

Evaluation of bovine polymorphonuclear
leukocyte function

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INTRODUCTION

Polymorphonuclear leukocytes (PMNs) are bone marrow derived white blood cells that play a central role in the defense of the host against infection. Through interactions with antibody, complement, and chemotactic factors, PMNs are attracted to invading microorganisms and are stimulated to phagocytose them. A complex series of metabolic events within the PMN leads to the intracellular killing of many infectious agents. Disorders of PMN function may lead to severe recurrent infection.

Many pathogenic agents possess the ability to impair or to evade host defense mechanisms; without this ability they presumably would not be pathogenic. When a pathogenic agent impairs a defense mechanism of the host in order to facilitate its own survival, it also facilitates the survival of other infectious agents within the host. In recent years there have been several reports of impairment of PMN function by infectious agents.

Bovine viral diarrhea (BVD) virus is an infectious agent which has been demonstrated to impair the host's immune defenses. In investigations at Iowa State University and elsewhere, the BVD virus has been demonstrated to have a marked effect on lymphoid tissue in vivo and in vitro (Peter et al., 1967; Muscoplat et al., 1973a, b; Truitt and

Shechmeister, 1973; Reggiardo, 1975), and to inhibit the clearance of bacterial organisms from the blood (Reggiardo, 1975). This inhibition of normal blood clearance mechanisms suggests that the BVD virus induces a defect in phagocytic cell function.

The purpose of the present study was to determine if a defect in PMN function occurred in cattle following infection with BVD virus. Techniques which had previously been reported for the evaluation of bovine PMN function were not felt to be adequate to screen for a defect in bovine PMN function following BVD virus infection. Therefore, it was necessary to adapt procedures which had been reported for use with human PMNs to use with bovine PMNs and to establish normal values for these procedures in the bovine system.

The objectives of the experimentation reported in this thesis were: (1) to develop new techniques for the evaluation of bovine PMN function and to establish normal values using these techniques; and (2) to determine if infection with BVD virus altered PMN function in cattle.

LITERATURE REVIEW

Polymorphonuclear Leukocytes

The polymorphonuclear phagocytic system is comprised of three types of cells found in blood: basophils, eosinophils, and neutrophils. These cells vary in size from 8-14 μm and are referred to collectively as polymorphonuclear leukocytes (PMNs). PMNs are produced in the bone marrow from a common stem cell, the myeloblast, which undergoes a series of cell divisions and maturational changes to eventually produce all three types of PMNs (Schalm, 1977). In response to infection, there can be a marked increase in production of PMNs by the bone marrow and a dramatic rise in the number of PMNs found in the blood.

Cells of the polymorphonuclear phagocytic system comprise the first line of defense against infectious agents. In response to infection, PMNs are rapidly released from the bone marrow into the circulation and are attracted to the site of microbial invasion where they will attempt to engulf and destroy potential pathogens. The importance of the polymorphonuclear phagocytic system is emphasized by the observation that animals which lack adequate numbers of normally functioning PMNs soon die of overwhelming bacterial infection. There are many excellent reviews dealing with polymorphonuclear leukocytes, their

phagocytic activity, and their role in host defense (Baehner, 1972; Stossel, 1974; Bellanti and Dayton, 1975; Murphy, 1976; Beeson and Bass, 1977; Babior, 1978).

The three types of PMNs in the circulation are distinguished on the basis of the staining properties of their cytoplasmic granules. Neutrophils are the most abundant and comprise about 70% of the normal circulating white blood cell population in man, but considerably less in domestic animals. Eosinophils and basophils possess less phagocytic activity, although they participate in other ways in the immunologic processes.

Basophils

The basophilic leukocyte is present in blood in an almost negligible quantity, usually forming less than 0.5% of the total number of leukocytes. The basophil has large granules which stain deep blue-black with Wright's stain. The granules of the basophil contain histamine and other substances which cause contraction of smooth muscle and an increase in the permeability of small blood vessels. The function of the basophil is not known in detail, but it does participate in allergic reactions and is essential in some types of tissue damage such as serum sickness. The basophil may be the precursor of the tissue mast cell; it is not thought to be active in phagocytosis.

Eosinophils

The eosinophil has granules that stain red or orange with Wright's stain. It has been demonstrated to have phagocytic potential, to ingest antigen-antibody complexes, and to have an important role in anaphylactic and allergic phenomena (Douglas, 1976). Eosinophils are capable of ingesting and killing bacteria in vitro, although they are much less efficient than neutrophils or macrophages (Cline, 1972). Eosinophils offer little or no protection against bacterial infection even when present in great numbers, as in children with congenital neutropenia. It may be that eosinophil phagocytosis is an in vitro phenomenon with limited relevance to the cell's activities in vivo. There are some data to suggest that eosinophils are geared more toward extracellular degranulation or "exocytosis" than toward phagocytosis. Two functions proposed for the eosinophil include modulation of inflammation and defense against metazoan parasites. These functions would be better served by a cell geared toward exocytosis than toward phagocytosis (Beeson and Bass, 1977).

Metabolic studies on essentially pure eosinophil preparations obtained from the ascitic fluid of a patient with eosinophilic gastroenteritis have demonstrated that

particle ingestion by eosinophils is associated with a marked increase in hexose monophosphate shunt activity, H_2O_2 formation, superoxide anion generation, chemiluminescence, thyroid hormone degradation, iodination, and estrogen binding. This postphagocytic metabolic burst by eosinophils was qualitatively similar to that observed in neutrophils, but for several parameters the eosinophil response was greater than the neutrophil response (Klebanoff et al., 1977).

Neutrophils

The neutrophil is the most common type of PMN found in the blood; its principal function is to combat bacterial infection. The neutrophil contains in its cytoplasm two types of membrane bound granules, which contain many different substances; some of these substances carry strong negative charges and others carry equally strong positive charges. The overall balance between the different chemical constituents gives a net staining reaction that is about neutral (Murphy, 1976).

Neutrophils are considered to represent a first line of defense against bacterial infection. They arrive at a site of bacterial invasion earlier than macrophages and serve to restrict the spread of invading microorganisms. They adhere to particles, and when functioning

normally, they ingest and destroy microorganisms by means of the enzymic contents of their lysosomes (Tizard, 1977). Neutrophils are much more important than eosinophils or basophils in phagocytosis (Cline, 1972), and more is known about neutrophil function and metabolism.

The mature human neutrophil is an end-stage cell which, once released from the bone marrow, circulates with a half-life of 6-7 hours before leaving the blood stream in a random (nonage related) fashion. In man about 10^8 neutrophils are turned over daily; most of these cells leave the body through the digestive and respiratory systems. The total blood granulocyte pool is composed of approximately equal numbers of circulating and marginated cells. Only the circulating pool is sampled by standard blood collection techniques. When there is a sudden demand for neutrophils, the marginated pool can supply cells almost instantly to double the numbers of cells in the circulating pool. The marginated pool is responsible for the rise in leukocyte numbers after exercise, excitement, fear, and feeding. If continued need for neutrophils occurs because of tissue damage, the large reserve of cells in the bone marrow is responsible for meeting this demand. Two to three times the circulating pool may be mobilized in one hour and 8-10 times the circulating pool may be mobilized in 6-7 hours (Medway, et al., 1969).

Phagocytosis by Polymorphonuclear Leukocytes

Historical aspects of phagocytosis

The idea that bacteria are disposed of by being taken up and digested by phagocytic cells is less than a century old, and was first clearly formulated by the Russian zoologist Elie Metchnikoff (Metchnikoff, 1893). In 1882 he studied the role of motile cells of a transparent starfish larvae in protection against foreign intruders. He introduced a rose thorn into these larvae and noted that a few hours later the rose thorn was surrounded by motile cells. This experiment can be considered the starting point of cellular immunology. Koch and Neisser had previously established that bacteria can be found in leukocytes, but they thought that this was the result of bacterial invasion of the leukocytes. Metchnikoff demonstrated that the leukocytes had in fact engulfed the microorganisms and called the process phagocytosis. He later demonstrated the existence of two types of circulating cells capable of phagocytosis, the polymorphonuclear leukocytes and the macrophages, and proposed the general term "phagocytes" for these cells (Grabar, 1976).

Phagocytosis by neutrophils is composed of four interrelated phases: chemotaxis, opsonization, ingestion, and degranulation (Drutz, 1976).

Chemotaxis

Chemotaxis is the process whereby phagocytic cells are attracted to the vicinity of invading pathogens. Chemotactic factors cause a change in the direction of leukocyte mobility but do not cause a change in the speed of leukocyte movement. The interaction of microorganisms and host tissue leads to the generation of chemotactic factors by several mechanisms (Stossel, 1974). Although some bacteria release substances that without further alteration have the capacity to attract phagocytes (Nelson et al., 1975; John and Sieber, 1976), most chemotactic factors are of host origin. The complement system appears to be the most important source of these leukotactic factors (Ward et al., 1965; Stossel, 1974). Complement components which are chemotactic include C3a, C5a, and C5⁶⁷; they may be generated via the classical or alternative pathway of complement activation or by the action of nonspecific proteases upon native complement components (Drutz, 1976; Repo, 1977). Other sequentially reacting protein systems (kallikrein system; fibrinolysis system) also contain factors with leukotactic activity (Drutz, 1976). Finally, leukocytes themselves contain factors that are directly or indirectly chemotactically active. Ingestion of particulate matter by neutrophils causes them to release a factor that has the capacity to attract other neutrophils in the

absence of serum (Zigmond and Hirsch, 1973), and lymphocytes responding to antigens elaborate lymphokines, which, among other properties, have chemotactic activity (Stossel, 1974). When chemotactic factors reach the surface of the neutrophil, an esterase on the cell surface is activated, the hexose monophosphate shunt is activated, calcium fluxes occur in the cell, and microfilaments (composed of actin) and microtubules (composed of tubulin) assemble, providing the motility needed to propel the cell toward the source of the chemotactic factor. Myosin also has been found in phagocytic cells, and it is likely that the motility of such cells may have a close molecular relationship to muscle contraction (Drutz, 1976). Prior phagocytosis of material has been shown to reduce a cell's ability to respond to a chemotactic attractant (Mowat and Baum, 1971).

Opsonization

Opsonins are serum components which react with microorganisms and make them more susceptible to ingestion by phagocytes. This process is termed opsonization and may occur by one of three mechanisms (Drutz, 1976).

(1) Specific antibody alone may act as an opsonin. When specific antibody combines with antigenic sites on

the surface of a microorganism through antibody combining sites located on the Fab portion of the immunoglobulin molecule, the Fc portion of the molecule is then free to attach to Fc receptor sites on the surface of phagocytes, thereby completing a bridge between the microorganism and phagocytic cell. Human PMNs are reported to have receptors for the Fc portion of certain IgG subclasses of antibody, but to lack an Fc receptor for IgM (Menzel et al., 1978). Bovine PMNs are reported to have an Fc receptor for IgG and for IgM (Grewal et al., 1978).

(2) Specific antibody acting in concert with complement via the classical complement pathway may promote opsonization. Here, a quantity of antibody apparently insufficient to opsonize on its own may react with bacteria and activate the classical complement sequence, resulting in the binding of complement components to the bacterial surface. Bovine and human PMNs have been demonstrated to have complement receptors (Grewal et al., 1978; Menzel et al., 1978).

Complement component C3b is believed to be the opsonically active component (Gigli and Nelson, 1968). The C3b on the bacterial surface apparently serves as a bridge between bacteria and phagocyte, prompting ingestion (Drutz, 1976).

(3) Opsonization can be nonspecific and involve the activation of complement via the alternative pathway. This method does not require the action of specific antibody and may play an important role in early pre-immune stages of infection prior to the production of specific antibody. Complement component C3b is again believed to be the opsonically active component (Drutz, 1976).

Ingestion may occur in the absence of opsonization by a method known as surface phagocytosis. Here, encapsulated bacteria are trapped between leukocytes themselves, between leukocytes and tissue surfaces, or along with leukocytes in fibrin clots. Surface phagocytosis is much less efficient in areas where leukocytes are not tightly packed (pleural, pericardial, synovial, and cerebrospinal fluids). Surface phagocytosis may also play an important role in the preimmune stages of infection prior to the production of specific antibody (Drutz, 1976).

The receptors for Ig and complement which have been referred to may not be actual membrane proteins that specifically combine with the opsonin. It has been postulated that the principal factor that determines whether phagocytosis of a particle can occur is the physical nature of the surface of the particle in comparison with

that of the phagocyte. Van Oss and Gillman (1972a) found that bacteria with surfaces that were more hydrophobic than the surface of phagocytes readily became engulfed, whereas bacteria with surfaces that were more hydrophilic than the surface of phagocytes resisted engulfment. Most nonpathogenic bacteria are hydrophobic and are readily ingested by phagocytes. Bacteria that are highly hydrophobic, such as Mycobacterium tuberculosis, are spontaneously ingested by phagocytic cells. These organisms owe their pathogenicity to their resistance to digestion by phagocytic enzymes. Bacteria like Diplococcus pneumoniae that possess a hydrophilic carbohydrate capsule are not normally subject to phagocytosis. Antibody to encapsulated organisms makes their surfaces more hydrophobic, and simultaneously they become easier to phagocytose. The addition of complement components C1, C4, and C2 has no effect, but when C3 is bound, both the hydrophobic character of the bacterial surface and the ease of phagocytosis increase sharply. It is, therefore, possible that antibody and complement aid phagocytosis because they change the character of the bacterial surface, rather than because there are specific receptor molecules for them on the phagocytic cell surface (Van Oss and Gilman, 1972a, b; Carpenter, 1975; Murphy, 1976; Van Oss, 1978).

Ingestion

Upon particle or microbial contact, cell pseudopodia are extended which fuse on the distal side of the material to be ingested. The particle becomes encased within a phagocytic vesicle, or phagosome, which is lined by inverted plasma membrane. The phagosome buds off from the cell periphery and moves centripetally, apparently through the mediation of microtubules. The mechanism for the triggering of ingestion is not clearly understood (Drutz, 1976).

Degranulation

The destruction of susceptible microorganisms within neutrophils is intimately associated with the process of degranulation, the release of granule contents into phagosomes. The neutrophil contains two types of membrane bound granules (lysosomes). In man, primary (azurophilic) granules contain abundant hydrolytic lysosomal enzymes, large amounts of myeloperoxidase, lysozyme, elastase, and cationic proteins. Secondary (specific) granules, which are smaller than primary granules, contain lactoferrin and lysozyme (Drutz, 1976). Bovine neutrophils have biochemical properties very similar to those of human neutrophils, although they appear to have a lower content

of primary granule enzymes and to virtually lack lysozyme (Rausch and Moore, 1975; Gennaro et al., 1978).

As the phagosome forms during microbial engulfment, neutrophil granules undergo violent movement in proximity to the phagosome, fuse with the phagocytic vacuole, and disappear from the cytoplasm (degranulate) (Stossel, 1974). Specific granules fuse with the phagocytic vesicle first; this process starts approximately 30 seconds after ingestion. Primary granules do not begin to discharge their contents until 3 minutes after ingestion, and usually only into the larger vesicles (Murphy, 1976). Neutrophils contain a skeleton of microtubules, which appear to direct the intracellular motion of the primary and secondary granules after phagocytosis. The degranulation phenomenon is prevented by treating neutrophils with either colchicine or vinblastine, both of which prevent the polymerization of microtubule subunits into functional tubules (Zurier et al., 1973; Weissmann et al., 1975; Wilson, 1975; Hoffstein et al., 1977). Degranulation is also prevented by agents that raise the intracellular levels of cyclic AMP (Goldstein et al., 1973; Zurier et al., 1973; Hawkins, 1974; Ignarro et al., 1974; Zurier et al., 1974; Weissmann et al., 1975; Goren, 1977). Neutrophils contain a protein kinase that can phosphorylate several proteins, probably including tubulin. It is hypothesized

that cyclic AMP may stimulate this protein kinase to phosphorylate the tubulin subunits of microtubules and prevent their aggregation (Murphy, 1976). Agents which are reported to raise intracellular levels of cyclic AMP and to block degranulation include beta adrenergic drugs (epinephrine, norepinephrine, isoproterenol), prostaglandin E₁, histamine, cholera enterotoxin, E. coli heat labile enterotoxin, theophylline, and exogenous cyclic AMP itself (Ignarro et al., 1974; Zurier et al., 1974; Ignarro and Cech, 1975; Weissmann et al., 1975; Hoffstein et al., 1977; Bergman et al., 1978). Agents which increase intracellular levels of cyclic GMP have been found to increase degranulation by PMNs. These agents include carbamylcholine chloride, phorbol myristate acetate, and exogenous cyclic GMP (Zurier et al., 1974; Goldstein et al., 1975a; Weissmann et al., 1975; Wright et al., 1977).

A low molecular weight component of complement, similar or identical to human C5a, has been demonstrated to be capable of interacting with human PMNs to induce degranulation, fusion of lysosomal membranes with plasma membranes, and transient assembly of microtubules associated with the release of endogenous myeloperoxidase. This component of complement has been called lysosomal enzyme-releasing factor because it provokes secretion of lysosomal hydrolases either into phagocytic vacuoles or into

extracellular spaces. Lysosomal enzyme-releasing factor may play an important role in the inflammatory response (Goldstein et al., 1973; 1975b).

Energy sources

The energy needed for PMNs to function in motility, ingestion, and degranulation is derived from their large reserve of glycogen through anaerobic glycolysis. This form of metabolism does not require oxygen, which is important for a cell which must operate in inflammatory sites where oxygen tension may be very low. Under normal conditions, the neutrophil takes up glucose from its environment, and its cellular reserve of glycogen remains relatively constant. During phagocytosis, the rates of glycogen breakdown, glucose uptake, and lactate output are markedly increased. Neutrophils may also metabolize glucose by the hexose monophosphate shunt which is important for the oxidative killing mechanisms of the PMN (Murphy, 1976).

Microbial killing mechanisms

PMNs possess a number of mechanisms for killing microorganisms. These mechanisms probably represent a functional redundancy; many microorganisms may be killed effectively by any one of the mechanisms, while some of

the mechanisms are likely to be active against only specific classes of microorganisms (Drutz, 1976).

Microbial killing mechanisms comprise two broad categories: nonoxidative and oxidative.

Nonoxidative killing mechanisms Many organisms are killed normally under anaerobic conditions (Mandell, 1974). The killing mechanisms which do not require oxygen are very important when neutrophils must function in an anaerobic environment. These killing mechanisms depend upon substances present in the primary and specific granules of the PMNs. A list of enzymes and other substances found within neutrophil granules is presented in Table 1 (Bellanti, 1978); some of the more important bactericidal substances are discussed below.

(1) Cationic proteins are a family of proteins found in the primary granules. They have a positive charge and therefore tend to stick to bacterial surfaces which are generally negatively charged. By some unknown mechanism, they are able to damage microbial membrane barriers and to kill some bacterial organisms (Drutz, 1976; Murphy, 1976).

(2) Lactoferrin is an iron binding protein which is found in the specific granules of PMNs. Most bacteria

Table 1. Enzymes and other substances found within neutrophils (Adapted from Cochrane, 1968).

Acid phosphatase	Hyaluronidase
Acid ribonuclease	Lysozyme
Acid deoxyribonuclease	Collagenase
Cathepsins B, C, D, E	Aryl sulfatases A and B
Phosphoprotein phosphatase	Phospholipases
Organophosphate-resistant esterase	Acid lipase
β -Glucuronidase	Lactoferrin
β -Galactosidase	Phagocytin and other related bactericidal proteins
β -N-acetylglucosaminase	Endogenous pyrogen
α -fucosidase	Plasminogen activator (Puro-kinase)
α -glucosidase	Hemolysin(s)
α -mannosidase	Mucopolysaccharides and glycoproteins
α -N-acetylglucosamidase	Basic proteins: (a) Mast cell-active (b) Permeability-inducing, independent of mast cells
α -N-acetylgalactosaminidase	
Myeloperoxidase	

such as Staphylococcus aureus, E. coli, Pasteurella multocida, and Mycobacterium tuberculosis require iron for growth. Lactoferrin may exert its antimicrobial function by binding and withholding required iron from ingested bacteria. Lactoferrin may be important in resistance to bacterial infection in the mammary gland. In response to bacterial invasion neutrophils release their stores of lactoferrin and, in this way, enhance the bactericidal activity of milk (Tizard, 1977).

(3) Lysozyme is an enzyme which is capable of destroying the peptidoglycan layer of the cell wall of certain bacterial organisms by breaking the bonds which join alternating units of N-acetylglucosamine and N-acetylmuramic acid. The destruction of the peptidoglycan layer alters the membrane barrier activity of the cell wall and leads to bacterial death. Lysozyme is present in both the primary and specific granules of human PMNs; it is completely lacking in bovine, caprine, ovine, and feline PMNs (Rausch and Moore, 1975; Gennaro et al., 1978). Lysozyme kills those few gram-positive bacteria that do not have some covering for their peptidoglycan layer; however, it can kill many gram-positive and gram-negative organisms if some other agent (antibody and complement) damages the cell wall and allows lysozyme access to its substrate. Because bacterial death often precedes the action of lysozyme, this enzyme may serve in a digestive rather than microbicidal capacity in the phagosome (Drutz, 1976; Murphy, 1976).

(4) Elastase from primary granules attacks mucopeptides of certain bacterial cell walls and may also be more important in digestion of bacterial organisms than killing.

(5) Lactic acid is generated in large quantities during phagocytosis because of the increased glycolytic

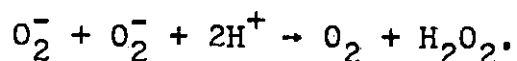
activity. In some unknown way, the excess hydrogen ions are concentrated in phagocytic vacuoles. In rat, mouse, and rabbit neutrophils the intravacuolar pH falls to about 4.0 following phagocytosis; in human neutrophils the intravacuolar pH falls to only about 6.0-6.5. The significance of the low pH is not clear. Few bacteria can continue to grow at pH 4.0; however, the cationic proteins and proteolytic enzymes of human neutrophil granules have a pH optimum near neutrality and may not function well under acidic conditions (Drutz, 1976; Murphy, 1976).

Oxidative killing mechanisms When neutrophils take up organisms under aerobic conditions, a series of related changes occur: increased oxygen consumption; increased hexose monophosphate shunt activity; and generation of hydrogen peroxide, superoxide anion, hydroxyl radical, and possibly singlet oxygen (Babior, 1978; Johnston, 1978). This increased oxygen consumption is not suppressed by cyanide, which is fairly good evidence that it is not being mediated by cytochromes (Johnston, 1978). It appears to be due to the activity of an oxidase enzyme, or enzyme complex, which is located in the plasma membrane, and therefore in the phagosomal membrane (Goldstein et al., 1977). The most likely substrates for the oxidase enzyme

in neutrophils are NADH and NADPH. There is conflicting evidence as to which of these molecules is the substrate. The hexose monophosphate shunt generates NADPH, but this may undergo a transhydrogenase reaction with NAD to form NADH, which may be the actual substrate (Stossel, 1974). Recent evidence strongly favors the hypothesis that NADPH is the electron donor in this reaction (Babior, 1978).

Oxidase enzymes do not necessarily, or even usually, mediate two-electron transfers to form H_2O_2 directly. Their reactions often proceed by one-electron transfer steps that result in the formation of unstable and highly reactive intermediates. In neutrophils there is evidence for the generation of superoxide anion, singlet oxygen, and the hydroxyl radical (Babior, 1978; Johnston, 1978).

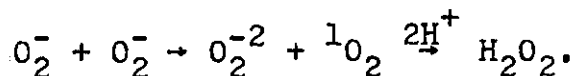
Superoxide anion is oxygen that has accepted one electron. It is formed either by the univalent reduction of oxygen or by the univalent oxidation of H_2O_2 and is a highly reactive radical which can act as an oxidant or as a reductant. When it functions as a reductant, as in the reduction of ferricytochrome C or nitroblue tetrazolium, the superoxide anion is oxidized to oxygen. When it acts as an oxidant, the superoxide anion is reduced to H_2O_2 . When two molecules interact, one is oxidized and the other reduced as follows:



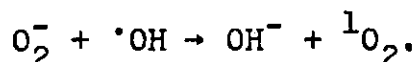
This dismutation occurs spontaneously and is also catalyzed by the enzyme superoxide dismutase (Rosen and Klebanoff, 1976). The growth of certain microorganisms is inhibited by superoxide anion (Johnston et al., 1975), and this inhibition is related to the content and distribution of microbial superoxide dismutase (Yost and Fridovich, 1974).

Singlet oxygen is an electronically excited state of oxygen. Ground state molecular oxygen has two valence electrons that are unpaired and have spins in the same direction. Singlet oxygen is formed when an absorption of energy shifts one of these electrons to an orbital of higher energy with an inversion of spin (Rosen and Klebanoff, 1976). The lifetime of singlet oxygen is short with dissipation of excess energy by thermal decay, light emission, or chemical reaction. Singlet oxygen is thought to be responsible, either directly or indirectly for the emission of light by PMNs during phagocytosis (Allen et al., 1972; Andersen et al., 1977). There is still no proof that singlet oxygen is actually formed during phagocytosis, because the means employed for detecting singlet oxygen in biological systems are nonspecific (Babior, 1978). There are several mechanisms proposed for the formation of singlet oxygen within the PMN (Webb et al., 1974; Rosen and Klebanoff, 1976; Babior, 1978). One proposed mechanism is that it is an

intermediate in the dismutation of superoxide anion, as follows:

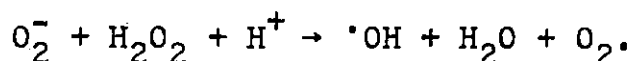


Another proposed mechanism is by the reaction of superoxide anion with the hydroxyl radical, as follows:



The hydrogen peroxide-myeloperoxidase-halide reaction is also proposed as a source of singlet oxygen. It is known to display chemiluminescence under the proper conditions. There is evidence that normal light emission by PMNs requires both superoxide anion and myeloperoxidase; therefore, more than one of the above mechanisms may be involved (Rosen and Klebanoff, 1976). It has been suggested that singlet oxygen may function in the bactericidal activity of PMNs by combining across the double bonds of unsaturated fatty acids (Krinsky, 1974; Murphy, 1976).

The hydroxyl radical is a highly unstable oxidizing species that reacts almost instantaneously with most organic molecules that it encounters. It appears that this exceedingly reactive substance can be generated biologically, and may participate in the microbicidal activity of phagocytes. Hydroxyl radical may be generated in the PMN by the following reaction (Webb et al., 1974; Babior, 1978):



A potent antimicrobial agent of neutrophils is hydrogen peroxide. Although hydrogen peroxide is bactericidal in its own right, its antibacterial, antifungal, and antiviral activity is markedly potentiated by myeloperoxidase in the presence of halide ions. The mechanism for this bactericidal activity is not precisely known; reactive aldehydes, which arise from the hydrogen peroxide-myeloperoxidase-halide-microbe interaction, may represent the actual bactericidal compounds (Stossel, 1974). The reaction of hydrogen peroxide and halide ions in the presence of myeloperoxidase results in the covalent bonding of halide anions to protein of ingested particles. The extent of this reaction may be determined by adding radiolabeled iodine to a phagocytic system and determining the amount of radioactivity which is covalently bound to protein (Pincus and Klebanoff, 1971; Simmons and Karnovsky, 1973; Klebanoff and Clark, 1977). Hydrogen peroxide for this reaction is formed in the phagosome by the action of the oxidase enzyme mentioned previously. Myeloperoxidase is found in the primary granules and is delivered to the phagosome by degranulation. Halide ions enter by diffusion; iodide can even be stripped from thyroid hormones by PMNs and subsequently fixed to bacteria (Stossel, 1974).

Polymorphonuclear Leukocytes as Mediators of Antiviral Immunity

Polymorphonuclear leukocytes are considered to be primarily involved in antibacterial rather than antiviral defense. However, some recent observations have suggested a role for PMNs in antiviral immunity (Rouse et al., 1978). There are three basic mechanisms suggested for mediation of antiviral immunity by PMNs: 1) phagocytosis and destruction of virus particles, 2) antibody-dependent cell-mediated cytotoxicity, and 3) release of a subcellular mediator, similar to interferon, which can render cells resistant to virus infection (Rouse et al., 1978).

Several viruses have been demonstrated to be engulfed by PMNs (Baratawidjaja et al., 1965; Gresser and Lang, 1966; Sommerville, 1966; 1968; Belding and Klebanoff, 1970). Some investigators have emphasized the protection afforded by intraleukocytic residence of viruses against specific antibodies and other nonspecific viral inhibitors present in blood and have pointed to phagocytosis as a means of disseminating viral particles (Gresser and Lang, 1966; Smith, 1972). However, others have reported a rapid decrease in the titer of certain viruses following ingestion by leukocytes (Sommerville, 1968). The nature of the intraleukocytic virucidal system is not known.

The myeloperoxidase-hydrogen peroxide-halide system has been shown to be virucidal for poliovirus and vaccinia virus in vitro and may have a role in antiviral defense in vivo (Belding and Klebanoff, 1970). If a virus is able to interfere with degranulation in the PMN, it will prevent the activity of this potentially virucidal system. The virus may then be able to survive in the PMN and be disseminated throughout the body.

Antibody-dependent cell-mediated cytotoxicity (ADCC) is the killing of antibody-coated target cells by an effector cell. PMNs have been demonstrated to be capable of mediating ADCC (Gale and Zighelboim, 1975; Rouse et al., 1976; Clark and Klebanoff, 1977; Russell and Miller, 1978), and in the bovine species, PMNs have been shown to be the most efficient of all cell types tested in mediating antiviral ADCC (Rouse et al., 1978). Furthermore, PMNs, in the presence of antibody, could prevent virus dissemination when added to virus-infected monolayers (Wardley et al., 1976b). This PMN mediated ADCC has been shown to be independent of DNA, RNA, and protein synthesis by the PMN; it is blocked by drugs which inhibit microtubule function, and it is subject to the control of cyclic AMP and cyclic GMP. Drugs which elevate cyclic AMP decrease cytotoxicity; drugs which decrease cyclic AMP or

increase cyclic GMP enhance cytotoxicity (Wardley et al., 1976a; Clark and Klebanoff, 1977). These properties of the regulation of ADCC are very similar to the regulation of degranulation within the PMN (Weissmann et al., 1975). This similarity suggests that degranulation may play a role in ADCC. Myeloperoxidase and cationic proteins, both of which are found in PMN lysosomes, appear not to be essential in the cytotoxicity system (Clark and Klebanoff, 1977); however, there are many other substances in the PMN lysosomes which may play a role.

There is recent evidence that PMNs may be involved in antiviral immunity by the release of subcellular mediators which can render cells resistant to virus infection (Rouse et al., 1977; 1978). These mediators have properties similar to interferon and have been tentatively called interferon 3 (Rouse et al., 1978). The release of this material can only be induced by exposure of PMNs to infectious bovine rhinotracheitis virus (IBR) infected Georgia bovine kidney cells (GBK). Exposure of bovine PMNs to GBK cells infected with other bovine viruses did not induce the release of the soluble factors (Rouse et al., 1978). More study is needed of this phenomenon before its role in antiviral immunity is elucidated.

Virus Induced Defects in Polymorphonuclear Leukocyte Function

There is very little known about the effects of virus infection on polymorphonuclear leukocyte function (Notkins, et al., 1970; Smith, 1972). There have been studies examining the ability of PMNs to ingest bacteria following virus infection. Kantoch et al. (1961) demonstrated that the phagocytic activity of polymorphonuclear leukocytes from patients with viral hepatitis was depressed. In vitro infection of polymorphonuclear leukocytes with mumps (Merchant and Morgan, 1950), influenza (Merchant and Morgan, 1950; Fisher and Ginsberg, 1956; Sawyer, 1969), and Coxsackie virus (Kantoch and Dubowska-Inglot, 1960) decreased the ability of these cells to engulf bacteria. No attempt was made to evaluate oxidative metabolism or degranulation in these studies.

A recent report describes depressed neutrophil motility in patients with recurrent herpes simplex virus infections (Rabson et al., 1977). The defective chemotactic response could be corrected in vitro by treatment of the neutrophils with 10^{-3} M levamisole.

More work needs to be done utilizing new techniques which are now available to study the interaction of viruses and PMNs. This may lead to a better understanding of the synergism between viral and bacterial infection (Degre', 1970).

Evaluation of Bovine Polymorphonuclear Leukocyte Function

A variety of procedures have been described for evaluating bovine PMN function. The most common method reported is the microscopic enumeration of ingested particles. The techniques utilized vary widely between laboratories.

Source of polymorphonuclear leukocytes

Evaluation of PMN function has been performed on PMNs in whole blood (Banas, 1974; Guidry et al., 1974; Guidry and Paape, 1976; Guidry et al., 1976; LaMotte and Eberhart, 1976), on PMNs isolated from blood (Naidu and Newbould, 1973; Renshaw et al., 1974; Paape and Guidry, 1975; Newbould, 1976; Beswick and Slater, 1977; 1978), and on PMNs collected from the bovine mammary gland (Newbould, 1973; Naidu and Newbould, 1973; Paape and Guidry, 1975; Guidry et al., 1976; Paape and Wergin, 1977; Jain and Lasmanis, 1978).

The most common procedure reported for isolation of PMNs from the blood was the method of Carlson and Kaneko (1973) involving centrifugation, removal of the plasma and buffy coat cells, and hypotonic lysis of the packed red blood cells. This procedure yielded a fairly

pure preparation of PMNs with good biological activity (Naylor and Little, 1975; Paape and Guidry, 1975; Beswick and Slater, 1978; Jain and Lasmanis, 1978). Other procedures reported for the isolation of PMNs from bovine blood included ammonium chloride lysis of the erythrocytes in whole blood (Renshaw et al., 1974), and sedimentation of the erythrocytes by adding 3 ml of a 6% solution of bovine fibrinogen to 1.7 ml of whole blood. This yielded a leukocyte rich supernatant, from which the leukocytes were harvested (Naidu and Newbould, 1973).

The isolation of PMNs from the mammary gland has been accomplished by infusing an irritant (saline, glycogen, or E. coli endotoxin) into the mammary gland, then obtaining milk from the gland a few hours to a few days later. Polymorphonuclear leukocytes isolated from milk have been found to be less active phagocytically than PMNs in the peripheral blood (Paape and Guidry, 1975; Jain and Lasmanis, 1978; Paape and Wergin, 1977). This depressed phagocytic activity may be due to the previous ingestion of milk fat globules and casein (Paape and Guidry, 1975; Paape and Wergin, 1977), or to the lower glycogen content of PMNs isolated from the mammary gland when compared to PMNs isolated from the peripheral blood (Naidu and Newbould, 1973).

Methods for evaluation of function

The most common method reported for the evaluation of bovine PMN function is the microscopic enumeration of ingested particles (Newbould, 1973; Guidry et al., 1974; Guidry and Paape, 1976; Guidry et al., 1976; LaMotte and Eberhart, 1976; Newbould, 1976; Paape and Wergin, 1977; Jain and Lasmanis, 1978). This is a time consuming, imprecise technique which does not make possible a distinction between particles merely attached to leukocytes and those actually ingested (Newbould, 1973). This is the same basic technique that was used by Metchnikoff in the late eighteenth hundreds (Metchnikoff, 1893). There is at least one report of the evaluation of ingestion by bovine PMNs utilizing ^{32}P -labeled Staphylococcus aureus as the ingested particle (Paape and Guidry, 1975). This procedure appeared to work fairly well; they reported a coefficient of variation between duplicate determinations of 5.6%. However, they did have problems with leaching of the ^{32}P from the bacteria. After a 5 minute incubation at 37°C , 33% of the ^{32}P was lost; after 60 minutes, 51% was lost.

Oxygen consumption by bovine PMNs following phagocytosis has been measured using a Clarke electrode (Beswick and Slater, 1977; 1978). This is a rapid

relatively simple procedure for the evaluation of one aspect of the oxidative metabolism of the PMN.

Probably the most thorough study of bovine PMN function was conducted by Renshaw et al. (1974). They evaluated PMNs isolated from the blood of normal cattle and cattle with the bovine homologue of the Chediak-Higashi syndrome of man. Bactericidal activity of the PMNs against six species of pathogenic bacteria was evaluated by incubating PMNs with the bacteria and determining bacterial survival utilizing standard plate counts. Electron microscopy and cytochemical techniques were used to detect myeloperoxidase activity in ultrastructural studies of the PMN, and for examining the sequence of events leading to fusion of primary granules with the phagosomes after ingestion of bacteria. Glucose oxidation was evaluated utilizing ^{14}C labeled glucose. Nitroblue tetrazolium reduction by PMNs was quantitated. They concluded from their study that PMNs from cattle with the Chediak-Higashi syndrome had a bactericidal defect that was associated with abnormal intracellular killing and not due to defective particle ingestion. The in vitro bactericidal defect was associated with a metabolic anomaly in the hexose monophosphate shunt, but not with an

alteration in the capacity to reduce nitroblue tetrazolium dye. Ultrastructural histochemical studies of phagocytosis and phagolysosome formation in polymorphonuclear leukocytes suggested that the impairment in bactericidal capacity is correlated also with either a delay or failure of primary granules to degranulate.

The histochemical nitroblue tetrazolium reduction test has been used to evaluate bovine PMN activity (Banas, 1974). In this procedure, nitroblue tetrazolium is mixed with a drop of whole blood on a slide and incubated for a defined period of time (15-30 minutes) at 37 C. The slide is then examined microscopically, and the number of PMNs containing intracellular deposits of formazan (formed by the reduction of nitroblue tetrazolium) is determined. Systemic bacterial infections resulted in an increase in the number of cells containing formazan. This test has been suggested for the early detection of systemic bacterial infection; however, false negative and false positive results do occur (Curreri et al., 1973; Stanford et al., 1974).

Bovine Viral Diarrhea-Mucosal Disease

Virus diarrhea, as originally described by Olafson et al. in New York in 1946, was an acute, highly contagious disease caused by a virus. The outbreaks in affected

herds were explosive with high morbidity but low mortality. Mucosal disease was described in 1953 by Ramsey and Chivers as a disease with a morbidity rate of 2%-50% and a mortality rate of approximately 100%. It was characterized by an initial febrile reaction, mucoid nasal discharge, anorexia, constant or intermittent watery diarrhea with feces often containing blood, rapid dehydration, and death. Erosions, ulcerations, and hemorrhages were always found in the alimentary canal (Jubb and Kennedy, 1970). These two disease syndromes are related and have a common etiologic agent (Tyler and Ramsey, 1965) called the bovine viral diarrhea (BVD) virus. It contains RNA as its genetic material and is ether sensitive. The virus is reported to be approximately spherical, to possess an envelope with few prominent surface projections, and to have a spherically shaped central core (Pritchett and Zee, 1975). Laboratory infections with the BVD virus in general do not yield the complete clinical picture of the disease. The typical response to challenge by the intravenous route is severe leukopenia, diphasic temperature curve, and decreased appetite (Tyler and Ramsey, 1965; Malmquist, 1968). After infection, an initial viremia occurs and the virus can be found in the blood at the height of the fever. Localization in the alimentary mucosa follows,

and the local lesions and mucosal edema which develop produce the clinical syndrome of stomatitis and enteritis. The gross lesions are primarily confined to the alimentary tract and are generally erosive in nature (Blood and Henderson, 1968). By far the most common form of infection by BVD virus is the subclinical case; this probably accounts for the high percentage of animals with serum titers (approximately 60%) (Malmquist, 1968).

There is evidence that BVD virus may be immunosuppressive. A consistent finding in clinical infections with BVD virus is lymphoid depletion to a greater or lesser degree (Tyler and Ramsey, 1965; Malmquist, 1968). The BVD virus has an apparent affinity for the cells of the immune system and destruction of lymphoid tissues can be observed in natural cases of the disease (Ramsey, 1956; Pritchard, 1963; Peter et al., 1967). Replication of the BVD virus in bovine lymphocytes and macrophages has been demonstrated. Stimulation of infected cells with the mitogen phytohemagglutinin (PHA) usually resulted in increased virus titers. Cells from both immune and susceptible cattle supported virus growth equally well (Truitt and Shechmeister, 1973).

Inhibition of bovine lymphocyte response to PHA after in vitro or in vivo infection has been reported

(Johnson and Muscoplat, 1973; Muscoplat et al., 1973a; Reggiardo, 1975). Animals chronically infected with the BVD virus characteristically do not produce detectable BVD serum-neutralizing antibodies (Peter et al., 1967; Johnson and Muscoplat, 1973; Muscoplat et al., 1973b). Reggiardo and Kaeberle (1979) noted that BVD virus infection resulted in inhibition of the normal blood clearance mechanisms as evidenced by the detection of an endogenous bacteremia in up to 85% of infected calves on the first 5 days following infection. This evidence suggests that the BVD virus is immunosuppressive and may predispose to secondary viral and bacterial infection.

PART I. EVALUATION OF BOVINE POLYMORPHONUCLEAR
LEUKOCYTE FUNCTION¹

¹Submitted for publication to Veterinary Clinical Pathology by J. A. Roth and M. L. Kaerberle.

IODINATION

Summary

Bovine polymorphonuclear leukocytes (PMNs) are capable of converting inorganic iodide to a trichloroacetic acid precipitable (protein bound) form during phagocytosis. A procedure for the determination of the ability of bovine PMNs to iodinate protein is described. The interpretation and significance of this test as a measure of PMN phagocytic activity is discussed and values obtained from normal animals are reported.

Introduction

A major function of the polymorphonuclear leukocyte (PMN) is the phagocytosis and destruction of invading microorganisms. A congenital or acquired defect in PMN function will result in an enhanced susceptibility to infection with bacterial or fungal pathogens (Baehner, 1972). When such a condition arises, it is desirable to be able to evaluate adequately the phagocytic capability of the circulating PMNs. The most common methods of evaluating phagocytic capability in cattle have been either the enumeration of ingested bacterial organisms or a determination of the ability of PMNs to kill a certain bacterial organism. The first method evaluates only the ingestion

phase of phagocytosis. It is not quantitatively accurate, it is time consuming, and it is difficult to differentiate ingestion from adherence. The bactericidal assays provide useful information, but their interpretation as to the overall integrity of PMN function must be approached cautiously. The PMN possesses a wide variety of bactericidal mechanisms, and different bacteria may be killed by different mechanisms. PMNs with a defect in a particular bactericidal mechanism may kill one bacterial organism normally while being totally unable to kill another bacterium (Holmes and Good, 1972).

Iodination is a measure of the ability of the PMN to convert inorganic iodide to a trichloroacetic acid (TCA) precipitable (protein-bound) form. This has been shown to occur in both neutrophils and eosinophils during phagocytosis under aerobic conditions (Klebanoff and Clark, 1977; Klebanoff et al., 1977). The iodide is covalently bound to a suitable acceptor molecule such as the tyrosine residues of protein in the phagocytic vacuole via the action of hydrogen peroxide (H_2O_2) and myeloperoxidase. This system has been found to exhibit a marked toxic activity toward bacteria, fungi, and viruses (Belding and Klebanoff, 1970; Simmons and Karnovsky, 1973). The iodination reaction is dependent upon a number of processes (Klebanoff and Clark, 1977). Hydrogen peroxide is generated

by the action of a membrane bound oxidase enzyme to convert O_2 to superoxide anion which may spontaneously be reduced to form H_2O_2 . The myeloperoxidase needed is present in the primary granules in the PMN cytoplasm. This enzyme must be delivered to the phagocytic vacuole by the process of degranulation. Ingestion of opsonized particles will stimulate H_2O_2 formation and degranulation in normal PMNs.

The iodination reaction has been shown to be useful for the evaluation of human PMN function and for the measurement of opsonic activity of human serum (Klebanoff and Clark, 1977). In this paper the use of the iodination reaction for the evaluation of bovine PMN function is reported.

Materials and Methods

Leukocyte preparation - PMNs were isolated using a modification of the method of Carlson and Kaneko (1973). Peripheral blood was obtained from apparently healthy 18-24 month old Holstein-Fresian steers and bulls by jugular venapuncture. ACD was used as the anticoagulant at a 50% greater concentration than the standard formula A. This was used at a ratio of anticoagulant to blood of 1:10.

The blood was placed in 250 ml silicon-coated glass centrifuge bottles and was centrifuged at 1,000 x g for 20 minutes. The plasma, buffy coat, and top few mm of packed red blood cells were aspirated and discarded. The packed red cells containing PMNs were then measured into an Erlenmeyer flask. The centrifuge bottle was rinsed with phosphate buffered (pH 7.2) saline solution (PBS) to remove PMNs which tend to pellet on the bottom. This PBS rinse was then added to the Erlenmeyer flask. The erythrocytes in the suspension were lysed by adding two volumes of cold phosphate buffered (0.0132 M, pH 7.2) deionized water with gentle mixing. Isotonicity was restored after 45 seconds by the addition of one volume of phosphate buffered (0.0132 M, pH 7.2) 2.7% NaCl solution. This preparation was poured into clean centrifuge bottles and centrifuged at 250 x g for 10 minutes. The supernatant fluid was poured off, and the pellet of cells was resuspended in 20 ml of PBS and transferred to 50 ml siliconized screw cap tubes and again centrifuged at 200 x g for 10 minutes. The supernatant fluid was poured off, and the cells were resuspended by vortexing in Hanks balanced salt solution without Ca^{++} and Mg^{++} (HBSS)¹ for counting. A 1:100 dilution was made and the cells

¹Grand Island Biological Co., Grand Island, N.Y.

were counted in a standard hemacytometer. A smear of the cells was made and stained with a modified Wright stain. A differential count was performed on 100 cells. The desired number of PMNs (neutrophils plus eosinophils) for use in the procedure were then transferred to a separate tube and pelleted by centrifugation. Immediately before using the PMNs, the HBSS was poured off and Earles balanced salt solution with Ca^{++} and Mg^{++} and without phenol red (EBSS)¹ was added to the PMNs to yield a concentration of 5.0×10^7 PMNs/ml. Holding the PMNs in a Ca^{++} and Mg^{++} free solution minimized clumping, but Ca^{++} and Mg^{++} are required in the medium for optimal phagocytosis during the assay.

Zymosan preparation - Zymosan² was suspended in cold EBSS at a concentration of 10 mg/ml by homogenization for one minute with a Potter-Elvehjem tissue grinder. This suspension was stored at 4 C for later use. Preopsonized zymosan was prepared by adding 100 ml of fresh bovine serum to an equal volume of the zymosan suspension and stirring for one hour at room temperature. The zymosan was sedimented by centrifugation at 250 x g for 10 minutes. Strong conglutination of the zymosan was reversed by washing

¹Grand Island Biological Co., Grand Island, N.Y.

²Sigma Chemical Co., St. Louis, Mo.

the zymosan pellet twice with 200 ml of 0.01 M sodium ethylenediamine-tetraacetic acid (EDTA) with stirring for 15 minutes between washes. The final pellet was resuspended in 100 ml of EBSS and frozen in aliquots at -20 C for later use. Prior to use the preopsonized zymosan was thawed and homogenized with a Potter-Elvehjem tissue grinder. When other concentrations of preopsonized zymosan were needed, this preparation was concentrated by centrifugation or diluted with additional EBSS to arrive at the desired concentration. Three batches of preopsonized zymosan were used for this experimentation with no detectable difference observed between them.

Serum preparations - Several apparently healthy adult cows were bled. The blood was allowed to stand at room temperature for 4 hours and the serum was removed and pooled. A portion of the pooled serum was immediately frozen in aliquots at -70 C; the remainder was heated at 56 C for 30 minutes, divided into aliquots and frozen at -20 C. Blood collected from experimental animals was allowed to stand at room temperature for 4 hours; the serum was removed and stored at 4 C until use within 1-2 hours.

Determination of Iodination - The iodination procedure was performed by a modification of the procedure of Klebanoff and Clark (1977). The standard reaction mixture

contained 40 nmole NaI, 0.05 μCi ^{125}I ,¹ 2.5×10^6 PMNs, and 0.05 ml preopsonized zymosan preparation (10 mg/ml) in a total volume of 0.5 ml EBSS. Zymosan was deleted for the determination of resting iodination. In certain experiments the amount of preopsonized zymosan was varied; in other experiments the preopsonized zymosan in the standard reaction mixture was replaced with 0.05 ml of unopsonized zymosan (10 mg/ml) with or without 0.05 ml of serum. All the components except the PMNs were placed in 12 x 75 mm polystyrene snap cap test tubes² and allowed to equilibrate in a 37 C incubator. The reaction was started by the addition of PMNs, and the mixture was incubated for 20 minutes at 37 C with end over end tumbling approximately 20 times per minute. The reaction was terminated by the addition of 2.0 ml of cold 10% TCA. The precipitate was collected by centrifugation at 1,000 x g for 5 minutes at 4 C and washed twice with 2.0 ml of cold 10% TCA. The counts per minute (CPM) of radioactivity remaining in the precipitate was determined in a Gamma Counter.³

¹Carrier free in 0.1 M NaOH, New England Nuclear, Boston, Ma.

²#2058 Falcon, Oxnard, Ca.

³Autowell 11, Picker Nuclear, North Haven, Ct.

A blank containing all components except serum and leukocytes was run with each experiment and the results subtracted from the experimental values. A standard containing the total amount of ^{125}I in the reaction mixture was counted, and the nanomoles of iodide converted to a TCA-precipitable form per 10^7 PMNs per hour were calculated as follows:

$$\frac{\text{CPM experimental} - \text{CPM blank}}{\text{CPM standard}} \times 40 \times 4 \times 3$$

The total amount of iodide in the reaction mixture was 40 nmole, and the results were standardized to 10^7 PMNs and 60 minutes by multiplying by 4 and 3 respectively. All reaction tubes were run in duplicate and the average value used. Less than 0.5% of the total added radioactivity was TCA-precipitable in the blank. It was found to be essential to allow the TCA solution to have no contact with metal automatic pipettors. The metal ions from the pipettor resulted in a 20 fold increase in the amount of radioactivity precipitated in the blank.

Results

Leukocyte preparation - The PMN isolation procedure performed on 50 separate blood samples from apparently healthy animals yielded the results shown in Table 2. An average of 1.4×10^6 PMNs were recovered per ml of blood;

Table 2. Yield and purity of PMNs isolated from 50 samples of blood from healthy adult cattle (Mean \pm S.D.).

Percent PMNs in the final preparation	93.6 \pm 6.9
PMN yield per ml of whole blood ($\times 10^6$)	1.4 \pm 0.7
Percent recovery of PMNs	45.8 \pm 15.5
Percent eosinophils in the PMN preparation	16.9 \pm 9.0

this represented an average recovery of 45.8% of the PMNs present in whole blood. The final PMN preparations contained an average of 76.7% neutrophils, 16.9% eosinophils, and 6.4% mononuclear cells.

Effect of different quantities of opsonized zymosan -

The effect of varying the quantity of opsonized zymosan available for phagocytosis is illustrated in Figure 1. Iodination increased with increasing quantities of preopsonized zymosan. Nearly maximal levels of iodination were obtained with a preopsonized zymosan preparation of 5 mg/ml; additional preopsonized zymosan beyond 5 mg/ml did not markedly increase iodination.

Effect of method of opsonization of zymosan - The effects of different methods of opsonization of zymosan on iodination by normal PMNs is illustrated in Table 3.

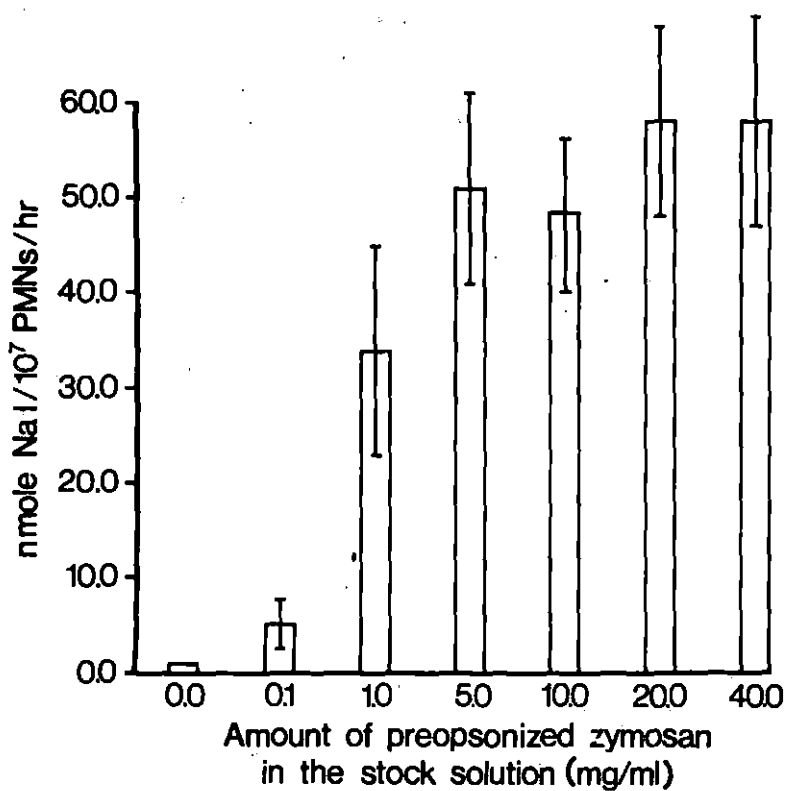


Figure 1. Influence of varying the quantities of preopsonized zymosan available for phagocytosis on iodination by bovine PMNs. Fifty microliters of the respective stock solution was added to each reaction tube.

Table 3. Influence of method for opsonization of zymosan on iodination by bovine PMNs. "Individual serum" refers to 50 different serum samples. "Serum from pool" refers to aliquots from one pool of bovine serum.

	nmole NaI/10 ⁷ PMNs/hr ^a
PMNs (resting)	2.2 ± 0.3 (50)
PMNs + zymosan	3.6 ± 0.6 (50)
PMNs + serum	2.4 ± 0.2 (50)
PMNs + individual serum + zymosan	13.6 ± 1.0 (50)
PMNs + serum from pool + zymosan	15.9 ± 1.3 (50)
PMNs + heated serum from pool + zymosan	12.3 ± 1.7 (29)
PMNs + preopsonized zymosan	48.3 ± 2.5 (56)

^aMean ± SEM of (n) experiments

There was very little iodination by resting PMNs or by PMNs in the presence of either zymosan or serum alone. Maximal iodination was attained in the absence of serum when preopsonized zymosan was used as the ingested particle. Heat inactivation of the serum did not abolish iodination.

Range of normal values - The points in Figure 2 illustrate the range of the data obtained. The wide range of values from normal individuals is similar to the

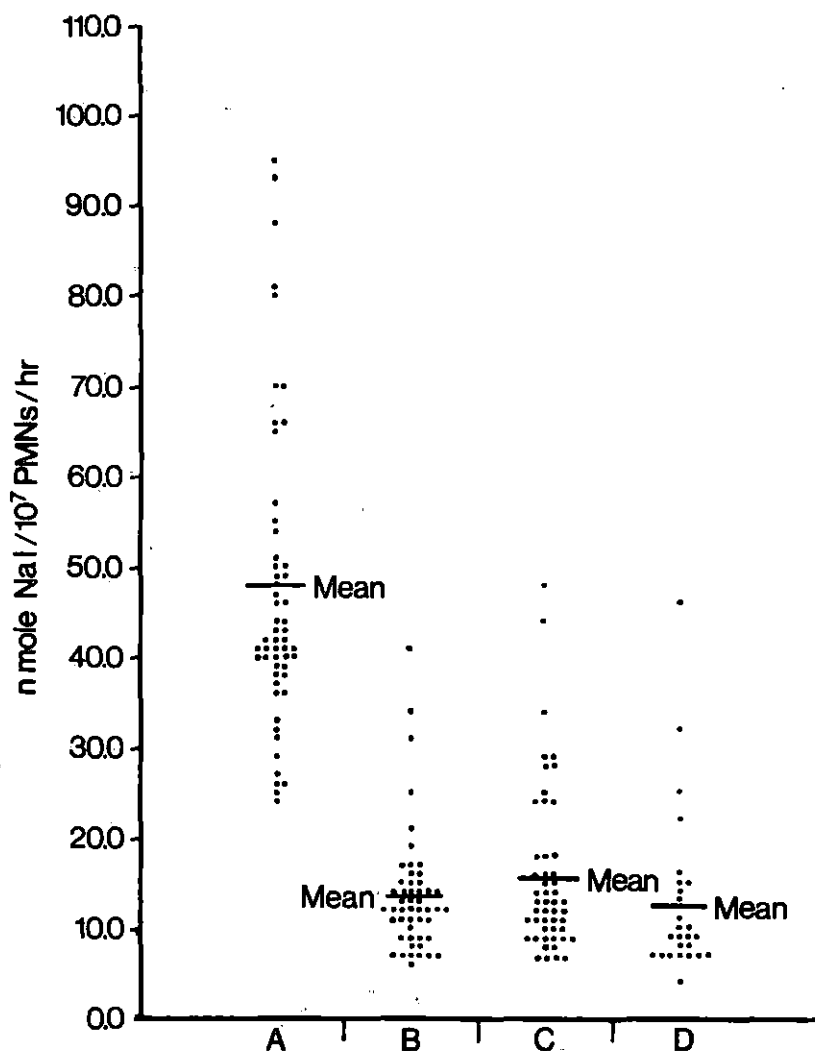


Figure 2. Range of iodination values obtained using different methods for opsonization of zymosan. Each point represents a single determination. Mean values are indicated by a bar. (A) Values obtained when preopsonized zymosan was used to stimulate iodination. (B) Values obtained when 50 individual serum samples and zymosan were used to stimulate iodination. (C) Values obtained when 50 aliquots from a single pool of serum and zymosan were used to stimulate iodination. (D) Values obtained when 29 aliquots from a single pool of heat-inactivated serum were used to stimulate iodination.

data reported in man (Klebanoff and Clark, 1977). The range of values obtained when a single pool of serum was used was similar to the range of values from fifty individual serum samples.

Discussion

The PMN isolation procedure was rapid and reproducible and yielded a cell preparation with good biological activity and no observable clumping. In preliminary experimentation clumping was observed when PMNs were held in suspension in the presence of Ca^{++} and Mg^{++} . This procedure worked equally well in animals that were neutropenic; however, the total yield of PMNs was, of course, reduced. Carlson and Kaneko (1973) reported a PMN recovery of $90.0\% \pm 28.9\%$ yielding a cell preparation of $87.8\% \pm 7.0\%$ PMNs. This is a higher percent cell recovery but a lower purity of the PMN preparation than in this report. Our reduced recovery was probably due to the more liberal removal of the upper portion of the packed erythrocyte layer before lysis. This area contains a mixture of red blood cells, PMNs, and mononuclear cells. By liberally removing the upper portion of the packed red blood cells, the purity of the PMN preparation can be increased, but many PMNs will be lost. The percent of eosinophils in the PMN preparations varied considerably. There was no apparent

correlation between the percent of eosinophils in the PMN preparation and the iodination value. Eosinophils have been demonstrated to be capable of iodination in man (Simmons and Karnovsky, 1973).

The iodinating capacity of bovine PMNs appears to be very similar to that reported for human PMNs. Klebanoff and Clark (1977) reported an iodination value for human PMNs in the presence of preopsonized zymosan of 52.9 ± 3.0 (Mean \pm SEM, $n = 4$) nmole NaI/ 10^7 PMNs/hr. Bovine PMNs in the presence of preopsonized zymosan with the same ratio of zymosan to PMNs to total volume of reaction mixture yielded an iodination value of 48.3 ± 2.5 (Mean \pm SEM, $n = 56$) nmole NaI/ 10^7 PMNs/hr. When human PMNs were incubated with zymosan in the presence of human serum, an iodination value was obtained higher than that for preopsonized zymosan (64.1 ± 2.5 nmole NaI/ 10^7 PMNs/hr) (Mean \pm SEM, $n = 27$). In contrast, in the presence of bovine serum, the iodination value for bovine PMNs was reduced by over 60% from that obtained with preopsonized zymosan (15.9 ± 1.3 nmole NaI/ 10^7 PMNs/hr) (Mean \pm SEM, $n = 50$). This lack of optimal iodination in the presence of serum and zymosan may, at least partially, be due to the presence of conglutinin in bovine serum. Conglutinin reacts exclusively with a determinant on the third

component of complement (C3b) and is responsible for the powerful clumping of complement coated particles in bovine serum. The determinant is a mannose peptide and is found not only in the fixed C3b of apparently all mammalian species, but is also found in the cell walls of yeast, and therefore, on zymosan. The reaction of the polysaccharide determinant with conglutinin requires calcium ions but the binding of C3b to a particle is not calcium dependent. The preopsonized zymosan was washed in EDTA to chelate the calcium, and consequently, remove the conglutinin. Following the EDTA wash C3b should still be present on the zymosan surface for opsonization (Lachmann, 1975).

Klebanoff and Clark (1977) reported that heating human serum to 56 C for 30 minutes reduced the mean iodination value from 64.1 to 1.8 nmole NaI/10⁷ PMNs/hr, which is equivalent to the level of iodination by resting PMNs. This demonstrates that only heat labile factors in human serum are able to opsonize zymosan. In the bovine system heating of the serum to 56 C for 30 minutes, reduced the mean iodination value from 15.9 to 12.3 nmole NaI/10⁷ PMNs/hr. This indicates that in addition to a heat labile opsonin there is also a heat stable opsonin for zymosan present in bovine serum.

Klebanoff and Clark (1977) concluded that when normal cells and patient's serum are employed, the iodination

reaction is an indirect measure of the opsonic activity of the patient's serum. They were able to demonstrate decreased opsonic activity for zymosan of human serum deficient in the fourth or third component of complement. Due to the presence of conglutinin and a heat stable opsonin for zymosan in bovine serum, the iodination reaction as described here cannot be used to detect opsonic defects in whole bovine serum.

The results in Figure 1 indicate that a zymosan preparation of 5 mg/ml will yield nearly optimal values for iodination. Additional zymosan beyond 5 mg/ml does not markedly increase iodination. Experimental evidence reported elsewhere utilizing nitroblue tetrazolium (NBT) reduction and chemiluminescence indicates that this level of zymosan does not give optimal stimulation of the oxidative metabolism of the bovine PMN (Roth and Kaeberle, 1979b, c). A concentration of 40 mg of zymosan per ml yielded over 150% more NBT reduction and chemiluminescence but only approximately 10% more iodination than 5 mg of zymosan per ml. Chemiluminescence and NBT reduction are measures of the oxidative metabolism of the PMN; therefore, as the opsonized zymosan concentration is increased to 40 mg/ml, there is increased oxidative metabolism without a corresponding increase in iodination.

Iodination has been demonstrated to occur both in the phagocytic vacuole, and extracellularly due to release of myeloperoxidase and H_2O_2 into the extracellular environment (Klebanoff and Clark, 1977). The extent of the extracellular iodination may depend upon the conditions under which phagocytosis is conducted. In an experimental system similar to the one described here, normal iodination was shown to be dependent upon ingestion (Klebanoff and Clark, 1977).

A depressed iodination value may be due to lack of ingestion, a lack of normal oxidative metabolism within the PMN, a failure of degranulation, a reduced amount of myeloperoxidase in the primary granule, destruction of H_2O_2 in the phagosome, or interference with the myeloperoxidase catalyzed reaction. Other experimental procedures are required to differentiate these defects. Because the iodination reaction is dependent upon a complex chain of events, it is a good screening test to detect PMN dysfunction.

A wide range of values were obtained from normal animals. This is typical of PMN function tests in general. Thus, in order to demonstrate a defect in PMN function, repeated testing is necessary and normal control PMNs must be evaluated concurrently. The iodination reaction has been used in man to demonstrate a defect in the PMNs from

patients with chronic granulomatous disease where there is an enzymatic defect and no H_2O_2 generation, and in PMNs from patients with myeloperoxidase deficiency (Pincus and Klebanoff, 1971; Klebanoff and Clark, 1977). Both of these conditions are characterized by severe recurrent bacterial infections. The iodination reaction has also been used to demonstrate a defect in PMNs from dogs with cyclic neutropenia (Chusid et al., 1975). In work reported elsewhere, the authors have used the iodination reaction to demonstrate a defect in bovine PMNs following bovine virus diarrhoea infection in cattle (Roth et al., 1978).

NITROBLUE TETRAZOLIUM REDUCTION

Summary

A procedure for the quantitative determination of nitroblue tetrazolium reduction by bovine polymorphonuclear leukocytes is described. The interpretation and significance of this test as a measure of polymorphonuclear leukocyte activity is discussed, and values obtained from normal animals are reported.

Introduction

The ability of polymorphonuclear leukocytes (PMNs) to reduce the yellow dye, nitroblue tetrazolium (NBT), to an insoluble purple formazan has been used by a number of investigators to evaluate the function of PMNs (Baehner and Nathan, 1968; Park et al., 1968; Medway, 1973; Stossel, 1973; Banas, 1974; Renshaw et al., 1974; Stossel and Taylor, 1976; Jarstrand, 1977; Persellin and Leibfarth, 1978). This reduction is dependent upon the ability of an oxidase enzyme to catalyze the formation of superoxide anion from molecular oxygen. The superoxide anion is capable of directly reducing NBT (Yost and Fridovich, 1974). The reduction of NBT, with the formation of intracellular deposits of formazan, is known to be increased during phagocytosis.

The NBT test has been used since 1968 in two basic procedures to evaluate PMN function. The original procedure of Baehner and Nathan (1968) involves the spectrophotometric quantitation of the amount of formazan formed by the cells. The histochemical procedure of Park et al. (1968) involves the microscopic observation of the proportion of cells containing intracellular formazan. The histochemical procedure has been used to demonstrate increased NBT reduction by unstimulated neutrophils during acute bacterial infection. It has been proposed as a diagnostic test to differentiate bacterial infection from other disease processes. However, a number of false negative and false positive results have been reported in both man and domestic animals (Medway, 1973; Banas, 1974; Fuenfer et al., 1976).

The spectrophotometric procedure quantitates NBT reduction more accurately than the histochemical method. It has been used successfully to demonstrate altered PMN function in chronic granulomatous disease of children (Baehner and Nathan, 1968), in adult carriers of chronic granulomatous disease (Baehner and Nathan, 1968), in patients with Influenza A infection (Jarstrand, 1977), and in PMNs incubated in serum from pregnant women (Persellin and Leibfarth, 1978).

This paper reports a modification of the spectrophotometric procedure of Baehner and Nathan (1968) for use in the evaluation of bovine PMN function.

Materials and Methods

Leukocyte preparation - The PMNs were isolated from the peripheral blood of apparently healthy 18-24 month old Holstein-Fresian steers and bulls as previously reported (Roth and Kaeberle, 1979a).

NBT solution - The NBT solution was prepared by suspending NBT¹ (at a concentration of 2 mg/ml) in Earles balanced salt solution with Ca⁺⁺, Mg⁺⁺, and phenol red (EBSS). The mixture was stirred for one hour at ambient temperature. Insoluble NBT was removed and the solution was sterilized by filtration through a 0.45 µm filter. The NBT solution was stored in 5 ml aliquots at 4 C since freezing may result in precipitation of the NBT. Sterility was maintained to prevent bacterial contamination and prolong storage life since the NBT solution will support bacterial growth resulting in increased NBT reduction by "resting" PMNs.

Zymosan preparation - The zymosan suspension, serum preparations and preopsonized zymosan were prepared as previously described (Roth and Kaeberle, 1979a).

¹Grade III, Sigma Chemical Co., St. Louis, Mo.

Determination of NBT reduction - Tests were conducted in triplicate in 15 x 100 mm silicon-coated glass test tubes. The standard reaction mixture contained 0.2 ml of NBT solution, 5.0×10^6 PMNs, 0.1 ml preopsonized zymosan preparation (10 mg/ml), and sufficient EBSS to bring the total volume to 1.0 ml. Zymosan was deleted for the determination of resting NBT reduction. In certain experiments the amount of preopsonized zymosan was varied. In other experiments the preopsonized zymosan was replaced with 0.1 ml of unopsonized zymosan (10 mg/ml) with or without 0.1 ml of serum. All of the reactants, except the PMNs, were added to the tubes and allowed to equilibrate in a water bath at 37 C for 15 minutes. The tubes were left in the water bath, and the reaction was initiated by adding the PMN suspension. Cells and particles were kept in suspension by periodic shaking. After exactly 5.0 minutes, the reaction was stopped by adding 5.0 ml of cold 1mM N-ethylmaleimide¹ in saline. The cells and precipitated formazan were pelleted by centrifugation at 500 x g for 10 minutes. The supernatant fluid was discarded and the pellet was suspended in 5.0 ml of pyridine.² The formazan was dispersed by brief sonication of the pyridine. The

¹Sigma Chemical Co., St. Louis, Mo.

²Certified ACS, Fisher Scientific Co., Pittsburgh, Pa.

tubes were then placed in a boiling water bath for 10 minutes in a ventilated hood to extract the formazan. (The use of automatic pipettors with exposed metal parts to dispense any of the reagents may result in decolorization of the formazan upon boiling.) The pyridine-formazan was clarified by centrifugation at 500 x g for 10 minutes and the optical density (OD) at 580 nm was immediately determined in a spectrophotometer¹, using a pyridine blank. (The formazan in pyridine is not stable and will lose color if allowed to stand overnight before determining the OD.) The results are reported as OD/5.0 x 10⁶ PMNs/5 min in 5.0 ml of pyridine.

Quantitative reduction and extraction of formazan -

Nitroblue tetrazolium was quantitatively reduced to formazan by adding 0.5 ml of freshly prepared 1mM ascorbic acid² in 0.2N sodium hydroxide to 0.1 ml of the filtered NBT solution. Five ml of saline solution was added and the insoluble formazan was precipitated by centrifugation at 500 x g for 10 minutes. The supernatant fluid was discarded and the pellet was resuspended in 5.0 ml of either pyridine or 1,4-dioxane.³ The formazan was dispersed by brief

¹Beckman DBG, Beckman Instruments, Inc., Irvine, Ca.

²Sigma Chemical Co., St. Louis, Mo.

³Certified ACS, Fisher Scientific Co., Pittsburgh, Pa.

sonic treatment and extracted by heating in either a boiling water bath or in an 85 C water bath for the indicated length of time. The solution was clarified by centrifugation at 500 x g for 10 minutes and the optical density at 580 nm was immediately determined using the appropriate pyridine or dioxane blank.

Results

The results of the quantitative reduction and extraction of NBT are shown in Figure 3. Pyridine was found to be superior to dioxane for the extraction of formazan. Prolonged boiling in either pyridine or dioxane resulted in a decrease in optical density. Heating to 85 C for 15 minutes or 30 minutes resulted in incomplete extraction of the formazan.

The effects of different methods of opsonization of zymosan upon NBT reduction by normal PMNs are shown in Table 4. There was very little NBT reduction by "resting" PMNs or by PMNs in the presence of serum or zymosan alone. Optimal NBT reduction occurred when preopsonized zymosan was used as the particle for ingestion. PMNs stimulated by zymosan in the presence of fresh serum reduced only approximately 50% as much NBT as PMNs stimulated by preopsonized zymosan. Heat inactivation did not markedly diminish the opsonic activity of the serum.

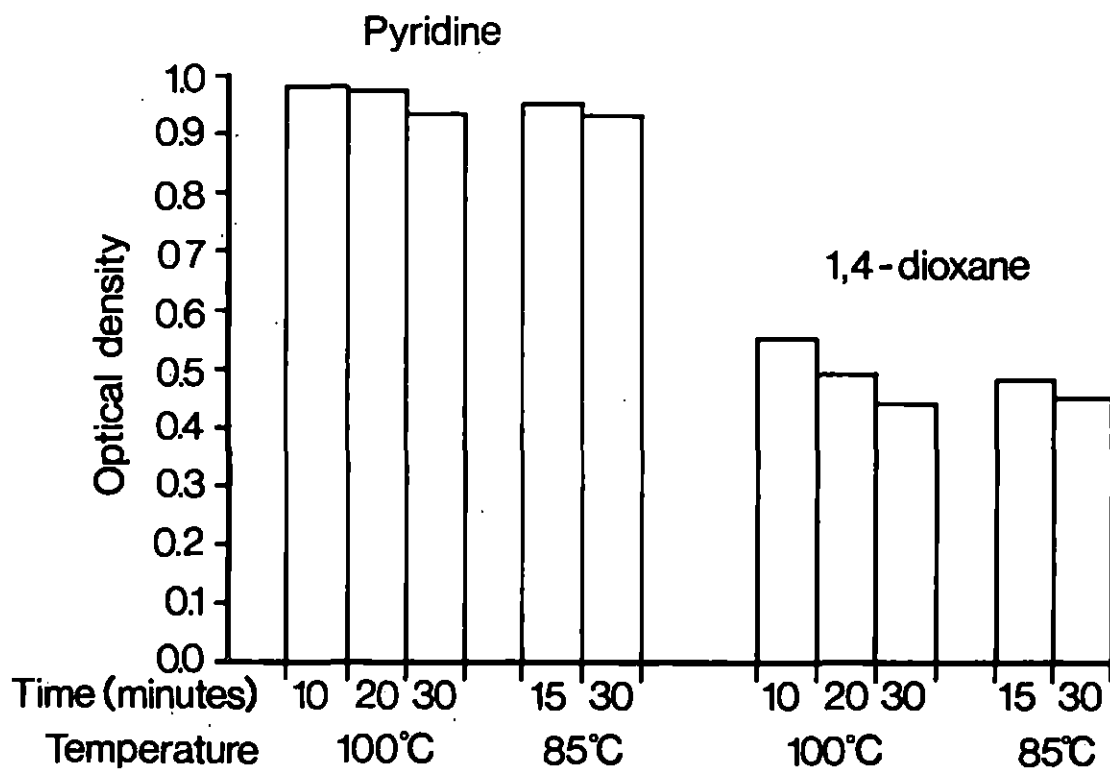


Figure 3. Comparison of different methods for the extraction of formazan. The effect of heating pyridine or dioxane at different temperatures and times for the extraction of a standard amount of formazan are shown.

Table 4. Influence of the method for opsonization of zymosan on NBT reduction by bovine PMNs. The amounts of formazan produced by 5.0×10^6 PMNs in 5 minutes are expressed as optical densities in 5.0 ml of pyridine (Mean \pm SEM of 4 experiments).

	O.D.
PMNs (resting)	0.04 \pm 0.003
PMNs + zymosan	0.04 \pm 0.014
PMNs + serum	0.02 \pm 0.003
PMNs + serum + zymosan	0.17 \pm 0.02
PMNs + heated serum + zymosan	0.14 \pm 0.02
PMNs + preopsonized zymosan	0.34 \pm 0.02

Stimulation of PMNs with graded amounts of preopsonized zymosan resulted in a positive correlation between the amount of preopsonized zymosan added and NBT reduction. The NBT reducing capacity of the PMNs was apparently not exceeded, even at high concentrations of preopsonized zymosan (Figure 4).

The points in Figure 5 illustrate the mean and range of NBT reduction obtained from 56 determinations on PMNs from normal animals, using preopsonized zymosan as the stimulating particle. A mean OD of 0.49 with a standard

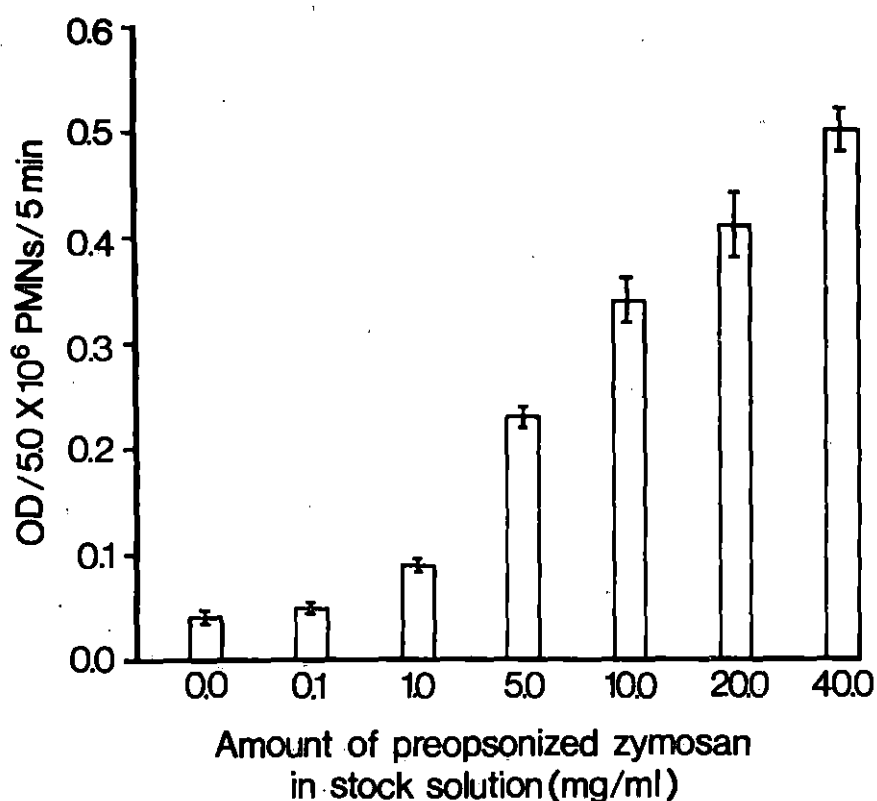


Figure 4. Effect of varying the quantity of preopsonized zymosan available for phagocytosis. Fifty μ l of the respective stock solution was added to each reaction tube. The amounts of formazan produced by 5.0×10^6 PMNs in 5 minutes are expressed as optical densities in 5 ml of pyridine. (Mean \pm SEM of 4 experiments, each experiment conducted in triplicate.)

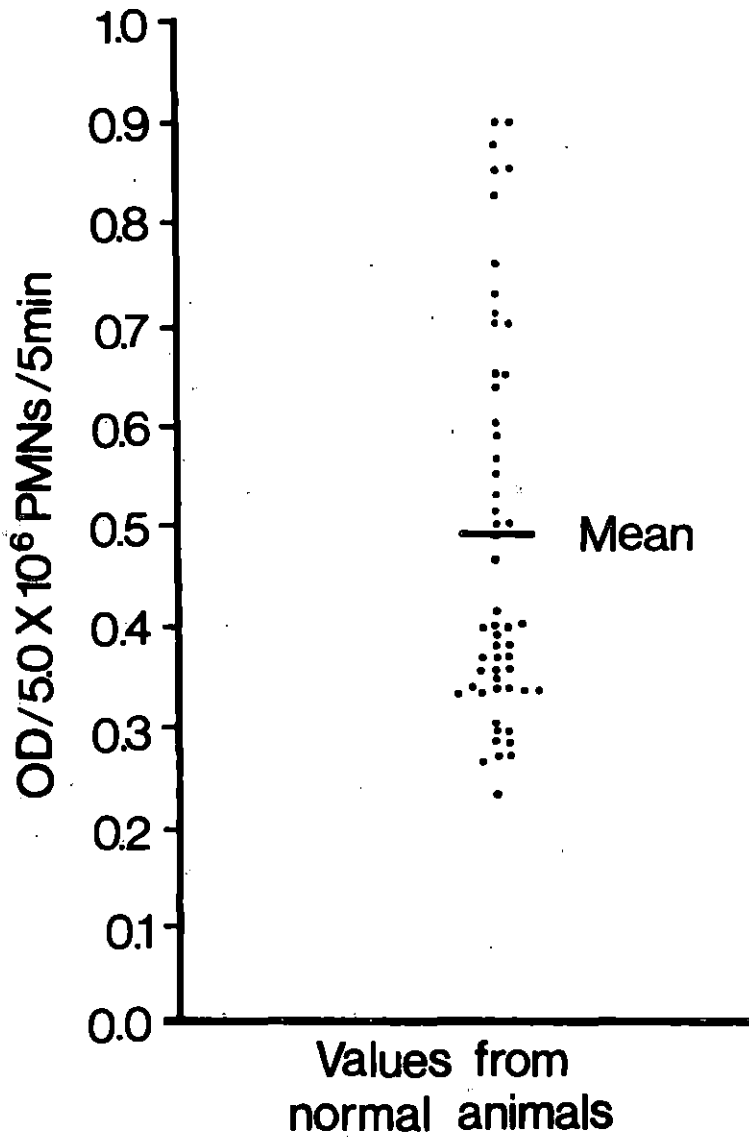


Figure 5. Mean and range of values obtained from normal animals. Each point represents the mean of triplicate values.

deviation of 0.20 and a standard error of the mean of 0.03 was obtained. Each point in Figure 5 represents the mean of triplicate values. Agreement among triplicate values was good; the majority of the time they differed by less than 5% from their mean.

Discussion

The use of both pyridine and dioxane have been reported for the extraction of formazan. It has been recommended that the insoluble formazan be extracted twice by boiling in pyridine (Baehner and Nathan, 1968). This would appear to be unnecessary since if the formazan is first dispersed by brief sonic treatment, a single extraction by boiling is sufficient. Other workers have recommended that the formazan be extracted with dioxane by either heating to 85 C for 15 minutes or boiling for 15 to 30 minutes. Neither of these procedures was found to be satisfactory due to the incomplete extraction of the formazan and loss of color with excessive boiling. Dioxane is prone to the formation of peroxides. This can result in the oxidation of formazan and the complete loss of color upon boiling. Occasionally, this tendency was observed with pyridine also, although it was not as marked. This can be monitored by quantitatively reducing a standard amount of NBT with ascorbic acid, then boiling this

formazan in the reagent. The optical density obtained should be similar from day to day.

Nitroblue tetrazolium is directly reduced by superoxide anion to an insoluble purple formazan (Yost and Fridovich, 1974). Goldstein et al. (1977) demonstrated that the superoxide anion generating system appears to be associated with the outer surface of the PMN plasma membrane and that superoxide generation proceeds independently of phagocytosis and degranulation. They found that superoxide anion recovery in the medium surrounding PMNs was in fact enhanced when the formation of phagocytic vacuoles was inhibited by treatment of cells with cytochalasin B. This superoxide anion in the medium will reduce extracellular NBT to formazan. The insoluble formazan formed in the extracellular medium will be pelleted with the cells by centrifugation and will contribute to the observed OD. The quantitative NBT reduction test cannot, therefore, be interpreted to be a valid measure of ingestion by PMNs. In normal PMNs the stimulation of the cell membrane by suitable particulate material results in the ingestion of the particle. Ingestion involves the invagination of the cell membrane to form a phagosome containing the particle. The outer

surface of the cell membrane becomes the inner surface of the phagosomal membrane; therefore, the superoxide anion generating system will also be associated with the inner surface of the phagosomal membrane. This allows for the formation of superoxide anion within the phagosome. Superoxide anion is believed to be involved in the formation of hydroxyl radicals, hydrogen peroxide, and singlet oxygen within the phagosome. All of these components are believed to play a role in the killing of microorganisms by PMNs under aerobic conditions. The oxidative metabolism of the PMN is an important aspect of its bactericidal activity. The quantitative NBT reduction test appears to be a valid measure of one aspect of the oxidative metabolism of the bovine PMN.

A wide range of values were obtained from normal animals, as depicted in Figure 5. A wide range of normal values have also been reported for human PMNs (Baehner and Nathan, 1968; Jarstrand, 1977; Persellin and Leibfarth, 1978). Thus, in order to demonstrate a defect in PMN function, repeated testing is necessary and normal control PMNs must be evaluated concurrently.

Renshaw et al. (1974) have reported the use of the quantitative NBT reduction test to evaluate neutrophil function in cattle with the Chediak-Higashi syndrome. They found no significant differences in NBT reduction

between animals with this syndrome and control animals. Their technique utilized 2.5×10^6 PMNs, latex spheres, and a 15 minute incubation period. They extracted the formazan in 40 ml of pyridine and read the OD at 515 nm. For 10 animals they reported a mean OD (\pm SEM) of 0.202 ± 0.110 . There are many differences in technique between that report and this one. The higher value reported here probably is due primarily to greater activity of PMNs toward preopsonized zymosan than toward latex particles.

As seen in Table 4, maximal NBT reduction occurred when preopsonized zymosan was used as the stimulating particle. Zymosan in the presence of fresh serum stimulated only approximately 50% as much NBT reduction as preopsonized zymosan. This may have been at least partially due to the presence of conglutinin in bovine serum as previously discussed (Roth and Kaeberle, 1979a). Zymosan in the presence of heat inactivated bovine serum stimulated nearly as much NBT reduction as zymosan in the presence of fresh bovine serum. This may be due to the presence of a heat stable opsonin for zymosan in bovine serum, as previously discussed (Roth and Kaeberle, 1979a).

CHEMILUMINESCENCE

Summary

Polymorphonuclear leukocytes are capable of emitting light during phagocytosis (chemiluminescence). A procedure for the detection of chemiluminescence by bovine polymorphonuclear leukocytes is described and values obtained from normal animals are reported. The interpretation and significance of a chemiluminescent response by polymorphonuclear leukocytes is discussed.

Introduction

The emission of light by polymorphonuclear leukocytes (PMNs) during phagocytosis was first described by Allen et al. in 1972. They originally proposed that electronically excited singlet oxygen is produced during phagocytosis; this is known to be capable of producing chemiluminescence. In displaying chemiluminescence, singlet oxygen may decay to ground state oxygen, releasing its energy as light, or it may react with other molecules, forming products that subsequently emit light (Andersen et al., 1977). Rosen and Klebanoff have provided experimental evidence that singlet oxygen is produced during phagocytosis. Singlet oxygen may be formed during phagocytosis by the spontaneous dismutation of superoxide anion, by the reaction of

superoxide anion with hydrogen peroxide or the hydroxyl radical, or by the reaction of myeloperoxidase with hydrogen peroxide and a halide (Rosen and Klebanoff, 1976). Which of these mechanisms occur and the extent of their contribution to chemiluminescence by the PMN is still unclear (Andersen and Brendzel, 1978); it may vary with the experimental conditions. Proof that the chemiluminescent event is directly bactericidal is lacking, but the light emitting reactions are related to bactericidal mechanisms (Allen, 1977). Thus, the measurement of chemiluminescence affords a sensitive, continuous, and nondestructive means of quantitating one parameter of microbicidal metabolic activity. The measurement of phagocytosis-associated chemiluminescence has been used successfully to detect opsonic activity of serum (Allen, 1977; Grebner et al., 1977; Stevens and Young, 1977), virulence factors in bacteria (Griffith et al., 1978), decreased metabolic activity of myeloperoxidase deficient neutrophils (Rosen and Klebanoff, 1976), abnormality of neutrophils from patients with chronic granulomatous disease (Stjernholm et al., 1973), and increased metabolic activity in activated peritoneal macrophages (Schleupner and Glasgow, 1978). This paper describes a procedure for the detection of chemiluminescence by bovine PMNs and reports normal values.

Materials and Methods

Leukocyte preparation - The PMNs were isolated from the peripheral blood of apparently healthy 18-24 month old Holstein-Fresian steers and bulls as previously reported (Roth and Kaeberle, 1979a).

Zymosan preparation - The zymosan suspension and preopsonized zymosan were prepared as previously described (Roth and Kaeberle, 1979a). A preopsonized zymosan stock solution of 10 mg/ml was used for all experimentation except where indicated.

Determination of chemiluminescence - Chemiluminescence was measured in a liquid scintillation spectrometer¹ at ambient temperature with the coincidence circuit switched off. The reaction was conducted in 20 ml glass scintillation vials.² Test vials contained 0.5 ml of preopsonized zymosan stock solution and 1.0×10^7 PMNs in a total volume of 10 ml of Geys balanced salt solution with Ca^{++} and Mg^{++} and without phenol red³ (GBSS). Zymosan was deleted for the determination of resting chemiluminescence.

¹Model DPM 100, Beckman Instruments, Inc., Irvine, Ca.

²Fisher Scientific Co., Pittsburgh, Pa.

³Grand Island Biological Co., Grand Island, N.Y.

In certain experiments the amount of preopsonized zymosan was varied, and in other experiments the preopsonized zymosan was replaced with 0.5 ml of unopsonized zymosan. The zymosan and GBSS were added to the scintillation vial; PMNs were added 1 minute before the vial was placed in the counting chamber. The vial was capped and mixed well before placing in the counting chamber; it was then left undisturbed for the remainder of the experiment. Each vial was counted for 1 minute at approximately 10 minute intervals. Counting was continued for at least 70 minutes. A vial containing 10 ml of GBSS with no PMNs or zymosan was counted each cycle to determine the background activity. The results were graphed as the number of counts per minute at each 10 minute interval after the addition of PMNs, as shown in Figure 6. Due to differences in the height and shape of this graph with different PMN preparations, it was decided to report the majority of results as total net counts per hour (cph). This was estimated by calculating the area of the bar graph shown in Figure 6 as follows: The first value obtained for a sample was multiplied by 6 minutes, the next 5 values were each multiplied by 10 minutes, the seventh value was multiplied by 4 minutes. These products were summed to give an estimate of the total counts for the

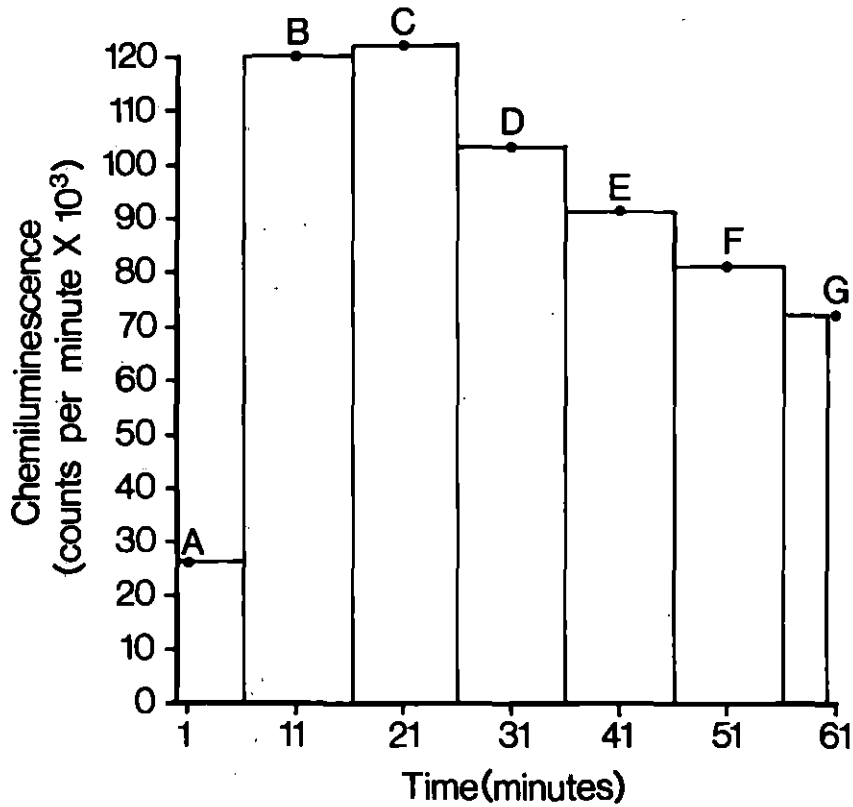


Figure 6. This figure illustrates the method used to estimate the counts per hour. The sum of the areas of the rectangles was determined by the following formula:

$$(6)(A) + (10)(B) + (10)(C) + (10)(D) + (10)(E) + (10)(F) + (4)(G).$$

This is an estimate of the area under the curve for the first 60 minutes, and, therefore, of the counts per hour.

first 60 minutes, or counts per hour. The net cph of a test vial was calculated as follows:

$$\text{net cph} = \text{cph of test vial} - \text{cph of background vial.}$$

All experiments were conducted in duplicate and the average net cph are reported.

Results

Figure 7 illustrates the effect of varying the quantity of preopsonized zymosan in the reaction mixture. Increasing the amount of preopsonized zymosan increased light emission. This did not reach an end point under the conditions employed. Resting PMNs, in the absence of serum or particles, did not demonstrate any significant chemiluminescence.

Figure 8 illustrates the difference in chemiluminescence by PMNs stimulated with either unopsonized zymosan or preopsonized zymosan. This experiment was conducted four times; a typical set of curves is depicted. The preopsonized zymosan elicited a much more rapid chemiluminescent response than the unopsonized zymosan; however, the unopsonized zymosan did stimulate chemiluminescence.

The range and mean of values obtained from normal animals are illustrated in Figure 9. Each point represents the mean of duplicate values. A wide range of values was obtained from normal animals. Thirty-eight

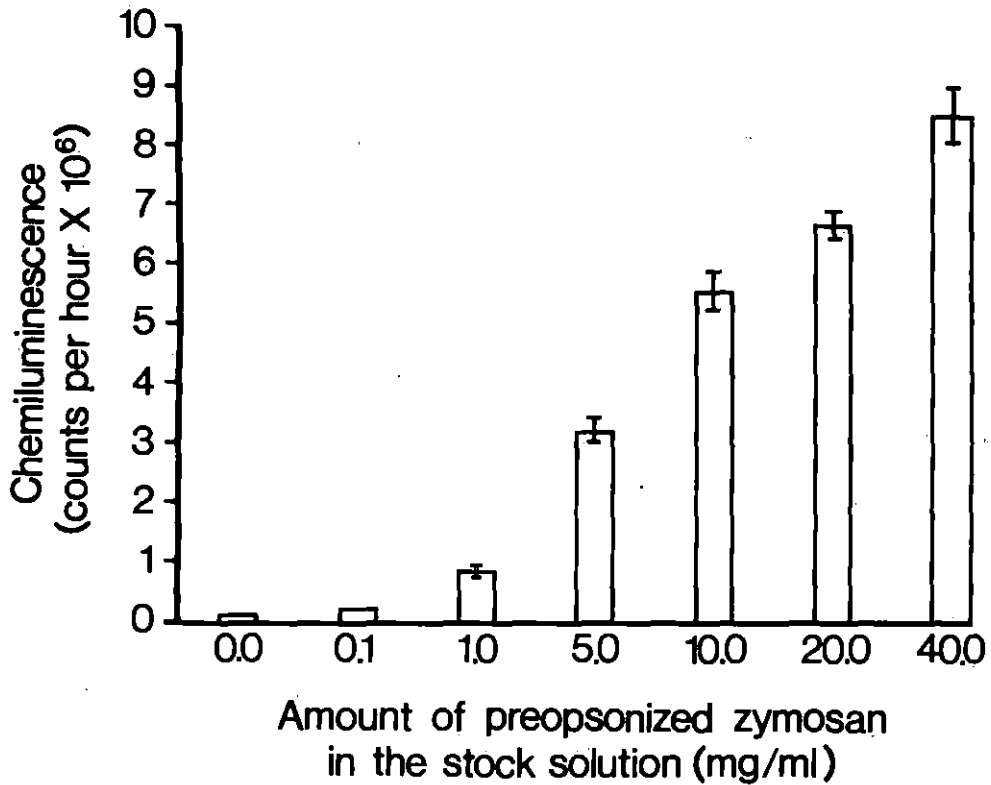


Figure 7. Effect of varying the quantity of preopsonized zymosan available for phagocytosis. One half ml of the respective stock solution was added to each reaction tube. The amount of chemiluminescence produced by 1.0×10^7 PMNs in 60 minutes is expressed as counts per hour. (Mean \pm SEM of 4 experiments.)

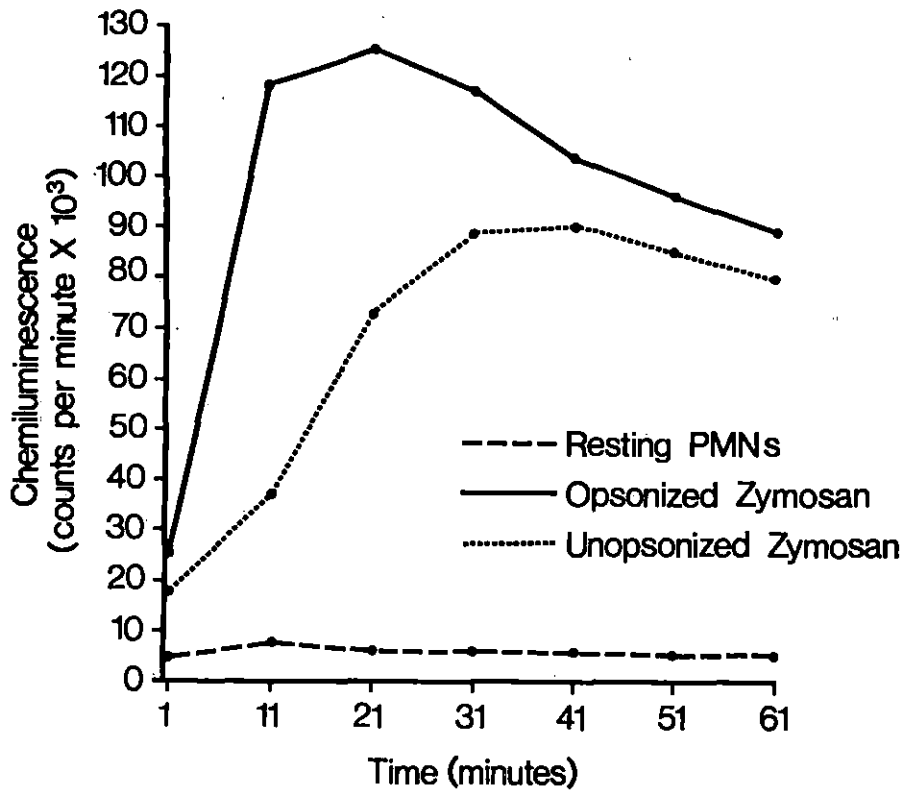


Figure 8. A comparison of the chemiluminescent response of bovine PMNs to opsonized zymosan and unopsonized zymosan is illustrated. Resting values are from PMNs that were tested in the absence of particles. The experiment was conducted 4 times; typical results are shown.

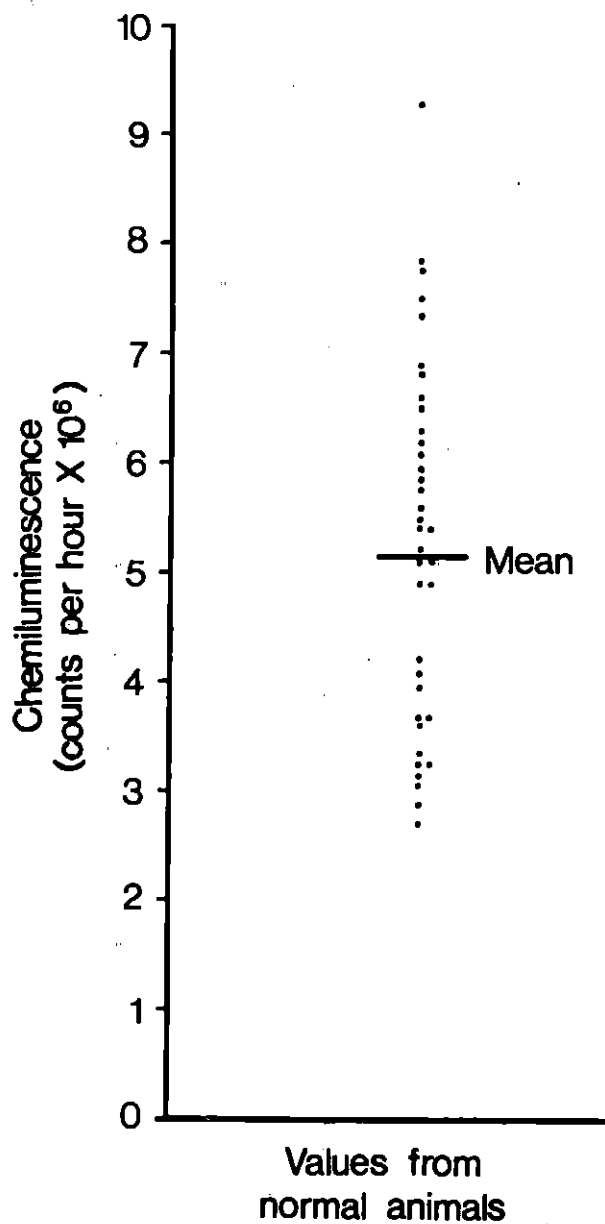


Figure 9. This figure illustrates the range and mean of chemiluminescence values obtained from 38 evaluations of PMNs from normal animals. Each point represents the mean of duplicate values. The amount of chemiluminescence produced by 1.0×10^7 PMNs in 60 minutes is expressed as counts per hour.

determinations on normal PMNs yielded a mean of 5.21×10^6 cph with a standard deviation of 1.57×10^6 cph, and a standard error of the mean of 2.8×10^5 cph.

Discussion

Unopsonized zymosan is reported to not stimulate chemiluminescence by human PMNs (Nelson et al., 1977). The data presented here indicates that unopsonized zymosan does stimulate chemiluminescence by bovine PMNs, although the kinetics of this stimulation are much different than for opsonized zymosan. The slower chemiluminescent response to unopsonized zymosan may reflect the necessity for the PMNs and zymosan to settle to the bottom of the vial, where the PMNs may be able to "trap" the unopsonized zymosan for ingestion. The authors have previously reported that unopsonized zymosan does not significantly stimulate iodination (Roth and Kaeberle, 1979a) or NBT reduction (Roth and Kaeberle, 1979b) by bovine PMNs. However, these tests were conducted over a shorter time period with agitation of the reaction mixture.

A wide range of values were obtained from normal animals. This is typical of PMN function tests in general. In order to demonstrate a defect in PMN function, repeated testing is necessary and normal control PMNs must be evaluated concurrently.

Many substances have been shown to influence the chemiluminescence response if they are present in the reaction medium (Nelson et al., 1977; Andersen and Brendzel, 1978). Phenol red, hemoglobin, or red blood cells will decrease the amount of chemiluminescence which is detected by the scintillation counter. This effect may be due to absorption of some of the light produced. These substances were also found to decrease chemiluminescence of bovine PMNs (data not shown). Nelson et al. (1977) demonstrated that soluble proteins have a marked effect on chemiluminescence. Supplementation of the medium with gelatin at a level of 3.0 gm/100 ml increased the peak counts per minute by 150%. Addition of greater amounts of gelatin resulted in a progressive decrease in peak chemiluminescence. They also demonstrated an increase in chemiluminescence by adding amino acids and tested 20 common amino acids individually. Of these, the only amino acids observed to influence significantly the amount of chemiluminescence measured were tyrosine, tryptophane, and cysteine. Addition of tyrosine increased the chemiluminescence response of PMNs by more than 75%. The maximal increase in chemiluminescence observed with tryptophane was approximately 35%. Unlike tyrosine and tryptophane, addition of cysteine was found to decrease the PMN

chemiluminescence detected by a maximum of approximately 60%.

Nelson et al. (1977) also demonstrated that addition of zymosan in excess of that required to achieve a maximal rate of phagocytosis significantly increased the amount of light measured. They did further studies to show that unphagocytosed extracellular zymosan will increase the magnitude of chemiluminescence. This is probably partially explained by the experimentation of Goldstein et al. (1977) who demonstrated that surface stimulation of PMNs by opsonized particles will result in the generation of superoxide anion by an enzyme complex on the cell surface, and the release of superoxide anion into the medium surrounding the cell. Superoxide anion is theorized to form singlet oxygen by a number of possible mechanisms (Webb et al., 1974; Rosen and Klebanoff, 1976). Electronically excited singlet oxygen may dissipate its energy in various ways, two of which are the direct emission of a photon of light or the transfer of its energy to another molecule which subsequently will emit light in a secondary light producing reaction. Singlet oxygen may also dissipate its energy in nonlight producing reactions. There is good evidence to support the suggestion that a major portion of the light measured in cellular

chemiluminescence reactions is the product of secondary light producing reactions in which soluble protein, or the phagocytosed particle, provides the necessary substrate (Nelson et al., 1977). This explains the increasing chemiluminescence measured as the level of opsonized zymosan was increased in the reaction mixture as shown in Figure 7. This is very similar to the results obtained using high levels of preopsonized zymosan when nitroblue tetrazolium (NBT) reduction by bovine PMNs was measured (Roth and Kaeberle, 1979b). The high values obtained for NBT reduction and chemiluminescence when high levels of preopsonized zymosan were used are probably both due to the extracellular generation of superoxide anion. Because of this extracellular generation of superoxide anion, chemiluminescence can proceed in the absence of phagocytosis (Goldstein et al., 1975b; Goldstein et al., 1977) and the chemiluminescence reaction cannot be used as a valid measure of ingestion. However, when normal PMNs and the proper particle to cell ratio is used, there is a good correlation between chemiluminescence and ingestion (Grebner et al., 1977).

Since phenol red, hemoglobin, soluble proteins, certain amino acids, and excess zymosan can all influence the chemiluminescence measured, it is very important to

standardize these components in the reaction mixture. When comparing different sera in the reaction mixture for their effect on opsonization, caution must be used in the interpretation of the results. All sera may not influence the chemiluminescent response to the same extent. However, if experimental conditions are well-controlled, the measurement of the chemiluminescent response affords a sensitive, continuous, and nondestructive means of quantitating a component of bovine PMN oxidative metabolism.

PART II. EXPERIMENTATION INVOLVING BOVINE
VIRAL DIARRHEA VIRUS

EFFECTS OF BOVINE VIRAL DIARRHEA VIRUS INFECTION
ON BOVINE POLYMORPHONUCLEAR
LEUKOCYTE FUNCTION¹

Summary

Four procedures were used to evaluate the function of polymorphonuclear leukocytes (PMNs) isolated from the blood of cattle experimentally infected with bovine viral diarrhea (BVD) virus: (1) uptake of an emulsion of paraffin oil and E. coli lipopolysaccharide, (2) nitroblue tetrazolium reduction, (3) chemiluminescence, and (4) iodination, or the conversion of iodide to a trichloroacetic acid-precipitable form. A marked impairment of iodination was consistently observed following BVD virus infection. A corresponding decrease in paraffin oil uptake, nitroblue tetrazolium reduction, and chemiluminescence was not observed. This defect was compounded by a decrease in the number of circulating PMNs following BVD virus infection. The impairment of PMN function may partially explain the increased susceptibility of cattle to secondary bacterial infection during infection with BVD virus.

¹Submitted for publication to the American Journal of Veterinary Research by J. A. Roth, M. L. Kaeberle, and R. W. Griffith.

Introduction

Bovine viral diarrhea virus is the causative agent of viral diarrhea-mucosal disease in cattle (Malmquist, 1968). The BVD virus is frequently incriminated in the so-called "bovine shipping fever complex" along with other viruses and bacterial organisms. Concurrent infection with two or more infectious agents commonly occurs in this disease syndrome. Pathogenic agents are presumably able to evade or impair the hosts immune defenses in order to facilitate their own survival within the host. This impairment of the resistance mechanisms of the host may result in an increased susceptibility to secondary infection with other infectious agents.

In earlier experimentation Reggiardo and Kaeberle (1979) demonstrated that 1-5 days following experimental infection with BVD virus a bacteremia could be detected (Reggiardo, 1975). This implied a defect in the bacterial clearance mechanisms of the host. Removal of bacteria from the blood stream is primarily the responsibility of the polymorphonuclear phagocytes (PMNs) and the mononuclear phagocytic system.

The purpose of this experimentation was to evaluate the function of bovine peripheral PMNs to determine if a defect in function could be demonstrated. Four procedures were utilized to evaluate different aspects of PMN function:

(1) Ingestion was evaluated by measuring the uptake of paraffin oil droplets which had been impregnated with oil red O and emulsified with bacterial lipopolysaccharide.

(2) Oxidative metabolism of the PMN was evaluated by quantitation of nitroblue tetrazolium (NBT) reduction by PMNs when stimulated by a phagocytosable particle.

(3) An additional aspect of the oxidative metabolism of the PMN was evaluated by the measurement of the amount of chemiluminescence emitted by PMNs when stimulated by a phagocytosable particle. (4) The fourth procedure, iodination, is dependent upon ingestion, oxidative metabolism, release of lysosomal enzymes into the phagocytic vacuole, and the presence of myeloperoxidase within the lysosomes. These parameters were evaluated in animals which were experimentally infected with BVD virus and in control animals.

Materials and Methods

Animals - Apparently healthy 18-24 month old Holstein-Fresian steers and bulls were used as control and challenge animals.

BVD virus - The NADL challenge strain (lot 70-2)¹ was used for intranasal challenge. Strain 1015-74 was used

¹Kindly supplied by Biologics Virology Section, NVSL, National Animal Disease Center, Ames, Ia.

for intravenous challenge. This strain was isolated at the Iowa Veterinary Diagnostic Laboratory from a typical case of bovine viral diarrhoea-mucosal disease. The isolate was plaque purified and used at the sixth bovine testicle passage as a challenge strain.

Experimental procedure - Four animals were inoculated intravenously with 10 ml of virus preparation containing 1.0×10^6 TCID₅₀ of the 1015-74 strain of BVD virus. Five animals were inoculated intranasally with 2.4 ml of virus preparation containing 1×10^5 TCID₅₀ of the NADL challenge strain of BVD virus. Six animals were used as controls. The research was conducted using experimental blocks consisting of one or two infected animals and one noninfected control. PMN function, body temperature, and white blood cell (WBC) counts of these animals were monitored 1-2 days prior to infection and at nearly daily intervals for the first 10 days following infection. For the succeeding 2 weeks the parameters were evaluated at irregular intervals.

Hematologic studies - Total and differential WBC counts were performed by standard procedures on heparinized blood obtained by jugular venapuncture.

PMN preparations - Blood was collected into ACD solution by jugular venapuncture. The PMNs were isolated

as previously described (Roth and Kaeberle, 1979a). The blood was centrifuged at 1,000 x g for 20 minutes; the plasma and buffy coat material containing predominantly mononuclear cells were removed. The red cells in the cell sediment, which contained the majority of the PMNs, were lysed by treating with two volumes of cold phosphate buffered (0.0132 M, pH 7.2) deionized water. Isotonicity was restored after 45 seconds by the addition of one volume of cold phosphate buffered (0.0132 M, pH 7.2) 2.7% NaCl solution. The PMNs were pelleted by centrifugation and washed twice with Hanks balanced salt solution without Ca^{++} and Mg^{++} (HBSS).¹ This yielded a leukocyte preparation of approximately 85%-100% PMNs (neutrophils plus eosinophils).

Preopsonized zymosan - Preopsonized zymosan was prepared as previously described (Roth and Kaeberle, 1979a). One gram of zymosan in 100 ml of Earles balanced salt solution with Ca^{++} and Mg^{++} and without phenol red (EBSS)¹ was added to 100 ml of fresh bovine serum and incubated at room temperature for one hour. The zymosan was then washed twice in 0.1 M EDTA solution. The pelleted zymosan was resuspended in EBSS to a concentration of 10 mg/ml and frozen in aliquots until use.

¹Grand Island Biological Co., Grand Island, N.Y.

Paraffin oil uptake - Paraffin oil uptake was performed by a modification of the method of Stossel and Taylor (1976). Heavy paraffin oil¹ was impregnated with oil red O dye², then emulsified with E. coli lipopolysaccharide (cold phenol extracted) by sonic treatment. The lipopolysaccharide coated paraffin oil droplets were then opsonized with complement via the alternative complement pathway by incubating 75.0 μ l of paraffin oil suspension with 0.1 ml of fresh bovine serum at 37 C for 15 minutes. Ingestion was initiated by adding 1.5×10^7 PMNs in 0.8 ml of EBSS to the paraffin oil suspension. After exactly 5.0 minutes, 5.0 ml of cold 1.0 mM N-ethylmaleimide² in saline (NEM) was added to terminate further ingestion. The cells were separated from the uningested paraffin oil by centrifugation, and washed with an additional 5.0 ml of NEM. The oil red O in the cell button was extracted with 1,4-dioxane¹, clarified by centrifugation, and the optical density (OD) at 525 nm was determined. The OD was converted to μ g of paraffin oil/ 10^7 PMNs/min by the method of Stossel and Taylor (1976). The procedure was performed in triplicate, and the average value is reported.

¹Fisher Scientific Co., Pittsburgh, Pa.

²Sigma Chemical Co., St. Louis, Mo.

Nitroblue tetrazolium reduction - Nitroblue tetrazolium reduction by PMNs was quantitatively determined as previously described (Roth and Kaeberle, 1979b). The procedure was performed in 15 x 100 mm silicon-coated glass test tubes. Each reaction tube contained 0.2 ml of NBT solution (2 mg/ml), 5.0×10^6 PMNs, 0.1 ml of preopsonized zymosan, and sufficient EBSS to bring the volume to 1.0 ml. All of the reactants except PMNs were added to the tubes and allowed to equilibrate in a water bath at 37 C for 15 minutes. The reaction was started by adding PMNs and was allowed to proceed with periodic shaking for exactly 5.0 minutes. The reaction was stopped by adding 5.0 ml of NEM. The cells and insoluble purple formazan formed by the reduction of NBT were pelleted by centrifugation and resuspended in 5.0 ml of pyridine.¹ The pellet was dispersed by brief sonic treatment, and the formazan was extracted by placing the test tubes in a boiling water bath for 10 minutes. The pyridine-formazan was clarified by centrifugation, and the OD of the supernatant fluid was immediately determined in a spectrophotometer,² using a pyridine blank. The results are

¹Fisher Scientific Co., Pittsburgh, Pa.

²Beckman DBG, Beckman Instruments, Inc., Irvine, Ca.

reported as OD/5.0 x 10⁶ PMNs/5 min in 5.0 ml of pyridine. The reported results represent the average values of triplicate determinations.

Chemiluminescence - Chemiluminescence was measured in a liquid scintillation spectrometer¹ at ambient temperature with the coincidence circuit switched off as previously reported (Roth and Kaeberle, 1979c). The reaction was conducted in 20 ml glass scintillation vials.² Test vials contained 0.5 ml of preopsonized zymosan suspension and 1.0 x 10⁷ PMNs in a total volume of 10 ml of Geys balanced salt solution with Ca⁺⁺ and Mg⁺⁺ and without phenol red (GBSS).³ The zymosan and GBSS were added to the scintillation vial, the PMNs were added 1 minute before the vial was placed in the counting chamber. Each vial was counted for 1 minute in each 10 minute cycle. Counting was continued for at least 70 minutes. All samples were run in duplicate; control vials containing 10 ml of GBSS and vials containing 10 ml of GBSS and PMNs were counted each cycle to determine the background activity and resting chemiluminescence. The results were calculated as

¹Model DPM 100, Beckman Instruments, Inc., Irvine, Ca.

²Fisher Scientific Co., Pittsburgh, Pa.

³Grand Island Biological Co., Grand Island, N.Y.

previously described and reported as net counts per hour (Roth and Kaeberle, 1979b).

Iodination - The procedure for the determination of iodination by bovine PMNs has been reported elsewhere (Roth and Kaeberle, 1979a). Each reaction tube contained 40 nmole NaI, 0.05 μ Ci Na 125 I (carrier free in 0.1 M NaOH),¹ 2.5×10^6 PMNs, and 0.05 ml of preopsonized zymosan in a total volume of 0.5 ml of EBSS with phenol red. All of the components except the PMNs were placed in 12 x 75 mm polystyrene snap cap test tubes² and allowed to equilibrate in a 37 C incubator. The reaction was started by the addition of PMNs, and the mixture was incubated for 20 minutes at 37 C with end over end tumbling approximately 20 times per minute. The reaction was terminated by the addition of 2.0 ml of cold 10% trichloroacetic acid (TCA). The precipitate was collected by centrifugation and washed twice with 20 ml of cold 10% TCA. The counts per minute of radioactivity remaining in the precipitate was determined in a gamma counter.³ Results are expressed as nanomoles of iodide converted to a TCA-precipitable form per 10^7 PMNs

¹New England Nuclear, Boston, Ma.

²#2058 Falcon, Oxnard, Ca.

³Autowell II, Picker Nuclear, North Haven, Ct.

per hour (nmole NaI/10⁷ PMNs/hr). All assays were performed in duplicate and the average value reported.

Determination of the effect of serum from BVD virus infected animals on iodination by PMNs from control animals -
Blood was obtained from the virus-infected and control animals by jugular venapuncture and allowed to clot at room temperature. The serum was removed and held at 4 C until it was used a few hours later. Normal serum for comparison was obtained from apparently healthy Holstein-Fresian cows. Blood was obtained from several cows and allowed to clot at room temperature. The serum was removed and combined into a pool of normal serum. This was divided into 2 ml aliquots and frozen at -70 C until use. The same pool was used throughout the experimentation. The effect of the different sera on iodination was determined by incubating 50.0 ul of each serum with unopsonized zymosan in an iodination reaction tube for 15 minutes at 37 C, then performing the iodination reaction as usual by adding 2.5×10^6 PMNs obtained from a control animal. Iodination in the presence of the serum from control animals and virus infected animals was expressed as a percent of the iodination in the presence of the pooled normal serum.

Statistical methods - All of the values from control animals were averaged and a mean and standard error of the mean were calculated. An analysis of variance procedure was performed to determine the level of significance of differences between the data from the control animals and that from the infected animals. The data from the intravenously infected and intranasally infected animals were considered separately. Only the data obtained from days 1 through 14 postinfection were included in the analysis of variance.

Results

Clinical response - Only mild clinical signs were induced by injection of virus either intravenously or intranasally. A diphasic temperature response was commonly observed; a dry nonproductive cough, mucous nasal discharge, and anorexia were often noted during the febrile period. Diarrhea was not observed. All of the animals readily recovered without treatment.

Hematologic response - The effect of BVD virus infection on numbers of total WBCs, lymphocytes, neutrophils, and eosinophils in peripheral blood is shown in Figures 10-13. There was a marked drop in number of all three cell types following BVD virus infection.

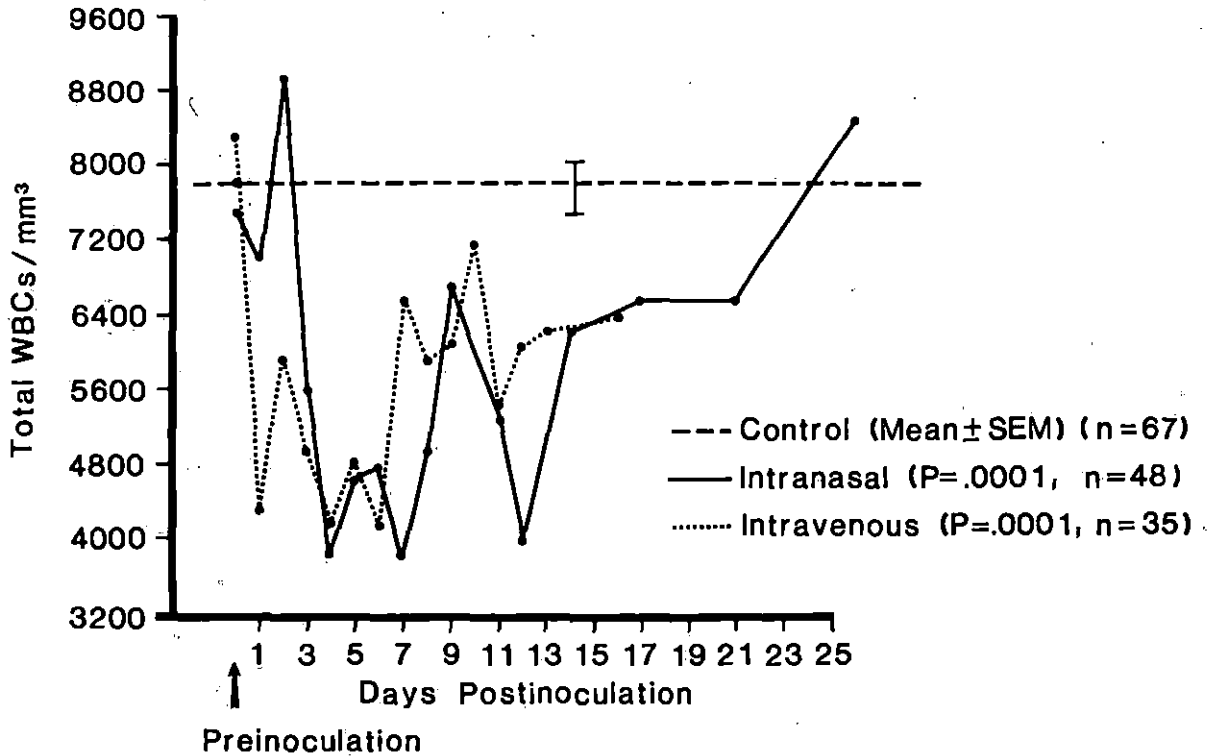


Figure 10. Mean total white blood cells (WBCs) in the peripheral blood of control and BVD virus infected cattle prior to and following challenge. "n" values from each experimental group were used in the analysis of variance to arrive at the probability statement.

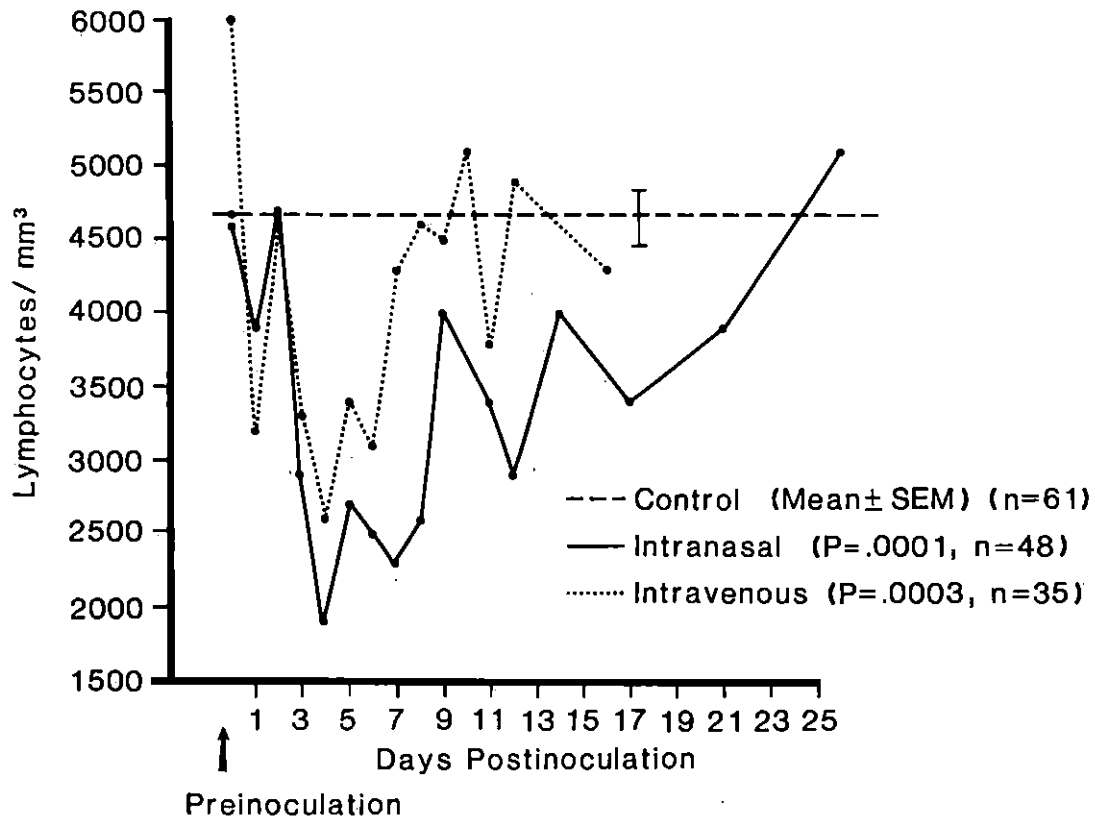


Figure 11. Mean total lymphocytes in the peripheral blood of control and BVD virus infected cattle prior to and following challenge. "n" values from each experimental group were used in the analysis of variance to arrive at the probability statement.

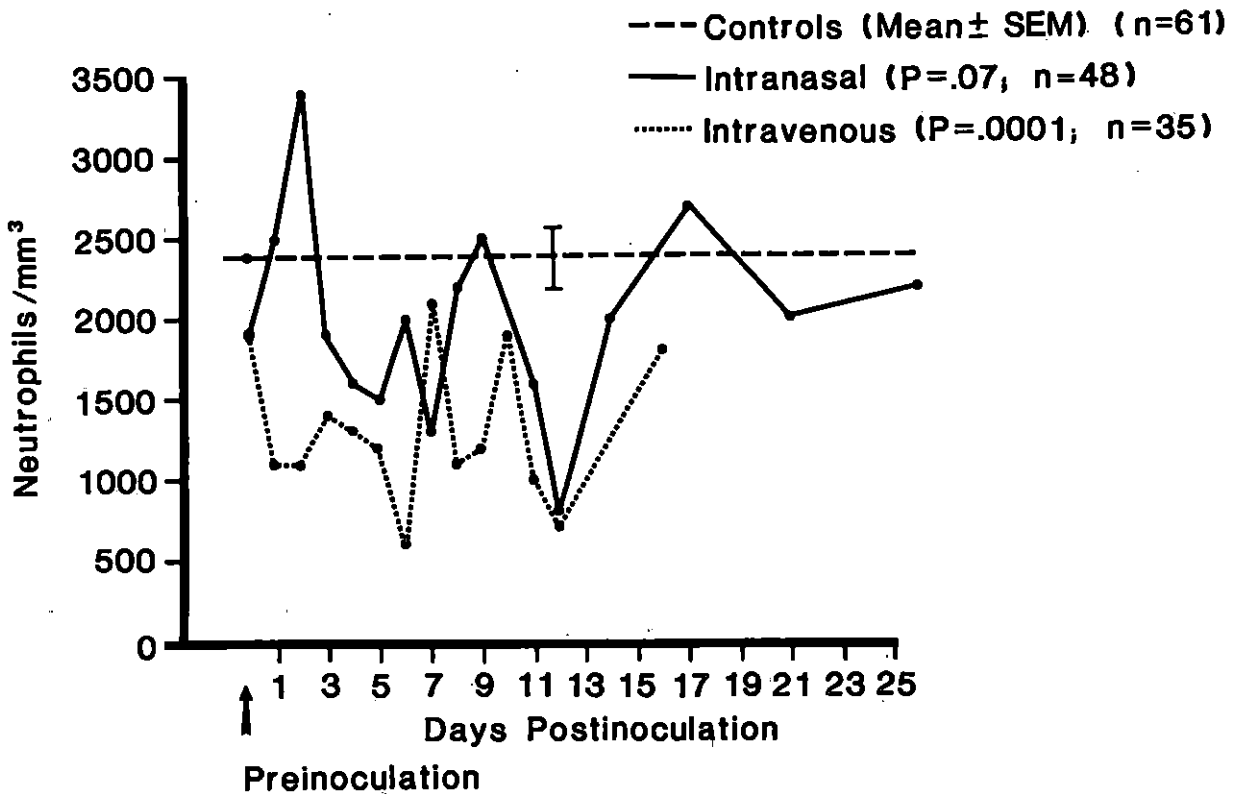


Figure 12. Mean total neutrophils in the peripheral blood of control and BVD virus infected cattle prior to and following challenge. "n" values from each experimental group were used in the analysis of variance to arrive at the probability statement.

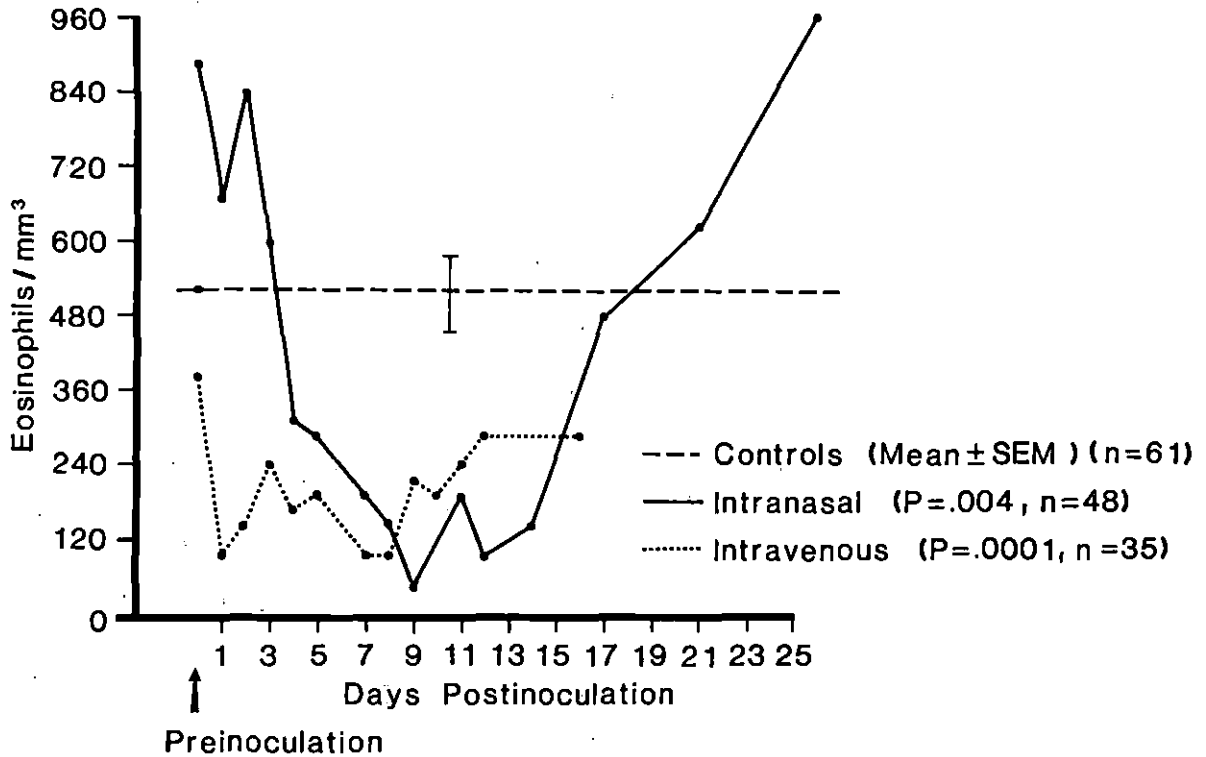


Figure 13. Mean total eosinophils in the peripheral blood of control and BVD virus infected cattle prior to and following challenge. "n" values from each experimental group were used in the analysis of variance to arrive at the probability statement.

Paraffin oil uptake - Figure 14 illustrates the effect of BVD virus infection on paraffin oil uptake. Statistical analysis indicated no significant difference in paraffin oil uptake between control and infected groups following BVD virus infection. However, there were indications of depressed paraffin oil uptake in three of the nine animals following inoculation of BVD virus.

Nitroblue tetrazolium reduction - The effects of BVD virus infection on nitroblue tetrazolium reduction by bovine PMNs are shown in Figure 15. There was no significant difference in nitroblue tetrazolium reduction by PMNs from normal and infected cattle. None of the individual animals had consistently depressed nitroblue tetrazolium reduction.

Chemiluminescence - The chemiluminescent response of PMNs following BVD virus infection is shown in Figure 16. There was no significant depression of chemiluminescence by PMNs following infection. None of the individual animals had consistently depressed chemiluminescence following BVD virus infection.

Iodination - The results of the iodination reaction by PMNs from control and infected animals are shown in Figure 17. There was a marked depression of iodination following BVD virus infection. This was noted consistently in each animal infected. Following intravenous inoculation with the 1015-74 strain of the virus, a marked depression

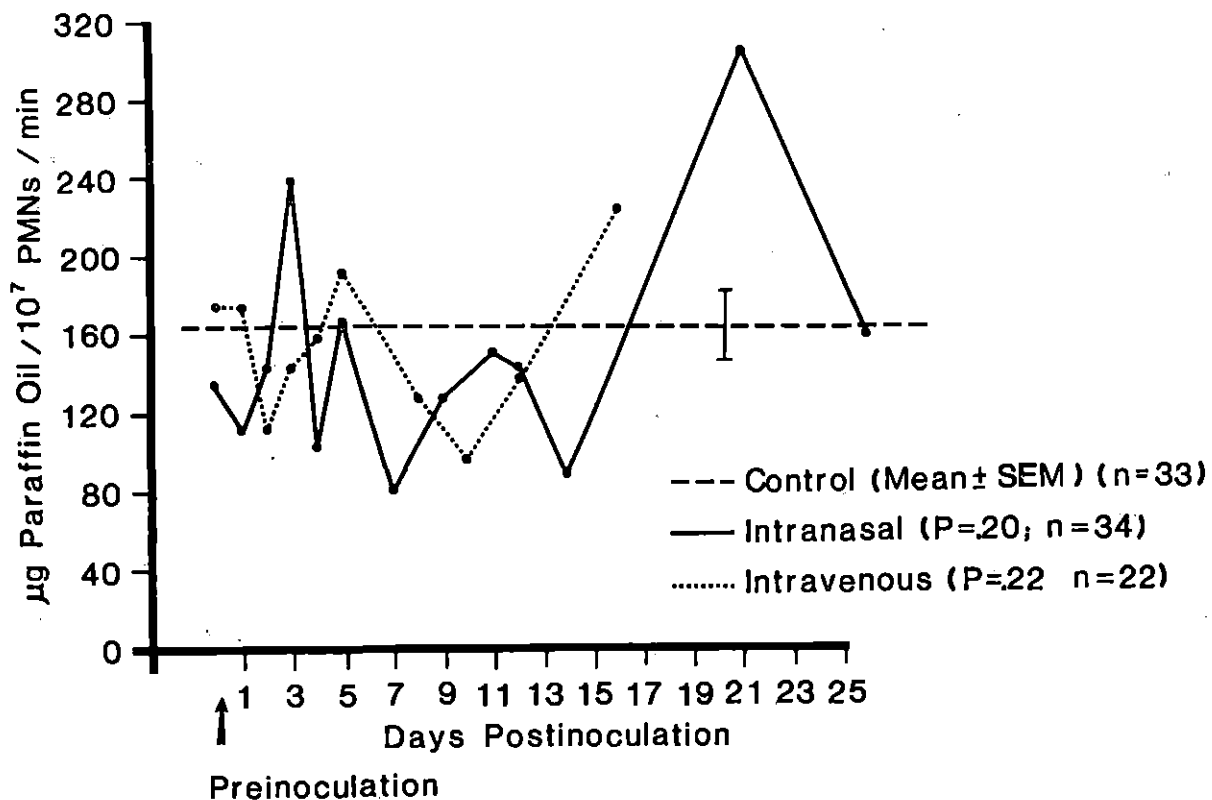


Figure 14. Mean values for paraffin oil uptake by PMNs isolated from the peripheral blood of control and BVD virus infected cattle prior to and following challenge. "n" values from each experimental group were used in the analysis of variance to arrive at the probability statement.

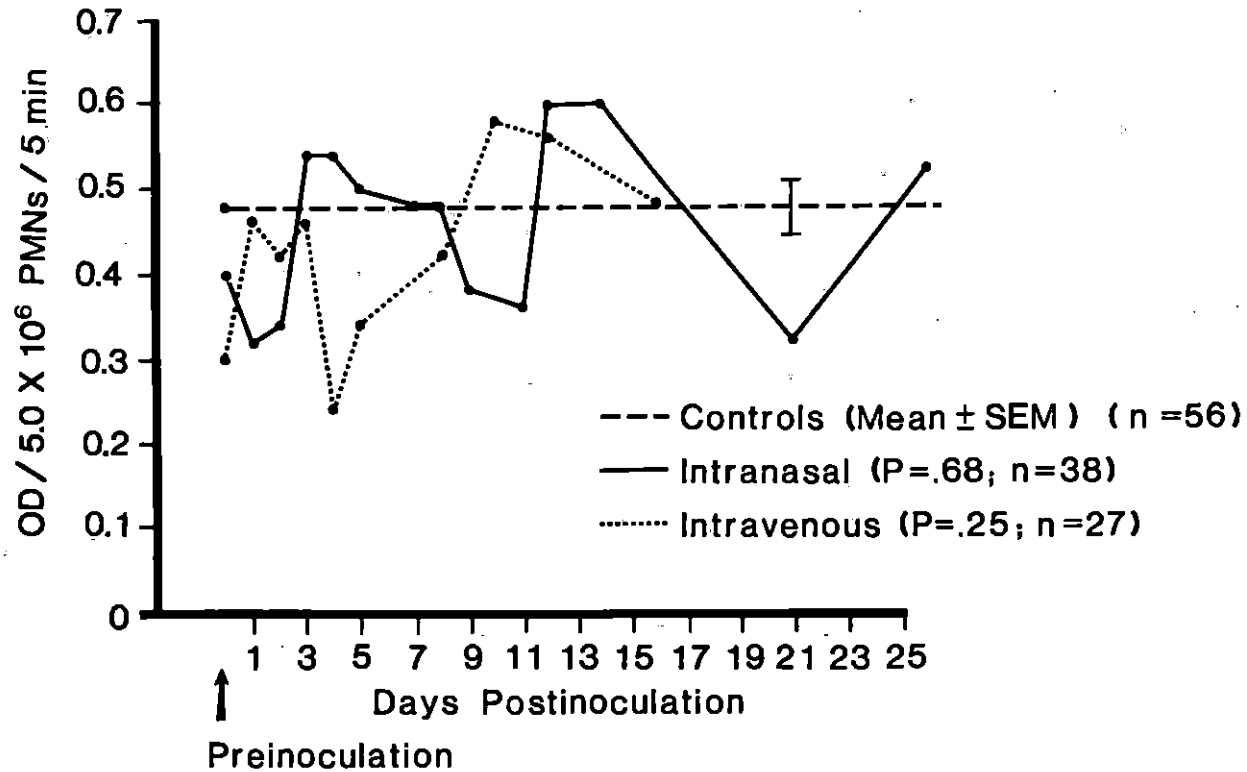


Figure 15. Mean values for nitroblue tetrazolium reduction by PMNs isolated from the peripheral blood of control and BVD virus infected cattle prior to and following challenge. "n" values from each experimental group were used in the analysis of variance to arrive at the probability statement.

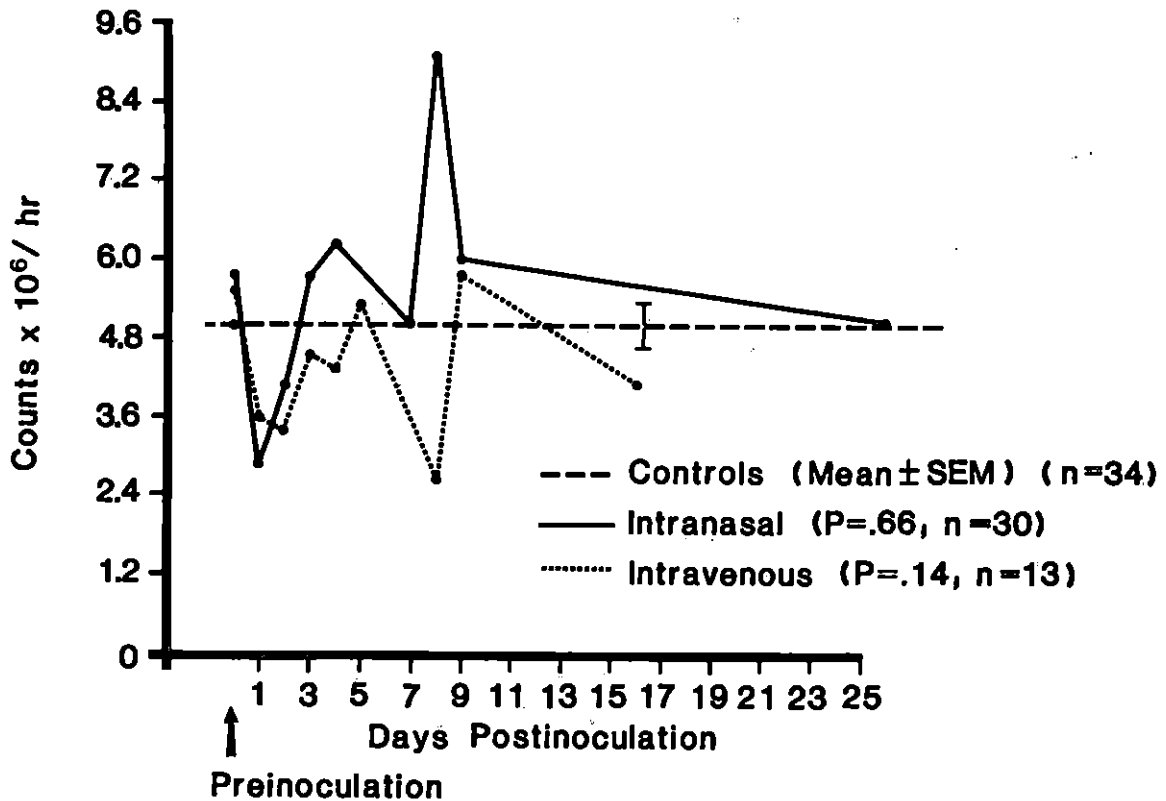


Figure 16. Mean values for chemiluminescence by PMNs isolated from the peripheral blood of control and BVD virus infected cattle prior to and following challenge. Chemiluminescence values were obtained in a liquid scintillation counter as counts per minute periodically during a 60 minute period, then extrapolated to counts per hour. "n" values from each experimental group were used in the analysis of variance to arrive at the probability statement.

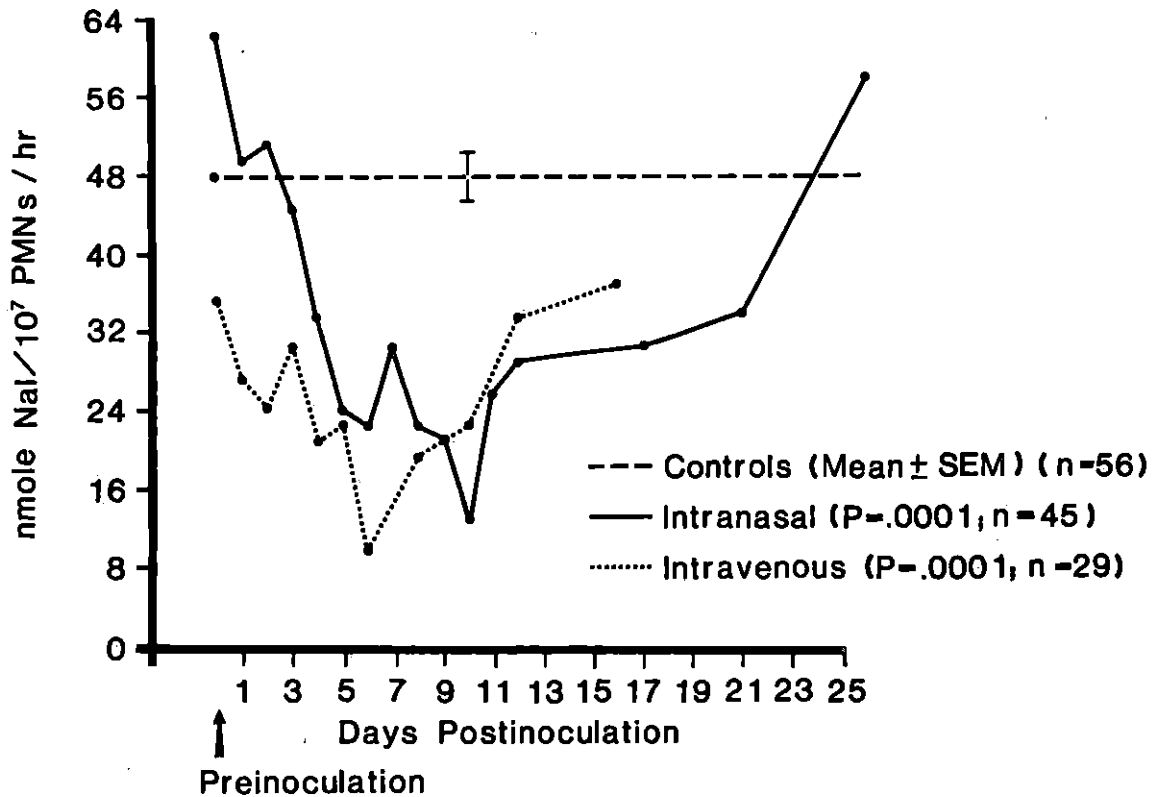


Figure 17. Mean values for iodination by PMNs isolated from the peripheral blood of control and BVD virus infected cattle prior to and following challenge. "n" values from each experimental group were used in the analysis of variance to arrive at the probability statement.

in iodination occurred by day one or day two postinfection. Following intranasal infection with the NADL challenge strain a depression of iodination was not noted until day three or day four postinfection.

Effect of serum on iodination - The effect of serum from BVD infected animals on iodination by PMNs from control animals is shown in Figure 18. Serum from BVD infected animals did not depress iodination by normal PMNs under the conditions employed.

Discussion

The clinical signs, diphasic temperature response, and severe leukopenia were typical of results reported for experimental infection with BVD virus (Malmquist, 1968; Tyler and Ramsey, 1965). While diarrhea has been reported following experimental BVD infection, it was not observed in this experimentation (Tyler and Ramsey, 1965). Bovine viral diarrhea virus infection was confirmed by the development of significant virus neutralizing antibody titers in all animals following challenge.

The results of the paraffin oil uptake procedure were equivocal. Three of the animals exhibited depressed paraffin oil uptake. However, statistical analysis of all data obtained from control and infected animals indicated no significant depression of paraffin oil

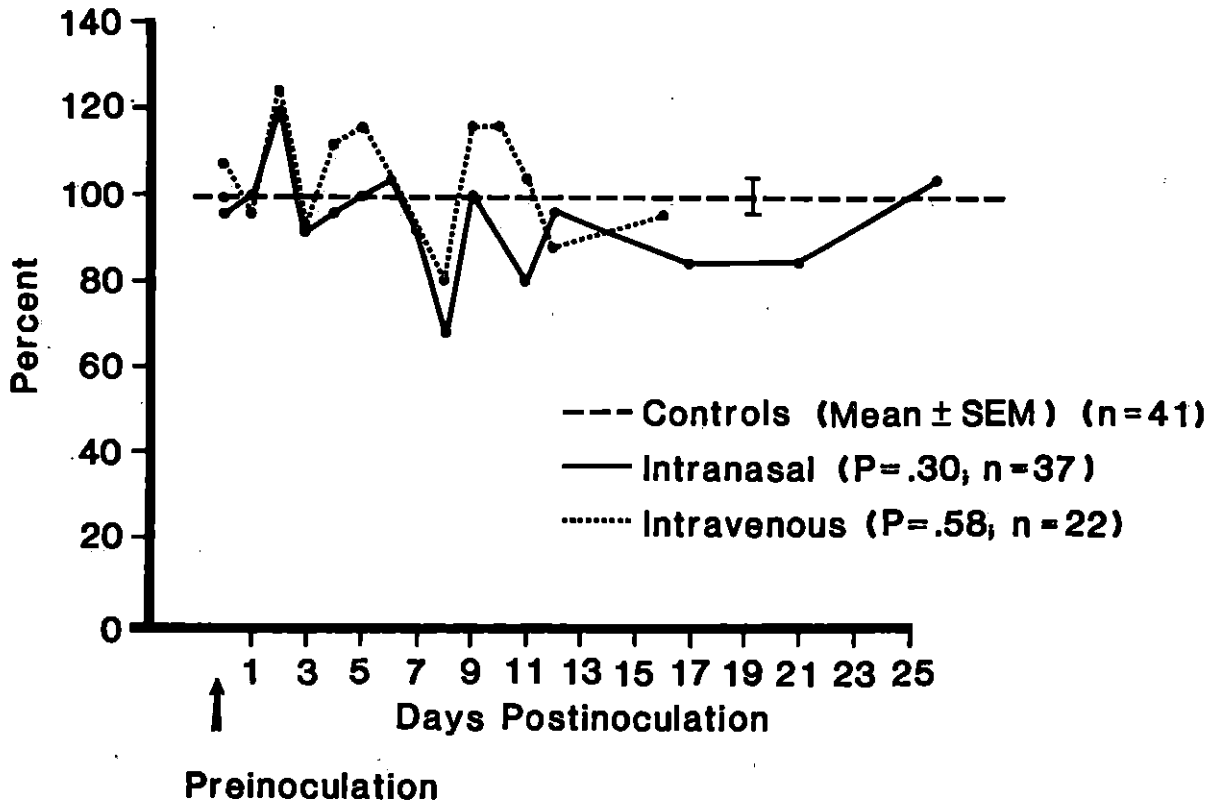


Figure 18. Iodination values of normal bovine PMNs in the presence of serum from control and BVD virus infected cattle, expressed as a mean percent of iodination by normal bovine PMNs in the presence of pooled normal bovine serum. "n" values from each experimental group were used in the analysis of variance to arrive at the probability statement.

uptake. This lack of significance may have been due to problems inherent in the test itself. Difficulty was encountered in separating the uningested paraffin oil from the cells containing ingested paraffin oil. Another problem was the standardization of the paraffin oil droplets from one day to the next. Paraffin oil uptake is a measure of the ability of the PMN to ingest particles. Due to the weaknesses in the test, it was not possible to conclude whether or not ingestion by PMNs was impaired during BVD virus infection.

There was no significant depression of NBT reduction or chemiluminescence by PMNs following BVD virus infection. These tests are both measures of the oxidative metabolism of the PMN (Roth and Kaeberle, 1979b, c); therefore, the results indicate that the oxidative metabolism of the PMNs following BVD virus infection is normal. Because oxidative metabolism of the PMN may be stimulated in the absence of ingestion (Goldstein et al., 1977), normal NBT reduction and chemiluminescence cannot be interpreted to demonstrate normal ingestion by PMNs. The iodination reaction was consistently and markedly depressed following BVD virus infection. Ingestion, the burst of oxidative metabolism, degranulation, and myeloperoxidase in the primary granules must all be present to obtain a normal iodination reaction (Klebanoff and Clark, 1977). Depressed iodination indicates

a defect in one of these parameters. The results of the NBT reduction and chemiluminescence tests indicate that the burst of oxidative metabolism which accompanies phagocytosis is normal. The depression of iodination could not be explained on the basis of stage of maturity or toxic changes of the PMNs. Microscopic examination of the whole blood smears and PMN preparations revealed only minor abnormalities in PMN morphology. The normal NBT reduction, chemiluminescence, and morphology indicated that the depression of iodination was not due to toxic changes or to lack of viability of the PMNs. Eosinophils are known to participate in the iodination reaction. The percent of eosinophils in the PMN preparations varied considerably with generally fewer eosinophils present in the PMN preparations following BVD virus infection. The drop in eosinophil numbers was not thought to be involved in the depression of iodination. This is supported by an observed lack of correlation between eosinophil numbers and iodination in PMN preparations from normal animals (Roth and Kaeberle, 1979a).

Depressed iodination could theoretically be due to inadequate amounts of myeloperoxidase in the primary granules of the PMNs. The fact that the depressed iodination was observed within 24 hours following intravenous infection

would indicate that the depressed iodination is probably not due to a lack of myeloperoxidase formation during granulopoiesis. Granulopoiesis in the bovine takes approximately 5-6 days (Schalm, 1977). Myeloperoxidase in the primary granules is formed fairly early in this period (Murphy, 1976). Inadequate amounts of myeloperoxidase in the primary granules could be due to previous intracellular or extracellular degranulation. This could be expected if significant previous contact with bacteria had occurred. Reggiardo and Kaeberle (1979) demonstrated that a bacteremia lasting for 1-5 days occurred following BVD virus infection. However, the depression of iodination lasted for 2-3 weeks following infection. Furthermore, ingestion, oxidative metabolism, and degranulation all require the expenditure of energy by the PMN. The normal values for NBT reduction and chemiluminescence indicate that the PMNs had not depleted their energy supply by the previous ingestion of particles and degranulation.

The depressed iodination may be due to inhibition of degranulation by the PMN following BVD virus infection. Degranulation is thought to be dependent upon the action of microtubules and actomyosin-like microfilaments and upon the fusion of the lysosomal membrane with the phagocytic vacuole (Goren, 1977). Agents which elevate cellular cyclic AMP levels or decrease cellular cyclic GMP

levels interfere with degranulation (Weissmann et al, 1975). In the case of BVD virus infection, this could theoretically be due to a direct interaction of the virus and the PMN, or it may be mediated secondarily through a humoral factor which is influenced by BVD virus infection. Humoral factors that could be responsible for an inhibition of iodination include histamine (Zurier et al., 1974), β adrenergic agents (Zurier et al., 1974), adrenal-corticosteroids (Mandell et al., 1970), prostaglandins (Smith, 1977), and toxic or inhibitory substances released by other cells. To test for the presence of inhibitory serum factors, serum from BVD infected animals was added to PMNs from normal animals and their iodinating ability was determined. Serum from animals with depressed iodination failed to depress iodination by normal PMNs under the conditions employed. This would indicate that the defect is inherent in the PMNs and not due to a serum factor.

It is not unreasonable to speculate that the depression of PMN function may facilitate BVD virus survival in the host animal. Bovine PMNs have been demonstrated to be the most active cell type tested in mediating antibody dependent cell mediated cytotoxicity (ADCC) against herpes virus-infected target cells (Rouse et al., 1978).

The exact mechanism of this cytotoxicity is not known, but it is not dependent upon DNA, RNA, or protein synthesis within the PMN (Wardley et al., 1976a; Clark and Klebanoff, 1977). The ADCC phenomenon is dependent upon microtubule activity and is enhanced by drugs which lower cyclic AMP or elevate cyclic GMP (Wardley et al., 1976a; Clark and Klebanoff, 1977). Degranulation is also dependent upon microtubule activity and is subject to the bidirectional control of cyclic nucleotides (Weissmann et al., 1975). This analogy suggests that degranulation may play a role in ADCC. The PMN lysosomal enzyme myeloperoxidase appears not to be essential in the cytotoxicity system (Clark and Klebanoff, 1977); however, there are many other substances in the PMN lysosome which may play a role. If the BVD virus does indeed block degranulation of the bovine PMN, it may be able to prevent or reduce PMN mediated ADCC of BVD virus-infected target cells. There is good evidence that PMN mediated ADCC is important in defense against infectious bovine rhinotracheitis (IBR) virus infection in cattle (Rouse et al., 1976; Wardley et al., 1976a). If infection with BVD virus does depress the ability of the bovine PMN to mediate ADCC, this would facilitate concurrent infection of the animal with IBR virus, a situation which occurs fairly commonly in cattle.

PMNs could also play a role in antiviral defense by phagocytosing virus particles. Many viruses are engulfed by neutrophils (Sawyer, 1969; Belding and Klebanoff, 1970). Phagocytosis normally results in a burst of oxidative metabolism, the generation of H_2O_2 , and the release of myeloperoxidase and other lysosomal enzymes into the phagocytic vacuole (Murphy, 1976). Myeloperoxidase and hydrogen peroxide, in the presence of chloride ions, have been shown to have potent virucidal activity (Belding and Klebanoff, 1970). This is the same system which is responsible for the iodination reaction. The depression of iodination by PMNs following BVD virus infection indicates that this potentially virucidal system is inhibited.

PMNs may also play a role in antiviral immunity by releasing a substance with interferon-like activity (Rouse et al., 1978). The effect of BVD virus infection upon this activity of PMNs is not known.

The data presented here indicate that following BVD virus infection a defect in bovine PMN function occurs. This may be a defect in ingestion of particles, degranulation and release of lysosomal enzymes into the phagosome, or an interference with the myeloperoxidase catalyzed reaction. The defect in PMN function is compounded by a

marked decrease in the number of circulating PMNs. This combination of factors could facilitate BVD virus infection and predispose the animal to secondary viral and bacterial infection.

There are reports of inhibited phagocytosis (ingestion) by PMNs following infection with mumps, influenza, and Coxsackie viruses (Merchant and Morgan, 1950; Kantoch and Dubowska-Inglot, 1960). Oxidative metabolism, degranulation, or iodination were not evaluated following infection with these viruses. It is not known if the PMN defect described here is unique to BVD virus infection or if it may occur with other infectious agents as well. Further study is needed to determine the exact defect induced in the PMN and the mechanism for the induction of this defect. Characterization of this deficiency theoretically could lead to a therapeutic approach to normalization of PMN function during BVD virus infection.

SUMMARY

New procedures for the evaluation of bovine PMN function were evaluated, and these procedures were used to study the effects of BVD virus infection on PMN function in cattle.

The paraffin oil procedure for the evaluation of ingestion by PMNs was found not to be satisfactory for use in cattle. Nitroblue tetrazolium reduction, iodination, and chemiluminescence were all found to be satisfactory for the evaluation of bovine PMN function, and normal values for cattle were reported.

BVD virus infection resulted in the depression of total numbers of lymphocytes, neutrophils, and eosinophils in the peripheral blood beginning at 1-3 days and lasting for 2-3 weeks following infection. Nitroblue tetrazolium reduction and chemiluminescence by bovine PMNs were normal following BVD virus infection; however, iodination was markedly depressed. The depression of iodination was noted as early as 24 hours following intravenous challenge with the 1015-74 strain of the BVD virus; it occurred 2-3 days following intranasal challenge with the NADL strain, and lasted for 2-3 weeks in both cases.

It was concluded that the depression of iodination may have been due to a defect in ingestion of particles, degranulation and release of lysosomal enzymes into the phagosome, or an interference with the myeloperoxidase catalyzed reaction. The defect in PMN function, compounded by a marked decrease in the number of circulating PMNs, could facilitate BVD virus infection and predispose the animal to secondary viral and bacterial infection.

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