Abstracts

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Topics in Discordant Xenotransplantation

F.H. Bach, J.L. Platt

The problem of discordant xenografting involves at least three areas of importance: first, the presence in the recipient of natural antibodies reactive with donor endothelial cells; second, the action of recipient complement which becomes activated consequent to the attachment of natural antibodies, and, third, the activation by the natural antibodies and complement of donor endothelium, leading to loss of vascular integrity, thrombosis. We have studied various topics in each of these and adherence of neutrophilic areas. With regard to natural antibodies, it appears that different individuals vary in terms of the levels of natural antibodies reactive with xenogenic endothelium; some individuals have no detectable natural antibodies, others have very high levels, detected by ELISA using pig endothelial cells as the target. It is possible to maintain low levels of natural antibodies in rats after depletion by plasma exchange using new immunosuppressive agents such as RS61443 and others. The area of complement is surely of great importance; it would appear that the mechanism of complement activation depends on either the classical and alternative pathways, or both. Our studies evaluating pigs as donors for primates would suggest that it is the classical pathway of complement that is primarily involved. A number of different approaches that might eventually be used in humans for the inhibition of complement are being tested; the use of membrane-associated inhibitors of complement will be discussed in this regard. As to the endothelial cell activation, it is the generation of pro-coagulation that would appear to be of the greatest importance in terms of hyperacute, or vascular, rejection. The role of natural antibody complement in eliciting procoagulation will be discussed as well as other manifestations of endothelial cell activation that could contribute to hallmarks of vascular rejection.

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Genetic Control of Hyperacute Xenograft Rejection

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Organ xenografts between distantly related species fail rapidly after revascularisation as a result of a collapse of the microvasculature involving thrombosis and interstitial haemorrhage. Heterotopic heart xenografts placed in guinea pigs genetically deficient for components of the complement cascade have demonstrated that the presence of functional complement is essential for this process of hyperacute rejection to occur. The rapid destruction of rabbit heart xenografts placed in new-born unsuckled antibody-deficient pigs suggests that the presence of anti-species antibody may not be essential for the initiation of hyperacute rejection.

tion. Membrane cofactor protein (MCP) and decay accelerating factor (DAF) are widely distributed complement regulatory membrane proteins postulated to protect autologous tissue by inhibiting C3 deposition. Human/mouse hybrids which express human DAF and MCP on their surface are not lysed by human complement in the presence of naturally occurring anti-mouse antibody but are lysed by rabbit complement. To investigate the cytoprotection from complement lysis directly attributable to MCP and DAF full length cDNAs (in forward or reverse orientation) were transfected into mouse fibroblast cell lines. Human complement mediated cytotoxicity in the presence of naturally occurring human anti-mouse antibody was assessed by ^{51}Cr release. Possession of human DAF and MCP provided almost complete protection from lysis by human complement. This protective effect was abrogated by the addition of monoclonal antibodies against either MCP or DAF at the start of the cytotoxic assay. Transgenic mice produced by microinjection of a DAF genomic construct express human DAF variably in their tissues. This DAF protects the cells of the mouse from lysis by human complement in a quantitative manner. They can be bred to homozygosity and survive normally. Such genetic manipulations on large animals could provide an organ donor source for man.

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Tolerance across Concordant Xenogeneic Barriers

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Previous studies from this laboratory have demonstrated that following a lethal dose of whole-body irradiation (WBI), transplantation of a mixture of rat bone marrow cells (BMC) plus T-cell-depleted host-type murine BMC leads to marked prolongation of donor-type rat skin graft survival, with normal rejection of skin from a different strain of rat [1]. More recently, we have demonstrated that mixed chimerism can be induced in this rat-mouse species combination using a nonmyeloablative conditioning regimen which involves thymic irradiation, low-dose WBI, and administration of monoclonal antibodies [MoAbs] against mouse T cells and NK cells prior to administration of T-cell-depleted rat BMC [2]. Such recipients did not develop graft-versus-host disease, showed excellent survival in a conventional animal colony, and demonstrated marked specific prolongation of donor-type skin graft survival (mean survival time > 100 days). Thus, mixed chimerism and donor-specific graft prolongation can be induced across this species barrier using a relatively nontoxic conditioning regimen. The rat-mouse xenograft system has generally been considered to be concordant on the basis of several criteria, including the absence of natural antibodies (NA) in the serum of potential recipients prior to transplantation. However, we were struck by the observation that much greater numbers of rat BMC than of murine BMC are required to achieve engraftment in mice, and we therefore considered the possibility that NA might exist in normal mouse serum with specific reactivity against rat BMC. Using flow cytometry and cytotoxicity assays, we have determined that normal mouse serum does indeed contain NA with specific binding to and cytotoxicity against rat BMC [3]. These NA were predominantly of the IgM and IgG3 classes, and activity toward BMC was much greater than that toward spleen cells. To evaluate in a more direct manner the effect of these NA on engraftment of rat BMC in mice, we performed adoptive transfer studies using T- and B-cell-deficient SCID mice as recipients. Because of their immunodeficiency, SCID mice accepted rat BMC readily, with only a low dose of WBI being necessary for conditioning. Passive transfer studies showed that normal mouse serum could markedly inhibit the engraftment of rat BMC, consistent with the hypothesis that NA provide a barrier to xenogeneic bone marrow engraftment, even in this phylogenetically close species combination. However, the barrier posed by NA could apparently be overcome by administration of excess rat BMC [4]. Mixed rat-mouse chimeric animals prepared by the nonmyeloablative conditioning regimen eventually lost detectable chimerism among peripheral lymphocytes (> 100 days), but remained specifically hyporesponsive to donor rat strain skin grafts [2]. In addition, no anti-rat serum antibodies were detected in these animals even after chimerism had fallen to undetectable levels [5]. These data therefore suggest that the tolerance induced by mixed xenogeneic reconstitution of the lymphohematopoietic system extends to both T cell and B cell compartments of the immune system. Induction of even transient xeno-

geneic chimerism may thus provide a means both for avoiding the return of NA and for inducing long-term cellular tolerance to xenogeneic tissues.

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Gene Transfer in Domestic Animals

G. Brem

The development of transgenic domestic animals which will be suitable donors of organs and tissues could revolutionize xenotransplantation. Hitherto, it was discussed to change transplantation antigens or to block rejection lysis by gene transfer in donor animals. Gene transfer by DNA microinjection has been reported in laboratory animals, i.e. mice, rats and rabbits, as well as in farm animals, i.e. sheep, goats, pigs and cattle. Theoretically, every mammalian species can be modified by gene transfer. However, various factors cause great differences in the efficiency and costs of gene transfer in different species: the ease of collecting fertilized oocytes, the number of oocytes available per donor, the visibility of pronuclei for microinjection, the survival and development rate of oocytes, the number of recipients required, the length of generation interval, the costs for animals and maintenance, the manpower for embryo/genettransfer work and the expenses for biological security. The choice of the transgenic animal or species for use in xenotransplantation must include the following considerations: ethical aspects, size and physiology of organs, availability of a large number of young animals and logistic and medical factors (specific pathogen-free or germ-free animals). Summarizing the factors listed above, the pig seems to be the most favorable animal species for transgenic xenotransplantation.

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Islet Xenotransplantation Using Immunoexclusion Membranes

A.P. Monaco

The impact of diabetes mellitus will be reviewed in terms of incidence and prevalence, morbid complications, mortality and cost to society. Existing and proposed alternatives for treatment of diabetes, including whole pancreas and islet transplantation, will be reviewed. Whole pancreas and islet transplantation currently requires nonspecific chronic immunosuppression with attendant high morbidity.

Islet transplantation without immunosuppression can be done using immunoexclusion membranes. The various immunoexclusion device designs will be reviewed. The biologic problems associated with interaction of host and membrane and donor tissues will be enumerated. Experiments will be described in which islet xenografts (bovine, porcine) have been successfully transplanted into streptozotocin-diabetic rats using cylindrical chambers ('straws') made from fabricated

permselective acrylic membranes (normal molecular weight exclusion of 50-80 kD). Also, long-term survival of canine islet allografts implanted in diabetic, pancreatectomized dogs will be described using similar cylindrical chambers fabricated from these acrylic membranes (nominal molecular weight exclusion of 50-80 kD). Canine islets were isolated from the pancreata of outbred mongrel dogs by collagenase digestion. Chambers containing $316 (\pm \text{SEM})$ 63K islet equivalents were implanted into the peritoneum of 6 totally pancreatectomized dogs, and the animals monitored for glycemic control by fasting and postprandial blood glucose determinations, and by responses to both intravenous glucose [intravenous glucose tolerance test, 0.5 g/kg and oral glucose (oral glucose tolerance test, 1 g/kg). All animals required appreciably lower doses of exogenous insulin for blood glucose control, with the mean daily insulin dose dropping

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from $38 \pm 7 \pm 5 \pm \text{IU}$ during the first week. Currently, three recipients required no insulin for > 94, > 80 and > 63 days. Excellent blood sugar control by oral and intravenous glucose tolerance testing has been achieved. These preliminary results are encouraging, and represent an important step in determining the feasibility of using this type of diffusion-based hybrid artificial pancreas as treatment for diabetes mellitus in humans.

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Beating Cardiomyocytes for Detection of Xenogeneic Rejection

C. Hammer, Ursula Müller, K. Werdan

Preformed natural antibodies (PNAb) are supposed to be the trigger of the hyperacute rejection of xenogeneic organs. Together with complement they are supposed to activate the coagulation cascade which, due to immediate cessation of microcirculation and blood flow, destroys the organ.

In vitro studies show that these PNAb bind not only to endothelial cells but at the same time to other parenchymal cells and even malignant cells without showing complement dependent cytotoxic reactions.

Method. In vitro cultures prove, that such activated endothelial cells release large amounts of mediators, like thromboxanes, prostaglandins and others within minutes. In order to investigate the PNAb effect, endothelial cell cultures were compared with cultured beating fetal rat myocytes. Porcine endothelial cells were incubated with different animal sera after fixation (solid phase); the binding capacity of PNAb was measured by an ELISA. Supernatants of living cells were investigated for mediators released. Under controlled conditions, the myocytes beat synchronously as a monolayer. When incubated with sera obtained from animals of different zoological relationship or human IgM and IgG concentrates or albumin as control, they show distinct signs of cellular disturbance. Depending

on the antibody concentration, the contractile cells increase the beating frequency and contraction velocity to slow down and stop for different time intervals. The standstill of contraction was reversible in all cases within minutes. Absorption of PNAb eliminates the inhibitory effect, decomplexation alone does not. All cultures recover after seconds to minutes to beat regularly again. Long-term incubation with high-titer sera diluted 1:1 did not affect the contraction frequency over a period of 48 h.

Conclusion. We assume that PNAb bind to the cell membrane but do not develop lethal cytotoxicity. They activate cell function in terms of liberation of mediators by endothelial cells or change the beating frequency of the heart cells. This suggests that PNAb do not destroy the cells but rather activate various functions which lead to secondary deleterious mechanisms like blood clotting and thrombosis, thus depriving the whole organs of their vital substrates, which concentration dependently leads to the termination of graft function.

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Rejection of Xenogeneic and Allogeneic Skin Grafts by Athymic Nude Rats: Potential Role of CD4⁺ T Cells

K. Wonigeit, R. Schwitzer, H.J. Hedrich

We and others have previously described that athymic nude rats are able to reject xenogeneic skin grafts from a variety of concordant and discordant species [1-3]. Rowett nude rats (rnu/rnu) 6-10 weeks of age have been shown to reject first set grafts of mouse ear skin after 17.7 ± 5.1 days ($n = 6$) and second grafts after 9.0 ± 0.6 days. Human skin was rejected after 10.4 ± 1.5 days ($n = 7$). This is in marked contrast to athymic nude mice which always accept xenogeneic skin permanently. In order to understand this interspecies difference, we have performed a detailed analysis of the T cell system of athymic nude rats and compared the results to published observations in nude

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mice. Flow cytometry analysis and detailed in vitro and in vivo studies of the alloreactive potential of T-like cells found in nude rats lead to the following findings: (a) Nude rats contained a remarkably high number of lymphocytes expressing T cell markers. These cells could be detected in all lymphatic organs already at the age of 8-12 weeks. The absolute numbers increased with age. (b) In animals older than 3 months, marked responses to PHA and Con A were obtained. (c) In aged animals (> 12 months) clear-cut in vitro alloreactivity could be observed in mixed lymphocyte cultures stimulated by a lymphocyte pool representing multiple MHC differences. MHC disparate allogeneic skin grafts were acutely rejected by about 50% of the animals in this age group. (d) The detailed analysis of the subset structure of nude rat T cells demonstrated the presence of both CD4⁺ and CD8⁺ T cells. Compared to euthymic animals, even a shift towards a higher proportion of CD4⁺ cells was seen. The comparison of these results to known features of nude mice revealed several major differences. (1) The thymus-independent pathway of T cell development is much more efficient in nude rats than in nude mice. (2) This results in stronger alloreactivity in vitro and even in the capability of aged animals to reject allogeneic skin grafts. (3) Whereas in nude mice CD4⁺ T cells do not develop in substantial numbers, they are the predominant T cell subset in nude rats. The latter feature may be of particular relevance for the difference in xeno-reactivity between the nude mutants of mouse and rat. Since a major role of CD4⁺ cells in xenogeneic responses has been demonstrated in other model systems, one could speculate that the high frequency of CD4⁺ cells may contribute to the strong xenoreactive potential of nude rats. The underlying mechanism could be the induction of a particular cytokine response activating nonspecific effector cells and/or a marked helper cell activation eliciting a strong humoral rejection response.

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Cytokine Gene Expression during T Cell Ontogeny and during the Induction of Peripheral Tolerance

M. Dallman

Cytokines not only control the magnitude of immune responses, but also appear to be involved in determining the outcome of immune responses. In certain parasitic infections and models of autoimmunity, particular cytokine profiles are associated with persistent or fatal disease. We have been interested in determining the role of cytokines in vivo in the induction of tolerance, both during T cell ontogeny and during peripheral tolerance induction. We also wanted to see whether any of the

events associated with thymic maturation were also associated with peripheral tolerance induction. Initially, we examined expression of mRNA for a variety of cytokines and their receptors using a semi-quantitative polymerase chain reaction (PCR) or Northern blotting. The semi-quantitative PCR enabled us to detect 5- to 10-fold differences in the level of specific mRNAs. This analysis, together with some functional data allowed us to establish that an alteration in the regulation of the IL-2 pathway was associated with transplant tolerance that had been induced by pretreatment with alloantigen. Further, in this and other models of transplantation tolerance, down-regulation of IL-2 expression was associated with up-regulation of IL-10 expression, resulting in an early imbalance in the normal ratio of IL-2 to IL-10 levels that are seen during graft rejection. In our analysis of thymic ontogeny, we found that many cytokines were expressed by fetal day 14 (IL-1β, IL-4, IL-5, IL-6, IL-7, TNFβ, IFNγ), whereas IL-2 gene expression did not occur before fetal day 16. Interestingly, we also demonstrated that thymocytes could potentially express high-affinity IL-2 receptors at a time when IL-2 itself was not being produced, since both chains of the IL-2 receptor are expressed by fetal day 14. The implications of these patterns of cytokine expression will be discussed.

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In vivo T Cell Activation and Cytokine Release Induced by Triggering of the CD3/TCR Pathway

Lucienne Chatenoud, J.-F. Bach

Monoclonal antibodies (MoAbs) to CD3 are highly potent immunosuppressors widely used in clinical transplantation both to treat and prevent acute organ allograft rejection. In addition, anti-CD3 MoAbs are very interesting tools to study the cytokine cascade, linked to the monocyte-dependent T cell activation that follows in vivo triggering of the CD3/TCR pathway. In fact, in contrast with their immunosuppressive activity, all anti-CD3 MoAbs display activating properties; in vitro this is reflected by their mitogenic effect on T cells. In vivo, both in humans and in mice, the first and anti-CD3 MoAb injection induces a dramatic T cell activation promoting the massive, although self-limited (lasting few hours after the first administration), release of several cytokines such as tumor necrosis factor (TNF), interferon gamma (IFNγ), interleukin (IL-)2, IL-3, IL-4, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Importantly, this cytokine release constitutes the pathophysiological basis of one major side-effect linked to clinical anti-CD3 use, namely the complex acute clinical syndrome that regularly follows the first administrations of the MoAb, which includes: high fever, chills, headaches, vomiting, diarrhea and, with lower frequency, more severe and eventually life-threatening symptoms, including meningeal syndrome, hypotension and respiratory distress linked to pulmonary edema.

The availability of the hamster MoAb 145-2C11 specific for the ε-chain of the murine CD3 complex was of great help to analyse in detail the fine characteristics of the biological and physical reaction. In pathogen-free mice, the physical syndrome is well evidenced by significant hypothermia, hypomotility (quantified by actimetry), diarrhea and piloerection; a dose-dependent high mortality rate is evidenced within 24-48 h when single doses of anti-CD3 higher than 50 µg/ mouse are used. The murine model allowed further insight into (1) the variability among different murine strains of the in vivo anti-CD3 activation properties; (2) the precise cell source of the released cytokines (T lymphocytes or monocyte/macrophages); (3) the ana-tomopathological lesions following the cytokine release, especially on privileged target organs such as the lung and the liver; (4) the definition of therapeutic strategies (including the administration of specific anti-cytokine MoAbs) to prevent the syndrome, and

(5) the respective role of each released cytokine in the induction of the syndrome and the modulation of the cascade. The most salient results deal with: the T cell origin of most of the released cytokines including TNF, and the central role of TNF in modulating the anti-CD3-induced cytokine cascade. In addition, anti-TNF MoAbs are major therapeutic tools to prevent the anti-CD3-induced syndrome both in mice and patients. Importantly, anti-TNF acts not only via direct neutralization of the TNF biological activity, but also by pretranscriptional regulation of other cytokines in the cascade, such as IFNγ.

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n-3 Fatty Acids and Cyclic Nucleotides in the Regulation of the Synthesis of Interleukin-1 and Tumor Necrosis Factor

S. Endres

The anti-inflammatory activity of n-3 fatty acids lends importance to investigating their effect on the production of IL-1 and tumor necrosis factor (TNF). Healthy volunteers supplemented their usual western diet with 18 g of fish oil concentrate (3.2 g eicosapentaenoic acid) a day for 6 weeks. Peripheral blood mononuclear cells (PBMCs) were incubated for 24 h with 1 ng/ml endotoxin. After n-3 fatty acid supplementation, synthesis of IL-1 and TNF was significantly reduced. As long as 10 weeks after cessation of the supplementation, production of IL-1 and TNF was reduced by 58%. It returned to pre-diet levels after 20 weeks. These data demonstrate an effect of dietary modification on the synthesis of cytokines in humans. They may explain, in part, the anti-inflammatory effect of n-3 fatty acids. The findings of this study have been confirmed in other study cohorts and in animal models. Most recently, we have also found a suppressive effect of n-3 fatty acids on IL-2 production and T cell proliferation.

With respect to the regulation of cytokine synthesis by cyclic nucleotides, we showed that theophylline and pentoxifylline suppress the LPS-induced synthesis of TNF α by human mononuclear cells. This effect is selective for TNF- α since up to several-fold higher con-

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Concentrations of these phosphodiesterase (PDE) inhibitors do not affect the production of IL-1 β in the same system. The observed effect of PDE inhibitors appears to be mediated by accumulation of cAMP since (1) addition of PDE inhibitors increases cAMP while cGMP levels are only marginally elevated; (2) raising cAMP by another mechanism (enhanced formation induced by prostaglandin E₂) leads to a similar suppression of TNF α production, and (3) raising cGMP does not inhibit TNF α synthesis. Raising cGMP levels by addition of nitric oxide (NO/EDRF) or related nitrovasodilators leads to suppressed synthesis of IL-1 β . Suppression of TNF α synthesis by PDE inhibitors may contribute to their beneficial effects in animal models of septic shock or lung injury, and may thus have clinical implications.

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Interleukin-6: Its Hepatic Receptor and the Acute-Phase Response of the Liver

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Interleukin-6 (IL-6) is an N- and O-glycosylated cytokine with a pleiotropic spectrum of action. It is involved in hematopoiesis, immune defense and in the regulation of acute-phase proteins in the liver. Studies on the structures of N- and O-linked carbohydrate chains and their location within the polypeptide backbone of human IL-6 synthesized and secreted by NIH/3T3 cells stably transfected with a human IL-6-cDNA have shown that only one (Asn 45) of the two potential N-glycosylation sites carries an oligosaccharide side chain. Ser 138 was identified as an O-glycosylation site. At the cDNA level we constructed human IL-6 mutants, lacking 1, 2, 3 or 4 amino acids from the C terminus. In the mouse B9 cell proliferation assay as well as in the γ -fibrinogen induction assay with the human hepatoma cell line HepG2 the removal of the last amino acid (Met 184) resulted in the loss of 80 % of biological activity. When 3 amino acids were removed from the C terminus, IL-6 activity was completely abrogated. Studies with various point mutations at the C-terminus of IL-6 indicate that an α -helical structure and a positive charge (Arg 182) are essential for biological activity [1]. The domain of human/mouse IL-6 responsible for species-specific receptor recognition has been identified by construction and expression of a whole series of chimeric human/mouse IL-6 proteins. Binding and activity studies with these chimeras will be presented. In previous studies with rat and human hepatocyte primary cultures as well as in the rat in vivo we have identified IL-6 as the major inducer of acute-phase protein synthesis [reviewed in ref. 2]. IL-6 acts via a specific hepatic cell surface receptor. Using a cDNA cloning approach, we could show that hepatocytes express an identical IL-6 receptor as leukocytes. Affinity cross-linking of ¹²⁵I-rhIL-6 to human hepatoma cells (HepG2) allowed the detection of three IL-6-containing complexes with molecular masses of 100, 120 and 200 kD. For the mRNA of the 80-kD IL-6 receptor subunit, a time- and dose-dependent up-regulation was observed after treatment of HepG2 cells with dexamethasone. Under

these conditions of IL-6 receptor up-regulation by dexamethasone γ -fibrinogen-mRNA induction by IL-6 is stronger and occurs earlier than without dexamethasone. At present, not much is known about the signal transduction of IL6. As a first approach, we have stably expressed the cDNA coding for the human IL-6 receptor in HepG2 cells. We have shown that ¹²⁵I-rhIL-6 is internalized by these cells. A genetically engineered soluble human IL-6 receptor was found to act as an agonist on HepG2 cells in acute-phase protein induction. Furthermore, the ability of the soluble IL-6 receptor to bind its ligand has been exploited to study receptor binding of IL-6 analogs (agonists/antagonists). When the 5'-end of the gene for the major acute-phase protein of the rat, α_2 -macroglobulin, linked to the reporter gene CAT was transfected into HepG2 cells, stimulation of CAT activity by IL-6 was observed. By use of deletion mutants in the promoter region of the rat β_2 -macroglobulin gene, we were able to identify an IL-6-responsive element consisting of two adjacent heptanucleotides (underlined): -196

5' CAGTAACTGGAAAGTTCTTCT-TAATCCTTCIGGGAATTCTGGC3'

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Synthesis Patterns of Proinflammatory Cytokines – Transcription and Protein Release under Stressful Conditions

E. Faist, C. Schinkel

In order to obtain deeper insight into the relevant intracellular behavioral changes of mononuclear leukocytes (PBMCs) in the course of disseminated inflammatory injury following trauma, close cell monitoring is warranted. Functional PBMC alterations of various states of trauma with uneventful or septic courses are best defined through sequential studies of their capacity to release soluble mediators during host defense responses. Rather than to continue with serial measurements of mediator levels in serum or in supernatants of in vitro cultures of a single cytokine, our strategy of investigation was as follows: We looked for comparable patterns of functionally related cytokines, like the proinflammatory monokines IL-1, IL-6 and IL-8 in comparison to TNF, or for the lymphokines IL-2 and IFN γ , in the individual patient, under close consideration of the internal serum environment and clinical course. We analyzed the cytokine de novo synthesis upon stimulation in patients with burns and major mechanical trauma, in individuals with a septic syndrome as well as in normal subjects. The quantity of cytokine protein released in in vitro cultures was compared to the amount of the corresponding cytokine mRNA detectable, following cytolysis. In our comparative analyses we found inadequate transcription signals following trauma for all cytokines investigated. While for IL-6 and IL-8 the depression of protein release seemed to be regulated mainly at the level of transcription for IL-1 and for TNF- α , the major regulatory factor seems to be located at the posttranscriptional level. Following conventional mitogenic stimulation (PHA, Con A), a major depression of IL-2 protein release and of IL-2 mRNA expression was found in the PBMC cultures of trauma patients. This suppression could be corrected when besides PHA a costimulant, phorbol myristate acetate (PMA), known to mimic the action of essential second messengers, was used. We concluded that the expression of the IL-2 mRNA and IL-2 molecules following trauma is not constitutionally defective, but most likely attributable to alterations in the transmission of signals from the cell membrane to the nucleus. These studies promise also to provide valuable information for the design of immunoaugmenting therapies in patients with compromised host defense mechanisms.

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Nitric Oxide: A New Kind of Signalling and Effector Molecule

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A few years ago, in 1987, nitric oxide was identified as an 'endothelium-derived relaxing factor'. Since then it became apparent that this nitrogen radical species plays an important role in various other biologic processes as well. It is now well accepted that nitric oxide regulates vascular tone, suppresses platelet function, modulates the immune response, inhibits cell proliferation and serves as a neurotransmitter. These different actions suggest an involvement of the nitric oxide pathway in

many pathological conditions, such as arteriosclerosis, diabetes mellitus, cancer and stroke. The physical and chemical characteristics of this molecule set the basis for its unique way of transmitting signals and acting directly as an effector substance. Nitric oxide is lipophilic and can easily cross the plasma membrane. Therefore, signal transduction by nitric oxide does not depend on a receptor, but is delivered by the intracellular action of nitric oxide itself. Since it is a highly reactive radical, nitric oxide binds vigorously to oxygen or reactive oxygen intermediates and to metal ions such as the iron in heme groups. This explains its short half-life of only several seconds in aerosol medium and many of its biological effects. The binding of nitric oxide to the iron of prosthetic groups of enzymes may either enhance or inhibit their activity. The soluble guanylate cyclase for example is activated by nitric oxide which results in the production of cGMP with its various effects on the cellular metabolism. The NADH-ubiquinone oxidoreductase – the first complex of the mitochondrial respiratory chain – on the other hand is inhibited by nitric oxide, which thereby suppresses aerobic energy metabolism. Many other direct effects of nitric oxide on enzyme activity have been described so far and many more will certainly follow. The production of nitric oxide from the amino acid L-arginine can be inhibited by various analogs of L-arginine, which appear to be selective for specific cell types. Stimulation of nitric oxide production

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by different mediators, such as endotoxin and cytokines, is also cell specific. These observations nourish the hope for sophisticated pharmacological manipulations of nitric oxide production in the different biological systems where it seems to play a major role.

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Eicosanoids as Mediators/Modulators of Acute Inflammation

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Since the discovery that aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit prostaglandin biosynthesis, interest has been devoted to actions and roles of arachidonic acid derivatives in microvascular function in general and inflammation in particular. Here are summarized some recent observations pertaining to the potential of leukotrienes (LT), prostaglandin E₂ (PGE₂) and lipoxin A₄ (LXA₄) to mediate or modulate significant events in acute inflammation. The results are based on intravital microscopy of the terminal vascular bed of the hamster cheek pouch, which has been adopted for quantitation of mediator release and observation of dynamic microvascular changes during antigen-induced inflammation. In this in vivo model, LTC₄, LTD₄ and LTE₄ provoke arteriolar constriction and plasma leakage from the venules, and LTB₄ causes diapedesis of leukocytes with associated extravasation of plasma. The vasodilating PGE₂ causes a marked enhancement of LT-induced plasma leakage and leukocyte emigration, despite being virtually devoid of effects on these parameters per se. The lipoxin representative, LXA₄, is also a vasodilator, but unlike PGE₂, it inhibits the microvascular actions of LTB₄. In immunized hamsters, topical antigen challenge of the cheek pouch causes a mast-cell-dependent inflammatory response which is characterized by vasoconstriction, plasma leakage, leukocyte emigration, and in vivo release of histamine as well as LTs. The mediator release and microvascular changes in response to antigen challenge are substantially enhanced by NSAIDs, while PGE₂, in nanomolar concentration, inhibits these effects. Of greater impact is the observation that the antigen reaction is profoundly attenuated by inhibitors of LT formation and action. Thus, the high-affinity antagonists for the LTC₄-E₄ receptor, ICI-198,615 and SKF-104,353, inhibit antigen-induced vasoconstriction completely and plasma leakage substantially, and the selective inhibitor of LT biosynthesis, MK-886, additionally inhibits both the accumulation of leukocytes as well as the release of leukotrienes. In conclusion, our results provide evidence for LTs being prominent mediators of allergic inflammation, and PGE₂ being a modulator of inflammation at two distinct levels with opposite effects. The findings further imply that LXA₄ is an inhibitor of leukocyte-dependent inflammation.

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Cytokine Inducible CAMs on Endothelial Cells of the Mouse

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The endothelial cell adhesion molecules which mediate the binding of leukocytes need to be carefully regulated in their cell surface expression. The surface expression of ICAM-1, VCAM-1 and E-selectin has been demonstrated to be transcriptionally upregulated within hours upon induction of human umbilical vein endothelial cells by different cytokines like IL-1 or tumor necrosis factor (TNF). In contrast, the surface expression of intracellularly stored P-selectin is induced within minutes by histamine or thrombin, which mobilize the content of storage granules to the cell surface. We have cloned full-length cDNAs for mouse E- and P-selectin and have generated polyclonal antibodies against both proteins. With these tools we found a new regulation mechanism for cell surface expression of P-selectin on several mouse endothelioma cell lines. This mechanism is the same as that for E-selectin: transcriptional upregulation induced by IL-1 or TNF, leading to a transient increase of both selectins at the cell surface with a maximum level after about 3 h and a rapid decrease to basal levels again within the next 16 h. In contrast, the TNF-induced increase of ICAM-1 and VCAM-1 on the cell surface of the mouse endothelioma cells was stable for more than

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20 h. We have established several in vitro cell adhesion assays in which lymphomas, monocytic cell lines and polymorphonuclear granulocytes (PMNs) specifically bind to the monolayer of TNF-activated mouse endothelioma cell lines. Based on antibody inhibition studies, we found that one of these assays defines a new TNF-inducible endothelial surface molecule which is most likely not identical with the four CAMs mentioned above. In this assay, the monocytic cells J774 bind to sEND 1 endothelioma cells (at 4 ° C) after 4 and 16 h TNF-treatment. E- and P-selectin can be excluded as candidates in this assay because they are dramatically decreased again after 16 h TNF treatment. ICAM-1 can be excluded, since antibodies against the β_2 -integrins did not block J774 binding. Also, the monoclonal antibody MK-2 against mouse VCAM-1 (kindly provided by Dr. Paul Kincade) did not inhibit the binding of J774. Surprisingly, the monoclonal antibody RI-2, which binds the α_5 -integrin chain (kindly provided by Dr. Bernhard Holzmann) blocks J774-binding for up to 70%. We conclude, that J774 cells bind with an α_5 -integrin to a new, not yet identified TNF-inducible CAM on mouse endothelioma cells.

In an attempt to identify this CAM, we produced monoclonal antibodies against TNF-inducible cell surface molecules on the endothelioma cells. In the course of these experiments we obtained three antibodies against mouse VCAM-1 and one against mouse ELAM-1, the latter being able to block PMN binding to TNF-induced endothelioma cells. The antibodies against VCAM-1 allowed us to analyse the tissue distribution of VCAM-1 in mice, which had been treated with human recombinant TNF- α (20 μ g per animal). This treatment causes induction of VCAM-1 expression on venules as well as arterioles, but capillaries do not express VCAM-1. Interestingly, we did not find VCAM-1 expressed in lymph node endothelium. This suggests that VCAM-1 is not the in vivo ligand for the homing receptor LPAM-1 (integrin $\alpha_4\beta_1$), although VCAM-1 clearly binds to the $\alpha_4\beta_1$ -integrin (LPAM-2 or VLA-4).

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Regulatory Mechanisms in Lymphocyte Adhesion Receptors

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Lymphocytes have contrasting but equally fundamental functions, namely continuous circulation in the blood followed by migration through lymphoid areas and peripheral tissues, and strong cell-to-cell interaction to perform immune surveillance and specialized effector functions. Mechanisms and structures have evolved to adapt lymphocytes to such a flexible behavior in a highly regulated and tissue-specific fashion.

(1) Antigen-dependent adhesion. Lymphocyte adhesiveness can be regulated by antigen recognition. Upon contact with cell-bound antigen, the lymphocyte undergoes dramatic morphologic changes, implying cytoskeletal rearrangement, which ultimately lead to increased area of contact, adhesion strengthening and reorientation of the lymphocyte protein secretory apparatus toward the bound target cell. It has been demonstrated that signalling through the antigen receptor promotes yet undefined allosteric transitions on a number of membrane structures involved in intercellular adhesion, resulting in increased avidity for their ligands.

(2) Antigen-independent adhesion. A variety of physiologic lymphocyte activities require adhesion in the absence of direct antigen recognition. These include homing to selected lymphoid organs, transendothelial migration into peripheral tissues and locomotion of mature, antigen-receptor positive lymphocytes, but also strong cell-to-cell and cell-to-matrix interactions by immature, antigen-receptor negative lymphocytes nesting in stromal areas of the bone marrow and the thymus early in the ontogenetic process. Thus, it is clear that external stimuli other than cell-bound antigen are capable of transiently conferring an adhesive phenotype to circulating lymphocytes. Immediate adhesion through professional adhesion receptors also occurs in pathologic conditions. These include: (a) increased surface expression of leukocyte adhesion receptors or their

cell-bound ligands, such as ICAM-1, VCAM and ELAM-1, at sites of inflammation; (b) expression of a ligand secluded from leukocyte recognition under physiologic conditions, for which adhesion molecules display constitutively high avidity. This appears to be the case for certain extracellular matrix proteins, such as laminin and collagen; (c) imbalanced concentration of extracellular divalent cations (such as Mn^{2+} and Mg^{2+}) which are capable of increasing the avidity of leukocyte integrins of both the β_1 and β_2 -subfamily for their respective ligands, by interacting

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with divalent cation binding sites located in the extracellular domain and promoting an energy-independent conformational change in the molecule. Finally, adhesion molecules have the ability to primarily promote a variety of cellular events which extend beyond simply providing the necessary contact described above. As membrane receptors, many leukocyte adhesion molecules are signal transducers. The use of monoclonal antibodies (MoAbs) to cross-link individual antigens, or purified, immobilized ligands to interact with their specific receptors has allowed definition of those molecules capable of triggering molecular events which commonly accompany cell activation. CD2, LFA-1, VLA-4, and CD44 all have transmembrane signalling properties resulting in elevations of cytosolic calcium. For example, LFA-1 cross-linking with MoAbs to the LFA-1 α chain and a secondary anti-Ig antibody consistently results in a calcium influx as well as phosphatidylinositol turnover. One important consequence of this signalling could be to exceed a critical activation threshold for cellular proliferation. Immobilized MoAbs to LFA-1 or VLA-4 synergize with anti-CD 3 antibodies to induce T cell proliferation, as do their respective cellular ligands, ICAM-1 and VCAM.

In conclusion, adhesion receptors, which encompass several distinct molecular classes, are examples of membrane glycoproteins which potentially have numerous roles, contributing to morphogenesis, differentiation, cell proliferation and, of course, adhesion. A given receptor's dominant role at any point in time depends upon a variety of factors, including the immediate microenvironment and the state of cell activation.

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White Cell-Endothelium Interaction in Rat Liver Transplantation

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In vivo fluorescence microscopy is an attractive tool to study pathomechanisms of hepatic ischemia-reperfusion injury. It allows to examine hepatic micro-vascular flow, leukocyte (WBC)-endothelium interaction, phagocytic activity of sinusoidal lining cells, structure and function of hepatocytes. Following the first application of this technique in transplanted rat livers [1], we were able to improve visualization by administration of sodium fluorescein and rhodamine G [2]. The aim of the present study was to analyze the contribution of reperfusion injury to WBC adherence after liver transplantation in the rat.

Material and Methods. Orthotopic syngeneic liver transplantation with arterial reconstruction was performed in 80 Lewis rats following cold storage in UW solution for 24 h; 10 nontransplanted livers served as normal controls. 30-90 min after reperfusion the liver lobe was exteriorized and examined by fluorescence microscopy with epiillumination as described previously [3]. 10 experimental groups (n = 8 each) differed in the conditions of reperfusion (additives to rinse solution and medication of recipient). WBC accumulation was quantified off-line by frame-to-frame video analysis. WBCs adhering for at least 20 s were counted as 'stickers'. Bile production was measured during the first 90 min after reperfusion. Statistical analysis was performed by two-way nested design analysis of variance with Dunnett's test for multiple comparisons with controls.

Results. In all cases WBC accumulation in sinusoids and postsinusoidal venules increased significantly after liver transplantation compared with normal controls. No changes could be observed in distributing portal venules and arterioles. Addition of thromboxane A₂ receptor antagonists and synthesis inhibitors to the rinse solution together with intravenous injection in the recipient were unable to modify WBC adherence compared with untreated transplants (rinse with Ringer's lactate or human serum albumin). Leukotriene synthesis inhibition or prostaglandin D₂ receptor blockade decreased WBC sticking in sinusoids but not in postsinusoidal venules. Addition of adenosine to the rinse solution used to flush the graft

immediately before reperfusion was able to decrease sticking both in sinusoids and postsinusoidal venules significantly. Most effective in this respect was the combination of adenosine, allopurinol, desferrioxamine, glutathione, and nicardipine in 'Carolina rinse' [4]. WBC accumulation in sinusoids ($p < 0.0001$, $r = -0.52$) and postsinusoidal venules ($p < 0.0002$, $r = -0.46$) correlated with bile production after reperfusion.

Conclusions. Marked WBC accumulation occurring after reperfusion in transplanted rat livers may be modified by additives to rinse solutions. However, although WBC adherence correlated with hepatocellular

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lar excretory function, it remains to be established whether sticking WBC exert direct negative effects on hepatocytes after reperfusion or whether they just accumulate preferentially in areas damaged by non-WBC-dependent mechanisms. In addition, further studies should address the question whether WBC accumulation in sinusoids is due to interactions of WBC with endothelium, Kupffer cells, or hepatocytes.

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White Cell-Endothelium Interaction and Reflow Paradox in Striated Muscle

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Restitution of blood flow after prolonged ischemia triggers a variety of events ('reflow paradox'), resulting in aggravation of ischemic organ injury. Reflow paradox is characterized by activation and adhesion of white cells (WBCs), release of mediators such as leukotrienes, platelet-activating factor, and increase of microvascular permeability [1]. Oxygen-derived free radicals are proposed to play a central role in the development of ischemia/reperfusion-induced microvascular injury [2]. The aim of the study was (1) to quantify reflow-paradox in postischemic striated muscle in vivo, including analysis of microvascular perfusion, WBC-endothelium interaction and extravasation of macromolecules, and (2) to assess whether superoxide dismutase (SOD) and allopurinol have the potential to attenuate these microvascular phenomena.

Methods. Using the dorsal skinfold chamber of Syrian golden hamsters [3] ($n = 48$), microhemodynamic analysis was performed by means of intravital fluorescence microscopy prior to and 30 min, 2 h and 24 h after 4 h of tourniquet ischemia. Analysis included functional capillary density (FCD), heterogeneity of capillary perfusion, microvascular accumulation of WBCs and WBC-endothelium interaction as well as extravasation of macromolecules (FITC-dextran

150,000) from capillaries and postcapillary venules. SOD was injected 5 min prior to reperfusion (i.v. bolus: 10 mg/kg) and over the first 20 min of reperfusion (i.v. infusion: 10 mg/kg, $n = 16$). An additional 16 animals were treated with allopurinol (50 mg/kg) at identical time points. Animals treated with 0.9% NaCl i.v. served as controls ($n = 16$).

Results. In controls FCD decreased significantly ($p < 0.01$) to 40% of baseline during postischemic reperfusion, presenting with highly heterogeneous capillary perfusion. In addition, microvascular WBC count was found significantly increased. WBC adherence to the endothelium of postcapillary venules amounted to $9.2-795.1$ cells/mm² endothelial surface ($p < 0.01$). Furthermore, extravasation of macromolecules from capillaries and postcapillary venules was prominent during the entire postischemic reperfusion period. Postischemic capillary perfusion failure was only minimally affected by SOD and allopurinol; however, both substances were efficient in significantly reducing WBC accumulation and WBC-endothelium interaction, and in abolishing the increase in microvascular permeability ($p < 0.01$).

Conclusion. Reperfusion after 4 h ischemia in striated muscle is characterized by capillary perfusion failure (no reflow), and WBC accumulation, WBC-endothelium interaction and increase in microvascular permeability (reflow paradox). While reactive oxygen metabolites, generated during postischemic reperfusion may not account for the defects in capillary perfusion, they seem to play a central role in triggering WBC activation and adhesion, resulting in increased microvascular permeability.

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