11
Anaphylatoxins

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In addition to the basic functions, opsonization and cytolysis, associated with the complement system there are some physiological activities elicited by complement peptides which contribute significantly to antimicrobial defence in infectious diseases and to the physiological and pathological consequences of immune complex formation, in short to the benefit and burden of inflammation. These effects depend on the cleavage of low-molecular-weight activation peptides from C4, C3 and C5: the anaphylatoxic peptides or anaphylatoxins C4a, C3a and C5a. The known primary structures[1] are given in Table 11.1. Generated from the N-termini of the α-chains of C4, C3 or C5 via activation of the classical or alternative pathways of complement, they interact with specific receptors on a number of cell types of the phagocytic system, mainly granulocytes and monocytes/macrophages. In addition, endothelial cells, possibly some T-cell subsets and, in some species, platelets express anaphylatoxin-receptors. Although the C5a receptor is often expressed with the C3a/C4a receptor, they are distinct species. The ligand–receptor interaction triggers G-protein coupled signal transduction pathways and results in a number of cellular responses[2–15], the most significant of which are documented in Table 11.2. Some of these biological effects are in general use as highly sensitive and specific *in vitro* detection systems for anaphylatoxins, e.g. smooth muscle contraction, enzyme release, stimulation of the respiratory burst, histamine release, ATP release, chemotaxis. However, these methods are too laborious for routine use and therefore quantification of anaphylatoxins using ELISA and RIA with monoclonal or polyclonal antibodies has been developed.[16–21] Two natural control mechanisms regulate the biological responses to anaphylatoxins and prevent excessive responses with pathological sequelae. First, a serum carboxypeptidase-N rapidly cleaves off the C-terminal arginine from all three anaphylatoxins and reduces circulating C3a and C4a activities to zero and C5a activity to 1–2%. Nevertheless, this residual C5a des Arg possesses significant biological activity. The second control mechanism works at the level of responder cells. A stimulus-specific receptor-mediated reversible deactivation

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Table 11.1 Primary structures of human C3a, C4a and C5a

	1	10	20
Human C3a	NH_2-Ser-Val-Gln-Leu-Thr-Glu-Lys-Arg-Met-Asn-Lys-Val-Gly-Lys-Tyr-Pro-( ) -Lys-Glu-Leu-Arg-Lys-		
Human C4a	NH_2-Asn = Asn-Phe-Gln-Lys-Ala-Ile-Asn-Glu = Leu = Gln = Ala-Ser-Pro- Thr-Ala-Lys-Arg-		
	1	10	20
Human C5a	NH_2-Thr = Gln-Lys = Ile-Glu-Glu-Ile-Ala-Ala = = Lys-His-Ser- Val-Val-Lys =		
	30	40	
Human C3a	Cys-Cys Glu-Asp-Gly-Met-Arg-Gln-Asn-Pro-Met-Arg-Phe-Ser Cys Gln-Arg-Arg-Thr-Arg-Phe- Ile-Ser-		
Human C4a	= = Gln = = Val-Thr-Arg-Leu- = = Met-Arg = = Glu-Gln = Ala- Ala-Arg-Val-Gln-		
	30	40	
Human C5a	= = Tyr = = Ala-Cys-Val = Asn-Asp-Clu-( )- Thr Glu-Gln = Ala-Ala-Arg = =		
	50	60	
Human C3a	Leu-Gly-Glu-Ala Cys Lys-Lys-Val-Phe- Leu-Asp Cys-Cys Asn-Tyr-Ile-Thr-Glu-Leu-Arg-Arg-Gln-His-		
Human C4a	Gln-() -Pro-Asn = Arg-Glu-Pro = = Ser = = Cln-Phe-Ala-Glu-Ser = = Lys-Lys-Ser-		
	50	60	
Human C5a	= = Pro-Arg = Ile = Ala = Thr-Glu = = Val-Val-Ala-Ser-Gln = = ( )–		
	70	77	
Human C3a	Ala-Arg-Ala-Ser-His- ( )-Leu-Gly-Leu-Ala-Arg-COOH		
		77	
Human C4a	Arg-Asp-Lys-Gly-Gln- ( )-Ala = = Gln = COOH		
	(CHO) 70	74	
Human C5a	= Asn-Ile = = Lys-Asp-Met-Gln = Gly = COOH		

Table 11.2 Test systems for biological activities of anaphylatoxins

Cells/organs to be tested	*Products/functions measured*	*C3a*	*C5a*
Rat mast cell	Histamine release	+[2]	+[2]
Guinea pig platelet	Aggregation	+[3]	+[3]
	Serotonine release	+[4]	+[4]
	ATP-release	+[5]	+[5]
Guinea-pig macrophage	Thromboxane A_2 release	+[6]	n.d.
Guinea-pig ileum	Isotonic contraction	+[7]	+[7]
Guinea-pig skin	Vasopermeability	+[8]	+[8]
Human PMNL	Chemotaxis	—	+[9]
	Enzyme release	—	+[10]
	Oxidative burst	—	+[11]
Human monocyte/macrophage	IL-1 release	+[12]	+[13]
Cell line U 937	β-Glucuronidase	n.d	+[129]
Human basophil	Histamine release	+(IL-3)[14]	+[15]
	Leucotriene B4 release	+(IL-3)[14]	+(IL-3)[15]

n.d. = not determined; superior numbers = references; + = active in the test; — = not active in the test

process is effective *in vivo* and *in vitro*[22, 23] and clearly discriminates between C5a and C3a/C4a respectively. This phenomenon is long known as tachyphylaxis.

STRUCTURE–FUNCTION RELATIONSHIPS OF ANAPHYLATOXINS

The information available at the moment on the essential structural features of anaphylatoxins has been obtained from two experimental approaches: synthetic peptides and recombinant peptides. In addition, removal of C-terminal arginine by serum carboxypeptidase-N from these reagents has permitted investigations into the essential role of the C-terminal arginine. The synthetic peptide strategy has been mainly successful for short peptides up to 21 amino acids from the C-terminus of C3a, and has allowed definition of optimal and minimal peptide length and amino acid sequence requirements. In addition, certain amino acids have been substituted by non-peptidic spacer and anchor elements[24,25]. In contrast, until now, the recombinant peptide strategy has been focused exclusively on C5a[26]. Site-directed mutagenesis and expression in *Escherichia coli* has been used to generate mutant peptides with changes of single or multiple amino acids[27]. In summarizing the state of the art, the structural requirements for ligand specificity of C3a are a C-terminal arginine, followed N-terminally by a short group of 4–8 hydrophobic amino acids, which are conserved in all species examined so far.

A non-peptidic N-terminal acyl residue, like the aromatic fluorenylmethoxycarbonyl (FMOC) group, coupled to the aminohexanoyl (Ahx) group potentiates activities of short synthetic peptides 10–50-fold and, in some cases, above that of natural C3a[24,25]. On the other hand, it was possible to shorten the minimal

sequence for a specific C3a activity to the tripeptide LAR[28] with this coupling strategy. The C3a peptide 57–77 previously reported to exhibit 100% acitivity[29] recently was shown to exhibit only 10% activity in both guinea-pig ileum contraction assay and in the platelet ATP release assay[30]. The C3a receptor, although until now only characterized from guinea-pig platelets[31,32], can be predicted to have only one binding pocket for properly presented arginine.

On the other hand, the synthetic peptide strategy was not successful for investigation of C5a: even the large C-terminal C5a peptides 55–74 failed to exhibit significant biological activity. Surprisingly, the FMOC membrane anchor and an amino hexanoic spacer (e.g. FMOC–Ahx–KDMQLGR) permit short synthetic C-terminal C5a peptides to trigger the C3a receptor[30]. Thus, site-directed mutagenesis of recombinant C5a has become the method of choice to study the interaction of C5a with the C5a receptor. These experiments have shown that C5a interacts with its receptor by multipoint binding, the crucial amino acid clusters[27,33] being Arg 74, Leu 72, Lys 68, a region around Arg 40 and Arg 37 and possibly His 15. The construction of a recombinant C3a–C5a hybrid peptide (C5a 1–69–LGLAR) representing the five C-terminal C3a amino acids, with both C3a (1%) and C5a (100%) activity, favours the interpretation that the carboxyterminal sequence, LGLAR, is able to bind to two different receptors, the C3a receptor as well as the C5a receptor.[34]

ANAPHYLATOXIN RECEPTORS

Until recently, the knowledge about anaphylatoxin receptors and receptor-mediated events was restricted to binding and crosslinking studies with C5a receptors on human polymorphonuclear leukocytes or macrophage-like cell lines[35,36], guinea-pig platelets[37] and to guinea-pig C3a receptors on platelets[31,32]. In early 1991, the human C5a receptor was finally cloned by an expression cloning strategy and identified as a member of the rhodopsin supergene family[38,39], in line with e.g. the formyl peptide receptor[40] or the β-adrenergic receptor[41]. The common features of this supergene family are seven membrane spanning helices and four short membrane protruding segments. By analogy with the β-adrenergic receptor, where the aspartic acid 82 of the receptor sequence represents a conserved binding site for the ligand, a first binding site for the C5a molecule (Arg 74) was postulated in a hypothetical receptor model (Figure 11.1)[33]. As the Asp 82 is about 20 Å below the outer membrane, not only the Arg 74 but also the adjacent residues, Gly 73 and Leu 72, would have to enter into this pocket. Because of the flexible random coil conformation of the eleven C-terminal amino acids of C5a, this interaction is conceivable. In consequence, the proposed second C5a binding site around Arg 40[27] would find a corresponding counterpart on the receptor at position Glu 179,180.

In the near future, the molecular genetic approaches will elucidate the receptor structure of the ligand binding pockets, the function of the different intra- and extracellular domains, signal transduction and association with regulatory GTP binding proteins, distribution pattern on different cell types and finally

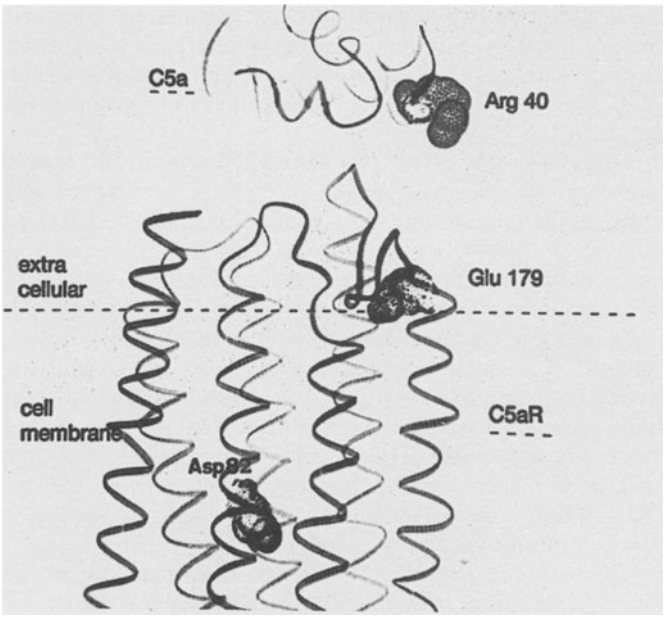

Figure 11.1 Ribbon representation of the C5a receptor model (in violet) viewed in the direction of the membrane plane. The extracellular site is on the top. Helices A–D are in the foreground from left to right, helices E–G in the background from right to left. The side chains of Asp 82 (B) (near the bottom on the left and Glu 180 (DE) (near the top on the right) suggested to bind the functionally essential arginines of C5a (74 and 40, respectively) are shown in dotted surface representation. On top, a ribbon representation of C5a with the side chain of Arg40 is added. (Taken from Grotzinger, J., Engels, M., Jacoby, E. and Wollmer, A. (1991). A model for the C5a receptor and for its interaction with the ligand. *Protein Engineering*, **4**, 767–71)

parallels to the putatively similar C3a receptor. Studies on receptor antagonists and receptor blockade by domain-specific antibodies will influence strategies to cope with the undesirable side-effects of anaphylatoxins (see below).

IN VITRO ASSAYS FOR ANAPHYLATOXINS

Due to the high inflammatory potential of the anaphylatoxins, there are diverse properties which in principle can be determined (see Table 11.2) in an *in vitro* assay. Of the biological activities, enhanced vascular permeability is measured using Evans blue in an *in vivo* skin test in guinea-pigs. Another organ assay using guinea-pigs is the ileum contraction assay. Although these two assays have been used frequently over the last 15 years, the data derived have been limited by the lack of good quantitation, and low sensitivity and considerable variability[30].

For qualitative determination of the biological activities of anaphylatoxins and synthetic anaphylatoxic peptides, new assays have been developed. In gene-

ral, four homologous and two heterologous cell types have been used for C5a assays: human neutrophils, basophils, monocytes and the promyelotic cell line U937 on the one hand, and the rat mast cell and the guinea-pig platelet on the other. For C3a there exist three heterologous but only two homologous cell types (Table 11.2).

Of these cell assays, only a few can be applied as standard procedures for quantitative estimations. Dose–response curves for C5a activity can be easily obtained with enzyme-release tests from neutrophil[10] and the U937 cell line[42]. Other biological properties of neutrophils can be estimated only semi-quantitatively, e.g. by chemotaxis[9], or display a considerable variability, e.g. the oxidative burst assay using luminol-enhanced chemiluminescence[11]. All assays with human basophils are difficult because of the small number of basophils in peripheral blood[14,15]. An ideal heterologous test system represents the guinea-pig platelet. Cell preparation is easy to perform with a high yield, so that some dose–response curves can be obtained within a day. At least three different reaction types can be measured: ^{3}[H]serotonin release[4], platelet aggregation[3] and ATP-mediated bioluminescence[5]. Each type can be used for determination of C3a and C5a activities since all reactions are mediated by specific receptors which have been described for both anaphylatoxins[31,32,37]. Biological activities of sythetic C3a analogue peptides have been quantitated in this cell assay because of its high sensitivity and reliability[30]. The only reservation to be made is that all data of structural requirements for an ideal C3a peptide are now well adapted to the guinea-pig C3a receptor. Whether these data can be transferred to the human situation remains to be shown with an homologous cell type in which quantitative responses can be obtained.

For the detection of anaphylatoxins in body fluids, two different test principles exist: commercially available radio-immunoassays (RIAs) and different enzyme-linked immunoassays (ELISAs), which differ in the specificity of their monoclonal antibodies. For detection of C3a two different types of antibodies have been described. The first was generated by immunization with intact C3[61] or C3a[16] and reacts with C3a and C3. Therefore, C3 has to be removed by a precipitation step prior to the assay. The second was raised to denatured C3[19] or a synthetic C-terminal C3a octapeptide (69–76)[17] and reacts only with C3a and C3a des Arg, respectively but not with C3. The advantage is obvious as no precipitation step is necessary. The detection limit of these assays is about 1–10 ng/ml. For C5a, the situation is quite similar as monoclonal antibodies which react with both C5a and C5[16] and those which exclusively detect C5a and C5a des Arg, are available[20,21]. The detection limit of these ELISAs is $\leq$ 1 ng/ml.

THE ROLE OF ANAPHYLATOXINS IN THE IMMUNE RESPONSE

Besides the inflammatory potential of the anaphylatoxins, there exist a variety of reports about the immunoregulatory activities of anaphylatoxins[43–49]. In contrast to the similar inflammatory properties of C3a and C5a, they behave in totally different ways in the humoral response. As C3a was found to be a potent supressor of antigen-specific and polyclonal antibody response, C5a was able to augment both *in vitro* humoral and cell-mediated immune response[43]. The

suppression of the polyclonal antibody response mediated by C3a seems to depend on the activation of non-specific $CD8^+$ suppressor T cells[44]. In addition, C3a is capable of activating human macrophages to release PGE_2 via the cyclooxygenase pathway of arachidonic acid metabolism, which is known to inhibit polyclonal antibody secretion[45]. These C3a effects could be mimicked by C-terminal oligopeptides while C3a des Arg was inactive[46,47]. C3a was not able to suppress antigen- or mitogen-induced B and T cell proliferation, whereas C5a was capable of potentiating antigen-induced but not mitogen-induced T cell proliferative responses.

The stimulating effect of C5a involves helper T cells, demonstrated with soluble T cell-replacing factors (Fc)TRF, which rendered lymphocyte cultures refractory to the enhancing properties of C5a[48]. In contrast to C3a, the C-terminal arginine of C5a is not essential for the immune response. C5a des Arg was shown in a recent study to induce a similar level of IL-6 from peripheral blood-derived mononuclear cells as C5a[49]. IL-6 is thought to be in part responsible for the immunoregulatory activities of C5a.

FROM PHYSIOLOGICAL TO PATHOLOGICAL ACTION

The anaphylatoxins are mediators of inflammatory reactions and important messenger molecules between the humoral and cellular branch of the early immune response, shifting a localized or systemic humoral cascade reaction to the cellular level of phagocytic cells with their non-specific and specific immune functions. The genetic deficiencies of C3 and C5 are complicated by severe localized and systemic pyogenic infections (see Chapter 6) due to lack of opsonization (in the case of C3), assembly of the cytolytic membrane attack complex (for both C3 and C5) and lack of anaphylatoxins. Thus, one should be cautious before declaring that anaphylatoxin activities are unnecessary and undesirable. Inflammation is an important host–defence mechanism and not a disease. However, occasionally excessive production of anaphylatoxins produces pathological consequences, which will be discussed in detail below. In the remainder of this chapter, we will focus on the role of anaphylatoxins in the pathogenesis of the adult respiratory distress syndrome (ARDS) and other diseases. In addition, we will discuss possible implications of the therapeutic anaphylatoxin blockade.

THE ADULT RESPIRATORY DISTRESS SYNDROME (ARDS)

The anaphylatoxins play a major role in the pathogenesis of ARDS and measurement of their plasma concentrations has been used as a predictive marker for its development and severity.

The first clinical description of this acute pulmonary disorder more than twenty years ago led to an overwhelming interest in this disease. In the USA, 150 000 cases of ARDS occur each year with a mortality above 50%. Thus, understanding the pathogenesis of this disorder is one of the major goals of pulmonary research[50–52].

ARDS is a disease never 'sui generis' but the result of direct parenchymal lung injury or of an excessive inflammatory response of the whole body after polytrauma, septic shock, burns, etc. (Table 11.3). In the early stages, increased vascular permeability is the striking feature of the disease, followed by necrosis and remodelling of the lung architecture which leads to impaired oxygenation and decreased compliance, often with a lethal outcome. Interaction of humoral mediators with polymorphonuclear leukocytes, microvascular endothelial cells, alveolar macrophages and type II epithelial cells seems to be responsible for the vascular leakage. The anaphylatoxins, as potent humoral mediators, are thought to play an important role in initiating the inflammatory cascade reaction, although, alone, they are not sufficient to induce a complete ARDS. Based on data from different animal models and the finding of elevated levels of inflammatory mediators in plasma and bronchoalveolar fluid in man, it seems likely that mediators must act in concert to induce severe ARDS. Thus, blockade of inflammatory cascade at key points may have therapeutic potential. The main humoral and cellular mediators taking part in this inflammatory concert are listed in Figure 11.2. Subsequently, we will try to outline a pathogenetic concept focusing on the early events in ARDS based on *in vitro* and *in vivo* findings, mostly in animal models. Because of the mass of *in vitro* data we will discuss only those results which correlate with *in vivo* findings in animals or humans. We will discuss first the adherence of neutrophil polymorphonuclear leukocytes (neutrophils) to vascular endothelial cells as the initial step in ARDS and secondly the activation of neutrophils as a key event in the increase in vascular permeability which leads to pulmonary oedema. We will not focus on the later phenomena which are linked to the irreversible lung remodelling because they are only poorly understood and the role of anaphylatoxins, if any, is unknown.

Pathogenesis

Adherence of neutrophils

In the case of all events listed in Table 11.3, the plasma enzyme systems in inflammation, i.e. the clotting, kinin, fibrinolytic and the complement systems, are activated. One of the most potent inflammatory mediators released is C5a. As a neutrophil chemoattractant, it is able to induce neutrophil aggregation and adhesion to vascular endothelium, respectively. This was investigated in a guinea-pig *in vivo* model[53,54]. After intravenous injection of purified C5a or C3a,

Table 11.3 Risk factors for development of an ARDS

Direct	*Indirect*
Aspiration pneumonia	Shock
Pulmonary contusion	Sepsis
Inhalation of toxic gas	Multiple blood transfusions
Infection (pneumonia)	Polytrauma
	Acute pancreatitis

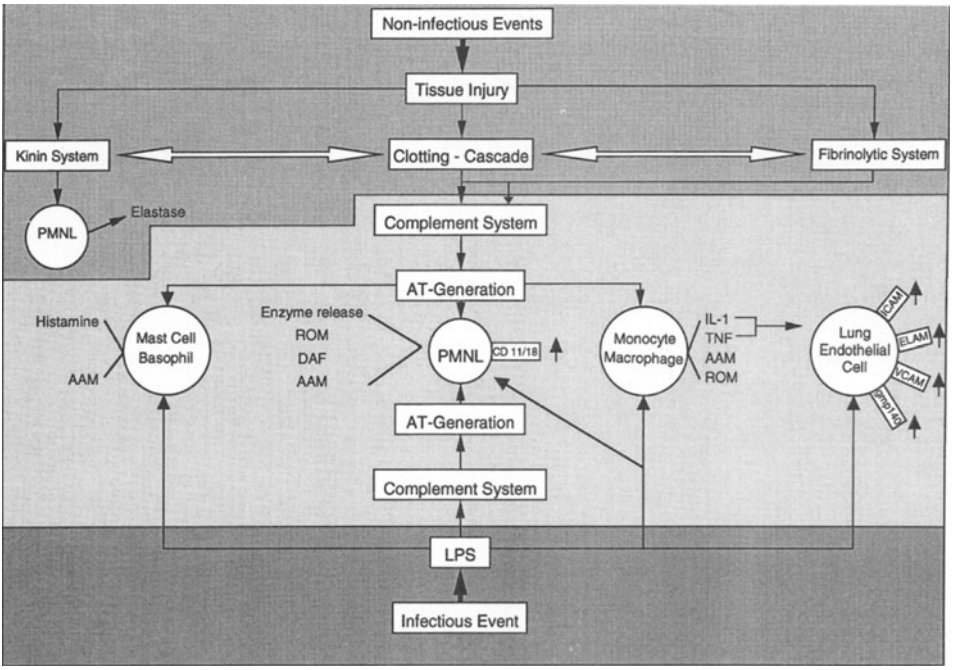

Figure 11.2 Network of main humoral and cellular mediators at the early stage of ARDS. At the top, the consequences of non-infectious (polytrauma, multiple blood transfusions, pancreatitis and other) events on the humoral enzyme and cellular system in inflammation are pictured. At the bottom, the pathway of LPS interaction in Gram-negative sepsis is shown. ROM, reactive oxygen metabolites; PAF, platelet activating factor; AAM, arachidonic acid metabolites; IL-1, interleukin 1; TNF, tumour necrosis factor; ICAM, intracellular adhesion molecule; ELAM, endothelial leucocyte adhesion molecule; VCAM, vascular cell adhesion molecule; GMP 140, granule membrane protein 140; < or > secretion.

granulocytes and platelets disappeared from circulating within a minute, returned to starting values after 2 min and increased to approximately double the normal level after 1–4 h (Figure 11.3). Simultaneously with the fall in the circulating neutrophils, the neutrophil content of the lung increased about three-fold (Figure 11.4), but gradually declined over the next 24 h. All the neutrophils were in the blood vessels with no evidence of migration towards the interstitial or the alveolar spaces. Recent *in vitro* data indicate that this adhesion process is mediated by members of the CD11/CD18 family of leukocyte integrins[55–58]. C5a as well as LTB_4 can induce a rapid increase (maximum in 2 min) of CD11/CD18 glycoproteins on neutrophils. In contrast, the adhesivity of endothelial cells, depending on upregulation of endothelial–leukocyte adhesion molecule-1 (ELAM-1) and intracellular adhesion molecule-1 (ICAM-1), increased after 4–6 h, requiring *de novo* RNA and protein synthesis. Thus, upregulation of CD11/CD18 on neutrophils appears to be responsible for the rapid margination of neutrophils in the pulmonary microcirculation following the intravenous injection of C3a or C5a. Furthermore, flow cytometric measurements with

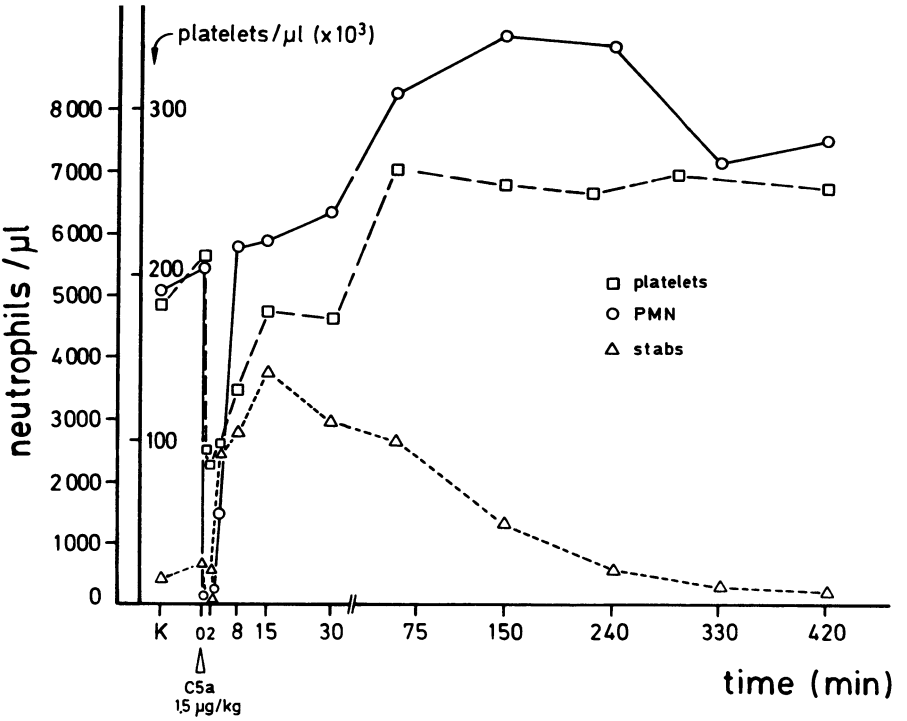

Figure 11.3 Reaction of circulation PMNS, stabs and platelets after injection of C5a. Dose of AT peptide: 1.5 µg kg^{-1}. At time −10 and 0 min, blood samples, serving as controls, were removed. All values are corrected for dilution. Data of representative animals are given (Taken from Hoffmann, T., Bottger, E. C., Baum, H. P., Dennebaumm, R., Hadding, U. and Bitter-Suermann, D. (1986). Evaluation of low dose anaphylatoxic peptides in the pathogenesis of the adult respiratory distress syndrome (ARDS). Monitoring of early C5a effects in a guinea-pig *in vivo* model after i.v. application. *Eur. J. Clin. Invest.* **16**, 500–8, with permission)

fluoresceinated C5a indicate that C5a does not bind specifically to human vein endothelial cells[59] showing that the anaphylatoxin C5a is not directly responsible for the upregulation of ELAM-1 or ICAM-1 on endothelial cells; this process involves other cell types, such as monocytes, which release IL-1 in response to C5a and C3a[60,12].

Although this animal model is a good example for the adhesion-promoting properties of the anaphylatoxins, it demonstrates that elevated plasma levels of anaphylatoxins alone are not sufficient to induce increased microvascular permeability in the lung. Only the administration of anaphylatoxins by unphysiological routes, like intratracheal instillation[62], the use of extremely high doses (double the maximal amount of C3a which could be generated in the animal) intravenously, combined with the administration of an inhibitor of serum carboxypeptidase-N to prevent degradation of C3a[63], produced histological evidence of lung injury. Although these models do not mimic clinical situations, they do show that, in circumscribed areas, high anaphylatoxin concentrations may produce tissue damage.

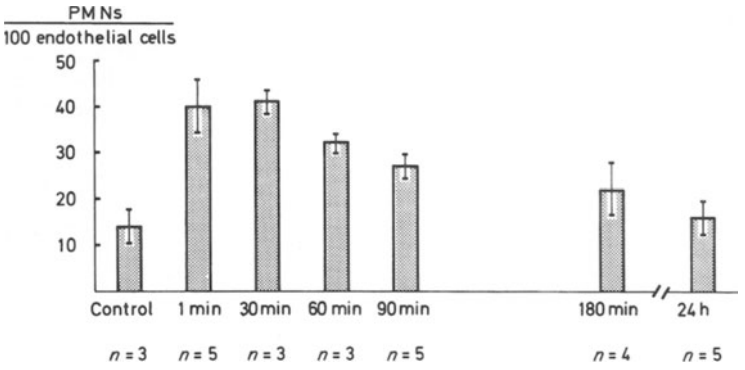

Figure 11.4 Number of PMNs per 100 endothelial cells in the lung at different times after C5a injection. Abscissa: time after C5a injection when the animal was sacrificed. (for details see Hoffmann, T., Bottger, E. C., Baum, H. P., Dennebaum, R., Hadding, U. and Bitter-Suermann, D. (1986). Evaluation of low dose anaphylatoxic peptides in the pathogenesis of the adult respiratory distress syndrome (ARDS). Monitoring of early C5a effects in a guinea-pig *in vivo* model after i.v. application. *Eur. J. Clin. Invest.*, **16**, 500–8, with permission)

Vascular leakage mediated by neutrophils

Because of the high frequency of ARDS following Gram-negative sepsis and of septic shock with lipopolysaccharide (LPS) as the causative agent, most animal models of ARDS depend on the administration of LPS.

As a result of these studies on animal models and humans with ARDS, it is evident that humoral mediators other than anaphylatoxins play a role in the development of ARDS (Figure 11.2). LPS stimulates monocytes to release cytokines, for example IL-1, IL-6 and TNFα, and to trigger neutrophils and endothelial cells to undergo an oxidative burst. However, as LPS can activate both the classical and alternative pathways of complement, the role of anaphylatoxins in LPS-induced ARDS has been investigated in different animal models. Primates[64–66] infused with *E. coli* showed an increase in C3a, C4a and C5a plasma levels associated with sequestration of neutrophils in the lung and an increase of extravascular fluid in the lungs. Thus, in addition to a neutrophil margination, increased vascular permeability occurred. LPS-primed adherent neutrophils are probably responsible for the plasma leakage, as neutrophil depletion by nitrogen mustard[67] or the use of oxygen radical scavengers reduced the lung injury.

Furthermore, it has been shown that adherent neutrophils produce more reactive oxygen species than non-adherent neutrophils[68]. The reactive oxygen-derived species include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), which can be released from adherent granulocytes upon stimulation with C5a[68,69]. These reactive oxygen species are considered to be the major neutrophil products responsible for acute microvascular damage in the lung[70], which leads to enhanced permeability and pulmonary oedema.

Recently, however, the role of the neutrophil as the main effector cell in vascular leakage has been questioned as ARDS may also occur in patients with

neutropenia[71–74]. It is therefore likely that other cells, such as alveolar macrophages or alveolar endothelial cells, may contribute to vascular leakage, an area requiring further investigation. Experimental and clinical studies have shown that activated neutrophils release proteases, such as elastase and collagenase[75–77]; their roles in the pathogenesis of ARDS are unclear.

Sequestration of neutrophils from blood vessels in the alveolar space

Anaphylatoxins are not involved in the events leading to neutrophil adherence and activation but are also thought to be responsible for the transalveolar traffic of granulocytes into the alveolar space. In principle, neutrophils have the ability to penetrate the endothelial/epithelial barrier by diapedesis, but migration is greatly enhanced by chemotaxis. Both C5a and C5a des Arg are chemotactic factors and have been detected in the bronchoalveolar lavage fluids of ARDS patients.[78] This C5a could be generated locally within the extravascular space or may have diffused from the plasma (as C5a des Arg), although most C5a generated intravenously binds rapidly to circulating neutrophils. Intact C5 could be present in the extravascular space as a result of diffusion from the microcirculation, secondary to increased vascular permeability, or may be synthesized locally. In this regard, it has been shown that human alveolar type II cells in culture *in vitro* synthesize C5, as well as C2, C4, C3 and factor B[79]. As the incubation of radio-labelled C5 with bronchoalveolar lavage fluid from ARDS patients results in the generation of C5a, enzymes capable of activating C5 must be present. Whether these enzymes are the C5 convertases of the complement system or the neutrophil proteases, or both, is unknown. However, the generation of C5a locally in the extravascular space will permit the establishment of a chemotactic gradient so that neutrophil migration from blood vessels into the alveolar space occurs. C5a will also stimulate secretion of neutrophil proteases so that more C5a is generated and a vicious circle has developed. In addition to C5a, transendothelial traffic of neutrophils is also stimulated by the actions of IL-1 or TNFα on endothelial cells, without any chemotactic gradient[80]. Thus, there are at least two mechanisms which contribute to the extravascular emigration of neutrophils in ARDS. Interestingly, it has been shown that C5a leads to shedding of soluble 20 kDa TNFα receptors by neutrophils[81,82]. As these receptors retain their ligand-binding capacity, it is possible that this process may limit neutrophil emigration as TNFα in the circulation and extravascular fluid would be bound by these receptors. Studies of the effects of C5a on TNF receptor expression by endothelial cells are required.

Therapeutic implications

According to the presented pathogenetic concept for the early events in ARDS, different therapeutic approaches are conceivable. There are at least two different neutrophil products which are able to induce the vascular leakage in ARDS: the proteinases and the reactive oxygen species. Although elastase is present at sites of inflammation[83] and increases vascular permeability *in vitro*, because of the

presence of α_1 antiprotease *in vivo*[75], it is unlikely to increase vascular permeability in ARDS. Thus, therapeutic blockade of the neutrophil oxidative burst appears to be an attractive option. Free radical scavengers, such as dimethylurea, dimethylsulphoxide and N-acetylcysteine, have been used in animal models to try to prevent the toxic effects of reactive oxygen species and resulted in the extent of pulmonary oedema being reduced. Despite this apparent success, the use of free radical scavengers in patients must be tempered with the knowledge that the ability of phagocytes to kill bacteria depends principally on reactive oxygen species. This is best demonstrated in chronic granulomatous disease patients who suffer from recurrent bacterial infections as their neutrophils cannot undergo a normal respiratory burst.

Perhaps a better therapeutic approach would be to arrest the generation of ARDS at an earlier stage, perhaps inhibiting the generation or activity of anaphylatoxins or the cytokines IL-1 and TNFα. In a mouse model, prevention of death has been observed with an anti-TNFα antibody after LPS challenge[84]. Recently, a human IL-1 receptor antagonist was tested in a rabbit model of ARDS, and shown to prevent the massive transudation and cellular infiltration of alveolar spaces and to reduce mortality[85,86]. These *in vivo* experiments indicate that blocking of IL-1 and TNFα could effectively interrupt the inflammatory cascade after LPS challenge.

The earliest event in ARDS appears to be the adhesion of neutrophils to the pulmonary vascular endothelium. As the anaphylatoxins represent key mediators in this initial phase of ARDS, their blockade should result in a decreased neutrophil adhesion, activation and, in consequence, a reduced vascular permeability of the lung. The blockade of the most powerful anaphylatoxin, C5a, was achieved by administration of an anti-C5a antibody in a primate model prior to the infusion of *E. coli*. This reduced the increase in pulmonary vascular permeability, the decrease in blood oxygenation and mortality[65]. Similar results could be obtained[87] from a rat model after LPS challenge using $F(ab')_2$ fragments of rabbit anti-rat C5a. These data. suggest that blocking the C5a peptide at an early stage of the disease could be a practical approach of great benefit.

In summary, some early intervention strategies show great promise in the prevention of ARDS. Antagonists of mediators such as anaphylatoxins and cytokines could be given prophylactically to high-risk patients to prevent ARDS and to decrease mortality.

Anaphylatoxin levels in patients at risk from ARDS

As anaphylatoxins are involved in the pathogenesis of ARDS, measurement of C3a and C5a levels in plasma and bronchoalveolar fluid could be important in the management of these patients. However, although the results of studies performed over the past decade have been somewhat inconsistent, they show that anaphylatoxin levels are elevated during episodes of sepsis and that persistent elevation tends to be associated with the development of complications[85–95].

In a more recent study, C3a and C4a levels were shown to be elevated and a clear correlation between mortality and anaphylatoxin levels was documented[96].

However, there was no relationship between anaphylatoxin levels and the development of ARDS or deterioration in lung function. C3a/C4a plasma levels were elevated both in patients with ARDS and those without ARDS and the mean levels in each group did not differ significantly. Thus, these findings are surprising as the high mortality of septic shock correlates with anaphylatoxin plasma levels. In contrast, the results of a recent study of 48 patients with trauma and sepsis, in which plasma levels of phospholipase A_2, the macrophage stimulation marker neopterin, elastase and C3a des Arg were measured, showed that only elastase and C3a des Arg levels could be used to distinguish between patients with uncomplicated sepsis and those with ARDS[97]. In addition to these differences, patients who developed ARDS had circulating C5a-deactivated neutrophils, with impaired migratory responses and reduced degranulation following C5a exposure[98].

Apart from the differentiation of those sepsis patients with ARDS from those without ARDS, plasma anaphylatoxin levels have been used to detect pulmonary deterioration. High-risk patients, with worsening arterial blood gases, had significantly higher C3a and C5a levels than those with stable blood gases. The authors concluded that monitoring arterial blood gases and anaphylatoxin levels in ARDS-risk patients permitted earlier and more precise diagnosis of ARDS[99].

The results of a recent multicentre study have verified the importance of measurement of plasma anaphylatoxin levels for the early identification of ARDS in high-risk polytrauma patients at an early stage (within 6–12 h) of the

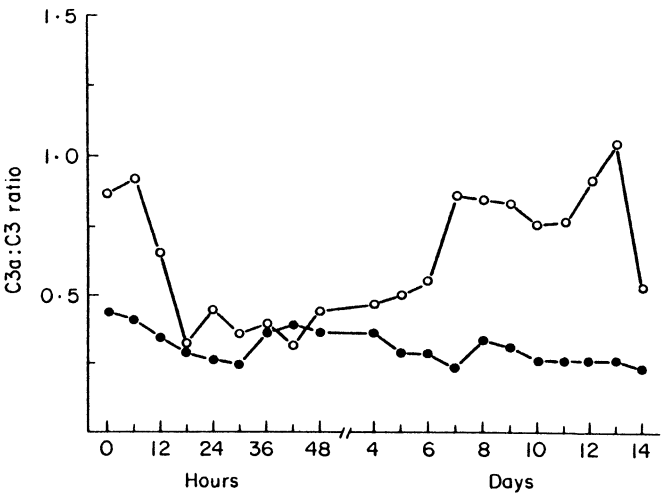

Figure 11.5 C3a:C3 ratio in 14 polytrauma patients with adult respiratory distress syndrome (ARDS) (open circles) and 17 patients without ARDS (closed circles). Differences in the first 12 h and from day 4 to 14 between the two groups are significant. (Taken from Zilow, G., Sturm, J. A., Rother, U. and Kirschfink, M. (1990). Complement activation and the prognostic value of C3a in patients at risk of adult respiratory distress syndrome. *Clin. Exp. Immunol.* **79**, 151–7, with permission).

disease. C3a and the C3a:C3 ratio proved to be important parameters in distinguishing between those patients who will develop ARDS and those who will not[100] (Figure 11.5). The ARDS group had significantly higher C3a levels 6 h after admission to the intensive care unit with a second rise on days 4–14. In contrast, the non-ARDS group showed only minor increases. The same was true for the C3a:C3 ratio. These data suggest that monitoring plasma anaphylatoxin levels with a minimum of two blood samples a day may serve as an early indicator for the development of ARDS in critically ill patients. Changes in anaphylatoxin levels can be confirmed by measurement of the C5b-9 terminal complement complex[101], plasma elastase[97] or IL-6[102].

Measurement of plasma anaphylatoxin concentrations by RIA or ELISA is time consuming with considerable delay between blood sampling and obtaining the final result. If anaphylatoxin assays are to be used in patient management, rapid on-line procedures must be developed.

ANAPHYLATOXIN GENERATION DURING HAEMODIALYSIS AND CARDIOPULMONARY BYPASS

Complement activation through the alternative pathway is an event which is independent of antigen–antibody complexes. A wide range of agents from LPS of Gram-negative bacteria, virus-infected cells and the cell wall of yeasts are able to activate this pathway. Besides these natural activators, iatrogenic activation occurs, most commonly during haemodialysis and during cardiopulmonary bypass using bubble oxygenation. Patients undergoing either of these procedures may develop complications associated with a systemic inflammatory reaction, either immediately at the onset of extracorporeal circulation or during the postoperative period. The symptoms range from urticaria and angio-oedema to life-threatening profound organ dysfunction, often manifest in the lung with neutrophil sequestration and increased microvascular permeability[103].

The striking similarities between the early events in ARDS and these observed clinical findings suggests a pathogenetic role for anaphylatoxins released during complement activation on the surface of the biomembranes. As a consequence, C3a, C4a and C5a plasma levels have been measured: C3a, but not C4a, was found to be elevated during both haemodialysis and cardioplumonary bypass[104]. These findings indicate that the alternative pathway is predominantly responsible for complement activation in these patients. When radio-immunoassays were used to quantitate plasma anaphylatoxin levels, C5a appeared to be normal whereas elevated C5a levels were demonstrated when the ELISA technique was used[16,105] (Figure 11.6).

In the recent literature, concentrations of the terminal complement complex have been used as a measure of bioincompatibility. With respect to the investigated biomembranes (Cuprophane, Hemophane and Polysulphone), the results seem to be analogous to those found using anaphylatoxin assays[106,107].

Since haemodialysis was first practised with Cellophane membranes, great progress has been made with regard to a better biocompatibility of the material. Today, at least three groups of dialyser membranes can be distinguished[108]: the first with high complement-activation potential, e.g. the classical regenerated

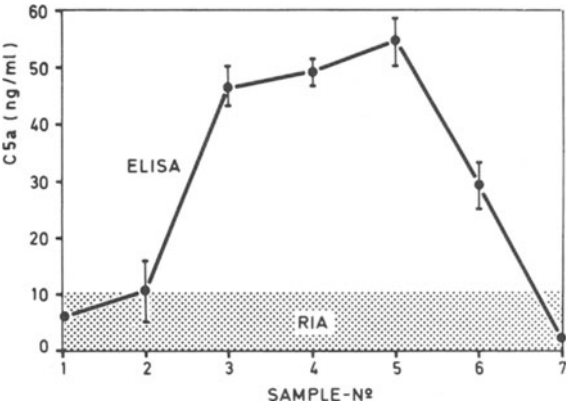

Figure 11.6 Time course of C5a plasma levels in patients with cardiopulmonary bypass. C5a plasma levels of two patients detected by ELISA (filled circles) and RIA. Threshold of detection of the C5a RIA for EDTA plasma samples is shown by the shaded area. 1: preoperative sample; 2: 5 min after partial bypass; 3: 5 min after complete bypass; 4: 30 min after complete bypass; 5: at the end of complete bypass; 6: 2 h postoperative; 7: first postoperative day. (Taken from Klos, A., Ihrig, V., Messner, M., Grabbe, J. and Bitter-Suermann, D. (1988). Detection of native human complement components C3 and C5 and their primary activation peptides C3a and C5a (anaphylatoxic peptides) by ELISA with monoclonal antibodies. *J. Immunol. Meth.*, **111**, 241–52, with permission).

cellulose (Cuprophane); the second with moderate activation potential, e.g. modified cellulose (cellulose acetate); and the third with a low activation potential, e.g. polycarbonate, polysulphone, polyacrylonitrile and modified cellulose (Hemophane)[109,110]. The polyacrylonitrile membranes which were characterized originally as weak activators of the complement system have recently been shown to produce marked complement activation but the majority of generated C3a binds to the membrane and evades fluid-phase detection[111,112]. Nevertheless, it is conceivable that specific interaction with neutrophils with membrane-bound anaphylatoxins may occur and lead to inflammation. In order to reduce complement activation, dialyser membranes have been reused as it has been shown that these membranes activate complement less effectively than new ones[113].

In addition to complement activation by the alternative pathway, there are reports of classical pathway complement activation occurring during extracorporeal circulation. Such activation does not depend on activation biomembranes but is due to C1 activation by protamine–heparin complexes.[114] Heparin is used routinely to prevent coagulation in the extracorporeal circulation (EC). On completion of the operation, protamine is used to reverse the anticoagulation action of heparin rapidly. The heparin–protamine complexes, which bind to red blood cells, activate the classical pathway of complement[115].

Protamine also inhibits plasma carboxypeptidase-N[116], which inactivates anaphylatoxins and kinins. Thus, by inhibiting the carboxypeptidase while simultaneously triggering complement activation and anaphylatoxin release, anaphylatoxins will not only be generated but remain active following protamine administration. As a result, some authors advise against the use of protamine to reverse heparin anticoagulation, particularly in patients with ischaemic heart disease[117].

Another problem is the increased risk of infection in patients with chronic renal failure undergoing either chronic haemodialysis or continuous ambulatory peritoneal dialysis (CAPD). This may be in part due to the recurrent C5a generation which has been shown to downregulate specific C5a receptors on phagocytes[118,119]. Neutrophils and monoctyes which have been deactivated by C5a have their bactericidal activity reduced markedly as both chemotactic responsiveness and the oxidative burst are reduced.

In summary, the anaphylatoxins play an important role in the clinical complications of extracorporeal circulation. At present, all known biomembranes activate complement, although Hemophane or Polysulphone membranes produce low levels of activation. Investigations to improve those membranes should be continued to minimize the occurrence of acute problems during extracorporeal circulation and to prevent impairment of antimicrobial defence mechanisms.

ANAPHYLATOXINS IN ATHEROMA AND MYOCARDIAL INFARCTION

Myocardial infarction is normally the result of severe coronary artery atheroma. The disease originates in early childhood when diffuse thickening of the musculo-elastic intima is seen. During adolescence and adulthood, the lesions progress through the formation of fatty intimal streaks, which contain smooth muscle cells surrounded by deposits of lipids (mainly cholesterol oleate), fibrous plaques, which contain lipid-laden (mainly cholesterol linoleate) macrophages and smooth muscle cells. This leads to a complicated lesion, in which the fibrous plaque has undergone calcification, cell necrosis, mural-thrombosis and possibly haemorrhage. Endothelial injury is thought to be the initiating event followed by the migration of smooth muscle cells and monocytes into the lesion. Complement activation occurs in atheromatous lesions and cholesterol-rich liposomes have been shown to activate complement activation in atheromatous lesions resulting in the formation of anaphylatoxins.

Anaphylatoxins and other chemotactic agents will stimulate the migration of phagocytes into the atheromatous lesions and amplify the inflammatory response. Thus, anaphylatoxins may play a significant role in the pathogenesis of atheroma. Anaphylatoxins also play a role in the pathogenesis of tissue injury occurring in response to myocardial ischaemia. *In vivo* studies have shown that cardiac muscle constituents are released from ischaemic and infarcted tissue. One of these, a mitochondrial constituent, will bind C1 and activate the classical pathway. The importance of C5a in the subsequent inflammatory process has been emphasized by the demonstration that, following the intracoronary perfusion of C5a, neutrophils accumulate rapidly in the coronary blood vessels, coronary blood flow decreases and myocardial contractility is reduced. The effects of C5a on myocardial ischaemia and myocardial contractility are likely to depend on members of the lipoxygenase (leukotriene D/E_4) and the cyclooxygenase (thromboxane A_2) pathways[120]. The use of antagonists to the arachidonic acid metabolites following C5a challenge has shown that both cyclooxygenase and lipoxygenase metabolites contribute to the negative inotropic effects and

myocardial ischaemia, while they do not affect neutrophil accumulation. Thus, the pathophysiological findings after intracoronary C5a infusion depended mainly on release of arachidonic acid metabolites[121,122]. Neutrophil migration is probably the result of direct chemotactic response to C5a. These findings were confirmed for human C3a in a guinea-pig model[123].

In addition to the experimental evidence suggesting a role for anaphylatoxins in myocardial infarction, a recent study has shown that plasma levels of C3a are elevated for up to 10 days after acute myocardial infarction[124]. However, further evidence is required to confirm a pathogenetic role of anaphylatoxins in this disease.

OTHER DISORDERS

Psoriasis

Although the classification of this dermatosis as an immunological disease is still open to discussion, the inflammatory nature of psoriasis is well documented. There are two pathological features which characterize the clinical picture: the increased cell proliferation and the infiltration of the epidermis by neutrophils. These inflammatory responses are probably mediated through anaphylatoxins since large amounts of C3a, C4a and C5a were found in scale extracts of psoriatic patients[125,126]. Nevertheless, C5a is not the only chemotactic factor present in scale extracts[127] as LTB_4[128] as well as neutrophil-activating peptide[129] have been isolated. The latter mediator has properties which are similar to the cytokine IL-1. The finding of elevated C4a levels indicates the occurrence of classical pathway activation. Recent evidence suggests that this activation is due to complexes of psoriasis-associated antigen, pso p27, with IgG[130].

In addition to complement activation occurring locally in dermal lesions, systemic complement activation may occur, since plasma levels of C3a and C4a are increased in psoriatic patients compared with non-psoriatic controls[131,132]. Thus, the continuous local and systemic generation of anaphylatoxins could, at least in part, be responsible for the ongoing transepidermal migration of neutrophils in psoriatic disease.

Rheumatoid arthritis

The initial event of this autoimmune systemic disorder of connective tissue is still unknown but there is no doubt that significant complement activation occurs via both the classical and alternative pathways. Within the joints' immune complexes (IgG–IgG and IgG–IgM), complement activation products and a predominant neutrophil infiltration are found[133]. Neutrophils are thought to play an important role in the inflammatory process in the joint because of their great destructive potential. The levels of anaphylatoxins in synovial fluid are seven-fold higher in patients with rheumatoid arthritis than in patients with degenerative joint disease[134,135]. This suggests that anaphylatoxins have a role in the persistent neutrophil influx and activation[136]. However, because neutrophils will

be deactivated after stimulation with anaphylatoxins[137], the role of other phlogistic mediators has to be considered, e.g. IL-1[138]. This cytokine has been found in high concentrations in knee-joint effusions of patients with rheumatoid arthritis.

Plasma anaphylatoxin levels, like C-reactive protein levels and erythrocyte sedimentation rate, correlate with disease activity and are therefore useful in patient management[134]. The best correlations were seen when two of these three parameters were compared with disease activity. Therefore, it is probably more useful to measure plasma anaphylatoxin levels in association with one other parameter in order to monitor disease activity in rheumatoid arthritis.

Systemic lupus erythematosus (SLE)

There is extensive evidence which shows that activation of complement by both the classical and alternative pathways occurs in SLE and that complement activation increases as the illness relapses and decreases as it remits. A number of single case reports have shown that measurement of plasma levels of both C3a and C5a were useful indicators for a flare of SLE. Moreover, it could be demonstrated that plasma C3a levels were elevated 1–2 months prior to a clinical exacerbation of the disease and therefore could serve as a predictive marker[139,140]. Interestingly, the plasma levels of C3a in patients with central nervous system involvement were significantly higher than in those without central nervous system involvement. The cerebral blood vessels of patients who died of acute central nervous system disease were occluded with aggregated neutrophils which may have been mediated by anaphylatoxins.

CONCLUDING REMARKS

The anaphylatoxins play an important role in the mediation of acute inflammation and can be generated by activation of any of the plasma enzyme systems. The variety of activation pathways, either by direct cleavage through proteases (e.g. factor XIIa, neutrophil elastase) or as a consequence of alternative or classical activation of the complement system, ensures that anaphylatoxins participate in any inflammatory process. In most instances, this is beneficial to the organism and provides a mechanism for interaction between the humoral and cellular components of inflammation. However, in chronic inflammation due to autoimmune phenomena (SLE or RA) or in conditions of excessive complement activation (e.g. due to plasma coming into contact with a foreign surface or in patients with polytrauma or septic shock), anaphylatoxins produce disease.

The diverse biological properties of anaphylatoxins are due to specific cellular receptor/ligand interactions. Until now, the list of receptor-bearing cells is somewhat longer for C5a than for C3a but awaits further expansion for both peptides. The interaction of the anaphylatoxins with their corresponding receptors is well characterized for C3a through C-terminal oligopeptides but is only poorly understood for C5a, where a minimum of three different binding sites may be involved. As the C5a receptor has been cloned recently, it is to be

expected that genetic and immunological approaches will clarify the extra- and intracellular mechanisms leading to the release of the diverse cell-derived mediators which are ultimately responsible for the effects of anaphylatoxins *in vivo*. This information may permit the development of pharmacological agents which could block the release of mediators by interfering with ligand–receptor interactions or by blocking signal transduction.

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