Essential Role for Neutrophil Recruitment to the Liver in Concanavalin A-Induced Hepatitis

Claudine S. Bonder, Maureen N. Ajuebor, Lori D. Zbytnuik, Paul Kubes and Mark G. Swain

*J Immunol* 2004; 172:45-53; doi: 10.4049/jimmunol.172.1.45

http://www.jimmunol.org/content/172/1/45

**References**

This article cites 52 articles, 24 of which you can access for free at: [http://www.jimmunol.org/content/172/1/45.full#ref-list-1](http://www.jimmunol.org/content/172/1/45.full#ref-list-1)

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions**

Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Essential Role for Neutrophil Recruitment to the Liver in Concanavalin A-Induced Hepatitis

Claudine S. Bonder,* Maureen N. Ajuebor,† Lori D. Zbytnuik,* Paul Kubes,* and Mark G. Swain2†

Leukocyte infiltration into the liver is paramount to the development of liver injury in hepatitis. Hepatitis occurring after the administration of Con A in mice is felt to be a T lymphocyte-mediated disease. In this study, we report that neutrophils are the key initiators of leukocyte recruitment and liver injury caused by Con A. The objectives of this study were to investigate the involvement of neutrophils in Con A-induced hepatitis in vivo via intravital microscopy. After Con A administration, we observed a significant increase in leukocyte rolling flux, a decrease in rolling velocity, and an increase in leukocyte adhesion to the hepatic microvasculature. Fluorescence microscopy identified that within 4 h of Con A administration only a minority of the recruited leukocytes were T lymphocytes. Furthermore, immunohistochemistry showed a significant increase in neutrophils recruited to the liver post-Con A treatment in association with liver cell damage, as reflected by elevated serum alanine aminotransferase levels. Using flow cytometry, we observed that Con A could bind directly to neutrophils, which resulted in a shedding of L-selectin, an increase in $\beta_2$-integrin expression, and the production of reactive oxidants. Following neutrophil depletion, a significant inhibition of Con A-induced CD4+ T lymphocyte recruitment to the liver resulted and complete reduction in hepatic injury, as assessed by serum alanine aminotransferase levels. In summary, the present data support the concept that neutrophils play an important and previously unrecognized role in governing Con A-induced CD4+ T cell recruitment to the liver and the subsequent development of hepatitis. The Journal of Immunology, 2004, 172: 45–53.

The liver contains a large resident and migratory population of leukocytes that provide immunosurveillance against foreign Ags. This hepatic population can be rapidly expanded in response to infection or injury by recruiting leukocytes from the circulation. Moreover, acute and chronic hepatitis is pathologically characterized by a prominent infiltration of lymphocytes into the liver (1). Con A-induced hepatitis is commonly used as an experimental animal model of acute hepatitis (2, 3) and is felt to mimic human disease in many respects (4–6). Specifically, the cell types involved in the initiation and propagation of Con A-induced hepatitis (i.e., CD4+ T cells, NK T cells, neutrophils, Kupffer cells) have been directly implicated in a number of human liver diseases, including autoimmune, viral, alcoholic, and ischemia/reperfusion injury (1, 7–11). Therefore, we believe that the Con A model of hepatitis is an appropriate model to use to examine the role of neutrophils in an acute hepatitis classically felt to be due to hepatic damage mediated by activated T cells.

The direct demonstration that CD4+ T lymphocytes are effector cells for Con A-induced liver injury has been well described. A seminal study showed that athymic BALB/c (nu/nu) mice or BALB/c mice pretreated with a T cell-specific immunosuppressive drug, FK506, failed to develop massive cellular recruitment and hepatic inflammation in response to Con A (2). Furthermore, in vitro studies have shown that Con A directly activates T lymphocytes to: 1) proliferate; 2) produce proinflammatory cytokines such as IL-2, TNF-α, and IFN-γ; and 3) migrate specifically to the liver within hours of exposure (12, 13). These studies all support the suggestion that Con A-induced hepatitis is a T cell-mediated disease. However, there is increasing evidence that leukocytes other than the CD4+ T lymphocytes play a role in this lectin-mediated model of hepatitis. Several studies have shown that, within hours of Con A administration, there is a significant increase in the number of macrophages and neutrophils in the liver parenchyma (14–16). Although cell depletion studies have also identified Kupffer cells as effector cells in Con A-induced hepatitis (9), the role of neutrophils remains unclear.

Previous observations suggest that neutrophils are the first cell type to arrive at sites of inflammation and, as such, form the first line of immune defense. In response to chemotactic factors released at inflammatory sites, neutrophils migrate from the bloodstream through the vascular endothelium to their target (17). Once at the inflammatory site, neutrophils can act as effector cells in an attempt to resolve the inflammation (18–20). Alternatively, these cells may be cytotoxic and cause necrotic tissue destruction by proteases or reactive oxygen species (21–23). The precedent for the detrimental effect of neutrophil involvement in acute and chronic liver disease processes includes hepatic ischemia-reperfusion injury (24), endotoxemia (11, 25), sepsis (26), and alcoholic hepatitis (27). In these models of liver injury, neutrophils are activated and recruited into the liver vasculature, followed by transendothelial migration, and finally adherence to parenchymal cells in the sinusoids (28, 29).

Although there is evidence that neutrophils are key to the recruitment of other leukocytes (for review, see Ref. 30), very little

*Immunology Research Group, Department of Physiology and Biophysics, and †Gastrointestinal Research Group, Department of Medicine, Faculty of Medicine, University of Calgary, Alberta, Canada. Received for publication May 30, 2003. Accepted for publication October 17, 2003. 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.

1 This work was supported by Canadian Institutes of Health and a Canadian Institutes of Health group grant. M.G.S. is an Alberta Heritage Foundation Medical Research Scholar and a Canadian Institutes of Health/Health Canada Hepatitis C Initiative Investigator; P.K. is an Alberta Heritage Foundation Medical Research scientist and a Canadian Research Chair recipient; C.S.B. and M.N.A. hold Canadian Association of Gastroenterology Fellowships.

2 Address correspondence and reprint requests to Dr. Mark G. Swain, Department of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada. E-mail address: swain@ucalgary.ca

Copyright © 2004 by The American Association of Immunologists, Inc.
work has been done to investigate the role of neutrophils in T lymphocyte recruitment. The potential of neutrophils to control cell recruitment as well as their ability to produce potentially damaging mediators could have important implications in the pathogenesis of Con A-induced hepatitis and possibly hepatitis in humans. Therefore, in this study, we have investigated the possibility that neutrophils play a critical role in Con A-induced hepatitis. Using intravital microscopy, we evaluated the recruitment, trafficking, and role of neutrophils in Con A-induced hepatitis. Our results demonstrate that neutrophils are the major cell type recruited to the liver within 4 h of Con A administration, and that their depletion reduces CD4+ T lymphocyte infiltration and prevents the development of hepatitis. Moreover, these results suggest that neutrophils play a more important role than previously appreciated in acute hepatic injury due to Con A and possibly in patients with T cell-mediated hepatitis.

Materials and Methods

**Mice and reagents**

Con A was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). BALB/c mice were purchased from Charles River Laboratories (Montreal, Quebec, Canada). Green fluorescent protein (GFP) T cell mice (on a BALB/c background) were a generous gift from C. Weaver (University of Birmingham, Birmingham, AL). All mice were maintained in a pathogen-free facility until they weighed between 20 and 25 g and were between 6 and 10 wk of age, at which point they were used.

**Con A-induced hepatic injury**

Con A (13 mg/kg) was administered i.v. to BALB/c or GFP T cell BALB/c mice for 4 h before analysis. The dose of Con A used in these studies is slightly lower than that previously published to study Con A-induced liver injury (20 mg/kg for 8 h) (2, 3). We chose this dose of Con A (13 mg/kg) to ensure that the mice developed significant and reproducible liver injury, but were still well enough to endure anesthesia, surgery, and intravital microscopy. For untreated mice, 200 μl of sterile saline was injected.

**Blood analysis**

Blood was obtained by cardiac puncture at the time of sacrifice for analysis of serum alanine aminotransferase (ALT) as an index of hepatocellular injury. Measurements of serum ALT were made using a commercially available diagnostic kit (Sigma-Aldrich). Results are expressed as International Units per milliliter of serum.

Blood samples were also analyzed using a crystal violet or May-Grunwald-Giemsa stain (Criterion; Richard-Allan Scientific, Kalamazoo, MI) to quantify and characterize circulating white blood cells (WBC), respectively.

**Immunohistochemistry**

Mice were sacrificed and livers harvested, rinsed in PBS, and fixed in 10% Formalin (Sigma-Aldrich). Formalin-fixed tissues were embedded in paraffin. Four-micrometer-thick sections were stained for neutrophils with a chloroacetate esterase (Leder) stain (Sigma-Aldrich) and analyzed by light microscopy in a blinded fashion. Leukocyte numbers were determined by counting the number of positive-stained cells over 20 fields at a magnification of ×40. The number of positive cells per high power field (hpf) was then calculated.

**Intravital microscopy in the liver**

The experimental preparation for murine liver intravital microscopy has been previously described elsewhere (31). Animal protocols were approved by the University of Calgary Animal Care Committee and met the Canadian Guidelines for Animal Research. Mice were anesthetized by i.p. injection of a mixture of 10 mg/kg xylazine and 200 mg/kg ketamine hydrochloride, and the jugular vein was cannulated for maintenance of anesthesia during the experiment. Body temperature was maintained at 37°C using an infrared heat lamp. Mice were placed in a right lateral position on an adjustable Plexiglas microscope stage. A lateral abdominal incision along the costal margin to the midaxillary line was made to exteriorize the liver, and all exposed tissues were moistened with saline-soaked gauze to prevent dehydration. The liver was prepared for in vivo microscopic observation, as previously described (31). Briefly, the liver was placed on the viewing pedestal of an inverted microscope (Axiovert S 100; Zeiss, Oberkochen, Germany) with ×40 objective lens; Carl Zeiss, Calgary, Alberta, Canada) and continuously superfused with warmed bicarbonate-buffered saline (pH 7.4). The liver surface was then covered with Saran Wrap to hold the organ in position. The image of the microcirculatory bed was recorded using a video camera (Dage MTI N C-07, Michigan City, IN) and a video recorder (NV9590; Panasonic, Secaucus, NJ).

Postcapillary venules (25–40 μm in diameter) with associated sinusoids were selected for each study. The number of rolling and adherent leukocytes was determined offline during playback analysis. Rolling leukocytes were defined as WBC that moved at a velocity less than that of erythrocytes in a given vessel. The number of rolling leukocytes (flux) was counted using frame-by-frame analysis. To obtain a complete leukocyte rolling velocity profile, the rolling velocity of all leukocytes entering the vessel was measured. A leukocyte was defined as adherent to venular endothelium if it remained stationary for >30 s. Adherent cells were measured and expressed as either the number per 100 μm length of postcapillary venules or the number of cells adherent in the sinusoids per field of view.

**Neutrophil depletion**

To characterize the role of neutrophils during hepatitis, mice were depleted of granulocytes before Con A challenge. For depletion, we used RB6-8C5 mAb, a rat anti-mouse IgG2b, directed against Ly-6G (previously known as Gr-1), an Ag on the surface of murine granulocytes (32). The Ab was produced by TSD BioServices (Germantown, NY) by i.p. injection of RB6-8C5 hybridoma into nude mice and subsequent ascites collection. A total of 150 μg of RB6-8C5 was administered i.p. 24 h before Con A challenge. This resulted in peripheral blood neutropenia (reducing absolute circulating neutrophil counts by ~95%). For control mice, an IgG2b Ab (BD Biosciences, Mississauga, Ontario, Canada) was injected as an isotype control.

**Liver-derived lymphocyte isolation**

Liver-derived lymphocytes were isolated using a method previously described (33). Briefly, livers were excised and finely minced in a digestive medium containing 0.05% collagenase (Worthington Biomedical, Lakewood, NJ) and 0.002% DNase I (Life Technologies Invtrogen, Burlingame, Ontario, Canada) in HBSS (Life Technologies, Grand Island, NY). After gentle agitation at 37°C for 30 min, the concentrate was passed through a 40-micron nylon filter and washed twice with ice-cold PBS (pH 7.4) and centrifuged at 300 g for 10 min. Lymphocytes were purified by layering the cell suspension on Lympholyte-M (CedarLane, Hornby, Ontario, Canada) and centrifuged at 800 g for 20 min at room temperature. Lymphocytes were washed in PBS and counted in a trypan blue using a hemacytometer. The lymphocytes were then stained for CD4 using the CD4-PE mAb (BD PharMingen, San Diego, CA) and the manufacturer's instructions.

**Labeling of cells for flow cytometric analysis**

To investigate the binding of Con A to neutrophils, flow cytometric analysis was used. Briefly, whole blood was collected by cardiac puncture from BALB/c mice using a 1-ml insulin syringe precoated with heparin. One hundred microliters of whole blood were incubated without or with biotinylated Con A (10 μg/ml; Vector Laboratories, Burlingame, CA) for 30 min in a 37°C shaking water bath. RBC were lysed with OptiLyse B (Immunotech, Marseille, France), and leukocytes were incubated with streptavidin-PE (BD Pharmingen) for 30 min at room temperature. The cells were washed, resuspended in PBS/0.5% BSA/20 mM glucose solution, and read on a FACScan flow cytometer (BD Biosciences) using CellQuest Pro software. Data were compared with results from blood that did not receive Con A.

In a separate set of experiments, we examined Con A activation of neutrophils. The blood (100 μl) was incubated with or without Con A (10 μg/ml; Sigma-Aldrich) for 30 min in a 37°C shaking water bath. The blood was then stained with 1 μg of mAb against L-selectin (MEL-14 rat anti-mouse; BD Pharmingen), CD18 (GAME46 rat anti-mouse; BD Pharmingen), or nonspecific isotype controls (IgG2a and IgG1; BD Pharmingen) for 30 min at room temperature. OptiLyse B was used to lyse RBC, and leukocytes were then stained with FITC-conjugated polyclonal goat anti-rat Ig (BD Pharmingen) for 30 min at room temperature. After washing, cells were analyzed, as described above.

---

*Abbreviations used in this paper: GFP, green fluorescent protein; ALT, alanine aminotransferase; hpf, high power field; MIP, macrophage-inflammatory protein; WBC, white blood cell.*
Isolation of emigrated neutrophils

Six-week-old male BALB/c mice were injected i.p. with 1% oyster glycogen (Sigma-Aldrich) in saline. After 4 h, mice were euthanized, and a peritoneal lavage was performed with 3 ml of saline. Lavage fluid was placed on ice for 5 min and then centrifuged at 1000 rpm at 4°C for 6 min. Pellets were then resuspended in PBS at a concentration of 10⁷ cells/ml and kept at 4°C. This approach yielded a 99% pure population of emigrated neutrophils, as analyzed with Wright-Giemsa staining. In all experiments, neutrophils were kept on ice and used within 2 h of isolation.

Cytochrome c reduction assay

A cytochrome c reduction assay was used to measure the production of O₂⁻ from suspensions of emigrated polymorphonuclear cells. In brief, polymorphonuclear cells (10⁷/ml) were added to two cuvettes containing PBS with CaCl₂ (1.19 mM), MgCl₂ (0.54 mM), and cytochrome c (1.5 mM; Sigma-Aldrich). In one sample, superoxide dismutase (from bovine erythrocytes, 264 U/ml; Sigma-Aldrich) was added, and both samples were read at the same time in a spectrophotometer (model U-2000; Hitachi) at 550 nm. OD differences between the two samples were recorded on an online chart recorder (Johns Scientific, Toronto, Ontario, Canada). After 5 min of baseline measurements, 1 mg/ml Con A was added to both samples and OD was recorded for an additional 10 min. In a separate set of experiments, polymyxin B (Sigma-Aldrich) and Con A were mixed to give final concentrations of 10 µg/ml and 1 mg/ml, respectively.

Statistical analysis

All results are expressed as arithmetic mean ± SEM. An unpaired Student’s t test was used for comparisons between 2 means with a Welch’s correction, where necessary, and ANOVA followed by Newman-Keul post hoc test was used for comparisons between >2 means. A paired Student’s t test was used in the cytochrome c reduction assay. Statistical significance was set at p < 0.05.

Results

Determination of hepatic injury

To confirm hepatic injury after Con A administration, plasma ALT levels were measured. As shown in Fig. 1, ALT levels rose significantly within 90 min of Con A administration, peaked 4 h after Con A, and remained elevated until 8 h after injection.

Increased leukocyte recruitment to the hepatic vasculature of Con A-treated mice

Preliminary experiments with intravital microscopy indicated that at 8 h post-Con A treatment, the majority of livers exhibited exaggerated sludging in the sinusoids such that they were too damaged for study. Based on these observations, unless otherwise stated, all of the subsequent experiments were performed 4 h post-Con A treatment.

Using intravital microscopy, leukocyte trafficking was visualized in postsinusoidal vessels, as well as sinusoids, of untreated and Con A-treated mice. GFP T cell BALB/c mice were used to investigate what proportion of the leukocytes interacting with the hepatic vasculature of Con A-treated mice were T lymphocytes. Using light microscopy, we observed within any given postsinusoidal vessel of untreated mice 1.4 ± 0.4 leukocytes rolling every minute. In addition, these cells rolled at a relatively fast...
velocity of 79.1 ± 5.7 μm/s (Fig. 2, A and B, bar 1). Fewer than two adherent leukocytes were observed in the postsinusoidal venules and sinusoids of untreated mice (Fig. 2, C and D, bar 1). At 4 h post-Con A treatment, we observed a significant increase in leukocyte rolling flux in postsinusoidal venules (3.5 ± 0.8 cells/min), and leukocyte rolling velocity decreased to 12.3 ± 2.2 μm/s (Fig. 2, A and B, bar 2). As shown in Fig. 2, C and D (bar 2), we observed a significant increase in leukocyte adhesion in both the postsinusoidal venules (13.7 ± 1.8 cells/100 μm) and sinusoids (6.7 ± 1.5 cells/field of view) following Con A treatment.

To determine what proportion of the leukocytes recruited to the hepatic vasculature of these Con A-treated GFP T cell mice were in fact T lymphocytes, we used fluorescence microscopy. As shown in Fig. 2A (bars 3 and 4), when the mice were treated with Con A, there was a modest, but insignificant increase in GFP T cell rolling in the postsinusoidal venules (from 0.1 ± 0.1 to 0.8 ± 0.7 cells/min). In addition, given the low numbers of GFP T lymphocytes rolling in the hepatic vasculature of Con A-treated mice, we were unable to determine rolling velocity of the cells (Fig. 2B). In Con A-treated mice, we also observed a small increase in GFP T cell adhesion in the postsinusoidal venules (from 0.4 ± 0.1 to 1.2 ± 0.4 cells/100 μm; Fig. 2C, bars 3 and 4) as well as in the sinusoids (from 2.3 ± 0.3 to 4.9 ± 1.0 cells per field of view; Fig. 2D, bars 3 and 4).

These results suggest that within 4 h of Con A administration, there is a significant increase in leukocytes interacting and adhering in the postsinusoidal venules, and that only a small proportion (~20%) of these cells are T lymphocytes found almost exclusively in sinusoids.

FIGURE 3. Con A reduces leukocyte counts in peripheral circulation. Mice were treated without or with Con A for 4 h before whole blood being taken by cardiac puncture. A. The calculated circulating leukocyte counts; B, indicates the calculated neutrophil, lymphocyte, eosinophil, and monocyte levels in the peripheral blood from BALB/c mice. Data are expressed as the arithmetic mean ± SEM of six mice per group. *, p < 0.05 relative to untreated (□) vs Con A-treated (◼) mice.

FIGURE 4. Con A-induced neutrophil sequestration into the livers of BALB/c mice. Mice were treated with Con A for 4 h, and the livers were prepared for histology. Leder (esterase) stain of liver sections from untreated mice (A) shows basal numbers of neutrophils residing (arrows). Increased neutrophils are seen in Con A-treated mice (B) (arrows). C, Quantitative analysis of neutrophil sequestration into the liver where data are expressed as the arithmetic mean ± SEM of four mice per group. *, p < 0.05 relative to untreated mice.
Con A induces neutrophil recruitment to the liver

By crystal violet staining of whole blood obtained at conclusion of the experiments (~5 h post-Con A treatment), we observed that Con A administration resulted in significantly reduced circulating WBC counts. Compared with controls, Con A-treated mice exhibited a 55% drop in circulating WBC counts (Fig. 3A). As shown in Fig. 3B, further characterization of the circulating WBC by May Grunwald Giemsa staining identified a 70% reduction in the number of circulating lymphocytes as well as neutrophils. As Con A treatment may increase the circulating numbers of neutrophils by stimulating their mobilization from bone marrow or demarginalization, we investigated the accumulation of neutrophils in the liver by immunohistochemistry using a neutrophil esterase stain. As shown in Fig. 4, A and B, the livers of Con A-treated mice contained significantly more neutrophils compared with those from untreated mice. Quantification of the immunohistochemistry showed that with Con A treatment the number of neutrophils per hpf rose significantly from 3.0 ± 0.9 neutrophils/hpf to 20.4 ± 3.7 neutrophils/hpf (Fig. 4C). Moreover, the neutrophils were predominantly in either the sinusoids or parenchymal tissue.

Con A binds and activates neutrophils

To confirm that Con A can directly bind to neutrophils and activate them, whole blood was drawn by cardiac puncture from BALB/c mice and incubated without or with 10 μg/ml of Con A for 30 min. Flow cytometry, we observed a significant increase in the mean fluorescence intensity of neutrophils incubated with biotinylated Con A (Table I, n = 3). To investigate the ability of Con A to activate the neutrophils, we examined the expression of the adhesion molecules L-selectin and β2-integrin. As shown in Table I, as a mean of three experiments, neutrophils exposed to Con A for 30 min significantly shed L-selectin and expressed significantly more β2-integrin. No changes in mean fluorescence intensity were observed with the addition of isotype control Abs.

Con A stimulates neutrophils to produce reactive oxidants

To confirm that Con A can activate the neutrophils directly within a milieu, we isolated neutrophils and assessed their production of O$_2^-$ following Con A exposure. Emigrated neutrophils were isolated from BALB/c mice, and a cytochrome c reduction assay was performed. In three separate experiments, we observed a significant increase in the production of O$_2^-$ following Con A exposure. As shown in Fig. 5, untreated neutrophils produced 0.06 ± 0.03 nM/min/10$^6$ cells of O$_2^-$, and Con A-treated neutrophils produced 1.69 ± 0.52 nM/min/10$^6$ cells. This level is comparable to that produced by murine emigrated neutrophils stimulated with zymo-activated plasma (34) and less than half of that produced by fMLP-treated human neutrophils (35). Results obtained when Con A plus polymyxin B (10 μg/ml, an endotoxin inhibitor) were given to the neutrophils were equivalent to that of Con A alone. These results suggest a direct effect of Con A, and not an endotoxin contaminant, on superoxide production (data not shown). No changes in OD were observed when the Con A diluent alone was added (data not shown).

Neutrophil depletion reduces leukocyte trafficking in the livers of Con A-treated mice

To further characterize the involvement of neutrophils in Con A-induced liver injury, we depleted neutrophils using the RB6-8C5 anti-granulocyte mAb 24 h before Con A administration. As shown in Fig. 6, analysis of peripheral blood neutrophil counts confirmed that 95% of the neutrophils were depleted from the mice before Con A treatment. By contrast, the number of circulating lymphocytes did not change with RB6-8C5 administration (Fig. 6, bars 3 and 4).

Using intravital microscopy, we assessed whether Con A-induced leukocyte trafficking was altered in neutrophil-depleted mice. As shown in Fig. 7, neutrophil depletion significantly reduced leukocyte rolling and adhesion in the hepatic vasculature. Within the postsinusoidal vessels of Con A-treated, neutrophil-depleted mice, we observed less than one leukocyte rolling per minute (Fig. 7A, bar 4). This is significantly less than that observed in the Con A-treated mice (with or without the administration of an isotype control IgG2b Ab) (Fig. 7A, bars 3 and 2). Depletion of

---

**FIGURE 5.** Con A stimulates superoxide anion production from isolated neutrophils. Emigrated neutrophils were isolated from BALB/c mice and resuspended in buffer containing cytochrome c with or without superoxide dismutase. Con A (1 mg/ml) was added, and OD was recorded. Results represent the mean ± SEM of experiments from three mice. *p < 0.05 for untreated (●) vs Con A-treated (■) neutrophils.

**FIGURE 6.** RB6-8C5 treatment depletes neutrophils, but not T lymphocytes. Mice were treated without or with RB6-8C5 for 24 h before whole blood being taken by cardiac puncture and differential leukocyte counts calculated from peripheral blood from BALB/c mice. Data are expressed as the arithmetic mean ± SEM of four mice per group. *p < 0.05 relative to control (●) vs RB6-8C5-treated (■) mice.

---

**Table I.** Flow cytometric analysis of Con A binding and effects on L-selection shedding and β2-integrin expression

<table>
<thead>
<tr>
<th>Mitogen or Ab</th>
<th>Untreated (MFI ± SEM)</th>
<th>Con A (10 μg/ml) (MFI ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin-Con A</td>
<td>5 ± 1</td>
<td>13 ± 2*</td>
</tr>
<tr>
<td>Isotype control</td>
<td>21 ± 2</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>L-selectin</td>
<td>185 ± 7</td>
<td>157 ± 9*</td>
</tr>
<tr>
<td>β2-integrin</td>
<td>79 ± 9</td>
<td>116 ± 10*</td>
</tr>
</tbody>
</table>

*Results are expressed as the mean fluorescence intensity ± SEM from three experiments.

* p < 0.05 (vs untreated).
A significant reduction in Con A-induced neutrophil recruitment to the liver was also observed by immunohistochemistry using the esterase stain. In neutrophil-depleted mice, we observed that the Con A-induced neutrophil recruitment to the liver in these mice was 2.9 ± 1.3 neutrophils/hpf. By contrast, without neutrophil depletion, Con A administration induced the recruitment to 20.4 ± 3.7 neutrophils/hpf.

Neutrophil depletion ameliorates Con A-induced liver injury

In neutrophil-depleted BALB/c mice, we observed a significant reduction in Con A-induced liver injury, as assessed by serum ALT levels. As shown in Fig. 8 (bars 1 and 2), Con A treatment resulted in a significant elevation of serum ALT. Administration of an isotype control Ab IgG2b did not alter Con A-induced ALT levels (Fig. 8, bar 3). By contrast, with neutrophil depletion, Con A-induced liver damage was significantly reduced from 223 ± 14 to 55 ± 6 IU/ml for Con A and RB6-8C5 + Con A, respectively (Fig. 8, bars 4 and 5). Notably, administration of RB6-8C5 alone increased serum ALT levels slightly, but this was not further elevated with Con A treatment (Fig. 8, bars 4 and 5). Previous studies with antisera to cause neutropenia have shown that some of the inactivated neutrophils accumulate in the liver after treatment and are phagocytosed by the Kupffer cells (36). The initial activation and priming of the Kupffer cells are known to induce the release of inflammatory mediators such as TNF-α (37), and we hypothesize that this may have stimulated the small release of plasma ALT observed in these mice.

Neutrophil depletion reduces CD4⁺ T lymphocyte recruitment to the liver of Con A-treated mice

Using intravital microscopy, we assessed whether Con A-induced T lymphocyte adhesion was altered in neutrophil-depleted mice. In control Con A-treated mice, we observed 1.2 ± 0.4 GFP T lymphocytes adhering per 100 μm of post-sinusoidal vessel, and 4.9 ± 1.0 GFP T cells in sinusoids (bar 1 of Fig. 9, A and B, respectively). By contrast, with the depletion of neutrophils we observed significantly less GFP T lymphocytes adhering post-Con A treatment, with 0.1 ± 0.1 GFP T lymphocytes adhering per 100 μm of post-sinusoidal vessel, and 1.7 ± 0.5 GFP T lymphocytes adhering in the sinusoids (bar 2 of Fig. 9, A and B, respectively).

Previous work has demonstrated a role for CD4⁺ T lymphocytes in this model of hepatitis (2). As our experiments using GFP-neutrophils also significantly reduced the number of adherent leukocytes observed in the post-sinusoidal venules and sinusoids of Con A-treated mice. In Con A-treated neutrophil-depleted mice, we observed 0.9 ± 0.2 leukocytes adhering per 100 μm of post-sinusoidal vessel, and 1.1 ± 0.2 cells in sinusoids (Fig. 7, A, B, and C, bar 4). The addition of the isotype control IgG2b Ab had no effect on Con A-induced leukocyte adhesion (Fig. 7, B and C, bar 3). In separate experiments, we confirmed that neutrophil depletion did not simply delay Con A-induced liver injury. In neutrophil-depleted mice exposed to Con A for 8 h, we observed 0.3 ± 0.1 leukocytes rolling, 0.5 ± 0.2 leukocytes adhering in post-sinusoidal vessels, and 0.7 ± 0.3 cells adhering in sinusoids.
Neutrophils were isolated from the livers of mice treated 8 h previously with Con A. By contrast, the total number of CD4$^+$ lymphocytes isolated from the livers of neutrophin Con A-treated mice was $0.2 \pm 0.1 \times 10^5$ cells.

**Discussion**

Previous studies have implicated T lymphocytes as the key effector cells in Con A-induced hepatitis, as athymic mice and mice pretreated with a T cell-specific immunosuppressive drug did not develop liver injury (2). More recent work, however, has demonstrated a combinatory role for leukocyte subsets in the development of Con A-induced hepatitis (9, 13). In the present study, we show that Con A binds directly to neutrophils, activates them, and induces a significant increase in neutrophil recruitment to the livers of mice. Moreover, we show that neutrophil depletion significantly reduces Con A-induced CD4$^+$ T lymphocyte recruitment to the liver and completely abrogates liver injury in this model of hepatitis.

Neutrophils are a major component of the circulating blood and are well defined to constitute a rapid and first line of defense against most classes of pathogenic microorganisms. There is also increasing evidence that the contribution of neutrophils to host defense and natural immunity extends well beyond their traditional role as professional phagocytes in that they can mediate injury. Neutrophils can be induced to express a number of mediators that can influence inflammatory and immune responses. These include reactive oxygen species, complement components, proteases, and a variety of cytokines (e.g., TNF-α, IL-1β and IL-12) and chemokines (including macrophage-inflammatory protein (MIP)-1α, MIP-1β and IL-8) (23, 29). Murine MIP-2 is a chemokine considered to be functionally analogous to human IL-8 and rat neutrophil chemoattractant (38), and is primarily induced by TNF-α (39, 40). In a study by Bajt et al. (41), it was demonstrated that MIP-2 treatment increases murine sinusoidal neutrophil accumulation. More relevant to this study, Nakamura et al. (16) showed with blocking studies that TNF-α-mediated MIP-2 production is important in Con A-induced liver injury. Our work now extends these studies as we suggest that neutrophils are essential for the Con A-induced hepatic injury.

Cells recognize humoral immunologic reactants through receptors on their surface membrane. Con A has been shown to bind to a mannose receptor on a wide variety of cell types, including neutrophils (42), as well as the insulin receptor on liver-derived lymphocytes (43) and to the MHC of macrophages (44). The finding that the liver is the target organ of Con A-induced toxicity in vivo may be due to the fact that the aforementioned receptors can be found in the liver and that this organ contains the majority of macrophages in the body, namely Kupffer cells. The involvement of macrophages in Con A hepatitis has been shown by the absence of Con A-inducible lesions in macrophage-depleted animals and by the suggestion that T lymphocytes recognize a Con A-modified MHC structure of macrophages and become activated (2). The shedding of L-selectin and increased expression of the β$_2$-integrin on neutrophils clearly indicates their activation by Con A, as does their production of reactive oxidants. The regulation of these molecules may be important, as other studies have shown that neutrophil emigration is a β$_2$-integrin-dependent process (45) and that oxidant release is limited from neutrophils until after they transmigrate and reach the extracellular space (34). Our results suggest that a second mechanism of action may exist wherein Con A binds directly to neutrophils, presumably via a mannose-sensitive receptor (42), and subsequently activates them independent of other leukocytes.

Using intravital microscopy, we observed in vivo that Con A treatment increased leukocyte recruitment to the liver and that this
was characterized by an increase rolling flux, a decrease in rolling velocity, and an increase in adhesion of leukocytes in the postsinusoidal venules. We also observed an increase of leukocyte adhesion to the sinusoids of the liver. The current paradigm underlined for leukocyte rolling, adhesion, and extravasation into an inflamed area is not completely applicable to an inflamed liver, as the liver has two distinct vascular beds that recruit leukocytes; the postsinusoidal venules and the sinusoids. Leukocyte recruitment into the postsinusoidal vessels is due to adhesion molecules, whereas recruitment into sinusoids does not appear to be dependent on known adhesion molecules. Because in this study significant numbers of cells were recruited to both compartments, targeting adhesion molecules is unlikely to inhibit liver injury. Indeed, Massaguer et al. (46) observed that Con A-induced liver injury was significantly reduced in P-selectin-deficient mice; however, the evidence of residual liver injury in that study suggests that an adhesion-independent mechanism of recruitment may exist. It has been postulated that a combination of factors, such as vasocostriction, vascular lining cell swelling and injury, and reduced membrane flexibility after activation of the neutrophil, contributes to the mechanical trapping of these leukocytes in sinusoids (13, 29, 47, 48). Inhibiting a number of these factors may be quite difficult; however, based on our results, dampening neutrophil activation might be a viable therapeutic approach.

We have shown in this study that neutrophils are required for the recruitment of CD4+ T lymphocytes to the liver in Con A-induced hepatitis. Although some evidence suggests that neutrophils can affect T lymphocyte-mediated immunity, the mechanism remains unknown (49, 50). Two reports suggest that neutrophils may be required for the lymphocyte infiltration into tumors (51), as well as playing a critical role in the effector phase of the development of the T lymphocyte-mediated clinicopathologic expression of experimental autoimmune encephalomyelitis (52). More relevant to our work is a recent publication by Sitia et al. (8). In their study, Sitia et al. observed that neutrophil depletion in a mouse model of viral hepatitis did not affect the intrahepatic migration or antiviral activity of T lymphocytes, but did inhibit the recruitment of all Ag-nonspecific cells to the liver. In this study, we have demonstrated that recruitment of neutrophils is paramount for disease development and that neutrophils are an essential mediator for the recruitment of T lymphocytes to the liver in Con A-induced hepatitis.

In summary, we have shown that Con A induces a significant hepatic recruitment of neutrophils within 4 h of administration. We have demonstrated that Con A can directly bind to, and subsequently activate, the neutrophils such that their expression of adhesion molecules alters and they release reactive oxidants. Furthermore, we have shown that without neutrophils Con A is unable to induce the recruitment of CD4+ T lymphocytes to the liver and subsequent liver injury. Taken together, our results suggest that the recruitment of neutrophils is paramount for the development of Con A-induced hepatitis, and the activation of the neutrophils is an important step. It is, therefore, our contention that neutrophils are more important than previously appreciated in Con A-induced hepatitis and potentially in patients with T cell-mediated liver diseases.

Acknowledgments
We thank Carol Gwozd for technical assistance.

References


