The effect of C1 inhibitor on intestinal ischemia and reperfusion injury

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Intestinal ischemia and reperfusion (IR) is a common clinical event caused by many medical conditions, including acute mesenteric ischemia, small bowel transplantation, abdominal aortic aneurysm, hemorrhagic shock, necrotizing enterocolitis, septic shock, and severe burns. IR injury is a potent inducer of complement activation that results in the production of a number of inflammatory mediators. Complement proteins are activated in three pathways: classical, alternative, and lectin pathways. C1 inhibitor (C1INH), a serine protease inhibitor that belongs to the superfamily of serine proteinase inhibitors, is a glycosylated plasma protein that plays an important role in the regulation of complement activation. C1INH inhibits activation of the contact system and is endowed with anti-inflammatory properties that are independent of protease inhibition. The goal of these studies was to investigate the role and mechanism of C1INH in alleviating IR-induced intestinal injury. C57BL/6, C1INH-deficient (C1INH-/-), and C3-deficient mice (C3-/-) were randomized into three groups: sham operated control, IR, and IR + C1INH-treated groups. Ischemia was induced by occlusion of the superior mesenteric artery followed by reperfusion. IR resulted in intestinal injury in C57BL/6, C1INH-/-, and C3-/- mice with significantly increased neutrophil infiltration into intestinal tissue. In each mouse strain, C1INH treatment reduced intestinal tissue injury and attenuated leukocyte infiltration compared with the untreated IR group. C1INH inhibited leukocyte rolling in the mesenteric veins of both C57BL/6 and C3-deficient mice subjected to IR. C1INH treatment also improved the survival rate of C57BL/6 and C1INH-/- mice following IR. Similar findings were observed in the IR animals treated with iC1INH. These studies emphasize the therapeutic benefit of C1INH in preventing intestinal injury caused by IR. In addition to the protective activities mediated via inhibition of the complement system, these studies indicate that C1INH also plays a direct role in suppression of leukocyte transmigration into reperfused tissue.

C1 inhibitor (C1INH) is a heavily glycosylated plasma protein that belongs to the superfamily of serine proteinase inhibitors. It is a major inhibitor of both the complement and contact systems (12). C1INH, like most other serpins, reacts with target proteases (such as C1s) to form high molecular weight complexes. However, reactive center-cleaved C1INH (iC1INH) loses the ability to complex with target proteases (7, 37). A number of studies have previously shown that C1INH could limit tissue injury in IR; this effect has been assumed to result from its ability to inhibit complement activation (4, 13, 30). However, direct evidence for the exact mechanism by which C1INH provides its protection after IR has not been reported. We recently demonstrated that C1INH suppresses leukocyte transmigration across the endothelium in other models of acute inflammation and that this activity is not dependent on the protease inhibitory activity of C1INH (6). This effect very likely depends on the presence of the Lewis tetrasaccharide attached to one or more of the six N-linked carbohydrates on C1INH, which suppresses interaction of selectin ligands with both E- and P-selectins (5). The present study was designed to investigate the role and mechanism of C1INH-mediated suppression of reperfusion-induced intestinal injury.

MATERIALS AND METHODS

Animals. C57BL/6 mice and bradykinin type 2 receptor-deficient (Bk2R-/-) mice were purchased from Jackson Laboratory (Bar Harbor, ME). C1INH-deficient (C1INH-/-) mice were maintained in our laboratory (19), and C3-deficient (C3-/-) mice were obtained from Michael Carroll, PhD, Immune Disease Institute, Boston, MA. Experiments were performed with mice at 8–12 wk of age. All experiments were performed in compliance with relevant laws and institutional guidelines and were approved by the Immune Disease Institute Animal Care and Use Committee.

Reagents. C1INH (Berinert) was generously provided by CSL Behring (King of Prussia, PA). iC1INH was prepared by incubating native C1INH with trypsin agarose (no. T4019; Sigma, St. Louis, Missouri) and purified by the method of Chauhan et al. (10). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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C1INH was incubated with trypsin agarose (1 U/8 ml) at room temperature for 1 h and then subjected to centrifugation (5,000 g) at 4°C. The supernatants were collected and used immediately. The function of C1INH and iC1INH was characterized using the ability of complex formation with C1s. C1INH and iC1INH were incubated separately with C1s (1:1 molar ratio) at 37°C for 60 min, subjected to SDS-PAGE, and stained with Coomassie blue. Native C1INH reacts with C1s to form a 200-kDa complex. However, iC1INH loses the ability to complex with C1s and is therefore regarded as inactive. In addition, iC1INH migrates on SDS-PAGE with a slightly smaller apparent molecular weight than intact active C1INH (~105 kDa).

Surgical procedures. The animals were anesthetized with ketamine (100 mg/kg ip) and xylazine (10 mg/kg ip). A midline abdominal incision was performed to expose the superior mesenteric artery (SMA). Ischemia was induced by totally occluding the SMA with a surgical microclip for 30 min. C3-deficient mice were subjected to 45-min ischemia to obtain an injury of similar intensity to that induced in the other strains. The abdomen was covered with warm moist gauze during this period, and a heating pad was used to keep the animal warm. Sham-operated mice underwent the same surgical procedure without the SMA occlusion. Five minutes before reperfusion either C1INH (0.4 U/g body wt, 1 U = 160 μg) or normal saline was administered intravenously. In addition, in some experiments, iC1INH (0.4 U/g in 200 μl PBS, 1 U = 160 μg) was used. Mesenteric ischemia was confirmed when the mesenteric pulsation was lost and the intestines became pale. Perfusion was achieved by removing the clip. Reperfusion was confirmed by the return of pulsatile blood flow at the mesenteric vessels in the intestines that were directly subjected to IR injury following clamp removal. The wound site was then closed, and the mice were kept anesthetized and warm during reperfusion. For the biochemical and histopathological analyses, reperfusion was allowed to occur for 4 h, and the mice were then subjected to euthanasia. For measuring survival rates, reperfusion was reestablished after 30 min (45 min in the case of C3−/− mice) of ischemia, and the mice were monitored for 7 days. At least 10 animals were used for each group.

Histology. For histological analysis of mucosal damage, a section of jejunum (about 1.5 cm) was immediately taken after 4 h of reperfusion and fixed in 10% formalin PBS at 4°C overnight. The samples were dehydrated and embedded in paraffin. Intestinal tissue sections (6 μm) were cut and stained with hematoxylin and eosin and then scored to quantify the extent of mucosal damage. Mice subjected to intestinal IR developed mucosal injury that ranged from shortening and vacuolated villi to complete destruction of normal mucosal architecture. Mucosal injury was classified using a modified grading system as described before (11). Briefly, the average of villus damage is graded on a 0–5 scale. A score of 0 is assigned to a normal villus with the mucosal surface intact and villus height maintained; a score of 1 applies to villi with mild tip distortion or development of an apical subepithelial space; a score of 2 is assigned to villi with extended subepithelial spaces or patchy disruption of the epithelial cells; a score of 3 indicates the presence of exposed villi but intact lamina propria with epithelial cell sloughing; a score of 4 is assigned when there is disintegration of the lamina propria; a score of 5 indicates the presence of hemorrhage or denuded villi. At least 10 villi from the same section were scored by one pathologist in a blind manner.

Intestinal myeloperoxidase activity analysis. Myeloperoxidase (MPO) activity, an indicator of neutrophil infiltration, was measured in intestinal tissue homogenates by a modification of the method of Grisham (16). Briefly, a fragment of small intestine was weighed and homogenized in 1 ml of PBS. A sample (100 μl) of potassium phosphate buffer (50 mM) containing 1% hexamethylenediamine-N,N,N′,N′-tetraacetate detergent (no. H9151, Sigma) was added into 100-μl samples, and subjected to centrifugation (10,000 g) at 4°C. The supernatants were collected and reacted with o-dianisidine dihydrochloride (0.167 mg/ml) and H2O2 (0.005%) in potassium phosphate buffer (50 mM, pH 6.0). The change in absorbance was measured spectrophotometrically at 450 nm every 10 s for 3 min; the linear portion of the tracing was used for analysis. One unit of MPO activity was defined as the quantity of enzyme that hydrolyzed 1 μmol H2O2/min at 25°C. MPO activity was expressed as units per 100 mg of wet tissue.

Lactate dehydrogenase activity in serum. Mouse blood samples were obtained by cardiac puncture after 4 h of reperfusion. Samples were centrifuged for 10 min at 3,000 g, and serum were stored at −80°C until they could be used. Serum lactate dehydrogenase (LDH) levels were measured using a microtiter plate adaptation of a commercially available kit (BioAssay Systems, Hayward, CA).

Determination of the mucosal content of TNF-α. The concentrations of TNF-α in mucosal tissue were determined using mouse TNF-α ELISA kits (PeproTech, Rocky Hill, NJ) according to the manufacturer’s instructions. Briefly, a fragment of small intestine was weighed and homogenized in 1 ml PBS. Samples were then centrifuged for 10 min at 3,000 g, and supernatants were stored at −80°C until they could be assayed. The detection limit of the ELISA assay was 8 pg/ml. Results are shown as pg TNF-α per 100 mg of wet tissue.

Observation of leukocyte rolling by intravital microscopy. Intravital microscopy (IVM) was performed 30–60 min after reperfusion. Veins of 200–300 μm in diameter with a shear rate ranging from 100–150/s were studied as described (10). Briefly, the exposed mesentery was kept moist throughout the experiment by periodic superfusion of warmed (37°C) saline. The mesenteric veins were transilluminated with a 12 V, 100 W, direct current stabilized source and visualized using a Zeiss (Oberkochen, Germany) Axiovert 135 inverted microscope (objective ×32) connected to an SVHS video recorder (AG-6730; Panasonic, Tokyo, Japan) using a CCD video camera (Hamamatsu Photonic Systems, Hamamatsu City, Japan). The wall shear rate for mesenteric veins (200–300-μm diameter) was calculated on the basis of Poiseuille’s law for a Newtonian fluid, \( \gamma_w = \frac{8 \cdot v_{ave} \cdot d_v}{\pi \cdot d}_v \), where \(v_{ave}\) is mean blood velocity and \(d_v\) is the diameter of the vein. The centerline erythrocyte velocity \(v_{cel}\) was measured using an optical Doppler velocimeter (Microcirculation Research Institute). \(v_{mean}\) is estimated from the measured \(v_{cel}\) by multiplying with an empirical factor of 0.625. Leukocytes were stained by rhodamine 6G (0.1 ml, 0.5 mg/ml) injection. Leukocyte interaction with the endothelium of the vessel wall was recorded for 5 min each in four different veins per mouse. Recorded images for leukocyte rolling were analyzed as follows. The number of leukocytes passing through a plane perpendicular to the vessel axis during a 1-min interval was counted by the same observer in a treatment and genotype blind manner. Leukocyte rolling/min/vein for each mouse was determined by taking the average of five 1-min counts as observed on the video screen during the entire recording.

Statistical analysis. The data are expressed as means ± SE. Differences between groups were statistically analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison tests. A value <0.05 was considered statistically significant. For survival analysis, the differences were evaluated by log-rank (Mantel-Cox) analysis. Statistical analysis was performed with Prism5 (GraphPad, La Jolla, CA).

RESULTS

C1INH reduces local mucosal injury. The progressive mucosal damage that occurs following intestinal IR has been comprehensively characterized and individual scores for all groups are shown in Figs. 1 and 2. Histological features of normal gut tissues were observed in tissues prepared from sham-operated mice, which had no obvious intestinal damage,
with the mucosal surface intact and villus height maintained. Mice subjected to IR that received vehicle control (normal saline) showed damage to the intestinal wall with reduction in villus height and marked destruction of epithelial cells within the villi (Fig. 1).

C57BL/6, C1INH−/−, Bk2R−/−, and C3−/− mice (45-min ischemia) had comparable levels of intestinal injury; the tissue injury scores were 2.5 ± 0.22, 2.2 ± 0.29, 2.2 ± 0.25, and 2.7 ± 0.33, respectively. Animals treated with C1INH (at a dose of 0.4 U/g) were protected from injury (Fig. 1). Mucosal injury scores were significantly reduced in C57BL/6, C1INH−/−, Bk2R−/−, and C3−/− mice treated with C1INH compared with mice that underwent IR without treatment (1.00 ± 0.30, 0.70 ± 0.15, 0.50 ± 0.19, and 1.33 ± 0.33, respectively). C57BL/6 mice treated with iC1INH were also protected from injury, with a reduced mucosal injury score of 1.5 ± 0.19 (Fig. 2).

C1INH treatment results in reduced intestinal MPO activities. MPO activity, an index of neutrophil infiltration, was measured in intestinal tissue homogenates. As indicated by elevated MPO levels, IR led to increased neutrophil infiltration into the intestinal tissue. MPO levels were similarly elevated in C57BL/6, C1INH−/−, Bk2R−/− and, C3−/− mice. C1INH treatment of each of the four groups of mice resulted in significant attenuation of leukocyte infiltration. Similar findings were observed in the C57BL/6 IR animals treated with iC1INH (Fig. 3).

C1INH reduces serum LDH activity. LDH, an important biomarker of tissue injury, was analyzed in the serum of mice subjected to IR. Untreated control mice undergoing IR dem-
C1INH inhibits leukocyte rolling in the mesenteric circulation. IR markedly enhances leukocyte-endothelial cell interactions in mesenteric veins. IVM was used to visualize these interactions. The shear rate and diameter of the evaluated veins were similar for control mice, those treated with native C1INH, and those with cleaved C1INH (Table 1). Leukocyte rolling in the mesenteric veins was strikingly increased by 30 min after reperfusion (Fig. 6). Therefore, in these experiments, rolling cells were counted at this time point. Reduced numbers of rolling cells were detected in the mesenteric veins in both C1INH and iC1INH-treated groups (Fig. 6). The mean number of rolling cells was reduced from 109 ± 11 cells/min in control untreated mice to 52 ± 9 cells/min in mice treated with C1INH (P < 0.001) and 56 ± 8 cells/min in mice treated with iC1INH (P < 0.001). Leukocyte rolling was also evaluated in C3−/− mice. The elevation in number of rolling leukocytes in C3−/− mice subjected to 45-min ischemia followed by reperfusion was similar to that observed in C57BL/6 mice subjected to 30-min ischemia (125 ± 36 cells/min). This elevation was reduced to 45 ± 12 cells/min in mice treated with C1INH (P < 0.001) (Fig. 6).

C1INH improves survival of mice after IR. The survival rate of C57BL/6 mice subjected to 30 min of ischemia and 4 h of reperfusion was 28%. Treatment with either C1INH or iC1INH significantly improved survival rates to 82% and 75%, respectively (Fig. 7A). The survival of untreated C1INH−/− mice following IR was very similar to that of wild-type mice (27%), and treatment with C1INH resulted in a similar higher survival rate (78%) (Fig. 7B). In contrast, although both Bk2R−/− and C3−/− mice had revealed significant histologic damage (Figs.

Table 1. Hemodynamic parameters of mesenteric veins

<table>
<thead>
<tr>
<th></th>
<th>Diameter, μm</th>
<th>Centerline Velocity, mm/s</th>
<th>Newtonian Wall Shear Rate, s⁻¹</th>
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<tbody>
<tr>
<td>Control</td>
<td>269 ± 16</td>
<td>7.5 ± 2.1</td>
<td>140.0 ± 37.9</td>
</tr>
<tr>
<td>Native C1INH</td>
<td>274 ± 15</td>
<td>6.9 ± 1.8</td>
<td>127.1 ± 36.2</td>
</tr>
<tr>
<td>Cleaved C1INH</td>
<td>281 ± 17</td>
<td>6.4 ± 1.9</td>
<td>124.3 ± 15.6</td>
</tr>
</tbody>
</table>

Values are represented as means ± SD. n = 16/group. P = NS. Hemodynamic parameters were established before recording leukocyte rolling. C1INH, C1 inhibitor.
1 and 2), the survival rates of both of these deficient mice were much higher (77% and 83%, respectively), as expected from previous observations (8–10). For this reason, no significant effect of C1INH treatment on survival of these mice could be observed (Fig. 7, C and D).

**DISCUSSION**

In injury attributable to ischemia, two of the major components involved in events leading to injury are complement activation and neutrophil stimulation. The complement split product, C5a, and the terminal MAC, C5b-9, are believed to be primarily responsible for complement-mediated IR-induced tissue injury (42). C1INH is a heavily glycosylated plasma protein that belongs to the serpin superfamily of serine proteinase inhibitors. It inhibits activation of the classical and lectin pathways by inactivation of the early proteases involved in activation, C1r, C1s, and mannose-binding lectin-associated serine protease 2 (38, 40, 44, 49, 52). In addition, via a

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mechanism that does not involve protease inhibition, it also may play a role in regulation of alternative pathway activation (26).

Complement-depleted animals and animals treated with complement inhibitors have been shown to sustain significantly less injury compared with control animals when subjected to IR (14, 21, 41, 48). In confirmation of previous reports (25, 28, 30, 31), the studies described here demonstrated that treatment with C1INH exerts effective protection against IR-induced injury. Taken together, these data provide evidence that C1INH (1) inhibits leukocyte rolling, 2) reduces neutrophil infiltration, 3) attenuates tissue damage, and 4) improves survival rate. Treatment with C1INH uniformly resulted in improvement of histopathology scores and in other signs of intestinal damage and inflammation.

The beneficial effect of C1INH has been assumed to result primarily from its ability to suppress complement-mediated damage. However, several observations suggest that suppression of IR injury by C1INH is more complex. C3 \( \sim^{-} \) mice previously were shown to be less susceptible to intestinal IR injury (48). By prolonging the period of ischemia to 45 min, the C3 \( \sim^{-} \) mice showed significant early histologic damage (Figs. 1 and 2), together with other evidence of tissue damage (elevated LDH levels), neutrophil infiltration (leukocyte rolling and elevated MPO levels), and inflammation (elevated TNF-\( \alpha \) levels) (Figs. 3–6). The histologic damage and these other indicators were all reversed in the C3 \( \sim^{-} \) mice, as in other mice, by treatment with C1INH. This strongly suggests that C1INH suppressed injury by mechanisms other than inhibition of complement activation. However, the C3 \( \sim^{-} \) mice, even with the longer period of ischemia (45 min vs. 30 min) survived at a higher rate than did control C57BL/6 mice (Fig. 7D), which is consistent with previous observations (48). This high rate of survival obscures any potential positive effect of C1INH on survival of the C3 \( \sim^{-} \) mice. Interestingly, the survival of untreated C1INH \( \sim^{-} \) mice following IR was similar to, not worse than, the wild-type mice. This similarity suggests that the concentration of C1INH in normal wild-type mice is insufficient to provide protection from IR injury. With the dose of C1INH in this and other studies, blood levels of C1INH very likely are increased by three- to fivefold.

Regulation of the contact system via inactivation of coagulation factor XIIa and plasma kallikrein is another important action of C1INH (12). Even though some studies provide evidence that bradykinin, generated via contact system activation, plays a role in mediating damage in the pathophysiological processes of IR, others suggest that it has little effect, or even provides a protective effect (27, 50). The Bk2R is constitutively expressed on various cell types, including endothelial cells, nerve fibers, leukocytes, and mast cells. In the survival studies reported here, the untreated Bk2R \( \sim^{-} \) mice had a higher survival rate after IR than the wild-type mice, consistent with previous findings that bradykinin may be involved in the mediation of damage (1, 46). In the histological analyses, however, we found that, even though they survive, the Bk2R \( \sim^{-} \) mice after IR do have mucosal damage and that C1INH suppresses this damage. This indicates that, although the contact system may play a role in IR injury, the protection provided by C1INH is not a result of contact system inhibition.

In addition to the observations that C1INH was effective in both C3 \( \sim^{-} \) and Bk2R \( \sim^{-} \) mice, iC1INH, which is cleaved at its reactive center and therefore has no protease inhibitory activity, also was effective in C57BL/6 mice. Therefore, C1INH must have suppressed inflammation via some mechanism other than protease inhibition.

The gut can become a cytokine-liberating organ after injury, and these endogenous inflammatory cytokines may function as mediators in the development of remote organ damage in response to local ischemic insult (17, 18). Several studies have clearly demonstrated the importance of TNF-\( \alpha \) in mediating IR-associated tissue injury and lethality (8, 47). Expression of adhesion molecules is also induced by TNF-\( \alpha \) signaling, resulting in increased leukocyte-endothelial adherence and activation of leukocytes, which is likely to induce the production of additional inflammatory mediators including proinflammatory cytokines, free radicals, and chemokines. These vicious and self-sustained cycles enhance further tissue damage (51). Our previous studies have shown that C1INH limited TNF-\( \alpha \) production after endotoxin shock or sepsis (35). In this study, we found that intestinal IR resulted in increased tissue TNF-\( \alpha \) production, whereas C1INH significantly blunted this elevation.

LDH is an intracellular enzyme with high concentrations found in the liver, heart, skeletal muscles, and other organs. Elevated serum LDH concentrations are detected after tissue injury, which makes serum LDH activity a reliable marker for IR injury. Hepatic damage is consistently observed as a component of intestinal IR injury (23, 34, 39). Our results indicate that serum LDH increased after intestinal IR and that C1INH prevents these changes in LDH levels. C1INH, therefore, in addition to limiting damage to the reperfused intestine, may also suppress the associated hepatic injury.

Leukocytes are key mediators of intestinal IR injury (36). Activated neutrophils infiltrate through the intestinal epithelium, causing mucosal and submucosal injury. In this study, in the absence of treatment, reperfusion following 30-min ischemia led to neutrophil accumulation and increased MPO activity, which is consistent with previous studies (15, 45). Treatment with C1INH and iC1INH resulted in reduced intestinal MPO levels after 4-h reperfusion. These data demonstrate that C1INH may be beneficial in reducing tissue injury, possibly as a consequence of the reduction in the number of neutrophils accumulating in tissues.

One of the earliest phenomena occurring in IR is endothelial dysfunction, which is considered the “trigger” of reperfusion injury (32). Activation of endothelial cells by cytokines, bacterial products, and activated complement leads to increased leukocyte adherence and transendothelial migration. The rolling of leukocytes on the endothelium is the first step in leukocyte extravasation into the postischemic gut. Therefore, inhibition of the adhesion of neutrophils to the vascular endothelium may attenuate or prevent IR injury. In the present study, 30 min after reperfusion an obvious increase in the number of rolling leukocytes was detected in the mesenteric veins, indicating the onset of inflammation. The number of rolling leukocytes after a single dose of C1INH was significantly decreased compared with controls, indicating a pronounced anti-inflammatory effect. These data are consistent with the reduction in intestinal MPO activities in mice treated with C1INH, as discussed in the preceding paragraph. Previous studies also have shown that C1INH effectively suppresses leukocyte rolling and adhesion in IR-initiated injury (24, 33, 43). These studies assumed that the effect was primarily via C1INH-mediated abrogation of
complement activation. However, the observations described above and the experiments showing that C1INH and iC1INH each suppressed leukocyte rolling in both C57BL/6 and C57−/− mice indicate that the effect is not solely on inhibition of complement activation. Leukocyte rolling, a prerequisite to transmigration into damaged tissue, is predominantly supported by the selectin family of adhesion molecules (P-, E-, L-selectins). We have demonstrated that C1 inhibitor binds to selectins, interferes with leukocyte-endothelial adhesion, and suppresses leukocyte transmigration across the endothelium in other models of acute inflammation (5, 6). This activity is not related to its protease-inhibitory function because the reactive center-cleaved inactive form is as potent as the native form. However, it is dependent upon the presence of the Lewisx tetrasaccharide that is attached to one or more of the six N-linked carbohydrates on C1INH (5). Therefore, in addition to its anti-inflammatory activities mediated via inhibition of complement and contact system proteases, C1INH plays a direct role in modulating leukocyte adhesion during inflammation. This suggests that the effect of C1INH (and iC1INH) on leukocyte rolling in IR injury may be mediated largely via this selectin-binding mechanism. Although the data described here do not prove that C1INH directly inhibits leukocyte recruitment in intestinal IR injury, together with previous data, this study strongly suggests that this mechanism plays a role.

In conclusion, treatment of intestinal IR with C1INH limits the local tissue damage and improves survival. It accomplishes this by inhibition of neutrophil infiltration, with a consequent reduction in the local inflammatory response. The protective activities of C1INH are very likely mediated via inhibition of complement activation, inhibition of contact system activation, and via other mechanisms, in particular the inhibition of leukocyte transmigration.

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REFERENCES


