Complement factor C5a exerts an anti-inflammatory effect in acute pancreatitis and associated lung injury

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Complement factor C5a exerts an anti-inflammatory effect in acute pancreatitis and associated lung injury. Am J Physiol Gastrointest Liver Physiol 280: G974–G978, 2001.—Complement factor C5a acting via C5a receptors (C5aR) is recognized as an anaphylotoxin and chemoattractant that exerts proinflammatory effects in many pathological states. The effects of C5a and C5aR in acute pancreatitis and in pancreatitis-associated lung injury were evaluated using genetically altered mice that either lack C5aR or do not express C5. Pancreatitis was induced by administration of 12 hourly injections of cerulein (50 μg/kg ip). The severity of pancreatitis was determined by measuring serum amylase, neutrophil sequestration in the pancreas, and acinar cell necrosis. The severity of lung injury was evaluated by measuring neutrophil sequestration in the lung and pulmonary microvascular permeability. In both strains of genetically altered mice, the severity of pancreatitis and pancreatitis-associated lung injury was greater than that noted in the comparison wild-type strains of C5aR- and C5-sufficient animals. This exacerbation of injury in the absence of C5a function indicates that, in pancreatitis, C5a exerts an anti-inflammatory effect. Potentially, C5a and its receptor are capable of both promoting and reducing the extent of acute inflammation.

Materials and methods

All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. Breeding pairs of C5aR knockout mice, back-crossed to a C57/Bl6 background, were generated as previously described (11). The identity of C5aR-deficient (−/−) homozygotes was confirmed by Southern blotting (11). Wild-type C57/Bl6 mice were used as C5aR-sufficient [C5aR(+/+)] controls. C5-deficient mice (B10-D2-OSN) and the C5-sufficient controls (B10-D2-NSN) were purchased from Jackson Laboratories. All animals were bred and housed in standard shoe box cages in a climate-controlled room with an ambient temperature of 23 ± 2°C and a 12:12-h light-dark cycle. Animals were fed standard laboratory chow, given water ad libitum, randomly assigned to control or experimental groups, and fasted overnight before each experiment. Cerulein, the decapeptide analog of the potent pancreatic secretagogue CCK, was obtained from Research Plus (Bayonne, NJ). C5a was obtained from Sigma Chemical (St. Louis, MO). Other reagents were purchased from sources previously reported (1) and were of the highest purity available.

Blood and tissue preparation. Animals were given hourly intraperitoneal injections of saline (control) or saline containing a supramaximally stimulating concentration of cerulein (50 μg/kg) for 12 h (1). One hour after the last injection, mice were killed by an intraperitoneal injection of a lethal dose of pentobarbital, and blood and samples of the pancreas were rapidly prepared for study. Harvested blood was allowed to clot and was centrifuged, and serum was obtained for mea-
measurement of amylase activity and for in vitro studies. For these in vitro studies, pancreatic acini were freshly prepared by collagenase digestion as previously described (19). For morphological studies, the pancreas was removed rapidly, and complete random cross-sections of the head, body, and tail of the pancreas were fixed in 4% neutral phosphate-buffered formalin for histological study. Other samples of pancreas and lung were prepared for measurement of tissue myeloperoxidase (MPO) activity as described below.

**Morphological examination.** Paraffin-embedded pancreas samples were sectioned (5 μm), stained with hematoxylin-eosin, and examined by an experienced morphologist who was not aware of the sample identity. Acinar cell injury/necrosis was quantitated by morphometry as previously described (1). For these studies, 10 randomly chosen microscopic fields (6×125) were examined for each tissue sample, and the extent of acinar cell injury/necrosis was expressed as the percentage of the total acinar tissue that was occupied by areas meeting the criteria for injury/necrosis. Those criteria were defined as either (1) the presence of acinar cell ghosts or (2) vacuolization and swelling of acinar cells and the destruction of the histoarchitecture of whole or parts of the acini, both of which had to be associated with an inflammatory reaction.

**Assays.** Serum amylase activity was measured using 4,6-ethylidene(G1)3-p-nitrophenyl(G2)3-x11-d-maltohexoside (Sigma Chemical) as the substrate (17). Neutrophil sequestration in pancreas and lung was quantitated by measuring tissue MPO activity, as previously described (1, 2). For these measurements, tissue samples harvested at the time of death were stored at –70°C. The samples were thawed and homogenized in 1 ml of 20 mM phosphate buffer (pH 7.4) and centrifuged (10,000 × g for 10 min at 4°C), and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecylmethylammonium bromide (Sigma Chemical). The suspension was subjected to four cycles of freezing/thawing and further disrupted by sonication (40 s). The sample was then centrifuged (10,000 × g for 5 min at 4°C), and the supernatant was used for MPO assay. The reaction mixture consisted of this extracted enzyme, 1.6 mM tetramethylbenzidine (Sigma Chemical), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide. This mixture was incubated at 37°C for 110 s, and the absorbance at 655 nm was measured in a CobasBio autoanalyzer. This absorbance was then corrected for the dry weight of the tissue sample used, and results were expressed as activity per dry weight (degree of increase over control). Intra-acinar cell trypsinogen activation and lactate dehydrogenase release from acinar cells were measured as previously described (19).

**Measurement of pulmonary microvascular permeability.** These studies were performed as previously described (1, 2), and a separate group of animals was used for these studies. Two hours before death, each animal received an intravenous bolus injection containing 5 mg/kg FITC-labeled BSA (FITC-albumin; Sigma Chemical). Immediately after death, the trachea was exposed, and the lungs were lavaged three times with 1 ml of saline. The lavage fluid was combined, and FITC fluorescence was measured in the lavage fluid and serum (excitation = 494 nm; emission = 520 nm). The ratio of fluorescence emission in lavage fluid to blood was calculated and used as a measure of pulmonary microvascular permeability.

**Analysis of data.** The results reported represent mean ± SE values obtained from multiple determinations in three or more separate experiments. In Figs. 1, 3, 4, and 6, vertical bars denote SE, and the absence of such bars indicates that the SE is too small to illustrate. The significance of changes was evaluated using Student’s t-test when the data consisted of only two groups and one-way ANOVA when comparing three or more groups. If ANOVA indicated a significant difference, the data were analyzed using Tukey’s method as a post hoc test for the difference between groups. A P value ≤0.05 was considered to indicate a significant difference.

**RESULTS**

**Cerulein-induced pancreatitis and associated lung injury.** Mice given 12 hourly injections of a supra-maximally stimulating dose of cerulein develop acute pancreatitis and lung injury. As shown in Fig. 1, the severity of the pancreatitis can be quantitated by measuring serum amylase activity, pancreatic MPO (a reflection of neutrophil sequestration within the pancreas), and the extent of acinar cell necrosis. Morphological changes of pancreatitis (Fig. 2) include acinar cell vacuolization, intralobular edema, an inflammatory infiltrate, and acinar cell necrosis. Lung injury also occurs in this model as evidenced by a rise in lung MPO activity and a rise in pulmonary microvascular permeability (Fig. 3).

**C5aR modulates the severity of cerulein-induced pancreatitis and associated lung injury.** Genetic deletion of C5aR results in an increased severity of both the pancreatitis and the associated lung injury. As shown in Fig. 1, serum amylase activity, pancreatic MPO activity, and acinar cell necrosis are all increased in the C5aR(–/-) knockout animals compared with the C5aR(+/+) controls. Similarly, the morphological changes of pancreatitis (Fig. 2) are worse in the C5aR(–/-) group than in the C5aR(+/+) controls. Lung MPO activity and pulmonary microvascular permeability during pancreatitis (Fig. 3) are also increased in the C5aR(–/-) knockout animals compared with the C5aR(+/+) controls.
C5 modulates the severity of pancreatitis and associated lung injury. The role of C5a in regulating the severity of pancreatitis and lung injury was further evaluated using congenic mice that do not express C5 and that are therefore incapable of generating C5a. As shown in Figs. 4–6, the severity of pancreatitis (i.e., hyperamylasemia, increased pancreatic MPO activity, acinar cell necrosis) and of pancreatitis-associated lung injury (lung MPO activity, pulmonary microvascular permeability) is increased in the B10-D2-OSN mice, which do not express C5 when they are compared with the C5-sufficient B10-D2-NSN controls.

C5a does not alter in vitro cerulein-induced amylase secretion. Dose-dependent amylase secretion, studied in vitro using freshly prepared acini in the presence or absence of C5a, showed an identical biphasic relationship between cerulein concentration and amylase secretion (data not shown).

**DISCUSSION**

C5a is a potent anaphylotoxin and chemoattractant that is generated from C5 as a part of both the classic and alternate pathways of complement activation. C5a, acting via C5aR on target cells, is generally believed to serve as a proinflammatory mediator (5, 6, 9, 13, 15). Recent studies using C5aR-deficient knockout mice have supported this belief by demonstrating that these mice are unable to clear intrapulmonary-instilled *Pseudomonas aeruginosa* in spite of their ability to mobilize neutrophils to the lung (11). As a result, they succumb to overwhelming pneumonia. On the other hand, the C5aR-deficient mice are protected against a sterile form of lung injury, i.e., immune complex-mediated pulmonary inflammation (4).

We have evaluated the role of C5a in a model of pancreatitis and systemic injury after pancreatitis us-
ing two independent but complementary approaches. In the first, mice that do not express C5aR were used (11), whereas in the second set of experiments, mice that do not express C5 were employed (7). The results of both studies were similar; interruption of C5a action either by deletion of its receptor or by deletion of its parent protein resulted in a worsening of pancreatitis. That worsening was noted for each of the parameters used to quantitate the severity of pancreatitis, i.e., serum amylase level, neutrophil sequestration in the pancreas, and pancreatic acinar cell necrosis. The severity of pancreatitis-associated lung injury was also increased when C5a action was rendered inoperative. Whether this worsening of lung injury reflects involvement of C5a as an acute inflammatory mediator in the lung or, alternatively, merely reflects worsened pancreatitis in these animals cannot be determined from our studies.

Our results, which indicate that C5a exerts an anti-inflammatory effect in pancreatitis, are in conflict with those previously reported by Guice et al. (10) and by Merriam et al. (16). In the former study complement depletion reduced the severity of pancreatitis, while in the latter study C5-deficient mice developed less severe pancreatitis after bile-pancreatic duct ligation than did C5-sufficient animals. These differing results may reflect the non-C5a-related effects of complement depletion in the former study and the use, in the latter study, of a different and milder model of pancreatitis. Although our studies clearly indicate that C5a has an anti-inflammatory effect in the cerulein-induced model of pancreatitis, the mechanisms underlying that effect are not obvious. We considered the possibility that, during pancreatitis, C5a levels in C5aR-deficient mice might be higher than in C5aR-sufficient mice during pancreatitis and/or that C5a might act via receptors that differ from the classic C5aR, which is disrupted in the C5aR-deficient strain. That possible explanation would appear to be excluded by our experiments using mice that do not express C5 since, in these animals, C5a should not be generated during pancreatitis. The recent reports that exogenously administered interleukin (IL)-10 could reduce the severity of pancreatitis (14, 18, 20) suggested to us that C5a might act by promoting the endogenous release of IL-10. We administered anti-IL-10 antibody (a kind gift of Dr. Steve Kunkel, University of Michigan) to wild-type mice to evaluate this possibility but found that administration of anti-IL-10 antibody had no effect on the severity of secretagogue-induced pancreatitis (data not shown). We also considered the possibility that C5aR deletion might alter the acinar cell responsivity to cerulein, but this seems to be ruled out by our observation that the biphasic secretion/inhibition response to cerulein, in vitro, is identical in the presence and absence of exogenous C5a. Thus, for now at least, the mechanisms by which C5a acts to reduce the severity

![Fig. 5. Effects of C5 deficiency on morphological changes of pancreatitis. Representative hematoxylin- and eosin-stained sections of pancreas were examined by light microscopy in normal control animals not given cerulein (A), in wild-type B10-D2-NSN (C5-sufficient) animals given cerulein (B), and in B10-D2-OSN (C5-deficient) mice given cerulein (C). Note evidence of pancreatitis in C5-sufficient animals (B) and worsened changes in C5-deficient animals (C).](image)

![Fig. 6. Effects of C5 deficiency on pancreatitis-associated lung injury. Mice were given 12 hourly injections of cerulein (50 μg/kg ip) or saline (control). One hour after the last cerulein injection, mice were killed, and capillary leakage of FITC-albumin and lung MPO activity was measured as described in the text. Values are expressed as a percentage of the value obtained for wild-type animals given cerulein. Results shown are means ± SE for 10 or more animals in each group. *P < 0.05 when B10-D2-OSN (C5-deficient) animals given cerulein were compared with B10-D2-NSN (C5-sufficient) animals given cerulein.](image)
of pancreatitis and associated lung injury have not been determined.

We are intrigued by a potential explanation for our unexpected observation, i.e., Foxman et al. (8) indicated that neutrophil chemoattractants act in a hierarchical manner. Foxman and colleagues (8) studied complex gradients of the chemoattractants C5a, formyl peptide, leukotriene B4 (LTB4), and IL-8 and observed that C5a and formyl-methyl-leucine-phenylalanine (FMLP) were both capable of arresting the migration of neutrophils toward gradients of LTB4 or IL-8 when cells encountered the former ligand in advance of sensing the latter. Mechanistically, it had been previously demonstrated that C5a and FMLP are capable of inducing a heterologous desensitization response to each other and to so-called secondary chemoattractants such as lipid mediators and chemokines. Thus cells that sense C5a (or formyl peptide) in advance of chemokines or arachidonic acid metabolites would be less responsive to the latter.

By extension of this logic to our current observations, it follows that in an in vivo inflammatory response, early generation of C5a could limit the cellular recruitment and subsequent pathology induced by chemokines and other secondary mediators. Evidence pertaining to this has already been reported. Ischemia-reperfusion injury in rabbits and dogs has been shown to lead to an initial recruitment in the first several hours that is C5a dependent for both monocytes and neutrophils (3, 12). After this first initial phase, chemokines such as IL-8 and monocyte chemoattractant protein-1 appear. Thus the kinetics of a developing acute inflammatory response has C5a as perhaps the earliest mediator, with later phase influx being controlled by chemokines and other mediators. Thus we hypothesize that the absence of C5a or its receptors in an acute inflammatory event such as pancreatitis allows the so-called secondary mediators (such as chemokines and arachidonic acid metabolites) to recruit cells that have not been partially desensitized by C5a, and thus a more robust inflammatory response ensues. Testing of this hypothesis in vivo using C5-deficient and C5aR-deficient animals might be possible with antagonists of the chemokine and arachidonic acid metabolite pathways once appropriate targets are identified.

REFERENCES


