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ROLE OF INNATE DEFENCE MECHANISMS IN ACUTE PHASE RESPONSE AGAINST GRAM-NEGATIVE AGENTS

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Summary

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The role of innate defence mechanisms - humoral and cellular, in a Gram-negative infection is studied. The species-related contradictions about the changes in lysozyme, complement and phagocyte activities and the possibility for using them as important indicators for the severity of disease are discussed.

A particular attention was paid to microbicide mechanisms of phagocytes - oxygen radicals and nitric oxide as well as to the regulatory mechanisms for prevention of the negative effects due to their pathological increase.

The key role of cytokines for the formation of local and generalized manifestations of systemic acute phase response after a Gram-negative infection is outlined too.

Key words: lipopolysaccharide, complement, lysozyme, phagocytes, oxygen radicals, nitric oxide

INTRODUCTION

The infection agent provokes a complex set of disorders at molecular, cellular and metabolic level, associated with hormonal, functional and immunological systemic transformations, characteristic for the acute phase of infection (Ballmer et al., 1991; Van Miert, 1991).

A natural result of this phase is the mobilization of the early mechanisms of defence - cellular and humoral, that ensure the control upon the infection until the induction of adaptive immunity elements (Hoffmann, 1980; Paape et al., 1985; Van Deventer et al., 1990; Holmskov et al., 1994). Therefore, the effective function of the early defence mechanisms is crucial for systemic resistance. The knowledge of their principles, functional status and regulatory potential during the infection is an important prerequisite for determination of the approach for the early diagnostics and purposeful therapy.

On the other part, infection-induced acute phase response could influence significantly the absorption, disposition, metabolism and excretion of drugs, applied during this stage, to increase their toxicity or decrease their therapeutic efficacy (Monshouwer, 1996).

GRAM-NEGATIVE AGENTS AND HUMORAL FACTORS OF THE INNATE IMMUNITY

The key molecule during the realization of Gram-negative infection is the lipopolysaccharide (endotoxin) (LPS) that accomplishes the communications between the

cell and the structures of the host (Rietschel et al., 1994).

Van Leeuwen et al. (1994) showed that normally, in blood there are negligible amounts of LPS coming from the intestinal microflora via the portal vein or the lymphatic system. This translocation appears to be an important phenomenon stimulating the monocyte-macrophageal system. During an inflammation of intestinal mucosa, LPS could enter the blood circulation and to provoke an endotoxaemia (Van Deventer et al., 1988). In 1990, Van Deventer et al. proved that LPS, infused in various doses, triggers a series of reactions that determine the acute phase response (Baumann & Gauldie 1994; Van Miert 1991, 1995; Olson et al., 1995; Werling et al., 1996). Among the features of the latter are many non-specific protective defence reactions to disease – anorexia, somnolence, fever and changes in the metabolic profile of many tissues, organs and systems (Lohuis et al., 1990; Olson et al., 1995; Pannen and Robotham 1995).

An important part in the multiple events, involved in the systemic acute phase response following challenge with Gram-negative agents, is the activation of humoral and cellular components of the innate immunity.

The complement has a primary role in defence mechanisms against Gram-negative infections. In patients with sepsis, Kushner and Mackiewicz (1993) observed decreased haemolytic activity of complement while McCabe et al. (1983) registered a higher activity of $C_{3a,}$ $C_{4a,}$ and C_{5a} complement fragments. The endotoxin activates both the alternative complement pathway (related to the innate resistance) and the classical pathway (Wilson and Morrison, 1982).

As a result of the activated complement system, a bacterial membranolysis occurs, several biologically active substances – histamine, kinines, interleukins, prostaglandins are realeased. Grondahl et al. (1997) reported a significant reduction of phagocytosis during complement inactivation while Bernoco et al. (1987), Hietala and Ardans (1987) evidenced that neutrophil activity was directly related to complement activity.

In patients with congenital C3 deficiency, opsonization disorders that could be corrected *in vitro* via addition of C3 component of the complement, are present (Komatsu et al., 1988). Fresh plasma could be a source of C3 but there are no studies reporting any noticeable clinical effect of its administration.

In body fluids, *lysozyme* (*muraminidase*) is active against the microorganisms. It possesses a clear lytic effect against Gram-positive bacteria. Studies on bacteriolytic properties of lysozyme (Paape et al., 1985) showed that it is active not only against Gram-positive, but against Gram-negative bacteria among which *E. coli* was found to be sensitive. A source of this important humoral factor of defence are probably phagocytes, where it participates together with other hydrolytic enzymes in the destruction and opsonization of ingested bacteria. Therefore, the suggestion of Gordon et al. (1974) that lysozyme could be a parameter of the functional status of circulating neutrophils seems rational. During *in vitro* studies however, Goranov (1978) observed lysozyme activity only in neutrophils from the peripheral blood in laboratory animals (rats, mice, rabbits, guinea pigs) but not in farm animals.

Despite the contradictory data about the changes in serum lysozyme concentrations originating probably from species-

related differences, used methodo-logy and the kind of infection itself, it is obvious that this humoral component of innate immunity is very important for mucosal defence.

The coagulation system is another rapidly reacting element of innate immune system. In endotoxaemia, Collatos et al. (1994) reported an increased blood coagulation capacity. The same event is present in viral infections in the presence of circulating immune antigen-antibody complexes. According to Todoroki et al. (2000), neutrophils are the source of a factor that activates directly the coagulation system. The regulation of blood coagulation and fibrinolysis systems is very complex as there are many possibly influencing factors – Na^{2+}, cytokines etc. The endotoxin activates the elements of blood coagulation (Tsilibarry and Wissing 1977), as well as protease inhibitors (Jin et al., 1998), metal-binding proteins characterized as acute phase reactants. The clinical significance of the latter is related to limitation of the damage and a fast homeostatic compensation (Liu et al., 1991; Pannen and Robotham, 1995).

The analysis of the status of humoral factors of innate immune system provides the necessary information about the systemic resources for effective defense.

CELLULAR FACTORS OF INNATE IMMUNITY DURING A GRAM-NEGATIVE INFECTION

LPS activates not only humoral, but also the cellular components of innate immunity – monocytes (macrophages, natural killer cells, fibroblasts, endothelial cells) (Raetz et al., 1991). Hamawy et al. (1994) showed a mastocytic degranulation, Tsuchiya et al. (1999), Wechsung and Houvenaghel (1999) – a platelet activation and Smedly et al. (1986), Lo et al. (1992), Hailman et al. (1994), Hallet and Lloyds (1995), Opdenakker et al. (1998) – enhanced adhesive properties of endothelial cells.

The key role among the cellural elements of systemic defence mechaniums is that of phagocytes (monocytes, macrophages, neutrophils) (Celada and Nathan, 1994; Krause 2000).

Monocytes are a heterogenous cellular population (Lewis and McGee, 1992), on whose surface are expressed multiple structures of cell differentiation (CD) and molecules of the main complex of tissue compatibility (Barclay et al., 1994). The phenotype characteristics is a determining factor for the sensitivity of those cells against some infection agents (McCullough et al., 1993). During their differentiation into *macrophages*, they change their phenotype expression, lose some markers (Haverson et al., 1994; Prieto et al., 1994) and acquire other, that are not found in monocyte precursors (Poli et al., 1993; McCullough et al., 1997). Thanks to the variety of receptors (Fc, manose receptor, CD14; Mac 1 etc.) the chances of pathogens, passing through the epithelial membrane, for being not recognized, ingested and destroyed by tissue macrophages, are fairly low. Unlike the latter, *neutrophils* represent over 90% of circulating granulocytes and are the biggest population of blood phagocytizing cells. They could migrate from the circulation to the site of infection. Their considerable amount and high pagocytic activity make them an essential factor for elimination of pathogens, present in blood. They are not able however to react directly with Gram-negative bacterial LPS. This is possibly only when LPS is bound in blood plasma with LPS-binding protein (Mollinedo et al., 1999; Borregaard et al., 2000). Thus, a complex that is joined to

CD 14 molecules on neutrophil surface, is formed (Goyert et al., 1988; Grunwald et al.,1996). When defects in the production, maturation or the antibacterial functions of neutrophils occur, recurrent infections are observed while when neutrophils are completely lacking, those infections turn into septicemia.

Mobile phagocytes are the first line of defence against bacterial infections. They participate in both the afferent part of immune response (via mobilization of cellular and humoral immunity by synthesis and release of immunoregulatory cytokines) and in its efferent part (as effector cells, capable to perform an antigenic destruction and to release enzymes and cytotoxic components).

The functional activity of phagocytes is highly dependent on activation pathways, transcription factors, as well as on gene expression regulation in those cells, as reported by Celada and Nathan (1994), Reiner (1994), Aliprantis et al. (1996) and Moll (1996). According to the latter author, the resistance or sensitivity to intracellular pathogens is determined by a protein, located in the phagosomic membrane of macrophages.

Every phagocyte has a number of bactericide systems (Krause, 2000). They include two kinds of mechanisms - oxygen-dependent and oxygen-independent. In aerobic conditions, oxygen-dependent microbicide mechanisms increase oxygen consumption (a respiratory burst) that results in the release of reactive oxygen metabolites - superoxide anion, hydrogen peroxide, hydroxyl radical. Correlation between the capability of macrophages to produce hydrogen peroxide and to destroy microorganisms is observed (Nathan et al., 1983). Later is shown that the cytotoxic effect of hydrogen peroxide varies depending on the target cells, whose sensitivity is different (Nathan, 1987). This reactive oxygen metabolite is further viewed as the primary mediator of antibody-dependent cellular cytotoxicity (ADCC). Hypochloric acid, organic chloramines and aldehydes are other products with a strong potential of destruction (Cao et al., 1995). Those reactive oxygen intermediates ensure the effective performance of phagocytosis and their determination is an important indice. The congenital immune deficiency of respiratory mechanisms in stimulated phagocytes results in increased frequency of infections.

Apart phagocytosis, there are also other sources of reactive oxygen products. Their *in vivo* generation is an integral part of systemic metabolism (Cao et al., 1995). The pathological elevation of such products could result in cellular damage followed by DNA destruction. The oxidation of enzymes, the stimulation of antiinflammatory cytokines release from monocytes and macrophages as well as lipid peroxidation are other aspect of their activity (Chapple, 1997) Free oxygen radicals could impair the cation pumps (Na^+/K^+, Ca^{++}), increase the permeability of membranes, facilitate lysosome protease release and therefore, enhance cellular damage.

So, the natural development of antioxidant systems, interacting with oxygen radicals and reducing their negative effects in all aerobic organisms is explicable (Cao et al., 1995; Halliwell, 1995). Antioxidants are substances in small concentrtions but with a powerful inhibitory effect against oxygen radicals (Halliwell, 1995). Chapple (1997) categorizes antioxidants according to their mode of action to antioxidants, preventing the formation of new oxygen radicals (ceruloplasmin, myoglobin, ferritin, transferrin), to anti-

oxidants "cleaning" the organisms (glutathione, vitamin E, vitamin C, bilirubin) and to enzyme antioxidants whose function is to promote the oxidation of other molecules (glutathione-reductase, glutathione-peroxidase, catalase and metal enzymes).

However, plasma have not to be talked about as a simple chemical system with antioxidase activity. The big number of various antioxidants in it as well as their presence in other body fluids renders difficult their independent determination. The possibility of *in vivo* interaction between the various antioxidants is also an obstruction for the determination of a given antioxidant. That is why there are methods detecting the total antioxidant capacity in various biological samples (Cao et al., 1995).

Among the microbicide mechanisms of phagocytes is the nitric oxide (NO). This reactive molecule participates in many physiological functions as regulator of vascular tone and through its neurotransmitter function (Moncada et al., 1991). A key factor for the production of NO is NO synthase (NOS). Its iso-form (iNOS) is associated with macrophages, but could be isolated from smooth musculature and hepatocytes (Fukuto and Chaudhuri, 1995). The activity of the enzyme in macrophages of various animal species is not uniform. In rats (Keller et al., 1990), mice (Hibbs et al., 1987), cattle (Adler et al., 1996) and birds (Sung et al., 1991) it is high while in goats, rabbits (Schneemann et al., 1993), swine, dogs and humans (Schmidt & Walter 1994) – low. The mechanism (s) mediating those differences, are not known. One hypothesis suggests differences in the regulation of iNOS at transcription level. Aminoguanidine is a selective inhibitor of this isoenzyme (Laszlo et al., 1995). Some H_2-receptor antagonists (cimetidine) are also able to prevent the production of nitric oxide by influencing the forementioned enzyme (Hunter et al., 1999). Cyclooxigenase products (prostaglandin E_2), as well as antiinflammatory cytokines interleukin 1 (IL-1β), tumor necrosis factor α (TNF-α) are iNOS stimulators and increase NO production (Ianaro et al., 1994).

Arginin-dependent NO production determines to a considerable extent the microbicide potential of activated macrophages (Hibbs et al., 1988), evidenced by the fact that iNOS–deficient mice exhibit an ineffective defence (MacMicking et al., 1995; Wei et al., 1995).

The immunoregulatory properties of nitric oxide, discussed by Kolb and Kolb-Bachofen (1998) are involved in influencing the balance between T-helper 1 and T-helper 2 cells, affecting the programmed cellular death (apoptosis) as well as in the inactivation of P-selectins and thus, the leukocyte migration.

In blood, NO is rapidly metabolized into nitrite (NO_2) and further to nitrate (NO_3) via interaction with oxyhaemoglobin (Moshage et al., 1995). Both nitrites and nitrates are stable in serum. Their levels are not always indicative for NO metabolism, since NO_2/NO_3 production could be affected by changes in the volume of extracellular fluids, the renal function and diseases in animals (Zaballos et al., 1995). Plasma levels of those stable NO metabolites could be used as indicator of its systemic synthesis. In ruminants however nitrogen metabolism is very complex (Campos et al., 1993). The serum NO_2/NO_3 level is influenced by lactation and the quality of the diet, the water content of the organisms and the metabolic activitty of rumen microflora, the metabolism and utilization of nutrients

and their excretion with urine (Lohuis et al., 1990).

The role of NO is not completely understood (Cook et al., 1994). It is shown that endotoxin induces NOS and cyclooxygenase-2 in equine alveolar macrophages (Hammond et al., 1999). The importance of those enzymes in the pathophysiology of sepsis is the reason to look for new pathways of therapy of endotoxaemia via correction of enzymes with pharmacological means.

CYTOKINES – IMMUNOREGULATORS AND INDUCTORS OF ACUTE PHASE RESPONSE IN GRAM-NEGATIVE INFECTIONS

Activated cellular elements secrete soluble mediators – cytokines, whose action occurs at the same time and is acompanied by interactions among them. As a result, they increase, decrease or impair their activity one towards another and thus, the response of various cells could be altered in each moment (Brouckaert et al., 1993). This is the profound biological significance of control and rapid regulation of processes in various cells and their interactions. Together with those positive effects, the high reactivity of cytokines is responsible for the risk of several negative effects.

During the last decade, numerous authors (Van Deuren et al., 1992; Campos et al., 1993; Splitter et al., 1997) have focused upon cytokine profiles in bacterial infections trying to differentiate those mediators that could be used as diagnostic markers (De Bont et al., 1994; Buck et al., 1994). TNF-α , IL-1 are macrophage-produced cytokines in response of bacterial products (LPS). TNF-α is one of the first cytokines, found in circulation, that reaches peak values between hours 1–2 after the LPS challenge (Bautler et al., 1985; Michie et al., 1988). An hour after the production of TNF-α, IL-6 and IL-1 concentrations are raised (Van Deventer et al., 1990). The same sequence in cytokine release is reported also by Mackay et al. (1991). Subsequently, Brouckaert et al. (1993) discussed the associating role of TNF-α during the release of IL-1β and IL-6. During a LPS infusion, there is a biphasic increase in observed cytokines, the second peak being most probably due to the activation of T-, B-cells or fibroblasts whose cytokine profile includes the forementioned soluble mediators (Hoffman, 1980). Of course, the kind and the amount of released cytokines varies according to the animal species (Baumann and Gauldie, 1994) and to the character of stimulation signals of the pathogen (Billiau and Vandekerckhove, 1991; Splitter, 1997).

The cytokines TNF-α, IL-1 and IL-6, acting as "trigger signals", initiate a series of reactions, determining the acute phase response (Van Miert, 1991; Baumann and Gauldie 1994). They exert local effects affecting stromal cells, fibroblasts and endothelial cells (that also release cytokines). As a result, the chemotaxis of neutrophils (IL-8) and mononuclear cells is enhanced, a transendothelial migration is initiated, the vascular tonus is modified, the vascular permeability is increased (Le et al., 1987; Oppenheim et al., 1991). The vascular tonus changes are further mediated by oxygen radicals, NO, arachidonic acid metabolites, formed after the activation of cyclooxygenase and lipooxygenase (thromboxanes, prostaglandins, leukotrienes) (Baumann and Gauldie, 1994). The totality of local reactions is completed by the effects of released histamine, serotonine, platelet-activating factor (Billiau & Vandekerckhove, 1991; Pogrebniak et

al., 1992; Yamashita et al., 1994; Tsuchiya et al., 1999).

Apart the local changes, the beginning of infection is also characterized by generalized manifestations as fever, anorexia, decreased locomotion, metabolic alterations and by changes related to the hormonal background and liver protein synthesis.

These general signs are also mediated by cytokines: TNF-α, IL-1 and IL-6. TNF-α is related to haemorrhagic necrosis (Remick et al., 1990), anaemia (Tracey et al., 1988) and thirst (Calapai et al., 1991), observed after a LPS challenge. IL-1 is involved in the mechanism of anorexia and IL-1–induced prostaglandin synthesis (Nawroth et al., 1986). Prostaglandin-mediated cytokine involvement at the level of the hypothalamic centre of thermoregulation (Dinarello, 1991) triggers the febrile systemic reaction.

Opdenakker et al. (1998) discussed the molecular basis of leukocytosis, showing the crucial role of IL-1, TNF-α, IL-8 etc.

After a LPS challenge, Lang et al. (1985), Hargrove et al. (1988) determined a transient hyperglycaemia, most probably due to changes in plasma cortisol level, result of activation of the hypothalamus-pituitary-adrenal system. The effect of IL-1 by respect to this system is similar. Hyperglycaemia is followed by hypoglycaemia, the latter perhaps being result from the enhanced utilization of glucose in muscle cells (Lee et al., 1987). Endotoxaemia provokes a depletion of liver glycogen depots. IFN-α, IL-1β and IL-6 could also contribute to LPS-induced hypoglycaemia (Lee et al., 1987).

TNF-α, IL-1 and IL-6 are cytokines, controlling the production of *acute phase proteins* in liver (Baumann and Gauldie, 1994). They consist of *protease inhibitors* that neutralize proteinases and limit the extent of tissue damage; *coagulation proteins* that limit bleeding, *metal-binding proteins* as haptoglobin, ceruloplasmin. The three most widely covered acute phase proteins are: *C-reactive protein, serum amyloid A* and *serum amyloid P*.

After a LPS challenge, a marked increase in serum amyloid A (SAA) is observed (Heegaard et al., 1988; Fey et al., 1994; Chamanza et al., 1999). According to Alsemgeest et al. (1994) it is a very sensitive acute phase marker, in contrast to haptoglobin whose levels are not significantly changed. Plasma levels of acute phase proteins could have an important informative value with regard to the preclinical detection of infection or its severity as well as to the degree of development of inflammation (Sevelins & Andersson, 1995).

The use of acute phase proteins as disease markers in animals is complicated by the fact, that there are species-related differences in the amount and the quality of their production (Chamanza et al., 1999; Eskersall, 2000).

Through the synthesis of acute phase proteins however, the organism is enabled to bind a part of challenging bacteria within 24–48 hours. As acute phase proteins are with a standard structure, independent of antigenic specificity, the binding is non-specific.

In conclusion, reviewed data about the humoral factors of innate immunity (complement, lysozyme, coagulation system) reveal the direct relationship among their activity and this of cellular components - phagocytes. Despite the controversial results for changes in serum lysozyme activities due to species-related and methodology-related variances, the prodominating opinion is that is could be an useful indice for the development of Gram-

negative infections along with other parameters.

The main pathogenic element in Gram-negative bacteria – lipopolysaccharide, triggers not only humoral, but also cellular components of innate immunity (phagocytes). The efficacy of their performance determines greatly the course of infection and its outcome. Therefore, the phagocytic microbicide mechanisms – oxygen radicals and NO, as well as the systemic regulatory mechanisms for preventing the negative effects of their pathological elevation deserve a special attention. The multiple biological properties of NO and the difficulties of its determination in blood circulation because of its rapid transformation into nitrite and thereafter, into nitrate, must also be emphasized. The problem of the role of NO in endotoxaemia remains however open and the attempts for new solution with regard to endotoxaemia therapy continue.

The soluble mediators - cytokines, released from activated cellular elements, are in the basis of the formation of local and generalized manifestations of acute phase response. The latter, being a complex and dynamic process, contributes to the neutralization of the pathogen and to maintenance of systemic homeostasis. Acute phase proteins are parameters with a genuine information value for the preclinical detection of infection. Their use as disease markers in animals is impiared by the fact that there are differences in the quantity and quality of their synthesis in the various species.

Discussed data reveal the complexity of the problem for elucidation of pathogenic mechanisms of local and systemic manifestations during the acute phase response and the necessity of a scientific approach for influencing the early stage of Gram-negative infections.

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