Inflammatory ascites formation induced by macromolecules in mice and rats

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Baintner K. Inflammatory ascites formation induced by macromolecules in mice and rats. Am J Physiol Regul Integr Comp Physiol 297: R218–R223, 2009. First published May 20, 2009; doi:10.1152/ajpregu.00086.2009.—Different macromolecules were administered intraperitoneally to stimulate formation of protein-rich ascitic fluid in rodents. Stimulatory effect of plant lectins depended on the attachment to cell surface carbohydrates, Canavalia ensiformis (ConA) lectin was used in the majority of experiments. The time course of ConA-induced ascites was divided into an early (up to 4 h) and a late (from 6 h on) phase, with a transitional period between the two. Water and protein accumulation showed parallel time courses: volume of the ascitic fluid peaked at around 3 h, and fibrin threads appeared after 6 h. Viscosity of the ascitic fluid and its supernatant increased with time, reaching maximal fibrinogen concentration at around 16 h. Peritoneal permeability, followed by pleural and pericardial effusions, was elicited only by lectins that form soluble complexes with serum glycoproteins, whereas the effect of serum-precipitating lectins was restricted to the peritoneum. Macromolecules with serial positive charges (e.g., polylysine or polyethyleneimine) enhanced peritoneal permeability by ionic interactions with cell surface molecules. Viscosity of the polycation-induced ascitic fluid did not tend to increase with time and corresponded to the early phase of the ConA-induced ascites. Polyglutamate, a polyamionic macromolecule, inhibited the effect of polycations, but not that of ConA. The most efficient stimulatory macromolecules appear to induce ascites by noncovalent cross-linking of cell surface glycoproteins or glycosaminoglycans or both. A similar mechanism may operate in the maintenance of abdominal organs. The excess fluid is returned to the lymphatics through the so-called lymphatic stomata (36), located primarily at the diaphragm, but reabsorption also occurs across the mesothelium (32). The volume of peritoneal fluid is regulated primarily by the permeability of submesothelial vessels (10, 11), which may also provide antimicrobial substances and fibrinogen.

In the present work, we have tested a series of substances for the stimulation of inflammatory ascites formation. Only macromolecules were tested, because their slow absorption results in sustained stimulation and the accumulation of ascitic fluid.

The inflammatory effect of carrageenan (8) and zymosan (18) has been previously described. Ascites is also induced by intraperitoneally injected plant lectins (1, 2, 23, 26, 27). Previous investigations have focused on the migration of leukocytes into the serosal cavities, whereas the present work deals primarily with the accumulation of protein-rich ascitic fluid.

MATERIALS AND METHODS

Preparations. All lectin preparations were purified by affinity chromatography. Glycine max soybean (SBA), Triticum vulgare wheat germ (WGA), and Phaseolus vulgaris kidney bean (PHA) preparations were provided by P. Kiss (Szt. István University, Gödöllő, Hungary) and prepared according to Sandaltali et al. (31), Vretblad (42), and Bardocz et al. (3), resp. Galanthus nivalis (GNA) and Robinia pseudoacacia black locust bark (RPA-I) lectins were gifts of W. Peumans (Catholic University of Leuven, Belgium) and prepared according to van Damme and colleagues (37, 38). Agarose L-1000 was from Pharmacia; PHA-P and the other chemicals were Sigma products.

Animals and protocol. Specific-pathogen-free female NMRI mice (24 to 25 g, Charles River) and female Wistar rats (120–125 g) were used. The experiments complied with the Hungarian Animal Welfare Act XVIII/1998 and Edict 243/1998 and were approved by the local ethical committee and the Veterinary Office of Somogy County.

Animals were used in groups of six, and the experiment was repeated, if necessary. Test substances were dissolved in physiological saline and injected intraperitoneally in 0.1-ml volume. The usual dose of the inducer was 25 mg/kg body wt, which produced submaximal stimulation, as determined in preliminary experiments. Na poly-L-glutamate was used at 50 mg/kg body wt as an inhibitor. Gel-forming substances were dissolved in saline and injected intraperitoneally at 37°C.

Two and one-half hours postinjection (if not otherwise mentioned), the animals were decapitated under ether anesthesia, the abdomen was opened at the linea alba, and the ascitic fluid was carefully collected with a 50-μl pipette, performed always by the same person. The volume was measured by pipetting into another vessel and was expressed in microliters or as percentage of body weight. Poly-α-amino acids were neutralized with phosphate buffer before use.

BSA was used as a control protein. Evans blue was injected into the tail vein (0.5 mg dissolved in 0.05 ml saline), followed by intraperitoneal Canavalia ensiformis (ConA) lectin, 2 min later. Combined pericardial and pleural fluid volumes were measured in rats due to the greater response of this species and a lesser tendency for contamination with blood.

Determinations. Evans blue content of the centrifuged peritoneal fluid was measured with Hitachi U-201 spectrophotometer at 620 nm. Measurement of flow rate was performed with 0.5-ml centrifuged and heparinized samples in an Ostwald viscosimeter (VETLAB) and prepared according to Vretblad (42) and Bardocz et al. (3). Determinations were performed as seconds of flow-through time. Each of the pooled ascitic fluid samples (n = 7) was collected from two to four mice. Fibrinogen was measured in pooled and centrifuged ascitic fluid samples, using the classical method of Grannis (12), whereby fibrinogen was converted to fibrin by the addition of thrombin to the sample. After drying and washing, the fibrin clot was dissolved and measured photometrically. Purified fibrinogen was used as control. Activity of the plant lectin preparations was checked with the hemagglutination assay and the interaction between lectins and serum proteins was demonstrated with double diffusion in agarose gel (Ouchterlony).

Statistics. Significance of differences was calculated with ANOVA of the SPSS program, except for comparison of flow-through times, where t-test was used.

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RESULTS

A panel of plant lectins (Table 1) with different carbohydrate-binding specificities was tested for the ability to stimulate formation of inflammatory ascites in mice (Fig. 1). The lectins were applied below the maximally effective dose. Most of the lectins were effective, with the exception of *Arachis hypogaea* peanut (PNA), GNA, and *Tetragonolobus purpureas* asparagus pea (TPA). The stimulatory effect of succinyl-ConA was an order of magnitude less than that of parent ConA (Fig. 1). Little peritoneal fluid could be collected both from untreated or BSA-treated control animals, and this volume was taken as zero.

Enhanced permeability to serum albumin was indicated by the appearance of intravenously injected Evans-blue in the peritoneal cavity. Induced with ConA, the time course of ascitic fluid accumulation and appearance of Evans blue showed striking parallelism; the lack of parallelism in the first 15 min is accounted for by the volume of the injected solvent (Fig. 2). This parallelism indicates a close coupling between the permeability for water and protein. The appearance of the latter started within 10 min after injection (Fig. 2).

In the murine experiments, ConA-induced accumulation of peritoneal fluid peaked between 2.5 and 3 h and was free-flowing. Its viscosity increased with advancing time and fibrin threads were observed from 6 h on. After freezing and thawing, the fibrin formed a ball-like clot in the sample.

At 2.5 h the flow-through time (as a measure of viscosity) of ascitic fluid was significantly higher ($P < 0.01$) than that of the distilled water ($8.3 \pm 0.5$ and $6.7 \pm 0.2$ s, respectively) and showed a dramatic increase by 10 h ($26.2 \pm 3.3$ s; $P < 0.01$), in spite of the removal of cells and fibrin by centrifugation. Fibrinogen content of the fluid rose steadily, reaching a peak at 16 h (Fig. 3).

Increase of viscosity with advancing time could also be observed with other lectins, but not with poly-L-lysine or polyethyleneimine.

In rat experiments, the effect of intraperitoneally administered lectins on pleural and pericardial effusions, collected together, was investigated. When PHA was administered intraperitoneally, enhanced peritoneal permeability was followed, after some delay, by the accumulation of fluid in the thorax (distant effect), and the fluid volumes in the two compartments equalized by 16 h (Fig. 4). The lectins could be divided into three groups: 1) those producing distant effect

Table 1. Panel of plant lectins tested

<table>
<thead>
<tr>
<th>Abbrev.</th>
<th>Plant Name and Source</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ConA</td>
<td><em>Canavalia ensiformis</em> jack bean</td>
<td>α-β-mannoside</td>
</tr>
<tr>
<td>GNA</td>
<td><em>Galanthus nivalis</em> snowdrop bulb</td>
<td>terminal Man(1,3)Man</td>
</tr>
<tr>
<td>WGA</td>
<td><em>Triticum vulgare</em> wheat germ (25)</td>
<td>-GlcNAcβ(1,6)Gal(Neu5Ac)</td>
</tr>
<tr>
<td>SBA</td>
<td><em>Glycine max</em> soybean</td>
<td>NAcGalactosaminide</td>
</tr>
<tr>
<td>PNA</td>
<td><em>Arachis hypogaea</em> peanut</td>
<td>terminal Gal</td>
</tr>
<tr>
<td>TPA</td>
<td><em>Tetragonolobus purpureas</em> asparagus pea</td>
<td>α-L-fucoside</td>
</tr>
<tr>
<td>MAA</td>
<td><em>Maackia amurensis</em> leguminose tree</td>
<td>Neu5Acot(2,3)Galβ(1,4)GlcNAc</td>
</tr>
<tr>
<td>PHA</td>
<td><em>Phaseolus vulgaris</em> kidney bean</td>
<td>complex specificity*</td>
</tr>
<tr>
<td>RPA-I</td>
<td><em>Robinia pseudoacacia</em> black locust bark</td>
<td>complex specificity*</td>
</tr>
</tbody>
</table>

PHA was composed of structurally very similar E and L polypeptide chains. Glc, glucose; Man, mannose; Gal, galactose; GalNAc, N-acetyl-galactosamine; GlcNAc, N-acetyl-glucosamine; Neu5Ac, sialic acid. *Not inhibited by simple sugars. Data are from Ref. 39.

**Fig. 1.** Ascites-inducing effect of plant lectins (25 mg/kg body wt ip, 2.5 h) with different carbohydrate specificities in mice. Values are means \( \pm \) SD. *Groups that do not differ significantly from each other, but significantly ($P < 0.05$) differ from all other groups. Results obtained with inactive lectins (PNA, GNA, TPA) are not shown. ConA, *Canavalia ensiformis* jack bean; Succ, succinyl ConA; WGA, *Triticum vulgare* wheat germ; RPA, *Robinia pseudoacacia* black locust bark; PHA, *Phaseolus vulgaris* kidney bean; MAA, *Maackia amurensis* leguminose tree; SBA, *Glycine max* soybean.

**Fig. 2.** Ascites-inducing effect of intraperitoneal ConA (25 mg/kg body wt) in mice, shown on semilogarithmic time scale. Appearance of serum albumin is indicated by the Evans blue measurements. Values are means \( \pm \) SD.
(PHA, RPA-I, SBA); 2) those with local effect, i.e., the increase of permeability was restricted to the peritoneal cavity (ConA and WGA) (Fig. 5); and 3) those without effect (PNA, GNA). Polycations (poly-L-lysine and polyethyleneimine) did not exert a distant effect, and in this respect, resembled the second group of lectins.

Several polyamine-type macromolecules, referred to as polycations, were tested for induction of ascites formation. The synthetic, cationic poly-amino acids (poly-lysine and poly-arginine) had comparable effect to that of ConA (Table 2). The effect of D- and L-enantiomers of poly-lysine did not differ significantly. Polyethyleneimine (containing mostly secondary amines) and DEAE-dextran (tertiary amines) were also effective (Fig. 6). When compared at low dose (10 mg/body wt) polyethyleneimine was significantly \( P < 0.05 \) more stimulatory than ConA (not shown). Poly-L-asparagine, whose amide-N is without electric charge, and the negatively charged poly-L-glutamate Na were ineffective (Table 2).

Gastric mucin, an unusual substance in the peritoneal cavity, exerted little stimulation, while the gel-forming acidic and neutral poly-galactans showed wide variations in their effects (Fig. 6).

Fig. 5. Ascitic and pleural fluid volumes 6 h after intraperitoneal administration of lectin (25 mg/kg body wt) to rats. The two highest columns differed significantly \( P < 0.05 \) from the others. The effect of ConA was restricted to the abdominal cavity. Values are means ± SD.

<table>
<thead>
<tr>
<th>Test Substance</th>
<th>Molecular Weight, kDa</th>
<th>Ascitic fluid, % body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-( \alpha )-lysine</td>
<td>30–70</td>
<td>2.3±0.4</td>
</tr>
<tr>
<td>Poly-( \beta )-lysine</td>
<td>150–300</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Poly-DL-lysine</td>
<td>&gt; 70</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>Poly-( \gamma )-arginine</td>
<td>&gt; 70</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>Protamine, positive charges</td>
<td>4</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Lysozyme, slightly basic protein</td>
<td>15</td>
<td>none</td>
</tr>
<tr>
<td>Poly-L-asparagin, uncharged</td>
<td>5–15</td>
<td>none</td>
</tr>
<tr>
<td>Poly-( \gamma )-glutamate, negative charges</td>
<td>50–100</td>
<td>none</td>
</tr>
<tr>
<td>Poly-L-lysine + poly-( \gamma )-glutamate</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>ConA</td>
<td>2.5±0.2</td>
<td>2.5±0.6</td>
</tr>
</tbody>
</table>

Values are means ± SD; \( n = 6 \). Poly-\( \gamma \)-glutamate was used at 50 mg/kg body wt. “All or none” responses were obtained except for protamine, which differed significantly \( P < 0.05 \) from other groups.
However, it is also possible that some of the excess ConA crosses the mesothelial layer and reaches the submesothelial capillaries. Lectin-binding properties of mesothelial cells appear to be the same as those of most mammalian cells (14, 15, 21, 34). Most of the lectins also readily bind to different leukocytes and release cytokines and other factors from macrophages (19), lymphocytes (20), and mast cells (16). It was demonstrated in earlier experiments (2) that lectins aggregate the macrophages and attach them to the peritoneum. Corresponding to these patches, massive diapedesis of neutrophil granulocytes was observed during the late phase of permeability, presumably due to the release of cytokines from the activated macrophages. It is known that the diapedesis of leukocytes is associated with increased vascular permeability (7), as indicated by the accumulation of fibrinogen (a 340-kDa plasma protein) in the examined tissue (35). However, in the early phase of the increased peritoneal permeability, fully developed inflammation was not observed (2). The contribution of leukocytes and/or mesothelial cells to this early phase of permeability and the communication between mesothelium and endothelium require further investigation.

Previously, it was shown that lectins absorbed from the peritoneal cavity into the circulation increase the wet weight of liver and spleen, presumably due to the uptake of lectin-aggregated blood cells (2). The effect was specific to these organs; the weight of the kidneys tended to decrease. Generalized edema was not observed.

SBA, PHA, and a related lectin, RPA-I, produced a distant and apparently tissue-specific permeability effect from the peritoneal cavity to the other serosal membranes, while the effect of ConA and WGA was restricted to the injected compartment (Figs. 4 and 5).

Several lectins precipitate serum proteins (44), while others form soluble complexes with them. As the lectins used in this series of our experiments were tetravalent, the difference may be due to the valency of the serum glycoprotein receptors. In the present experiments, only the nonprecipitating lectins exerted distant effect, while the precipitating lectins, a local one. From soluble complexes the lectin may be transposed to the serosal membrane more readily than from a precipitate. Our experiments suggest that the absorbed lectin exerts a direct, distant effect; however, the contribution of humoral factors cannot be excluded.

**Polycations and other macromolecules.** In addition to lectins, several other macromolecules were tested for stimulation of peritoneal permeability. Polycations and related molecules were investigated for structural requirements of effectiveness (Table 2). For stimulation, an abundance of positive charges, in the form of primary, secondary, or tertiary amines, was required. Both L- and D-forms of basic poly-amino acids were required. Both L- and D-forms of basic poly-amino acids were equally stimulatory. The uncharged amide-N (poly-L-asparagine) and lysozyme, a protein with slight positive charge dominance, were ineffective; the reduced effect of protamine may be due to its small molecular mass. The polyanionic poly-L-glutamate did not stimulate permeability and inhibited the effect of polycations either by precipitation (poly-L-lysine) or without it (polyethyleneimine) (Table 2).

The polycationic molecules markedly dilate blood vessels (28, 33, 40). In the present experiments, polycations absorbed from the peritoneal cavity markedly dilated the abdominal

**DISCUSSION**

Due to rapid peritoneal absorption of small molecules, macromolecules were used as test substances. The BSA-treated control had zero stimulation.

**Plant lectins.** Glycosyl side chains of animal glycoproteins differ from those of the plant proteins, and the latter may be recognized by serum mannose-binding lectin (MBL), the start-differ from those of the plant proteins, and the latter may be control had zero stimulation. macromolecules were used as test substances. The BSA-treated ConA are the peritoneal leukocytes and the mesothelial cells.

The appearance of water and dye-labeled albumin paralleled changes with advancing time. These alterations suggest that the regulatory process itself was arbitrarily divided into an early (up to 4 h) and a late (from 6 h on) phase, with a transitional period between the two.
vessels, frequently resulting in blood-stained ascitic fluid samples. The vascular effect of lectins was much less prominent.

Zymosan A (particles of yeast cell wall lipopolyanamannan) specifically recognized by TLR2 receptors on the surface of both mesothelial cells (30) and peritoneal macrophages (24), significantly enhanced permeability (Fig. 6), but the participation of TLR2 receptor in the mediation of this effect remains to be investigated.

The gel-forming acidic and neutral poly-galactans showed wide variations in their stimulatory effect (Fig. 6). It is concluded, that the gel-forming property per se is not sufficient to induce inflammatory ascites.

Cross-linking and attachment sensing. Water molecules interact via H-bonds with each other and with hydroxyl-groups of cell surface polysaccharides. Neighboring glycosyl branches may be connected by water-bridges formed between a proton-donor and a proton-acceptor hydroxyl-group (6, 41). However, these water-bridges, because of their instability and continuous restructuring, do not interfere with the movement of cell surface glycoproteins in the liquid crystalline plane of plasma membrane.

Tetravalent plant lectins cross-link the glycosyl side chains, form lattices (4), and gradually restrict the mobility of membrane glycoproteins (13), as seen by the phenomenon of “capping” (22). When succinylated, the tetrameric ConA molecule dissociates to two dimeric units (43). This reduces its cross-linking capacity to a minimum and results in a dramatic decline of its stimulatory capacity (Fig. 1), despite the fact that the total number of carbohydrate-binding moieties remains the same. This finding underlines the importance of noncovalent cross-linking of cell surface glycosyl side chains in triggering permeability responses. Macromolecules with a series of positive charges (polycations) also triggered peritoneal permeability changes. They form cross-links through ionic interactions with terminal sialic acids of glycosyl branches, as well as with hyaluronate and sulfated glycosaminoglycans. All of these molecules were shown to be present on the surface of mesothelial cells (29, 45, 46), and the glycosaminoglycans also function as binding platforms of restricted specificity for a series of pro-inflammatory molecules (5).

Noncovalent cross-linking also takes place during exsiccation of serosal membranes: due to the disappearance of water bridges, progressively more H-bonds will be formed between the hydroxyl groups of neighboring and opposing glycosyl side chains (17), resulting in the well-known stickiness of semidry carbohydrates. This may explain the regulation of basal secretion of serosal membranes: the beginning of an exsiccation process will stimulate permeability until the restoration of the original wet state.

It is concluded that the serosal surface of body cavities functions as an attachment-sensing system triggered by cross-linking of cell surface molecules, although the existence of other signals cannot be excluded. Response of this system appears to be a common denominator in the regulation of both basal secretion and the early phase of stimulated permeability.

Perspectives and Significance

Investigation of adherent lectins and polycationic macromolecules may be used to clarify the regulation of ascites formation and to identify the released mediators, especially in the early phase, when the inflammatory response is not fully developed. The results may be used to refine existing models of peritoneal permeability and ascites formation during peritonitis and continuous ambulatory peritoneal dialysis. Future work should also clarify the communication among the three main players: the mesothelial lining, peritoneal leukocytes, and submesothelial endothelium.

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GRANT

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