" and "between group" variance with regard to the peak chemiluminescence, the highest CPM level measured for each response, was large. In this study, there was also a large "within group" variance in regard to the time when peak chemiluminescence occurred with the CHS mink neutrophils.

The reason for this large CHS "within group" variance was a bimodal pattern seen exclusively in the CHS neutrophil responses. As a result there were primary and secondary peaks observed with the CHS mink neutrophils, thought by this author to possibly represent the delayed interplay of peroxidase containing lysosomes with the phagosome.

This bimodal phenomenon, however did not occur in the CHS mink when the luminol concentration was increased to 8×10^{-6} M in subsequent studies. Therefore, in the final analysis of this first study only the peak with highest value in a bimodal response was evaluated to describe the neutrophil functional capability.

The bimodal chemiluminescence phenomenon has only been specifically reported to occur in normal human blood donors which did not have a documented neutrophil defect (96). This analysis did show that the first peak was of extracellular origin and required superoxide, hydrogen peroxide and myeloperoxidase for the oxidation of luminol and was also believed to occur in part from the formation of HOCl (hypochlorous acid) (97). The second peak was thought most likely to be an intracellular chemiluminescent event (96).

Traditionally, chemiluminescence assays are performed in a liquid scintillation counter in an out-of-coincidence setting meaning only one of the two photomultiplier tubes is activated. This often used method accounts for the fact that in chemiluminescent reactions one single photon is emitted in each luminescent relaxation. In theory photons reach both photomultiplier tubes at random making simultaneous signals on both sides rare at low light levels (68, 69).

Light emission per molecule of oxygen consumed and transformed into an oxygen derived radical, superoxide being one of those oxygen derived radicals, is much less in chemiluminescent neutrophils than the extensively studied bioluminescent phenomena of biochemical reactions. Superoxide production makes up the major part of oxygen consumption after phagocytosis and is measured at a maximal rate of 4.4×10^{-9} mol/min per 10^6 neutrophils. With a similar stimulation, a peak chemiluminescent count of

35×10^3 CPM is obtained (69). Chemiluminescence at this level is either a side reaction involving a minor part of the oxygen-derived radicals produced in oxidation of a substrate with high quantum yield or a light-emitting reaction of very low quantum yield not comparable with bioluminescent phenomena at all.

Amplification of neutrophil chemiluminescence with luminol, a substance with a high quantum yield upon oxidation, overcomes the problem of low light levels produced during chemiluminescence of neutrophils. However, this limits the accuracy of an out-of-coincidence system because of high background counts (69).

The advantages of utilizing a luminol enhanced "in-coincidence" chemiluminescence system for mink neutrophils were: 1) elimination of high background counts, 2) no requirement for working in dark-adapted conditions, 3) permitting the use of a lower number of cells, and 4) a shorter reaction time to peak chemiluminescence. Whereas other reported assay systems require 20 to 60 minutes to reach peak chemiluminescence (67, 81), peak times of under 5 minutes were common in these experiments. This also eliminated the need for continued mixing during the experiments and reduced the possibility of measuring chemiluminescence produced after the initial neutrophil phagocytic event. All of these advantages resulted in a more accurate evaluation of chemiluminescence produced during phagocytosis.

The second experiment was the examination of glutaraldehyde fixed cells by transmission electron microscopy two minutes after the addition of latex particles. Both CHS and normal mink neutrophils phagocytized the particles. Even though the particles per cell were not quantitated, the subjective evidence (Figure 3) showed a greater tendency for the normal mink neutrophils to have coalescence of the particles in greater numbers

within phagocytic vacuoles. Whereas, in the CHS mink neutrophils (Figure 4) the latex particles remained individualized within the cytoplasmic vacuoles.

In Experiment #3, to further explore the features revealed by the first chemiluminescence assay, another assay was done to compare the chemiluminescence mediated by soluble stimuli with that of the particulate stimuli. The assay was run with a higher concentration of luminol to amplify the CHS neutrophil response. The soluble stimuli, phorbol myristate acetate (PMA), a croton oil ester, is known to be the most potent membrane activator of O_2^- production in neutrophils. PMA stimulation of superoxide anion is a membrane receptor mediated event (98). Although PMA is not a "physiologic" stimulus, the compound causes marked stimulation of all parameters of leukocyte oxidative metabolism (99).

In all of the experiments using PMA the chemiluminescence response of the CHS mink neutrophils was significantly less than that observed with the normal mink neutrophils. The onset of the peak CPM induced with PMA, though not evaluated until later experiments, showed a greater lag time in both CHS and normal mink neutrophils than onset of the peak observed with latex particle induced chemiluminescence.

Technical problems encountered with the second set of chemiluminescence experiments required a variation of the isolation procedure. Cell activation and irreversible aggregation of the neutrophils in the isolated preparations became pronounced, particularly with the CHS neutrophils. Only 1 out of 10 CHS cell preps could be properly counted and/or had a low enough background to be assayed in comparison to the controls. This was thought to be secondary to use of bovine serum albumin and possibly the phenol component of the media RPMI. However, stress of

the animals, subjected to extremely cold environmental temperatures and repeated anesthesia could not be ruled out.

The irreversible aggregation of CHS neutrophils presented a problem that was not unlike that reported by investigators isolating bovine neutrophils (91). Excessive neutrophil hyperadhesiveness has also been reported in a human patient with CHS (100). Often, as in this protocol, the standard techniques for isolation of neutrophils involves sequential exposure of the cells to nonphysiologic environments of ficoll-hypaque and hypotonic conditions. It has been suggested that these procedures may be harmful to the recovered neutrophil and can alter in vitro activation (101). In an effort to correct the problems the BSA was eventually eliminated from the luminol and the animals were occasionally housed inside. The isolation procedure was only increased to an efficiency of 1 neutrophil isolation out of 8 preparations that could be properly assayed. The final procedural manipulation was elimination of RPMI from the isolation procedure and use of continued inside housing for the animals used in the remaining experiments. The net result was an isolation efficiency of 2 CHS mink neutrophil preparations out of 3 that could be properly counted and assayed.

In experiment #4, a third set of chemiluminescence experiments were designed to explore the possibility that a neutrophil-neutrophil interaction may exist. The reason for this experiment was, that during phagocytosis of latex particles by normal mink neutrophils regurgitation of neutrophil enzymes and mediators could possibly augment functional capabilities of unstimulated cells or CHS neutrophils in the surrounding medium. CHS and normal mink neutrophils were therefore mixed in varying ratios as outlined. Unlike the established platelet-neutrophil

interactions such as myeloperoxidase mediated release of platelet constituents (102) no positive influence was seen in three experiments using varying ratios of CHS and normal mink neutrophils. Figure 5 reflects these results.

The validity of the Chi square statistical analysis of these mixing experiments is of concern. The ranges for 100:0 and 0:100 Normal:CHS ratios had such large variation between the 3 separate times the mixing studies were done, coupled with the small number of repetitions, $n=3$, and the magnitude of the CPM numbers used in the calculations makes the statistical analysis of little value.

The design of Experiment #5 was intended to allow determination of the source of the light produced in this luminol enhanced system using enzyme inhibitors. Both of the inhibitors used, SOD and catalase, have been documented not to interfere with the neutrophils ability to ingest particles (43). Inhibition of chemiluminescence with SOD and catalase yielded data which was contradictory to most recent reports involving luminol enhanced chemiluminescence systems.

These reports state that myeloperoxidase (MPO) mediated reactions are the only ones important in the oxidation of luminol to an excited aminophthalate anion (103,104). The absolute requirement of the luminol system for MPH, H_2O_2 , and Cl^- suggested that the initial reaction involves formation of hypochlorous acid, which in turn, serves as the intermediate for the luminol (96). Superoxide dismutase caused marked inhibition of the luminol enhanced chemiluminescence response with normal mink neutrophils making the importance of the role of superoxide in chemiluminescence obvious. This remarkable need for O_2^- is consistent with a mechanism proposed by Misra and Squatrito which does not involve hypochlorous acid,

but requires both O_2^- and H_2O_2 for formation and oxidation of the luminol radical (105).

Catalase, which acts on H_2O_2 , inhibited normal mink neutrophil chemiluminescence substantially but less than the inhibition achieved with SOD or the combination of SOD and catalase. Inhibition of the PMA and latex induced chemiluminescence response of CHS mink neutrophils was almost complete with either catalase or SOD.

A large difference in PMA induced responses with CHS neutrophils in Experiment #5 compared to those observed in Experiment #3 was noted. The PMA induced chemiluminescence response of CHS mink in Experiment #3 was done with a different lot of PMA than that used in Experiment #5. Regardless of which lot of PMA was used, the response was significantly less than that observed with the normal mink neutrophils.

Oliver, who earlier studied PMA's effect on CHS neutrophils, in a controversial "capping phenomenon" experiment (106), attempted to document a microtubular defect in CHS cells as the cause of compromised host defense in CHS animals. Her work centered around the in vitro responses of leukocytes to concanavalin A, a synthetic lectin. If a microtubular defect were responsible for the alterations of CHS, then the sequela would be abnormal cell division. It is now known that CHS animals have no abnormal mitoses (107).

Recent work with PMA and human CHS cells involved an Epstein Barr virus transformed B cell line grown in cell culture. These non phagocytic cells mimicked the surge in oxidative metabolism seen in normal neutrophils after PMA stimulation (108). The summary of the study noted that both CHS and normal B cell lines responded with an increase in oxidative metabolism after stimulation with PMA.

PMA's effect on the neutrophil in morphologic studies with the transmission electron microscope documents that the ester has the capability to induce cytoplasmic vacuolar changes and degranulation similar to normal phagocytosis of particulates. The PMA induced morphologic change of the CHS neutrophil, a cell known to have delayed granule fusion (33), will probably show a distortion when compared to the normal neutrophil. This distortion can be visualized by the qualitative differences between CHS and normal mink neutrophils during phagocytosis of latex particles as noted in Experiment #2.

The extention represented by this research with PMA and CHS leukocytes when combined with a report of a recent examination of the role of calcium in the storage pool defect in CHS platelets (109) allows new insight into the problem. The platelet work done in part with calcium ionophore A 23187, which like PMA, is also a potent stimulus of neutrophil oxidative metabolism and an inducer of chemiluminescence (77), suggest that calcium ionophore A 23187 induced chemiluminescence of CHS and normal mink neutrophils may reveal additional useful information. The use of this ionophore with the CHS platelet has established a difference in the secretable and non-secretable calcium pools and suggests a metabolic defect which could explain both membrane fusion and granular extrusion delay characteristically observed in CHS neutrophils. Calcium has long been established as necessary cofactor in many biochemical reactions (110). If the cytoplasmic level of calcium is altered in other CHS cells manifesting functional abnormalities, then the implication is that a common mechanism may explain the defect in CHS.

The vast difference between the peak chemiluminescence of CHS neutrophils and normal mink neutrophils may be linked to other variables.

Luminol can be used in concentration higher than that used in the outlined experiments with no loss of cellular integrity (69), but inhibition of the assays with enzyme inhibitors is compromised. Briefly, the amount of superoxide dismutase that had to be used in the normal animal to maximally inhibit the response totally obliterated the CHS response. This was also a problem with catalase inhibition. Some authors state that catalase should be purified over a sephadex column (73) and then assayed for contaminating superoxide dismutase activity (111). In this study that could not be done because the resultant purified product was only usable in quantities that caused dilutional changes of the reaction mixture. However, the product utilized as purchased "Catalase" (Sigma Chemical Co., St. Louis, MO) was assayed as described (111) and found to be reasonably pure; therefore, the results should stand as reported: 1) SOD inhibited the generated O_2^- and 2) the unpurified catalase inhibited primarily the reactions involving H_2O_2 . Any overlap which may be the reason for the failure of the additive properties of catalase and superoxide dismutase to further inhibit latex induced chemiluminescence of normal mink neutrophils in Experiment #5, was not statistically significant.

There was also a reappearance of bimodal peak chemiluminescence in Experiment #5. This reoccurrence was seen in the latex induced chemiluminescence of 2 of the 5 normal mink neutrophils assayed. This phenomenon, as previously described, when statistically analyzed had no bearing on the mean outcome of events and was not considered a significant finding by this researcher.

One can conclude that the luminol enhanced "in-coincidence" chemiluminescence assay is a quick reproducible assay that can be used for the detection of neutrophil defects related to phagocytosis. Using this

assay it was shown that there is a marked distortion of the CHS neutrophils, exhibited by decreased luminol enhanced chemiluminescence, in response to latex particles phagocytosis and a decreased response to PMA induced chemiluminescence almost of equal magnitude. It was also shown that the chemiluminescence response was inhibited by SOD and catalase in both CHS and normal mink neutrophils.

Therefore, what ever the defect in CHS neutrophils is, there is still a portion of the normal mechanisms present. Whether it be a product or process qualitatively or quantitatively lacking it is inhibitable with SOD and catalase.

The question of which reactive species of oxygen is responsible for exciting the aminophthalate anion makes the assay adaptable to further studies, and definitive screening for the neutrophil defect in the CHS mink. If the distorted luminol enhanced chemiluminescence of CHS mink neutrophils, documented by this author in this dissertation, can be pinpointed to primarily involve the peroxidase containing granule, then CHS mink can serve as a model of neutrophil myeloperoxidase deficiency for assay purposes. Even if the defect can not be narrowed down to one of the interdependent neutrophil microbicidal systems at least the ground work is laid for a new examination of the neutrophil defect in CHS mink.

Further studies of the distorted chemiluminescence should involve parallel "in-coincidence" nonenhanced, "in-coincidence" enhanced, "out-of-coincidence" enhanced and "out-of-coincidence" nonenhanced chemiluminescence systems with both CHS and normal mink neutrophils. The relevant inducers of chemiluminescence should include PMA and calcium ionophore A23187. The author's original work with endotoxin challenge in CHS also warrants further study (17). The recent finding that interleukin 1

production from endotoxin treated macrophages results in a potent stimulation of prostacyclin synthetase in endothelial cells (112) suggests that an examination of CHS cells, other than CHS neutrophils, for interleukin 1 production may explain the abnormal susceptibility of CHS animals in response to endotoxin seen in the pilot experiment preceeding this study.

Given these insights into a defect that has been researched for years one must note that CHS mink have compromised host defense but are not totally deficient. They appear to have intact some of the normal mechanisms by which neutrophils kill microorganisms as evidenced by the fact that the chemiluminescence is inhibited by SOD and catalase. This may explain why it has been possible for mink breeders as well as researchers to maintain this animal model of CHS.

The use of animal model systems to study disease processes is a well established practice. However, the application of data across species lines is a problem and it is important that several species with a particular defect be studied for idiosyncratic differences. In CHS one is provided with a neutrophil defect in phylogenetically disparate species. The characterization of this neutrophil defect in CHS mink by using chemiluminescence questions the integrity of the oxidative metabolic pathways involved in phagocytosis when compared to normal mink neutrophils. Although the luminol enhanced system cannot substitute for assays of specific oxidative metabolism using radionuclides, it can lay important ground work for a more complete dissection of the CHS neutrophil defect.

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