Alteration of neutrophil function by factors secreted by normal and hydrocortisone-treated monocytes

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Native immune mechanisms are those which are naturally present in the body and have not been induced by vaccination or exposure to infectious agents. Native immune factors include serum components such as complement, lysozyme, B-lysins, and the phagocytic activity of neutrophils and macrophages. These factors are especially important in the early stages of infection, and are mechanistically complex since they involve the interaction of cellular and humoral components (75).

There are two main types of "professional" phagocytic cells. These are the mononuclear phagocytes, the monocytes and macrophages, and the polymorphonuclear leukocytes, the neutrophils and eosinophils (71). The neutrophil is a highly specialized cell whose primary function is to ingest and kill invading microorganisms (8,48,71). Neutrophils represent the first line of defense against foreign and pathogenic elements and are the first cells to arrive at a site of infection (71,73). They must be able to detect invasion of the body, move toward the site of invasion, and accumulate at sites of injury or infection. This phenomenon, begun by the detection of soluble factors, is termed directed locomotion or chemotaxis. Most neutrophil chemotactic stimuli are soluble factors which cause the neutrophil to move up a concentration gradient of the stimulating factor (71).

Circulating neutrophils accumulate at inflammatory sites because of increased endothelial stickiness, attachment to capillary walls, and migration into tissues. Chemotactic substances are produced by miroorganisms. The best known is the peptide N-formylmethionine which is similar to the synthetic peptide n-formyl-methionyl-leucyl-phenylalanine (fMLP) used in chemotactic studies. Most chemotactic substances are produced by either the classic or alternate complement pathways. The C5a fragment is the most potent chemotactic factor derived from the complement pathways (14).

At the site of infection, neutrophils phagocytize and destroy invading microorganisms. Phagocytosis is composed of four phases: chemotaxis, opsonization, ingestion, and killing. The migrating neutrophil has a characteristic morphology. The leading edge of the pseudopod is free of granules but filled with actin microfilaments. The actin extends as a thin layer around the cell and occurs as dense bundles in the trailing edge of the neutrophil. The actin provides structural support and together with myosin can contract and provide cell movement. The energy for cell movement comes from anaerobic glycolysis. Microtubules are present, but are not involved in motility. They increase cell rigidity and stabilize cell shape while serving as an internal "skeleton" (14,84). Microtubules may also be involved with movement within the cell itself (79).

Agents which impair microtubule function in vitro (e.g. colchicine) will cause an enhancement of neutrophil random migration and an inhibition of degranulation (71). The random migration of

neutrophils under agarose, as measured by the number of cells leaving the well, is enhanced when microtubule inhibiting agents are used. In one experiment, incubating neutrophils with concentrations of colchicine above 5×10^{-7} M increased the number of cells migrating and decreased the mean number of centriolar microtubules in a dose-dependent fashion from 16 to 4 per um^2 at 10^{-5} M, but the distance of migration from the well was not significantly different from non-colchicine treated human neutrophils. Colchicine acts by decreasing peripheral microtubules. The data in this experiment suggest that microtubules play a constraining role within the cell, limiting the ability of the cell to move and change direction (69).

The interaction of a microorganism with the neutrophil cell membrane is the crucial step in initiating the events which result in phagocytosis and elimination of that microorganism. The interaction can be facilitated by the presence of heat stable opsonins found primarily in the IgG fraction of serum (50). Neutrophils bear Fc receptors to which antibody-coated particles adhere (15). Opsonization makes bacteria more susceptible to ingestion by neutrophils. This can be accomplished by reaction of microorganisms with specific antibody alone (especially IgG), specific antibody (IgG or IgM) acting with complement in the classical pathway, or nonspecifically via the alternate complement pathway. In this third method, opsonization occurs without antibody by complement activation by bacterial polysaccharides resulting in fixation of C3 (14).

Ingestion occurs after opsonization of a microorganism. The motion involved in phagocytosis occurs by the same actin-myosin system described for chemotaxis. Since the energy needed comes from anaerobic glycolysis, phagocytosis can take place even under the anaerobic conditions of an abcess. The sequential interaction of opsonic ligands on the microbial surface and receptors on the phagocyte membrane results in the "zippering" up of particles as they are engulfed by pseudopodia. Pseudopodia fuse around ingested particles, forming a phagosome, and the microtubules are involved in its movement toward the center of the cell (14,84). Microtubules could provide a "tracking" mechanism for neutrophil phagosomes. There is a convergence of microtubules toward phagosomes and an alignment of granules along the microtubules. Several functional interpretations can be offered for this possible microtubule "tracking" mechanism: (1) granules from the center of the cell may move along microtubules towards the peripherally located phagocytic vacuole, (2) microtubules aid in the movement of the peripheral phagocytic vacuole towards the granule-rich center, (3) the phagocytic vacuole is carried into the microtubule granule network via another mechanism such as the actin-myosin contractile system. Phagolysosome formation could then occur by way of either passive collisions of the phagosome with the microtubule-associated granules, or via granule transport along the microtubule towards the phagosome (79).

After phagocytosis, the neutrophil discharges various lysosomes and granules into the phagocytic vacuole. These promote the

degradation and neutralization of the engulfed particle or organism (48,61,84). Neutrophils contain two types of granules. Primary or azurophilic granules contain hydrolytic enzymes, myeloperoxidase, lysozyme, elastase, and cationic proteins. Secondary or specific granules are smaller, stain poorly, are more numerous in the mature neutrophil, and contain lactoferrin and lysozyme. During the process of engulfment and digestion of microorganisms, secondary granules fuse with the forming phagosomes and discharge their contents. Thus lactoferrin and lysozyme can escape the neutrophil membrane. Primary granules fuse later, with completed phagosomes, so their contents stay within the phagolysosome (14). Following phagocytosis of bacteria, neutrophils usually disrupt and die, releasing their lysosomal enzymes (80).

Phagocytosis results in a burst of oxidative metabolism, causing the formation of highly reactive oxygen intermediates such as superoxide anion, hydrogen peroxide, hydroxyl radical, and probably singlet molecular oxygen. Simultaneously, the granules are discharged into phagocytic vacuoles. These two processes are essential for killing ingested microorganisms (48,51,61,84,92). The neutrophil has oxygen-dependent and oxygen-independent antimicrobial systems. The oxygen-dependent system includes the myeloperoxidase-independent systems (hydrogen peroxide, superoxide anion, hydrogen radical, singlet oxygen) and the myeloperperoxidase-mediated systems (hydrogen peroxide and an oxidizable co-factor such as iodine). The oxygen-independent antimicrobial system includes acid conditions within the

phagosome, lysozyme, lactoferrin, and granular cationic proteins (14,48,61,75,80,84). Oxidative metabolism or the respiratory burst is a metabolic pathway of the neutrophil which results in the formation of highly reactive microbicidal agents. These agents are formed by the partial reduction of oxygen. Any perturbation of the neutrophil membrane, such as contact with a microorganism, stimulates the respiratory burst (14,51). The mechanism of the respiratory burst is: (1) particle contact with the neutrophil membrane activates NADPH oxidase which catalyzes the one-electron reduction of molecular oxygen to superoxide anion, (2) two molecules of superoxide anion combine spontaneously with two protons to form hydrogen peroxide, (3) myeloperoxidase is deposited by degranulation into phagosomes where in the presence of hydrogen peroxide and a halide such as iodine it catalyzes microbicidal reactions, (4) superoxide anion escaped from the phagosome is reduced to hydrogen peroxide by superoxide dismutase and the hydrogen peroxide is detoxified by the catalase and glutathione-peroxidase-glutathione reductase systems, (5) the $NADP^+$ formed by NADPH oxidase and glutathione systems is converted back to NADPH by the hexose monophosphate shunt (14,48).

The fixation of inorganic iodide to bacteria has been shown to be a major mechanism by which the neutrophil kills bacteria (10,48,84). This iodination reaction reflects a complex sequence of events. Phagocytosis of the opsonized particle is associated with the formation of hydrogen peroxide by the neutrophil with the discharge of myeloperoxidase into the phagosome from adjacent azurophil granules.

When iodide is oxidized by myeloperoxidase and hydrogen peroxide in the presence of a suitable iodine acceptor molecule such as the tyrosine residues of protein, iodine-carbon bonds are formed (49).

The neutrophil is usually regarded as a cell whose sole function is to recognize, ingest and kill invading microorganisms, but the neutrophil also has the capacity for antibody-dependent cell-mediated cytolysis (ADCC) (12). Although ADCC is usually considered to be the province of mononuclear cells, when the two types of cells are compared, the neutrophil has been shown to be as effective as mononuclear cells in mediating ADCC (12,22,53). The mechanism of the cytolytic effect is unclear, but it has been shown that the formation of reduced oxygen radicals, which normally takes place during phagocytosis, is important (5,12). Experimentally, target cell specificity can be achieved by coating the target cells with specific antibody (8,53). Rosettes are formed as the effector neutrophils bind to the target cells in a divalent cation-dependent process (8,22). Little is known about neutrophil ADCC after rosette formation, and no particular neutrophil constituents have been definitely associated with the cytotoxic event (8). In ADCC, non-immune Fc receptor-bearing leukocytes bind and destroy antibody-coated target cells (53). Available evidence supports the following sequence of events in neutrophil ADCC: neutrophils attach to antibody-coated target cells in a divalent cation-dependent process that involves surface Fc receptors; perturbation of the neutrophil plasma membrane activates a cellular oxidase which causes the formation of toxic products that

damage the target cell (3). Although release of granular components might also occur, no evidence for the involvement of the myeloperoxidase system or granular cationic proteins has been found (8).

Neutrophil ADCC does not require complement synthesis, de novo protein synthesis, or DNA replication. The activity is time and temperature dependent, and requires close proximity between effector cell and target cell (22). Surface receptors on the effector cell for the Fc portion of the immunoglobulin molecule are necessary for cytolysis in neutrophil ADCC (15,22,53,78). The same requirement for Fc receptors is necessary for lymphocyte- and monocyte-mediated ADCC (22). Neutrophil ADCC requires larger amounts of target cell-bound antibody than lymphocyte- or monocyte-mediated ADCC (12,53,78). In human leukocyte ADCC for both erythroid and tumor target cell types, neutrophil ADCC requires 100 times more target cell-bound antibody than does ADCC by lymphocytes and monocytes (53). Although the mechanism for neutrophil ADCC is not known, it appears that the metabolic burst is essential for maximal ADCC (8), oxygen radicals play an essential role (5), and neutrophil mediated cytolysis can be modulated by intracellular cyclic adenosine 5'-monophosphate (cAMP) levels (22).

Both monocytes and neutrophils reduce oxygen for the production of superoxide and hydrogen peroxide during phagocytosis, and the processes of ADCC, phagocytosis, and post-phagocytic events are intimately correlated. Under anaerobic conditions, ADCC by human

monocytes and neutrophils is approximately 50% reduced, but phagocytosis and degranulation are unaffected (5).

In neutrophil ADCC, experiments with autonomic agonists and inhibitors suggest a cyclic nucleotide-dependent control mechanism. Increased neutrophil cAMP suppresses and increased cGMP enhances cytolytic activity. The inhibition of neutrophil ADCC by colchicine, an inhibitor of microtubules, suggests that microtubules may be important, but inhibitors of protein synthesis, nucleic acid synthesis, or complement do not depress neutrophil ADCC (22). B-adrenergic agonists, aminophylline, cholera toxin, and dibutyryl cAMP inhibit neutrophil-mediated ADCC. A neutrophil-to-target-cell ratio of 0.25:1 is effective in neutrophil-mediated ADCC. Using ^{51}Cr-labeled target cells, release of ^{51}Cr begins in 30 minutes and becomes maximal at 1 to 2 hours. Neutrophil ADCC is inhibited by the glycolytic inhibitors, iodoacetamide, fluoride, and 2-deoxyglucose, with the effect of the latter reversed by excess glucose. Since energy production (ATP) by the neutrophil largely depends on glycolysis, this suggests an energy requirement, presumably in the form of ATP, for cytotoxicity (8). In a microcytotoxicity assay, the antibody requirement for cytolysis was further substantiated by the absence of cytotoxicity by neutrophils toward nonantibody-coated "innocent bystander" cells. In "innocent bystander" experiments, no evidence for a soluble mediator of target cell lysis was found for effector leukocytes. Lysis was induced by neutrophils in the presence of antiserum, and their activity was greater than that of equivalent

numbers of mononuclear cells (53). Considerable ADCC activity was found in the lymphocyte and neutrophil cell populations (44).

The neutrophils circulating in peripheral blood are derived from granulocytic precursors in the hemopoietic marrow. At the time when the mature neutrophils are released into circulation, they are generally believed to be terminally differentiated end products, devoid of biosynthetic capacity. However, Granelli-Piperno et al. have shown that human peripheral blood neutrophils synthesize RNA and proteins during short term culture, and this synthesis can be modulated by low concentrations of glucocorticoids (29).

Stress and Neutrophil Function

Stress is a term used to describe a wide variety of external influences on an animal (71). It can increase susceptibility to disease and has long been associated with the occurrence of infectious disease (71,75). The increased susceptibility to disease caused by stress is partially due to alterations in immune function (71). Stressful conditions in cattle include weaning, transportation, handling, castration, dehorning, parturition, forced exercise, mastitis, diarrhea, extreme weather conditions, and pain (75). An important response of the body to stress is the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland which in turn stimulates the adrenal cortex to increase cortisol (hydrocortisone) secretion, resulting in elevated blood cortisol levels (71,75,76). Physiologic increases of plasma cortisol in response to stress is an important factor in the pathogenesis of

infectious diseases, including "shipping fever" or the bovine respiratory disease complex (BRD), the most costly of all diseases of feedlot cattle in the United States (75,76). Increased plasma cortisol levels in cattle decrease the ability of neutrophils to leave the circulatory system, enhance neutrophil random migration, and decrease the myeloperoxidase catalyzed reaction by neutrophils. This alteration of neutrophil function may be enough to give infectious agents time to colonize and produce disease before host defense mechanisms can bring them under control (71).

Glucocorticoids and Neutrophil Function

Clinical observations indicate that glucocorticoids can be detrimental to host defense mechanisms. Defining the specific effects of glucocorticoids, especially cortisol, on the immune system would help define the pathogenesis of stress-induced immunosuppression. The neutrophil is the phagocytic cell type which has received the most study in relation to cortisol effects (71). Natural as well as synthetic glucocorticoids are used extensively in bovine medicine (72,75). Dexamethasone is a potent glucocorticoid which is administered to cattle for the induction of parturition, the alleviation of physiological udder edema, the reduction of musculoskeletal inflammation, and the treatment of ketosis. Dexamethasone can cause recrudescence of infectious bovine rhinotraceitis virus (IBR), predisposition to a fatal viremia in bovine viral diarrhea virus infected calves, and a more severe disease course in cattle already suffering from bronchial pneumonia (72). The

inhibition of neutrophil-mediated ADCC may allow multiplication of IBR virus which has been recrudesced by administration of dexamethasone (71). The potentiation of infectious processes indicates that dexamethasone treatment suppresses the bovine immune system (72,75), and the concurrent administration of effective antibacterial agents is often recommended because of this association of glucocorticoids with exacerbation of infectious disease processes (75).

Glucocorticoids decrease levels of circulating peripheral lymphocytes and immunoglobulins, inhibit mitogen- and antigen-induced blastogenesis of cultured lymphocytes, and decrease bactericidal activity of granulocytes and other phagocytic cells (15). Neutrophils from dexamethasone-treated cattle display enhanced random migration under agarose and impaired ingestion of Staphylococcus aureus, nitroblue tetrazolium (NBT) reduction, chemiluminescence, iodination, and ADCC. Dexamethasone also suppresses mitogen-induced bovine lymphocyte blastogenesis (72). The decreased margination and migration of neutrophils into the tissues is probably related to the observation that glucocorticoids cause a decreased "stickiness" of the neutrophil membrane (71).

The most direct approach to evaluating the effects of cortisol on the immune system is to administer ACTH. The alterations in immune function can then be assumed to result from increased plasma cortisol concentrations. Cortisol is a relatively weak glucocorticoid and may induce subtle changes in immune function that are important in vivo but are below the limits of sensitivity of assay systems.

Administration of ACTH causes a neutrophilia in cattle, indicating that cortisol reduces the rate of efflux of neutrophils from the blood into the tissues. This neutrophilia usually occurs without an increase in band or other immature forms and is due to an increased influx of neutrophils from the bone marrow storage pool, decreased margination of neutrophils, and a decreased egress of neutrophils from the circulation into the tissues (71). Random migration of neutrophils is enhanced by in vivo ACTH treatment and the neutrophil iodination reaction is significantly impaired, but there is no effect on Staphylococcus aureus ingestion, NBT reduction or ADCC (76). Since ACTH administration does not inhibit the oxidative metabolism of the neutrophil, the inhibition of the myeloperoxidase reaction is probably due to an inhibition of degranulation, which is dependent upon normal microtubule function (71).

Glucocorticoid action classically depends on the combination of the steroid with a cytosolic receptor protein and the translocation of this drug-receptor complex to the nucleus (1,18,43). In the nucleus, by regulating transcription, hormone-receptor complexes stimulate the synthesis of particular kinds of messenger ribonucleic acid (mRNA) that, in turn, results in synthesis of specific proteins that mediate the hormone effects (17,57). Many effects of glucocorticoids are blocked or counteracted by inhibitors of RNA synthesis. Timed addition of inhibitors shows that the period of sensitivity to inhibitors of RNA synthesis coincides with the early phases of hormone action. This precedes the appearance of the hormone effect and the

period of sensitivity to inhibitors of protein synthesis. It has been hypothesized that glucocorticoids induce certain proteins by increasing the rate of transcription of the appropriate mRNA (57).

Reduction of Fc receptor numbers on neutrophil cell surfaces could explain the suppressive effects of glucocorticoids on phagocytosis (15). It has been suggested that corticosteroids inhibit the interaction of IgG sensitized erythrocytes with the neutrophil Fc receptor. The inhibitory effect is dependent on the presence of steroid before adherence, and is reversible and dose-dependent. It was shown that once sensitized erythrocytes were attached to the neutrophil membrane, steroids were unable to cause displacement (50). Glucocorticoids inhibit expression of the Fc receptor on the human promyelocytic cell line HL-60, but do not cause increased cell death, reduced proliferation or a general reduction in protein synthesis. After a 48 hour incubation of a human promyelocytic cell line in 1 uM cortisol, Fc receptor sites were reduced from 14,400 to about 8,000 per cell. Dexamethasone gave a half maximal response at 10u M, and in both cases the numbers of Fc receptor sites returned to normal within 48 hours of removal of the steroid (15). Two criteria have been applied for identification of cells with glucocorticoid receptors. The first is for a metabolic or morphologic effect induced by cortisol, corticosterone, dexamethasone, prednisolone, or other steroids with recognized glucocorticoid activity, at concentrations of about 1 ug/ml or less. The second is evidence for specificity of glucocorticoids, that is, they produce effects with significantly

higher activity than non-glucocorticoids. The effective concentration should not grossly exceed plasma levels of free hormone seen physiologically (57).

Corticosteroids are capable of reducing lysosomal enzyme release from neutrophils exposed either to a variety of phagocytosable particles or to immune reactants deposited on non-phagocytosable surfaces (26). By preventing release of "sticky" specific granule products, corticosteroids would eliminate some of the factors such as lactoferrin and other acidic products which contribute to and sustain granulocyte adhesion (64). Release of lysosomal enzymes follows fusion between the membranes of lysosomes and those of phagocytic vacuoles or the cell itself. Inhibition of lysosomal enzyme release and superoxide production may explain, in part, both the antiinflammatory actions of steroids and their deleterious effects on host defenses (26).

When comparing results obtained in an *in vitro* system to an *in vivo* situation, the concentration of glucocorticoids, the form of the glucocorticoid, the incubation time, temperature, composition of the media, and the various cell types present must be considered. For *in vitro* results to have *in vivo* relevance the concentration of glucocorticoid should at least be near pharmacologic plasma levels. For *in vitro* models of the effects of stress on the immune system, cortisol should be used at near physiologic levels. For example, Fleer et al. found a large discrepancy between the minimal concentration of hydrocortisone effective *in vitro* (0.5-1 mM) and the

much lower plasma concentration found effective during treatment in vivo (12.5 uM) for inhibition of monocyte ADCC in human cells (16). The form of glucocorticoid used in an in vitro assay may influence the results since the molecule may need to undergo in vivo modification for maximum effect. If a succinate form of glucocorticoid is used, the succinate must be split off in the liver by an esterase enzyme before the drug is active. Spontaneous cleavage of the succinate under in vitro conditions only occurs slowly. The time of in vitro incubation is important because at least some glucocorticoid effects require RNA and protein synthesis. The incubation should be conducted at physiologic temperature and pH (71).

If no effects with the glucocorticoid are observed, it may be because there are none or because the assay system lacked the sensitivity to detect the effect. The types and proportions of cells present in an in vitro system may determine the effects obtained. A major problem with assays of immune function involving living cells in vitro is significant day to day variability in assay results (71). The in vivo effect of glucocorticoids on a particular cell type may be indirectly mediated by products of other cells (71,72).

Animal species are classified as glucocorticoid sensitive or glucocorticoid resistant based upon the relative susceptibility to production of lymphopenia. Species vary greatly in their response to glucocorticoids. For instance, the rat, mouse, and rabbit are quite sensitive to corticosteroids, while man, monkey, and guinea pig are relatively resistant. The response of cattle to glucocorticoids is

more like that of the corticosteroid-resistant species, but differences do exist between glucocorticoid effects in cattle and other corticosteroid-resistant species (71). Lymphopenia develops when cattle are treated with dexamethasone, but no lymphopenia or a transient lymphopenia results with ACTH treatment (76). The results obtained in other species are informative and provide insight into basic mechanisms, but it cannot be assumed that the same effect will occur in cattle until it is confirmed by experiments using cattle (71).

Some workers reported that in vitro incubation of neutrophils with dexamethasone at a concentration of 10^{-6} to 5.8×10^{-6} M did not impair the phagocytic or bactericidal capacity of human neutrophils (55), others reported that a significant impairment of bactericidal activity occurred with concentrations of dexamethasome of 10^{-4} to 10^{-6} M (80). However, when dexamethasone was added to human neutrophils in vitro in whole blood with concentrations equaling 7×10^{-4} M, hexose monophosphate shunt (HMS) activity, ascorbate-induced HMS, phagocytosis, and iodination were inhibited. They found that human neutrophil migration following direct in vitro incubation with hydrocortisone at a concentration of 5×10^{-6} to 2.8×10^{-4} M was inhibited, but that enhanced migration was observed at a concentration of 5×10^{-7} to 2.7×10^{-6} M (55). In another study, in vitro incubation of human neutrophils in 0.08 mg/ml of hydrocortisone sodium phosphate was found to decrease the phagocytic activity of those neutrophils (34).

The in vitro effects of a number of steroids was found to include inhibition of both hexose monophosphate shunt activity and iodination of bacteria. Since inhibition occurred in resting as well as in phagocytosing cells, the steroids probably were not acting solely upon particle ingestion but were affecting an enzymatic reaction. Hydrocortisone at a concentration of 0.7 uM suppressed iodination of zymosan particles (10). Aggregation engendered in vitro by zymosan-activated plasma was inhibited by methyl prednisone and hydrocortisone at concentrations that approximated plasma levels that would be induced with therapy in man. Dexamethasone was found to be almost without effect. The relative inefficacy of dexamethasone was not explained, but its requirement for dephosphorylation and relatively slow cellular uptake may be involved (32).

Aggregated granulocytes have been found to disaggregate upon addition of corticosteroids with an order of potency of methyl prednisone:hydrocortisone:dexamethasone, with a methyl prednisone concentration of about 2-3 mg/ml required to effect complete disaggregation. High-doses of corticosteroids can cause disaggregation as well as block granulocyte aggregation, and the same concentrations alter binding of the synthetic chemotaxin, N-formyl-methionyl-leucyl-phenylalanine (fMLP), to its specific receptor on the neutrophil surface. This alteration in receptor binding is due primarily to a decrease in the association rate constant for peptide-receptor interaction without significant change in dissociation rate or receptor number. Dose-dependent inhibition of binding was observed

at corticosteroid concentrations paralleling plasma levels achieved with 30 mg/kg intravenous therapy; the order of potency was again methyl prednisone:hydrocortisone:dexamethasone (83).

Stevenson showed that culture of human mononuclear leukocytes in the presence of hydrocortisone results in the appearance in the culture medium of a factor which markedly stimulates the migration of human neutrophils in vitro. The steroid-mononuclear cell interaction is dependent on the duration and temperature of the culture, and on the concentration of hydrocortisone. He suggests that an active biological process is involved which may be relevant to the immunosuppressive action of corticosteroids. Cortisosteroids have long been known to depress the immune response, but the mechanism of their effect is obscure. Stevenson suggests that an enzymatic reaction may be involved, the effect of which can be augmented by culturing monocytes in the presence of hydrocortisone. He showed that hydrocortisone alone had a direct inhibitory effect on the migration of purified neutrophils. Corticosteroids have been shown to inhibit the normal responses of macrophages and neutrophils to antigenic challenge in vivo, and this study provided evidence both for a direct effect of hydrocortisone on neutrophils, resulting in migration inhibition, and for an indirect effect, mediated by monocytes, resulting in migration stimulation (87,88). It was found that steroid-treated lymphocytes did not produce the factor which gives this indirect effect on neutrophils (88).

Stevenson's results indicate that a neutrophil migration stimulator is produced *in vitro* by the interaction between hydrocortisone and glass-adherent mononuclear leukocytes, of which over 95% are derived from blood monocytes. He used 1-10 ug/ml of hydrocortisone in the medium used for this study. This neutrophil migration stimulating factor may be relevant to the *in vivo* effects of hydrocortisone on neutrophil kinetics and immunological responsiveness, and may relate to the granulocyte colony-stimulating factor also produced by monocytes. Neutrophil migration was marginally stimulated by hydrocortisone alone, probably due to the reaction between hydrocortisone and the mononuclear leukocytes in the migrating cell population, which produces a migration-stimulating effect on the migrating neutrophils (88).

Stevenson reported that corticosteroid therapy stimulates human neutrophil migration *in vitro*, but the effect is dependent on the presence of monocytes in the cell population. The monocytes may have reacted with steroid *in vitro* and subsequently liberated neutrophil migration stimulator in the migration chamber (89). It was shown that neutrophil migration stimulator is induced only by steroids with glucocorticoid activity and that spleen and bone marrow cells also react with corticosteroids to produce the neutrophil migration stimulating factor. Since the inhibition of protein synthesis during culture of steroid-treated monocytes prevented the production of neutrophil migration stimulator, the migration stimulating activity may therefore reside in a peptide moiety actively synthesized under

the influence of corticosteroids (90). Studies on the physical characteristics of neutrophil migration stimulator show that it is soluble and stable at high and low temperatures, and that its activity is reduced by acid and alkali treatment and destroyed by protease. Experiments involving dialysis, ultrafiltration, and gel filtration chromatography indicate that the molecular weight of the neutrophil migration stimulator is between 12,000 and 15,000 (92).

The results suggest that neutrophil migration stimulator acts by inhibiting the assembly of neutrophil cytoplasmic microtubules, an effect which may be mediated by cAMP in the same manner as other peptide hormones. An enhancement of neutrophil migration is produced by colchicine and vinblastine, drugs which inhibit the assembly of the microtubules on which the functional activity of neutrophils depends. Conversely, deuterium oxide which stabilizes microtubules inhibits migration (93). Pharmacological agents which inhibit microtubular assembly indirectly by increasing intracellular cAMP also stimulate neutrophil migration in vitro. These observations suggest that the antiinflammatory activity of glucocorticoids is mediated by a peptide hormone which inhibits neutrophil microtubular assembly. Many peptide hormones are believed to act by increasing the concentration of cAMP within target cells, and this mechanism may also be responsible for the inhibitory effect of steroids on phagocytic cells (91). Increased intracellular cAMP is associated with microtubular inhibition, and isoprenaline, theophylline, and dibutyryl cAMP are also found to stimulate "capillary tube" migration by neutrophils (93).

Mononuclear Cells

Freshly harvested monocytes and macrophages will adhere to and subsequently spread on a glass or plastic surface (9). There is evidence that macrophages are derived from peripheral blood monocytes that have undergone further differentiation in tissue. Monocytes in culture will mature to become macrophages following 3-7 days in culture (86). Monocytes or macrophages are usually maintained in stationary culture in glass or plastic petri dishes or tissue culture flasks. Tissue culture flasks are preferred for long term cultivation because they are not subject to evaporation (35). It is difficult to keep macrophages in suspension for long periods of time without extensive aggregation (9).

At present, there is no way to obtain a completely pure population of monocytes from a mixed-cell suspension. The best population that one can obtain is one highly enriched in monocytes. Adherence to glass or plastic allows for the separation of mixed-cell suspensions into adherent (monocyte-rich) and nonadherent (lymphocyte-rich) populations. Cell populations should be depleted of red blood cells and granulocytes prior to adherence by separation on a Ficoll-Hypaque gradient. Resultant cells should be counted and adjusted to a concentration of 2-4 x 10^6/ml in medium containing 5-20% fetal bovine serum. A one hour incubation period at 37 C with 5% carbon dioxide is used for adherence. Weakly adherent monocytes will be lost with this short incubation period. Larger yields require a 20-24 hour incubation period. Following incubation, the adherent cell layer

should be washed 2-3 times with warm medium to remove the non-adherent cells (68).

The types of cells contaminating the enriched monocyte population vary depending on the source of the original cell suspension. B-lymphocytes are known to be adherent, but the presence of higher concentrations of fetal bovine serum (10-40%) in the medium can limit B-cell adherence. Neutrophils are also adherent but survive less than 24 hours in culture. Fibroblasts are another cell type which is adherent but survives only a short time in culture (68). Everything which may come into direct or indirect contact with the monocytes must be sterile. The pH of the medium should be maintained at 7.2 with HEPES or 7.5% sodium bicarbonate. All cultures should be incubated at 37 C in a humidified atmosphere of from 5-7% carbon dioxide in air. Basal media such as MEM or M199 are used, supplemented with L-glutamine, penicillin (100 U/ml), streptomycin (100 ug/ml), and 5-20% fetal bovine serum (35).

Bovine lymphocytes and monocytes cannot be reliably differentiated by light microscopic observation of Wright's-stained blood smears (71). "Nonspecific esterase" is the most reliable cytochemical marker for macrophage or monocyte identification. In hematology, nonspecific esterases refer to those enzymes which hydrolyze acetate or butyrate esters of alpha-naphthol or complex, higher molecular weight, naphthol aniline sauer compounds (46).

Mononuclear phagocytes produce bioactive factors important in regulating functions of other cells (9,25). Such factors include

colony-stimulating activity, plasminogen activator, complement components, pyrogen, lymphocyte-activating factor, and prostaglandins (25). Human monocytes produce leukocytic pyrogen (13). Therefore, cells within the monocyte/macrophage series play a pivotal role in the complex mechanisms of host defenses. They originate ontologically in the yolk sac, and, in the adult, derive from a common stem cell in the bone marrow. The cells undergo a series of developmental steps and seed multiple organ systems. They display a broad range of functional capabilities, including defense against microbial invaders, removal of various forms of debris, participation in the cellular interactions of immunoregulation, secretion of a variety of bioactive materials, and control of neoplastically transformed cells. Cells of the mononuclear series are concentrated in organs of the reticuloendothelial system, which is represented by the spleen, liver, and bone marrow (25).

Vadas et al. found that media conditioned by human monocyte-enriched mononuclear cells stimulated ADCC by neutrophils and eosinophils, and the uptake and killing of Candida albicans by neutrophils. They found that different factors in the media were involved in the neutrophil and eosinophil activation. These factors were produced without intentional stimulation of the monocytes, and their production was prevented by an inhibitor of protein synthesis (94).

Yang and Zucker-Franklin have found that diffusates of human monocytes markedly decreased the killing function of natural killer (NK) cells, and diffusates of neutrophils enhanced killing by NK

cells. Indomethacin did not abolish the suppressive effect of monocyte diffusates, and it did not restore the ability of monocyte diffusate-treated NK cells to conjugate with melanoma targets (95).

Arachidonic Acid Metabolism

Arachidonic acid is a 20-carbon polyunsaturated fatty acid which is the precursor of prostaglandins and other metabolites (37). Arachidonic acid is widely distributed in mammalian tissues, usually incorporated into phospholipids and triglycerides but rarely exists as a free fatty acid. Prostaglandin or leukotriene synthesis must, therefore, be preceded by liberation of substrate (31,38,86). Arachidonic acid is very hydrophobic, so a large portion of it is picked up by the avid lipophilic binding sites on serum albumin (31). As in other cell types, there is essentially no intracellular pool of free arachidonic acid in neutrophils (86). Arachidonic acid is degraded by the so-called arachidonic acid cascade which is comprised of two distinct pathways. In one pathway, it is hydroxylated by fatty acid lipoxygenase which results in the evolution of such hydroxy acids as hydroxyeicosatetraenoic acid (HETE). In the other, it interacts with prostaglandin endoperoxide synthetase (also referred to as fatty acid cyclooxygenase), a membrane-bound multienzyme complex that specifically catalyzes the incorporation of molecular oxygen in polyunsaturated free fatty acids, thereby generating 15-hydroxyprostaglandin endoperoxides (PGG2 and PGH2 if the substrate is arachidonic acid). The liberation of free arachidonic acid causes evolution of prostaglandin endoperoxides and the subsequent formation

of prostaglandins, thromboxanes, prostacyclins, and a multitude of other arachidonic acid metabolites depending on the system under investigation (31,37).

Arachidonic acid metabolism generates three families of compounds with biological activities. The synthesis of two of these, the prostaglandins and the thromboxanes, is initiated by the cyclooxygenase, and the synthesis of the leukotrienes is initiated by the lipoxygenase enzyme. The relative importance of each of these pathways depends on the cell source. In some cells, such as platelets, arachidonic acid is metabolized by both pathways, while in other cells, such as neutrophils, the metabolism of arachidonic acid proceeds predominantly through the lipoxygenase pathway (11,36,43). Arachidonate cyclooxygenase and lipoxygenase products, generated either by leukocytes themselves or by other tissues involved in the inflammatory response, modulate the fundamental leukocyte functions of chemotaxis, phagocytosis, and lysosomal enzyme release. Leukocytes of all types contain arachidonic acid metabolizing enzymes which are activated by non-specific stimuli such as phagocytosis, immune factors, the calcium ionophore A32187, mitogens, endotoxin, or lymphokines (37). In most tissues and cells, fatty acids are incorporated into larger molecules, such as phospholipids, which form an integral part of structures such as cell membranes, and therefore prostaglandin synthesis must be preceded by liberation of substrate by phospholipase activity (37). Many, possibly all, cells release arachidonic acid when they are stimulated with ligands such as

hormones, neurotransmitters, and antibodies. The ligands induce physical and biochemical events in the membranes, which in turn make phospholipids, a major component of biomembranes, accessable to phospholipases, resulting in the release of arachidonic acid (41). The possibility that arachidonic acid metabolites derived from neutrophils may act as regulators of neutrophil function, in particular their movement, has interested scientists for several years (21). Neutrophils respond to the addition of exogenous arachidonic acid and metabolize it through one or more metabolic pathways depending on the species (58). When arachidonic acid is added simultaneously with the chemotatic peptide fMLP, the normal responsiveness of neutrophils to fMLP is inhibited (59).

Platelet activating factor (PAF) is a phospholipid first described as a mediator of anaphylaxis. This factor is released by circulating rabbit basophils. PAF is also produced by a variety of other cells, including human neutrophils. The action of PAF was first restricted to platelet activation, but it also affects neutrophil function, including induction of acute neutropenia, the formation of intravascular neutrophil aggregates, and the release of lysosomal enzymes (45).

Plasma membranes play an essential role in the establishment and the maintenance of the transmembrane conditions necessary for the transmission of biological signals. The lipid moieties of the plasma membrane play, in concert with or independent of the protein constituents, an essential role in many of these biological functions.

Various phospholipids modulate the reactivity of several membrane enzymes such as the ATPases and the transport activity of ion carriers (58). Prostaglandins appear to act by activating adenylate cyclase and raising cAMP levels (86).

The biological effects of arachidonic acid metabolites are species specific. At submicromolar concentrations, exogenous arachidonic acid induces chemotactic, secretory, and aggregatory responses from rabbit neutrophils, but essentially none from human neutrophils. These species differences are thought to be related to the endogenous metabolic activity of the various cell populations. For example, rabbit peritoneal neutrophils metabolize exogenous arachidonic acid to a significantly greater extent than human peripheral neutrophils (58).

Leukocytes are involved in all types of inflammatory, immune, and anaphylactic responses, and arachidonic acid metabolism apparently modulates the development of these reactions. There is extensive evidence that leukocytes are the source of inflammatory mediators derived from arachidonic acid (37). All types of leukocytes have been shown to release a variety of arachidonic acid metabolites in response to various stimuli. Leukocytes are present at all stages in the inflammatory response, and the leukotriene B4 derived from these cells is a putative inflammatory mediator (36). Although in small amounts, neutrophils release prostaglandins and thromboxanes during phagocytosis (36), and it has been shown that rabbit peritoneal neutrophils synthesize prostaglandins of the E and F series, an

activity enhanced by phagocytosis of killed bacteria (37). The major products synthesized by leukocyte cyclooxygenase are PGE2 and thromboxane B2. Phagocytic leukocytes are more avid sources of cyclooxygenase activity than lymphocytes, and both cyclooxygenase and lipoxygenase products found in the early stages of experimental inflammation are derived from neutrophils. Neutrophils appear to be an important source of inflammatory mediators derived from arachidonic acid. These oxygenation products can initiate and sustain both the vascular and the cellular responses characteristic of inflammation (38).

One or more of several lipooxygenases initiate a relatively newly discovered metabolic pathway for arachidonic acid (52,58). The best known of this family are the leukotrienes (30,38,58), a term proposed by Bengt Samuelsson for these compounds to indicate that they were derived from leukocytes and that they possessed a conjugated triene structure (21). Like the prostaglandins, the leukotrienes represent a group of potent arachidonic acid oxygenation products whose actions appear to be largely inflammatory in nature. However, in view of the requirement for recruitment of phagocytic cells to areas of infection and the possible involvement of leukotriene B4 in these processes, it seems likely that, as with the prostaglandins, the leukotrienes may also have important regulatory roles in cell function. The slow-reacting substance of anaphylaxis is representative of leukotrienes and is a mixture of leukotriene D4 and C4 (52). The leukotrienes have been shown to be of critical importance in various immediate

hypersensitivity reactions, to activate mast cells, vascular and pulmonary smooth muscle cells and several types of leukocytes (58). Since the neutrophil is essential for inflammatory processes, involvement of leukotriene B4 in this disease process is indicated (52). Leukotrienes are formed within neutrophils, eosinophils, both resting and elicited rat peritoneal macrophages, pulmonary interstitial macrophages, tissue mast cells and rat and mouse tumor cells after stimulation by a lipooxygenase-dependent oxygenation of arachidonic acid (17,21,54,58). These phagocytic cells are either resident or capable of migrating into tissue loci, which places these proinflammatory mediators at the sites of their effector functions (54). The generated epoxide leukotriene A4 is unstable and converts rapidly to leukotriene B4 by enzymatic hydrolysis (30).

The stereospecificity in the migration, adherence, and secretion responses suggest a physiological role for leukotriene B4. Palmblad et al. hypothesized that leukotriene B4 is generated primarily in order to recruit neutrophils to an inflammatory area and that leukotrienes stimulate neutrophil migration, adherence and secretion. As adherence is critical for attachment to endothelial cells, the first step in the emigration of neutrophils into the tissues and a prerequisite for locomotion, it is noteworthy that leukotriene B4 affects both of these functions in a similar way. Leukotriene B4 does not augment the production of cytotoxic oxygen radicals and causes only a marginal extrusion of lysosomal enzymes (65). Leukotriene B4 is the most chemotactic factor among the lipooxygenase products tested

assay systems (66,70). The non-biologically active isomers of leukotriene B4 were found to be considerably less active than the enzymatically formed leukotriene B4, and 5-HETE and leukotriene C4 did not show an effect on chemotaxis (65). It has been shown that leukotriene B4 is an order of magnitude more active than the mono-HETES and has comparable activity to the most potent chemotactic agents known, such as the complement-derived peptide C5a (21). It is also a potent aggregator of neutrophils and generator of chemiluminescence (54,59,65,66,70). Leukotriene B4 does not affect bactericidal mechanisms or oxidative metabolism (65), but does cause expression of surface C3b receptors in neutrophils (58).

Most of the agonist properties of arachidonic acid towards neutrophils are attributable to the generation of leukotriene B4 (58). It was demonstrated to be a potent chemokinetic and aggregating agent for neutrophils, and is chemotactic for neutrophils. Leukotriene B4 also stimulates the movement of other leukocyte populations in vitro, including guinea-pig eosinophils, rat macrophages and human monocytes. It is a potent mediator of leukocyte movement both in vivo and in vitro, and its presence in rheumatoid synovial fluid shows that it may be an important mediator of inflammation. Leukotriene B4 has similar biological properties to the complement-derived peptide C5a but differs from this compound in that it may be produced by the cell upon which it acts, suggesting that leukotriene B4 may have an intracellular role as a modulator of the action of other stimulators of neutrophil function (21). Leukotriene B4 is a potent and

multifunctional neutrophil stimulus at nanomolar concentrations (58). There is evidence for a role for leukotriene D4 in circulatory functions (52). Catabolic inactivation of the C-6-sulfidopeptide leukotrienes can result from the respiratory burst in human neutrophils (54).

"Classical" chemotactic factors such as fMLP induce, probably in a calcium-dependent manner, the generation and release of leukotriene B4. The fatty acid will then interact specifically with binding sites or receptors on or in neighboring cells. Activating essentially the same events as chemotactic factors, leukotriene B4 then increases calcium mobilization and cellular responsiveness. The essential function of leukotriene B4 would therefore be to be able to amplify or stabilize the chemotactic gradients (21,58). There are no specific inhibitors of leukotriene B4, but the production may be inhibited, at least in part, by steroids through induction of a phospholipase inhibitor (21) which prevents the release of arachidonic acid from phospholipids (38). Nonsteroid antiinflammatory drugs do not inhibit leukotriene synthesis (38).

Thromboxanes are formed by the degradation of cyclic prostaglandin endoperoxides (31). Thromboxane B2 is the major cyclooxygenase metabolite generated by neutrophils, monocytes, macrophages, and lymphocytes (37). It has been shown that stimulation of thromboxane A2 release by agents such as histamine, 5-hydroxytryptamine, and rabbit aorta contracting substance-releasing factor is inhibited by antiinflammatory steroids, and that the steroid

potency in this action closely parallels their antiinflammatory activity. The mechanism of steroid action involves the inhibition of phospholipase A2 activity, and thus of arachidonic acid release (18). The appearance of thromboxane in platelets as a result of arachidonic acid metabolism is accompanied by formation of malonyldialdehyde, a cytotoxic degradation product of the prostaglandin endoperoxide pentanone nucleus, and hydroxylated fatty acids such as 12-hydroxy-8-cis,10-cis-heptadecatrienoic acid (HHT) and hydroxyeicosatetraenoic acid (HETE). The amount of HHT and HETE or prostaglandins formed in any one system is dependent on the relative activity of prostaglandin cyclooxygenase and fatty acid lipoxygenase (31). It has been demonstrated that a number of HETEs have chemotactic activity for neutrophils (21).

Biologically active prostaglandin products may be involved in patho-physiological phenomena such as pain, fever, inflammation, bone resorption, fibroblast activation, and thrombosis. These products also potentiate the vascular effects of other agonists such as histamine or bradykinin. Prostaglandins are able to modify the secretion of mediators, such as histamine from human basophils, histamine and SRS-A from human lung fragments and, in inflammation, lymphokine from lymphoid cells. Some of these phenomena may be related to the potency of E-type prostaglandins and prostacyclin to stimulate adenylate cyclase (56). Membrane receptors have a specific role in inducing the monocyte to secrete prostaglandins, and membrane events are potent inducers of prostaglandin release (67).

The ability of monocytes and macrophages to regulate various aspects of immunologic responses may depend in part on their release of soluble substances such as prostaglandins (27). Macrophages appear to be the major site of prostaglandin synthesis in the immune response. They produce prostaglandins in response to some phagocytic stimuli, as well as in response to lymphokines and other soluble activators. Macrophage-derived prostaglandins have a physiologically important inhibitory role in the immune response (86). Macrophage phospholipids contain a high proportion of arachidonic acid (60). Among human peripheral blood mononuclear cells, only the macrophage and not the T lymphocyte has the capacity to synthesize prostaglandins or thromboxanes (28). The capacity of the human peritoneal macrophage for arachidonic acid metabolism is extensive (20). During phagocytosis macrophages produce prostaglandins, 6-oxo-PGF1a and thromboxanes (23,36). However, Glatt et al. found that PGE2 is the main product released from macrophages while other prostaglandins could only be obtained in trace quantities (23). In inflammed tissue, macrophages are likely to be a main source of inflammatory prostaglandins (6). Macrophages are a rich source of cyclooxygenase activity but stimulated macrophages release less PGE2 and 6-oxo-PGF1a and more thromboxane than do resident macrophages (36,56). This may explain why the concentrations of cyclooxygenase products in inflammatory exudates decrease as the inflammation progresses (36). It is known that inflammatory stimuli induce prostaglandin release from macrophages, but it is unclear how they trigger this event

(6,60). Monocytes in culture release small amounts of PGE into the medium (37,67). Addition of Fc portions of IgG to human monocyte monolayer cultures results in a marked increase in PGE release, and preincubation with indomethacin inhibits the stimulatory effect of both Fc fragments and Con A in human cells (67). Prostaglandin E2 was found to be the major prostaglandin synthesized in culture by human peripheral blood monocytes (27). Human monocytes produce prostaglandins when stimulated with zymosan, endotoxin, or IgG fragments (37).

The suppression of prostaglandin biosynthesis by glucocorticoids is by the "classical" pathway. This mechanism requires the occupation of a cytosol receptor, interaction of the receptor complex with the nucleus, the formation of new mRNA, and the generation of an enzyme or other protein whose specific function is to suppress phospholipase A2 activity. It was observed that this effect involved a short time lag and required intact, metabolically active cells for expression. Glucocorticoids can act rapidly to suppress prostaglandin biosynthesis by many types of cells. Unlike the aspirin-like drugs, they do not do so by inhibiting the cyclooxygenase pathway, but by suppressing the release of the fatty acid substrates necessary for biosynthesis, apparently by blocking phospholipase A2 (19).

Antiphospholipase A2 proteins

Lipomodulin Hirata et al. theorized that the antiinflammatory effects of glucocorticoids might reside in the capacity of cells to synthesize an antiphospholipase A2 protein (43).

Because the products of cyclooxygenase and lipoxygenase are known to have many physiological functions which could lead to inflammatory reactions, the presence of a phospholipase A2 inhibitory protein might provide a control mechanism for the activity of phospholipase A2 and the subsequent availability of its metabolic products. They found that a phospholipase A2 inhibitory protein is induced in many cells, including neutrophils, by glucocorticoids. Glucocorticoids inhibit the release of arachidonic acid from many cells by inducing synthesis of this protein which they named lipomodulin (41). This protein can mimic a variety of the biological activities of glucocorticoids such as antiinflammation, immunosuppression, and promotion of cellular differentiation (40,43) and can control phospholipase A2-mediated events such as lymphocyte mitogenesis, chemotaxis of leukocytes, histamine release of mast cells, desensitization of B-adrenergic receptors in C6 astrocytoma cells, and bradykinin action of fibroblasts. Lipomodulin is easily accessible to pronase, which acts on the outer surface of the cells, and appears to be secreted into the media, as is often the case with glycoproteins. The conditioned media obtained from such cultures are able to block chemotaxis of neutrophils and inhibit pancreatic phospholipase A2 (43).

In experiments using lipomodulin, a highly purified preparation inhibited NK and ADCC activities of human lymphocytes in a dose-dependent manner. The presence of lipomodulin during the early period of the cytotoxicity assay was necessary to obtain maximal inhibition. The inhibition of NK and ADCC activity was greater when effector cells

were treated with lipomodulin than when target cells were incubated with lipomodulin. As lipomodulin did not block binding of effector cells to target cells, it was suggested that lipomodulin inhibits the cytolytic phase of NK and ADCC after binding, and that phospholipases may be involved in NK and ADCC activities (33).

Antilipomodulin antibody can block the effects of both glucocorticoids and lipomodulin, and this is further evidence that lipomodulin is a mediator of glucocorticoid effects (40). Antibodies against lipomodulin were found in patients with rheumatic diseases. The titer of antilipomodulin antibodies was related to the severity of the disease symptoms (41).

When thymocytes are stimulated with Con A, lipomodulin is released into the medium, and it is thought that lipomodulin may be a suppressor T-cell inducing factor. In T-lymphocytes, lipomodulin causes maturation of presuppressor T-cells to suppressor T-cells, a process which has been postulated to be under the control of the major histocompatibility (MHC) genes. Lipomodulin bears the I-J determinant of the MHC gene complex. Since the glucocorticoid susceptibility of prostaglandin formation by mitogen-stimulated lymphocytes has been reported to be controlled by MHC genes, lipomodulin might be one of the MHC gene products (41).

Macrocortin Flower et al. have discovered a steroid-induced factor which mimics the antiphospholipase effects of steroid antiinflammatory agents (18). Antiinflammatory steroids such as dexamethasone are able to induce this exogenous inhibitor of

phospholipase A2, an effect apparently dependent on de novo protein synthesis (31). The factor was named "macrocortin", cortin from corticosteroids and macro because it was first found to be produced by macrophages. Macrocortin is a protein with antiphospholipase activity that is released and generated by macrophages and other cells in the presence of glucocorticoids. Its antiphospholipase activity enables macrocortin to reduce the generation by target cells of prostaglandins, and, by implication, other products of fatty acid oxidation. This protein has a molecular weight of 40,000 (19), and is a second messenger for the effect of steroids on prostaglandin generation (3). Flower found that dexamethasone and hydrocortisone induce the release of macrocortin into the peritoneal cavities of rats. ACTH also causes the release of these proteins in normal but not in adrenalectomized rats. The most likely source of the antiphospholipase proteins found in the first series of experiments was the resident peritoneal leukocyte, mainly the macrophage (2). Macrocortin derived from rat peritoneal leukocytes was later found to be very similar to that released from guinea pig lungs. Macrocortins from both sources have a number of properties in common. Both are stable at 70 C for up to 30 minutes but are destroyed by boiling. Acidification to pH 2 for 20 minutes results in less than 15% loss of activity. There is a substantial loss of activity when material is stirred overnight at 4 C with trypsin or papain (3). Flower proposed that at least some of the antiiflammatory effects of steroids are mediated by the release of macrocortin (15). Peritoneal and alveolar

macrophages from many species, including mouse, guinea pig, rabbit, horse and cow, also seem to secrete a protein similar to macrocortin (17). Flower suggests that macrocortin is an important second messenger for some of the acute antiinflammatory effects of steroids. This may be due solely to the ability of the protein to reduce eicosanoid synthesis or may be due to another, more subtle, effect secondary to inhibition of phospholipase A2. A monoclonal antibody to macrocortin was developed, using a crude mixture of lavage proteins obtained from steroid-treated rats as the immunizing agent, and this antibody reverses the effects of macrocortin (19). The inhibitory effect of macrocortin and the effects of steroids are reversed by the addition of exogenous arachidonic acid. It is possible that macrocortin inactivates membrane phospholipases by interacting with the enzyme from the outside of the cell. Glucocorticoids have no direct effect on these enzymes, but glucocorticoids can prevent prostaglandin and leukotriene synthesis by many intact cells (17).

Renacortin Rothut et al. report that rat renomedullary interstitial cells in culture, when treated with dexamethasone, release two proteins which they call "renacortins". These proteins, one of 15,000 MW and one of 30,000 MW, are both able to inhibit prostaglandin synthesis in untreated cells by an antiphospholipase effect. These researchers feel that the variety of cells which produce antiphospholipase proteins suggests a general physiological phenomenon (77).

Nonsteroidal drugs and arachidonic acid metabolism

Nonsteroidal drugs, such as aspirin, indomethacin, and acetaminophin and other substituted arylacetic acids, are inhibitors of fatty acid cyclooxygenase, the first enzyme in the metabolic conversion of arachidonic acid to prostaglandins (7,18,23,31). These aspirin-like drugs bring about much of their therapeutic action by blocking the prostaglandin forming cyclooxygenase and possibly also lipoxygenase (17). Kaplan et al. presented evidence that indomethacin interferes directly with endoperoxide formation from arachidonic acid by interacting with cyclooxygenase in rabbit neutrophils. They reported that indomethacin at 1.0 uM inhibits phospholipase A2 with apparent specificity because phospholipases from other sources were unaffected at 50 uM (47). Palmblad et al. showed that high concentrations of indomethacin reduced the chemotactic effect of fMLP, as well as the release of enzymes, whereas lower concentrations, inhibiting only the cyclooxygenase, stimulated migration (65). It is possible that the selective inhibition of cyclooxygenase by indomethacin enhances neutrophil migration by diverting substrate towards the production of chemotactic lipoxygenase products (39). However, Morley et al. showed that thromboxane generation in monocytes is inhibited by indomethacin (56). Using an assay system for prostaglandin release in vitro as an assay system for antiinflammatory drugs, Glatt et al. found that both dexamethasone, at a concentration of 10^{-6} to 10^{-7} M, and indomethacin, at a concentration of 10^{-7} to 10^{-8} M, inhibited prostaglandin release (23).

Macrophages and arachidonic acid metabolism

It is increasingly clear from work in animal systems that macrophages are a major source of arachidonic acid metabolites. These cells respond to inflammatory stimuli by the release of arachidonic acid metabolites into extracellular spaces. The in vivo site of macrophage differentiation seems to be important in regulating the quantity of arachidonic acid released by macrophages and the expression of enzymes for the synthesis of specific cyclooxygenase and lipoxygenase products. Activated macrophages have a reduced capacity to release arachidonic acid in response to stimuli. Scott et al. showed that the capacity for metabolism of exogenously supplied arachidonic acid was reduced in activated macrophage populations, implying that the host immune system may regulate the synthesis of proinflammatory lipids by these cells. They found that ^{3}H-arachidonic acid supplied in serum-containing medium is readily taken up by macrophages under conditions of in vitro cultivation. In the absence of a discernable phagocytic or pharmacologic trigger, the incorporated arachidonic acid is retained in cell phospholipids and insignificant amounts are metabolized to cyclooxygenase products. Oxygenation of exogenously supplied arachidonic acid yields qualitatively similar products to those generated from phospholipid stores following a phagocytic challenge, but the relative proportion of products differ considerably. After a 20 minute exposure of resting cells to arachidonic acid, 45% of the products are lipoxygenase and 22% of the products are cyclooxygenase. This ratio is the reverse of that

released by phagocytizing cells. HETEs and prostacyclins constitute the primary arachidonic acid metabolites generated from exogenous arachidonic acid by macrophages (81).

Neutrophils and arachidonic acid metabolism

O'Flaherty et al. found that lipoxygenase-dependent products were the predominant derivatives formed by arachidonic acid stimulated neutrophils. Incubation with arachidonic acid caused a dose and time dependent rise in the numbers of neutrophil aggregates, and blockers of arachidonic acid metabolism inhibited the aggregation response to arachidonic acid. A neutrophil aggregating activity factor was found when neutrophils were preincubated with cytochalasin B, Ca^{++}, and Mg^{++}, then incubated with arachidonic acid. Since the generation, but not the action, of this substance was inhibited by substances which inhibit arachidonic acid metabolism, they felt the substance may be closely related to, or actually be, an arachidonic acid metabolite. Neutrophil aggregating activity factor is stable and appears dependent upon the action of lipoxygenase rather than cyclooxygenase. This conclusion was reached since other workers had shown that lipoxygenase dependent products are the predominant derivatives formed by arachidonic acid stimulated neutrophils. In summary, they found that neutrophil aggregation proceeds by means of arachidonic acid metabolism and the consequential generation of a proaggregating activity which mediated the ensuing response (61).

In another study by O'Flaherty et al., human neutrophil aggregation response to 5,12-dihydrohydroxy-6,8,10,14-eicosatetraenoic

acid (diHETE) and the chemotactic stimuli C5a and fMLP was desensitized by preincubating the cells with small amounts of diHETE. They found that responsiveness to diHETE appears to be necessary for neutrophil aggregation response to C5a and fMLP. Endogenous diHETE, which forms rapidly in cells challenged with stimuli, may mediate their aggregating actions (62). Neutrophils release granule-bound enzymes when challenged under appropriate conditions. They found that the human neutrophil degranulation response to diHETE was completely desensitized by preincubating the cells with small amounts of this same fatty acid. The degranulating actions of C5a, fMLP, the calcium ionophore A32187, 1-0-alkyl-2-0-acetyl-sn-glycero-3-phosphocholine (AAGPC), and phorbol myristate acetate (PMA) appeared to be mediated by an arachidonic acid metabolite because arachidonic acid antimetabolites blocked the degranulation response to these stimuli. Each stimulus caused responding neutrophils to rapidly metabolize their arachidonic acid stores, and arachidonic acid metabolites could also cause degranulation of neutrophils (63).

Addition of dexamethasone to a stirred suspension of granulocytes with or without cytochalasin-B resulted in marked impairment of neutrophil aggregation in response to the chemotactic agent fMLP. Dexamethasone inhibited the ability of fMLP to increase granulocyte adherence to endothelium, but when human lactoferrin was added to the neutrophils exposed to 1.0 mM dexamethasone, the cells were able to adhere as avidly to endothelium as the cells exposed to either fMLP or lactoferrin but free of dexamethasone, which suggests that

dexamethasone has a selective effect on receptor-stimulus coupling. At concentrations found previously to inhibit neutrophil aggregation, hydrocortisone was found to block the release of lactoferrin to a much greater extent than lysozyme or B-glucuronidase from neutrophils upon stimulation with fMLP. The results appeared to show that the products of arachidonic acid metabolism are involved in neutrophil migration, aggregation, degranulation, and oxidative metabolism (64).

Retardation of fusion between neutrophil lysosomal and plasma membranes can limit the access of inflammatory and antimicrobial lysosomal constituents either to surrounding tissues or to ingested microorganisms. The possibility that endogenous monohydroxyeicosatetraenoic acid (HETE) derived from the lipoxygenation of arachidonic acid might serve a role in human neutrophil migration was examined by studying the effects of depletion of the intracellular HETEs on random migration and chemotaxis. The depletion of intracellular HETEs was associated with suppression of human neutrophil random migration and chemotaxis to several stimuli without evidence of cytotoxicity. Maximal suppression of migration was achieved by a 30-60 minute preincubation with the inhibitors of cyclooxygenase and lipoxygenase while inhibitors of cyclooxygenase activity enhanced random migration and chemotaxis to a lesser extent. Incubation with indomethacin at a concentration which inhibits cyclooxygenase, but not lipoxygenase activity, significantly enhanced the random migration of neutrophils. 5,8,11,14-eicosatetraynoic acid, which inhibits the activity of both lipoxygenase and cyclooxygenase,

and nordihydroguaiaretic acid, which preferentially inhibits lipoxygenase activity, significantly suppressed neutrophil random migration and chemotaxis to C5a in a dose dependent fashion with no evidence of cytotoxicity assessed by the trypan blue exclusion test. It was demonstrated that the suppression of neutrophil random migration and chemotaxis by lipoxygenase inhibitors is associated with a concurrent reduction in HETE content in the membranes of human neutrophils and is specifically reversed by the addition of purified neutrophil 5-HETE (24).

Hirata et al. found that when rabbit peritoneal leukocytes were treated with chemoattractants such as fMLP, degradation of methylated phospholipids increased. This fact suggests that the chemoattractant activated phospholipase A2, demonstrating a close association between chemotaxis in leukocytes and the degradation of phospholipids by phospholipase A2 (42).

It was shown that release of lysosomal enzymes induced by C3a, C5a, and fMLP was inhibited by the arachidonic acid antagonist indomethacin. Lipoxygenase products of aracidonic acid are thought to be necessary for changes in the neutrophil plasma membrane with respect to the increase in Ca^{++} permeability seen with chemotactic factors, or could be interfering with locomotion and degranulation because of this blockage. The inhibitory activity of indomethacin in this instance is probably a reflection of the capacity of this reagent to inhibit phospholipase A2 activity at high concentrations (82).

Summary

The neutrophil is a cell which is very important in host defense against invading microorganisms. The neutrophil attacks microorganisms by phagocytosis and ingestion and the bactericidal mechanisms of lysosomal enzymes, the highly reactive oxygen intermediates produced during oxidative metabolism, and fixation of inorganic iodide to bacteria. Neutrophil function can be adversely affected by stress, administration of glucocorticoids, and products of other cell types, including arachidonic acid metabolites.

ALTERATION OF NEUTROPHIL FUNCTION BY FACTORS SECRETED BY NORMAL AND HYDROCORTISONE-TREATED MONOCYTES

Research Objectives

The objective of this research was to elucidate the *in vivo* mechanisms of action of glucocorticoids on bovine neutrophil function. When dexamethasone (.04 mg/kg body weight) is administered intramuscularly (IM) to cattle, several *in vitro* neutrophil functions are adversely affected. Random migration is enhanced and ingestion, oxidative metabolism, the activity of the myeloperoxidase-hydrogen peroxide-halide antibacterial system (as measured by iodination), and the neutrophil's ability to mediate ADCC are all suppressed following *in vivo* dexamethasone treatment (72). When ACTH is given *in vivo*, causing the serum cortisol level to rise as it does during stress, random migration is enhanced and iodination is suppressed (76). The difference in the affects of dexamethasone and ACTH correlates well with the observation that dexamethasone is approximately 30 times more potent than serum cortisol as an anti-inflammatory agent (75).

Phagocytosis and killing of bacteria by neutrophils plays an important role in the body's defense against invading microorganisms. Factors which cause neutrophils to function less effectively leave the animal less able to withstand bacterial invasion. The first phase of experimentation involved examining the direct effect of glucocorticoids on neutrophils. This involved incubating neutrophils in hydrocortisone at various concentrations, and evaluating the effect

of hydrocortisone on neutrophils by means of in vitro neutrophil function assays. The second phase of experimentation involved incubating monocyte-enriched, adherent cell cultures with physiological to pharmacological concentrations of hydrocortisone. The supernatants produced by the monocytes were incubated with neutrophils. The effects of the supernatants on neutrophil function were determined using in vitro neutrophil function assays. The monocytes were found to produce two factors. One factor was found to be produced by resting monocytes during the first 24 hours of culture in medium which did not contain hydrocortisone. This factor enhanced ADCC of neutrophils and had no effect on the other functions tested. A second factor was found to be produced by resting monocytes during the first 24 hours of incubation in medium containing 5.0, 0.5, or 0.05 ug/ml of hydrocortisone. This factor enhanced the random migration of neutrophils, suppressed neutrophil ADCC, and had no effect on the other in vitro functions tested. The third phase of experimentation consisted of attempting to characterize the two monocyte factors which had been produced.

Introduction

This study is part of an ongoing effort to explain in vivo effects of glucocorticoids on neutrophil function in cattle. When cattle are treated in vivo with dexamethasone or have an elevated serum cortisol concentration, several in vitro neutrophil functions are suppressed (72,75,76). Following IM injection of dexamethasone (0.04 mg/kg body weight), neutrophil random migration is enhanced, and

iodination, NBT reduction and ADCC are suppressed (72). After IM injection of ACTH (200 IU IM) which stimulates the adrenal cortex to increase the synthesis and secretion of cortisol, resulting in a rise in the serum cortisol level, random migration is enhanced and iodination is suppressed (71,76). The difference in results may be due to the observation that dexamethasone is known to be 30 times more potent than hydrocortisone (cortisol) as an anti-inflammatory agent (75). As glucocorticoid therapy is common in cattle and stressful situations often exist, an explanation for the mechanism of action of glucocorticoids in cattle could suggest a method for overcoming these effects.

Materials and Methods

Isolation of neutrophils

Neutrophils were isolated as previously described (73). Briefly, bovine peripheral blood was collected into acid-citrate-dextrose solution and centrifuged at 1000 x g for 20 minutes. The plasma and buffy coat were discarded. The packed erythrocytes were lysed by brief exposure to hypotonic conditions and the remaining cells, which generally consisted of approximately 95% neutrophils, were washed in 0.015 M phosphate buffered saline solution (PBSS) (pH 7.2), counted using a Coulter Counter (Model F, Coulter Electronics, Inc., Hialeah, Fla.) and suspended in PBSS at a concentration of 5.0×10^7/ml for incubation with test materials and subsequent functional analysis.

Isolation of monocytes

Monocytes were isolated from bovine peripheral blood which was collected into acid-citrate-dextrose solution and centrifuged at 1000 x g for 20 minutes. The buffy coat was removed by aspiration and diluted 1:1 with PBSS. Thirteen ml of this mixture were layered over 6 ml of 1.077 specific gravity Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mo.) and centrifuged at 450 x g for 40 minutes. The mixed mononuclear cell (MMC) layer was collected by aspiration, washed twice in PBSS at slow speeds to reduce the numbers of platelets in the cell button (170 x g for 10 minutes), counted using a Coulter Counter and standardized to 5 x 10^6/ml in medium. This medium was composed of Medium 199 (Gibco, Chagrin Falls, Ohio) with 10% fetal bovine serum, 1% penicillin-streptomycin solution (KC Biological, Lenexa, Kansas) and 2.4% HEPES (KC Biological, Lenexa, Kansas) as buffer. This medium was used throughout the experimentation and will be referred to as monocyte medium. The isolation and standardization procedures were carried out in siliconized glass screw cap tubes, and sterility was maintained at all times.

Immediately following isolation of the MMC, the monocytes were separated from the other mononuclear cells by an adherence to plastic method (68). Falcon 75 cm^2 (Becton Dickinson Labware, Oxnard, Calif.) or Corning 25 cm^2 (Corning Glass Works, Corning, N.Y.) tissue culture flasks were used, and 5 x 10^6 MMC in 1 ml of monocyte medium were used per 5 cm^2 tissue culture flask area. All incubations during this experimentation were at 37 C in a humidified atmosphere containing 5%

CO_2. After a one hour incubation, the flasks were washed vigorously 3 times with warm M199 to remove the nonadherent cells, and the medium replaced with monocyte medium with or without hydrocortisone (No. H-4001 Cortisol, approximately 98%, Sigma Chemical Company, St. Louis, Mo.) for a 24 hour incubation period. The adherent cells were determined to be approximately 95% monocytes both by morphology and latex bead ingestion. Trypan blue exclusion showed more than 99% viability.

Production of monocyte supernatants

Supernatants were produced both by control cells incubated in monocyte medium which did not contain hydrocortisone and by cells incubated in monocyte medium which contained hydrocortisone at levels which approximated pharmacological to physiological serum levels (5.0, 0.5, 0.05 ug/ml). After a 24 hour incubation, the supernatants were removed from the flasks and frozen in 1 ml aliquots at -70 C until used. Flasks were examined for sterility, percentages of monocytes present, and viability of the cells. The supernatants from any flasks which were found to be contaminated were discarded as were the supernatants from flasks which were found to have low viability. Viability after the 24 hour incubation period was generally better than 95% using Trypan blue exclusion.

Heat and pH treatment of supernatants

To assess the heat stability of the monocyte-produced factors, supernatants with and without hydrocortisone were held at 100 C for 10 minutes and cooled to ambient temperature. To assess for low pH

stability, supernatants with and without hydrocortisone were brought to pH 3 with HCl, held 1 hour, then returned to pH 7.2 with KOH. For assessment of high pH stability, supernatants with and without hydrocortisone were brought to pH 11 with KOH, held 1 hour, then returned to pH 7.2 with HCl.

Incubation of monocytes with actinomycin D and puromycin

Mixed mononuclear cells were isolated from bovine peripheral blood, and monocytes were isolated from the MMC by adherence to plastic as described above. Following the adherence and washing procedures, monocyte medium containing 200 ug/ml of actinomycin D or 20 ug/ml of puromycin was added to the tissue culture flasks to which the monocytes were adhered. The cells were incubated for 1 hour, then an amount of monocyte medium equal to the amount of medium already in the flasks and containing 0.0, 10.0, 1.0, or 0.1 ug/ml of hydrocortisone was added to the flasks. This resulted in a final concentration of 100 ug/ml of actinomycin D, 10 ug/ml of puromycin, and 0.0, 5.0, 0.5 or 0.05 ug/ml of hydrocortisone. After a 24 hour incubation, the supernatants were collected and frozen in 1 ml aliquots at -70 C until used.

Incubation of monocytes and neutrophils with indomethacin

Monocytes were isolated from bovine peripheral blood and adhered to plastic tissue culture flasks as described above. Monocyte medium containing 100 uM indomethacin was added to the flasks for a 1 hour incubation period. Following incubation, the indomethacin-containing medium was removed from the flasks, the cultures were washed with warm

M199, and the medium was replaced with monocyte medium containing 0.0, 5.0, 0.5 or 0.05 ug/ml of hydrocortisone. After the cells were incubated for 24 hours, the supernatant was removed and stored at -70 C in 1 ml aliquots until used. To assay for effects of indomethacin on neutrophil function, 100 uM indomethacin was included with both control media and monocyte supernatants for the 1 hour incubation period prior to running the neutrophil function assays.

Examination of lipid fraction with thin layer chromotography

The lipid fraction of monocyte supernatants from monocytes prelabeled with 5 ul/ml of ^{14}C-arachidonic acid was extracted by a modification of the chloroform-methanol method of Bligh and Dyer (4). Briefly, 1 ml of monocyte supernatant was added to 6 ml of a chloroform-methanol mixture (1:1, v:v), the mixture stirred, refrigerated 18 hours, then centrifuged at 170 x g for 10 minutes. The chloroform phase was removed to a clean glass tube, evaporated to dryness under nitrogen, and reconstituted in 50 ul of ethyl acetate. The lipid-containing ethyl acetate was spotted on silica gel G plates (Analtech, Newark, Del.), and the bands separated using n-Hexane:Ether:Acetic Acid (80:20:1, v:v:v) as a solvent (85). The bands were visualized by autoradiography, scraped into scintillation vials, and the dpm determined for each sample.

Incubation of neutrophils with supernatants

One ml aliquots of 5.0 x 10^7 purified neutrophils suspended in PBSS were dispensed into siliconized sterile glass screw cap test tubes. Two ml of PBSS was added to each tube, the cells were pelleted

by centrifugation at 450 x g for 10 minutes and the supernatants were discarded. One ml of control medium containing 0.0, 5.0, 0.5, or 0.05 ug/ml of hydrocortisone or one ml of the monocyte supernatants which also contained 0.0, 5.0, 0.5, or 0.05 ug/ml of hydrocortisone was added to individual tubes. The neutrophils were suspended by using a vortex mixer, the screw caps loosened slightly and the tubes incubated for 1 hour. A control tube of neutrophils suspended in PBSS was also included. Following incubation, 5 ml of warm PBSS was added to each tube, and the cells were washed by centrifugation at 450 x g for 10 minutes. The neutrophils were resuspended in 1 ml of PBSS and used immediately in the neutrophil function assays.

Incubation of neutrophils with actinomycin D and puromycin

Neutrophils were isolated, standardized, and aliquoted as previously described. One ml of control medium containing 0.0 or 5.0 ug/ml of hydrocortisone or 1 ml of the monocyte supernatant containing 0.0 or 5.0 ug/ml of hydrocortisone and also containing 100 ug/ml actinomycin D or 10 ug/ml puromycin was added to the individual tubes. Following a 1 hour incubation, the neutrophils were washed in PBSS, resuspended to 5 x 10^7 in PBSS and used in the neutrophil function assays.

Neutrophil function assays

Random migration under agarose Evaluation of random migration under agarose was performed as previously described (73). The agar consisted of bicarbonate-buffered Medium 199 with Hank's salts (Gibco, Grand Island, N. Y.) containing 0.8% agarose (Pharmindustrie, Clichy,

France), 10% heat-inactivatd (56 C, 30 minutes) fetal bovine serum and 1% penicillin-streptomycin solution (KC Biologics, Lenexa, Kansas) in 60 x 15 mm tissue culture grade Petri plates (Falcon, Div. Becton, Dickinson and Co., Oxnard, Calif.). Cells were placed into wells cut into the agar, and the plates were placed into an incubator with a humidified 5% CO_2 atmosphere. Eighteen hours later the cells were fixed with 8% glutaraldehyde for 30 minutes, the agar removed from the Petri plate, and the cells adherent to the plastic were stained with modified Wright's stain. The area of migration was determined and expressed in mm^2.

Staphylococcus aureus ingestion Heat-killed ^{125}I iododeoxyuridine-labeled S. aureus cells are used in the antibody facilitated ingestion assay at a bacteria-to-neutrophil ratio of 60:1 as previously described (73). ^{125}I-labeled S. aureus, bovine anti-S. aureus serum and neutrophils (2.5×10^6) in a total volume of 0.5 ml of Earle's balanced salt solution were incubated together for 10 minutes at 37 C. The extracellular S. aureus was removed by lysostaphin treatment for 30 minutes at 37 C. The cells were washed twice in PBSS, and the neutrophil associated radioactivity was determined in a gamma counter. The results were expressed as the percentage of the total S. aureus ingested per test tube.

Iodination The iodination procedure evaluated the activity of the myeloperoxidase-hydrogen peroxide-halide antibacterial system as previously described (73). The standard reaction mixture for the determination of stimulated iodination contained 2.5×10^6

neutrophils, 0.05 uCi ^{125}I, 40 nmole NaI, and 0.5 mg of opsonized zymosan in 0.5 ml of Earle's balanced salt solution. For the determination of resting iodination, the opsonized zymosan was omitted. The mixture was incubated for 20 minutes at 37 C with end-over-end rotation. Following the incubation, the reaction was stopped by addition of 2.0 ml of cold 10% trichloroacetic acid. The resulting precipitate was washed one additional time in 10% trichloroacetic acid, and the amount of radioactivity in the precipitate was determined in a gamma counter. The results were expressed as nmole of NaI/10^7 neutrophils/hour.

Cytotoxicity The ADCC assay was performed utilizing ^{51}Cr-labeled chicken red blood cells (CRBC) as target cells (74). The ADCC reaction mixture contained 1.25 x 10^5 ^{51}Cr-labeled CRBC and 1.25 x 10^6 neutrophils (effector:target ratio of 10:1) in 0.3 ml of Medium 199 containing 2.5% bovine anti-CRBC serum. The assay was conducted in triplicate using a microtiter procedure. Triton-X controls, antibody controls, and neutrophil controls were included. After a 2 hour incubation at 37 C in a humidified 5% CO_2 atmosphere, the supernatants were harvested by absorption into filter pads using a microtiter supernatant collection system. The radioactivity in the fiber pads was determined in a gamma counter. The results were expressed as percent of specific release, and were calculated using the following formula: Percent specific release = (mean cpm test)-(mean cpm control)/(mean Triton-X treated cpm)-(mean cpm control) x 100. To determine the cytotoxic activity of the neutrophils in the absence of

specific antibody, the same procedure was used, except the anti-CRBC serum was replaced with fetal bovine serum.

Nitroblue tetrazolium reduction Nitroblue tetrazolium (NBT) reduction by neutrophils was quantitatively determined as previously described (73). 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 x 10^6 neutrophils, 0.1 ml of preopsonized zymosan, and sufficient Earle's balanced salt solution to bring the volume to 1.0 ml. All of the reactants, except neutrophils, 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 neutrophils, and was allowed to proceed with periodic shaking for 10 minutes. The reaction was stopped by adding 5.0 ml of cold PBSS. The cells and insoluble purple formazan formed by the reduction of NBT were pelleted by centrifugation and suspended in 5.0 ml of pyridine. 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 (580 nm) of the supernatant fluid was immediately determined in a spectrophotometer, using a pyridine blank. The results are reported as OD/5.0 x 10^6 neutrophils/10 minutes in 5.0 ml of pyridine. The results represent the average values of duplicate determinations.

Results

To assess the effects of medium with hydrocortisone and monocyte supernatants with and without hydrocortisone on neutrophil function, a

fixed number of neutrophils (5.0×10^7) was incubated for 1 hour in 1 ml of the appropriate supernatant or medium. Following the incubation, the neutrophils were washed by centrifugation at 450 x g for 10 minutes in 5 ml of PBSS, resuspended in 1 ml of PBSS, and used immediately in the neutrophil function assays.

As can be seen in Fig. 1, the functions of neutrophils incubated in supernatant from untreated monocytes were found to have an enhanced ability to mediate ADCC. Supernatant from untreated monocytes did not significantly affect the other neutrophil functions. Neutrophils incubated in monocyte supernatant containing 5 ug/ml of hydrocortisone were found to have enhanced random migration, and suppressed ability to mediate ADCC when compared to neutrophils incubated in supernatants from untreated monocytes. The other functions were not affected. Neutrophils incubated in medium containing 5 ug/ml hydrocortisone also had enhanced random migration, but not to the extent of those incubated in hydrocortisone-containing monocyte supernatant.

Figs. 2 and 3 show the results obtained with ADCC and random migration assays when 3 dilutions of hydrocortisone were contained in the medium used to incubate neutrophils and in the medium used to produce monocyte supernatants. Both the ADCC suppression and random migration enhancement were found to be affected in a concentration-dependent manner. The enhancement of ADCC by monocyte supernatant was blocked by incubating the monocytes in 5.0 or 0.5 ug/ml of hydrocortisone but not by 0.05 ug/ml of hydrocortisone. Random migration was enhanced when neutrophils were incubated directly with

only the highest dosage of hydrocortisone, however there was significant enhancement when both 5.0 and 0.5 ug/ml of hydrocortisone are used in the production of monocyte supernatants. There was no significant enhancement of random migration when neutrophils were incubated with supernatant from monocytes treated with 0.05 ug/ml of hydrocortisone.

Supernatants were prepared from the non-adherent mononuclear population in the same way as shown for the adherent population. Neutrophils were incubated in these supernatants, and the results compared to those from neutrophils incubated in medium with and without hydrocortisone. Supernatant from the non-adherent mononuclear cell population did not produce these same affects (data not shown).

In order to determine whether protein synthesis was required by the monocyte to produce the 2 factors found, monocytes were incubated with 100 ug/ml actinomycin D (RNA synthesis inhibitor) or 10 ug/ml puromycin (protein synthesis inhibitor). These inhibitors were contained in the medium with or without hydrocortisone for the 24 hour incubation period. Table 1 shows the results obtained when neutrophils were incubated in the resultant supernatants and when 100 ug/ml actinomycin D and 10 ug/ml puromycin were included when neutrophils were incubated in untreated monocyte supernatants. Protein synthesis was not found to be necessary for production of the ADCC enhancing factor by the monocyte nor for the neutrophil to respond to it. However, it was found that protein synthesis was required by the neutrophil for response to the ADCC suppressing and

random migration enhancing factor. It could not be determined if protein synthesis was required for the monocyte to produce this factor because the actinomycin D and puromycin were still in the monocyte supernatant when it was put on neutrophils.

Table 2 shows the results obtained when neutrophils were incubated in monocyte supernatants which had been heat- or pH-treated. Heat treatment destroyed the ADCC enhancing activity found in untreated monocyte supernatants. The enhancement of random migration of neutrophils incubated in supernatants from hydrocortisone-treated monocytes was reduced when the supernatants were heat-treated. Both acidifying and alkalinizing supernatants from untreated monocytes somewhat reduced the ADCC enhancement. Alkalinizing supernatants from hydrocortisone-treated monocytes reduced the enhancement of random migration to a small extent, but acidifying these supernatants did not affect their ability to enhance random migration.

Because the monocyte-produced ADCC enhancing factor did not appear to be a newly-synthesized protein and arachidonic acid oxidation products are important in initiating and propagating inflammation, the effect of hydrocortisone on arachidonic acid metabolism of monocytes was examined. Table 3 contains the results obtained when monocytes or neutrophils were treated with 100 uM indomethacin. Supernatants both with and without hydrocortisone from monocytes preincubated with indomethacin greatly enhanced the neutrophil's ability to mediate ADCC. When neutrophils were incubated with indomethacin and in untreated monocyte supernatants, the ability

of neutrophils to mediate ADCC was only slightly enhanced.

Because the indomethacin inhibition studies suggested that arachidonic acid metabolism might be involved with ADCC enhancement, the monocyte supernatants were examined for arachidonic acid and its metabolites. The lipid fraction was extracted from supernatants produced from monocytes prelabeled with ^{14}C-arachidonic acid, thin layer chromatography was used, and the bands visualized with autoradiography. Six bands were found. Bands 2-5 contained 98% of the radioactivity and are shown in Fig. 4. Band 4 comigrated with the arachidonic acid standard and is significantly increased in supernatants from monocytes incubated with hydrocortisone. Bands 2 and 3 show significantly higher amounts of radioactivity in the supernatants from monocytes incubated without hydrocortisone.

Discussion

Fig. 5 depicts in simplified form the results drawn from the data so far. The monocyte incubated in medium produces a factor tentatively called monocyte-produced ADCC enhancing factor (MAEF) which increases ADCC in neutrophils but does not affect the other functions evaluated. RNA and protein synthesis are not required for production of the factor by the monocyte or response to it by the neutrophil. The monocyte incubated with hydrocortisone produces a factor tentatively called hydrocortisone-induced monocyte factor (HIMF) which stimulates production of protein in neutrophils resulting in suppression of ADCC and enhancement of random migration. When HIMF is inhibited, MAEF is again expressed, suggesting MAEF is also

produced by the hydrocortisone-treated monocyte.

The results show that resting monocytes produce a substance (MAEF) during the first 24 hours of culture which when incubated with neutrophils for 1 or 3 hours (results shown for 1 hour incubation only) causes an enhancement of ADCC by these neutrophils but has no effect on the other neutrophil functions tested. Incubation of the neutrophil with actinomycin D or puromycin at the same time as incubation with monocyte supernatant does not prevent ADCC enhancement by the neutrophil. Preincubating monocytes with actinomycin D or puromycin for 1 hour before adding the incubation medium did not prevent the synthesis of this product, therefore this product is not a newly-synthesized protein but may be a preformed protein. Protein synthesis by the neutrophil is apparently not necessary for this factor to affect the neutrophil.

This ADCC enhancing factor or MAEF may be an arachidonic acid metabolite. Preincubation of the monocyte with 100 uM indomethacin not only did not suppress the synthesis of this substance, but apparently enhanced its production (Table 3). Indomethacin inhibits the cyclooxygenase pathway at low doses and both the cyclooxygenase and lipoxygenase pathways at high doses in other species (37), but it is not known whether or not this is true in the bovine. Possibly only the cyclooxygenase pathway was inhibited. Indomethacin (100 uM) enhanced production of MAEF and may have enhanced production of lipoxygenase products. Leukotrienes and HETEs, lipoxygenase metabolites, are known to be aggregators of neutrophils (61,62,70), an

event which occurred if the neutrophils were incubated with the monocyte supernatants for longer than 1 hour (results not shown). The results indicate that MAEF is not a cyclooxygenase product, may be a lipoxygenase product, or it may not be an arachidonic acid metabolite at all but a product whose production is regulated by arachidonic acid metabolism. After the indomethacin preincubation of monocytes, the supernatant produced did not have either an enhancing or suppressive effect on neutrophil random migration.

Monocytes in culture medium containing hydrocortisone produce a factor (HIMF) during the first 24 hours of culture which causes an increase in neutrophil random migration and a suppression of neutrophil ADCC. The neutrophil must be able to synthesize proteins in order for the effects to be expressed. Because the actinomycin D or puromycin used to preincubate monocytes were still present in the supernatant when it was used to incubate the neutrophils, it could not be determined if protein synthesis by the monocyte is necessary for production of HIMF. When neutrophils were preincubated with actinomycin D or puromycin and when monocytes were preincubated with the RNA and protein inhibitors the effects of HIMF were lost, and thus it could be determined that protein synthesis was necessary for the neutrophil to respond to HIMF. The hydrocortisone-treated monocyte continues to produce MAEF, the neutrophil ADCC enhancing factor. HIMF seems to override the effects of MAEF. When the response to HIMF is eliminated by inhibition of RNA and protein synthesis by the neutrophil, MAEF is expressed and ADCC becomes enhanced in neutrophils

incubated in supernatant from hydrocortisone-treated cells (Table 1). Indomethacin does not block the production of HIMF (Table 3), thus, HIMF is not a cyclooxygenase product.

The mechanism of action of glucocorticoids on neutrophil function is not clear. Various investigators have reported that glucocorticoids cause an inhibition of phospholipase A2 activity in the neutrophil membrane resulting in decreased release of arachidonic acid from the cell membrane upon stimulation and decreased production of arachidonic acid metabolites (1,2,3,19,40,42,43). These metabolites are important in initiation and regulation of neutrophil function. The results of this study show that hydrocortisone has an effect on the arachidonic acid metabolism of monocytes. Using thin layer chromatography, it was shown that there were changes in the arachidonic acid metabolites present in the supernatants from monocytes incubated with or without hydrocortisone. With the solvent which was used here, six distinct bands were found on autoradiography. The upper and lower bands showed very little radioactivity and were discounted. The four middle bands accounted for more than 90% of the radioactivity found. Band 4 comigrated with the arachidonic acid standard, and is therefore probably arachidonic acid. The large increase in the amount of arachidonic acid present in the supernatant of hydrocortisone-treated monocytes may have been due to decreased uptake of free arachidonic acid by hydrocortisone-treated monocytes or to an inhibition of arachidonic acid metabolism by hydrocortisone. Hydrocortisone significantly reduced the amount of radioactivity in

Bands 2 and 3 of the monocyte supernatants. These bands did not comigrate with the PGE^1 or PGE^2 standards used. Other metabolites may have been present in the supernatant, but failed to separate with this solvent system.

The composition of the monocyte products is not known, nor is it known if the monocytes are being stimulated to synthesize the products or if they are releasing preformed substances. The factor released by the hydrocortisone-treated monocytes has characteristics that are different from macrocortin or lipomodulin. These latter two factors are produced by a variety of cells when incubated with glucocorticoids and are proteins which inhibit phospholipase A2 (1,2,19,42,43). They are similar proteins, very likely the same substance, which have been found by two different groups of researchers. Flower et al. showed that de novo protein synthesis is not necessary in lung cells for there to be a response to macrocortin (19). However, neutrophils must be able to synthesize proteins in order to respond to HIMF. The neutrophil protein induced by HIMF could be the lipomodulin described by Hirata et al. Neutrophils are affected after only a 1 hour incubation with monocyte supernatant, but Hirata et al. reported that neutrophils required a 16 hour incubation with glucocorticoids before an effect was noted (43). The neutrophil preparations used by Hirata et al. contained 10% mononuclear cells (42). It may be that the 16 hour incubation period was needed in order for the monocytes in the cell culture to produce a factor which induced lipomodulin synthesis in the neutrophils. An interesting comparison between lipomodulin and

our neutrophil-produced factor is that lipomodulin from rabbit neutrophils treated with glucocorticoids, inhibited NK and ADCC activities of human peripheral blood lymphocytes in a dose-dependent manner (33). This effect is similar to the neutrophil ADCC suppressing factor induced in neutrophils by HIMF in this study.

When dexamethasone is given intramuscularly to cattle, the function of the neutrophils of those animals is affected. Random migration is enhanced, while iodination, NBT reduction and cytotoxicity are suppressed. A neutrophilia also results from treatment of cattle with steroids (72). When ACTH is given to cattle, resulting in an increase in serum cortisol, random migration is enhanced, iodination is suppressed, and the other neutrophil functions are unaffected. This correlates with the observation that dexamethasone is approximately 30 times more potent as an anti-inflammatory agent than cortisol (75). The serum cortisol level produced by ACTH treatment of cattle has been reported to average about 0.08 ug/ml which falls between the 0.5 and 0.05 ug/ml levels used in this study (76). The use of dexamethasone in vivo or 5.0 ug/ml of hydrocortisone in vitro would both be considered pharmacologic levels of glucocorticoid (72).

The in vivo treatment results differ from the in vitro effects we have found. The iodination assay is consistently suppressed by in vivo treatment with dexamethasone or ACTH. No effect on iodination was found when neutrophils were incubated directly in either dexamethasone or hydrocortisone for periods ranging from 1 to 24 hours

(only the results from 1 hour incubation with hydrocortisone are shown). We propose that glucocorticoid effects on neutrophils may be mediated by products of other cell types, rather than being direct effects on the neutrophil itself. The results presented in this study indicate that monocytes may mediate some, but not all of the suppressive effects which glucocorticoids have on neutrophils. Further characterization of the two factors described may give insight into the mechanism of glucocorticoid suppression of neutrophil function.

Summary

Hydrocortisone (5.0, 0.5, or 0.05 ug/ml) when incubated for 1.0 or 3.0 hours with neutrophils isolated from bovine peripheral blood had no significant effect on the ability of neutrophils to ingest S. aureus, reduce nitroblue tetrazolium (NBT), iodinate protein, or mediate antibody-dependent cell-mediated cytotoxicity (ADCC) against chicken erythrocytes. Random migration was significantly enhanced by the highest concentration of hydrocortisone, but was unaffected by the lower concentrations. Bovine monocytes were incubated for 24 hours in medium with or without added hydrocortisone (5.0, 0.5, or 0.05 ug/ml). Purified bovine neutrophils were then incubated for 1.0 or 3.0 hours with the resulting monocyte supernatant fluids. The supernatant from control monocytes significantly enhanced neutrophil ADCC but did not affect the other neutrophil functions evaluated. Supernatants from monocytes incubated with hydrocortisone produced a concentration-dependent blockage of the monocyte enhancement of neutrophil ADCC and

an enhancement of neutrophil random migration under agarose. The other parameters of neutrophil function were unaffected. The results indicate that the monocyte may mediate the enhancement of neutrophil random migration and inhibition of neutrophil ADCC which occurs in glucocorticoid-treated cattle.

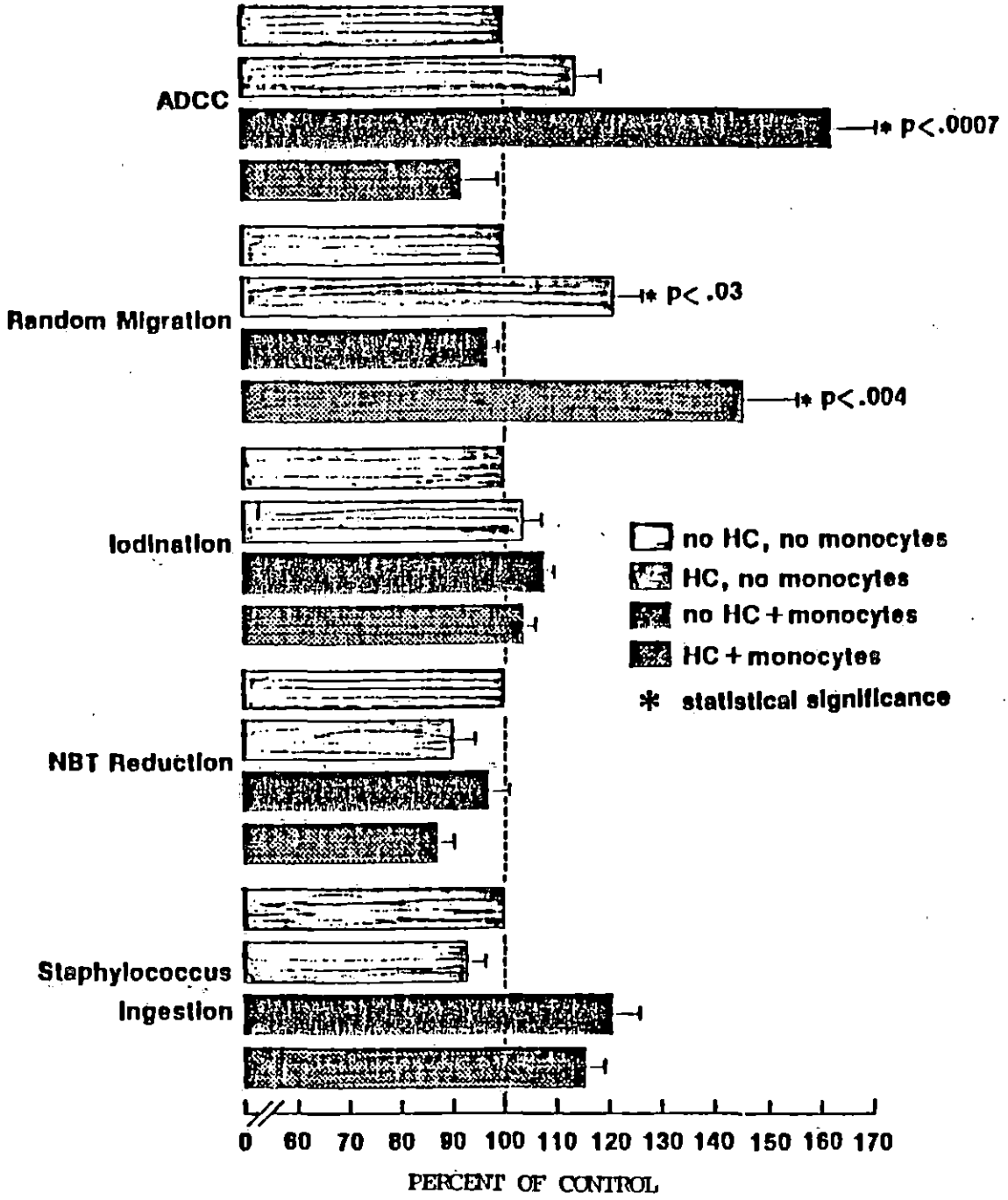


Fig. 1. Effects of incubation in medium containing 0.0 or 5.0 ug/ml hydrocortisone on various aspects of bovine neutrophil function[a]

[a]Results are expressed as a percent of the value for neutrophils incubated in medium alone (control) ± SEM. $n \geq 30$.

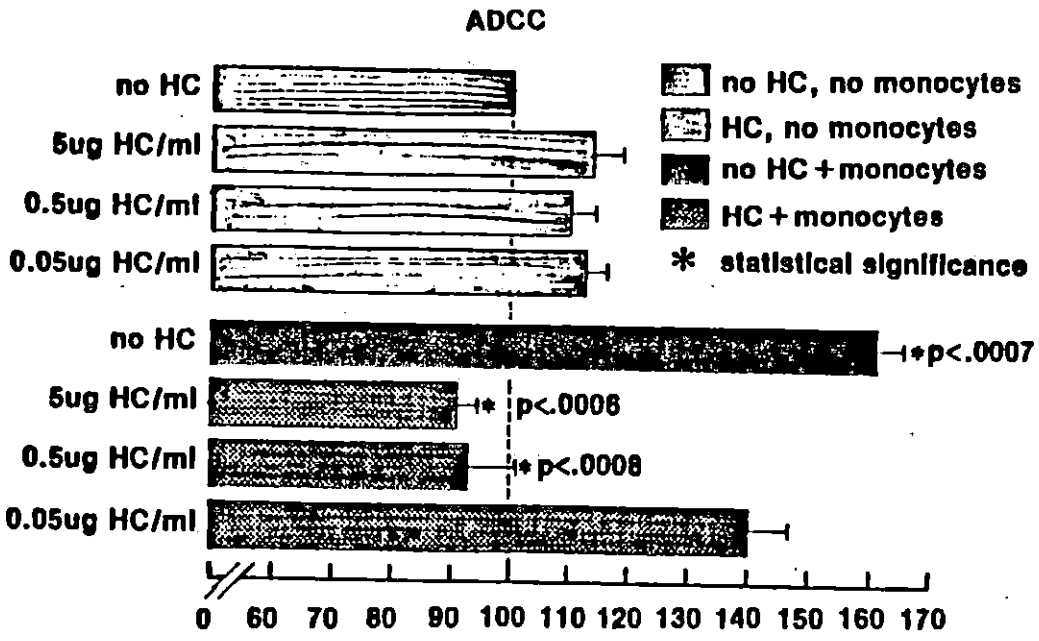


Fig. 2. Effects of incubation in medium or monocyte supernatant and three concentrations of hydrocortisone on bovine neutrophil-mediated ADCC[a]

[a]Results are expressed as a percent of the value for neutrophils incubated in medium alone (control) $\pm$ SEM. $n \geq 30$.

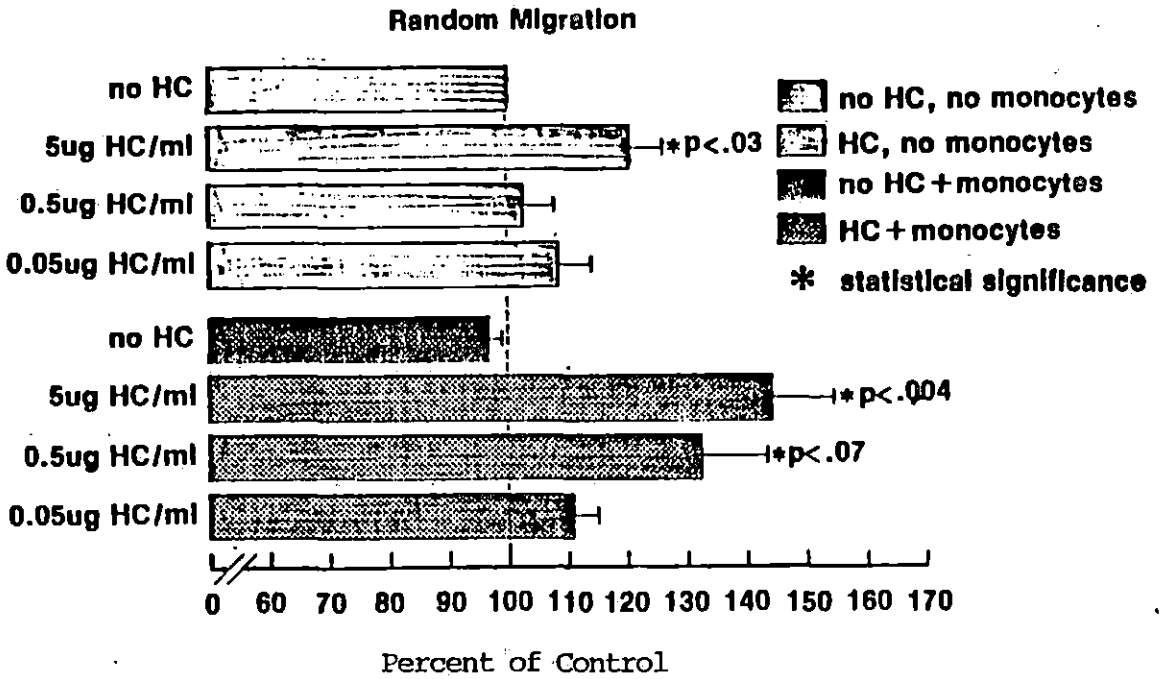


Fig. 3. Effects of incubation in medium or monocyte supernatant and three concentrations of hydrocortisone on bovine neutrophil random migration[a]

[a]Results are expressed as a percent of the value for neutrophils incubated in medium alone (control) ± SEM. $n \geq 30$.

Table 1. Effects of preincubation of neutrophils with inhibitors of RNA and of protein synthesis[a]

	ADCC		Random Migration	
Neutrophil incubant	0.0 ug/ml Hydrocortisone	5.0 ug/ml Hydrocortisone	0.0 ug/ml Hydrocortisone	5.0 ug/ml Hydrocortisone
Medium	100 percent	98.2 ± 13.0	100 percent	123.3 ± 7.1
Monocyte supernatant	154.3 ± 13.5	115.7 ± 8.2	88.8 ± 6.8	136.7 ± 9.7
Supernatant from monocytes incubated				
with Actinomycin D	150.9 ± 34.4	133.2 ± 33.2	88.7 ± 7.7	90.3 ± 7.8
with Puromycin	133.5 ± 34.6	147.1 ± 32.2	107.4 ± 9.6	113.1 ± 12.6
PMNs preincubated with				
Actinomycin D and supernatant	146.1 ± 6.1	136.2 ± 13.3	91.1 ± 13.1	109.4 ± 20.2
Puromycin and supernatant	154.9 ± 10.3	157.3 ± 9.8	75.8 ± 6.0	103.9 ± 11.2

[a]Results are expressed as a percent of the value for neutrophils preincubated in medium alone (control) ± SEM. n=18

Table 2. Results of incubation of neutrophils with monocyte supernatants which have been heat- or pH-treated[a]

	ADCC		Random Migration	
	0.0 ug/ml Hydrocortisone	5.0 ug/ml Hydrocortisone	0.0 ug/ml Hydrocortisone	5.0 ug/ml Hydrocortisone
Medium	100 percent	98.2 ± 13.0	100 percent	123.3 ± 7.1
Monocyte supernatant	154.3 ± 13.5	115.7 ± 8.2	88.8 ± 6.8	136.7 ± 9.7
Monocyte supernatant				
Heat-treated	102.0 ± 5.3	84.6 ± 6.7	98.3 ± 2.6	122.4 ± 4.8
pH-11-treated	120.0 ± 11.1	102.7 ± 10.0	98.7 ± 10.9	129.5 ± 6.8
pH-3-treated	127.7 ± 12.4	100.1 ± 7.4	97.8 ± 7.7	140.3 ± 9.4

[a]Results are expressed as a percent of the value for neutrophils preincubated in medium alone (control) ± SEM. n=18

Table 3. Results of incubation of neutrophils with supernatants from monocytes preincubated with indomethacin and of neutrophils incubated with indomethacin and monocyte supernatants[a]

	ADCC		Random Migration	
Neutrophil incubant	0.0 ug/ml Hydrocortisone	5.0 ug/ml Hydrocortisone	0.0 ug/ml Hydrocortisone	5.0 ug/ml Hydrocortisone
Medium	100 percent	98.2 ± 13.0	100 percent	123.3 ± 7.1
Monocyte supernatant	154.3 ± 13.5	115.7 ± 8.2	88.8 ± 6.8	136.7 ± 9.7
Supernatants from monocytes incubated with Indomethacin	183.4 ± 7.2	169.0 ± 8.0	90.9 ± 7.5	151.1 ± 7.1
Neutrophils incubated with Indomethacin and monocyte supernatant	122.1 ± 15.5	131.0 ± 24.9	75.2 ± 4.2	125.9 ± 14.7

[a]Results are expressed as a percent of the value for neutrophils preincubated in medium alone (control) ± SEM. n=18

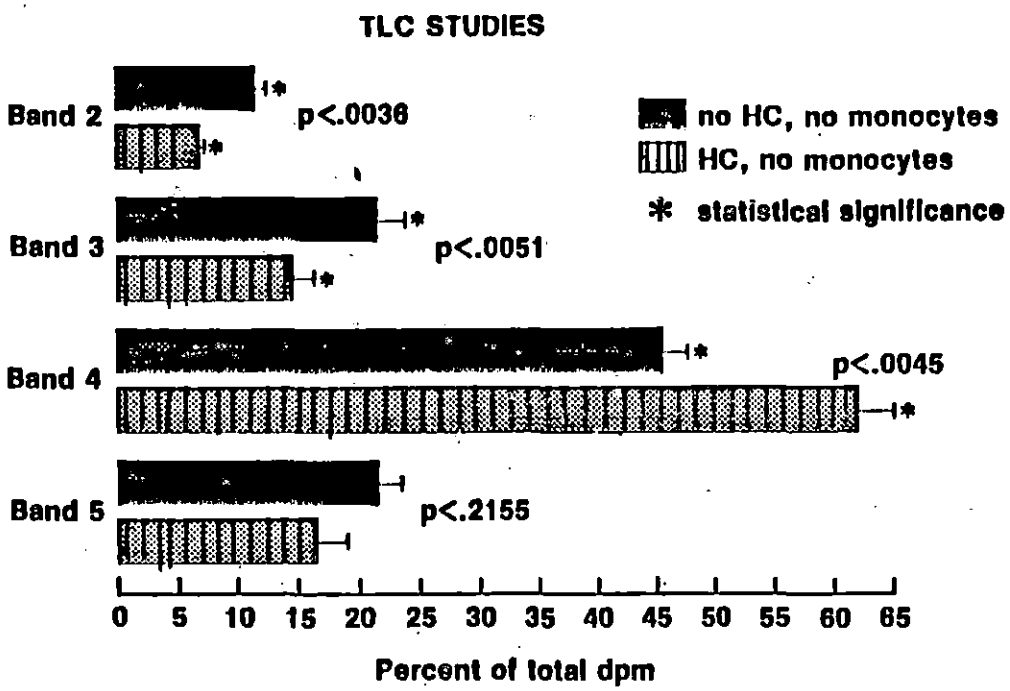
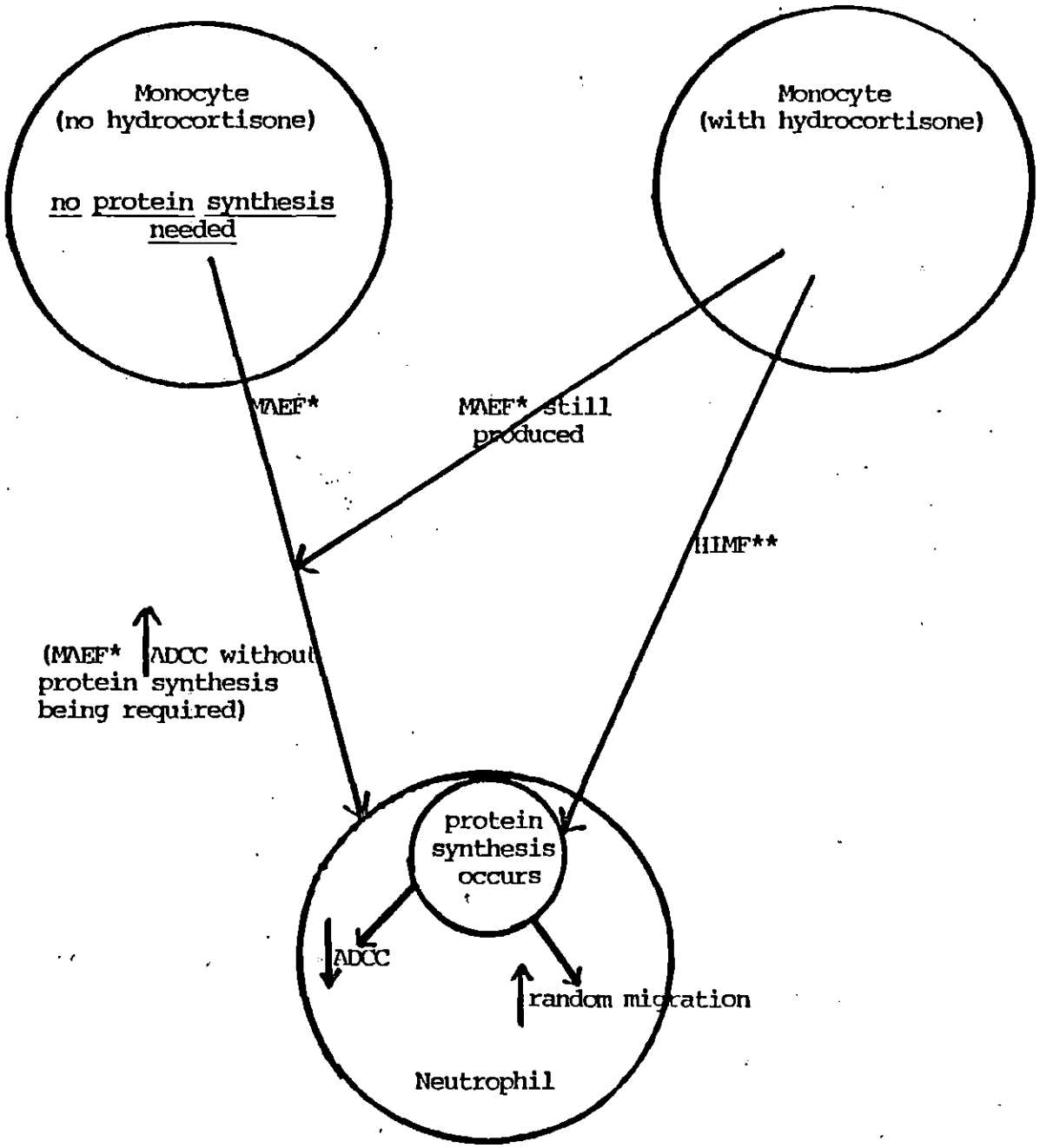


Fig. 4. Results of studies on the lipid fraction of monocyte supernatant containing 0.0 or 5.0 ug/ml of hydrocortisone[a]

[a]Results are shown as a percent of the total dpm ± SEM. n=6.



* MAEF - monocyte-produced enhancing factor
**HIMF - hydrocortisone-induced monocyte factor

Fig. 5. The conclusions from the data are summarized in the diagram above

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