The decrease in nonsplenic interleukin-6 (IL-6) production after splenectomy indicates the existence of a positive feedback loop of IL-6 production during endotoxemia in dogs

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The Decrease in Nonsplenic Interleukin-6 (IL-6) Production after Splenectomy Indicates the Existence of a Positive Feedback Loop of IL-6 Production during Endotoxemia in Dogs

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The spleen is involved in endotoxin-induced interleukin-6 (IL-6) production. To quantitate the relative contribution of the spleen to endotoxin-induced IL-6 production, we studied the effect of endotoxin (1.0 μg/kg of body weight) in control dogs (n = 7) and splenectomized dogs (n = 7). Blood for analysis of tumor necrosis factor (TNF) and IL-6 was sampled from the femoral artery and the portal, hepatic, and splenic (only in controls) veins. Arterial plasma endotoxin and cortisol levels were also measured. Whole-body IL-6 production was calculated by a deconvolution technique. Splenic IL-6 production in control dogs was measured from splenic blood flow and arteriovenous concentration differences. Endotoxin levels were higher in splenectomized dogs (P < 0.05) because of a decreased distribution volume (P < 0.05) and decreased clearance of endotoxin (P < 0.05). Endotoxin-induced plasma IL-6 levels were decreased by ~75% in splenectomized dogs (P < 0.01), and whole-body IL-6 production rates were severalfold lower (median of 8.7 mg/4 h and range of 3.9 to 11.4 mg/4 h versus a median of 32.3 mg/4 h and a range of 22.7 to 70.2 mg/4 h) (P < 0.05). However, in control dogs splenic IL-6 production (0.6 ± 0.2 mg/4 h) was only ~2% of whole-body IL-6 production. Plasma TNF levels increased in both groups (P < 0.01) but were not different between the groups. Plasma cortisol levels were slightly higher in splenectomized dogs than in control dogs (P < 0.05). In conclusion, splenectomy decreases the distribution volume and clearance rate of endotoxin. Spleenectomy results in decreased endotoxin-induced IL-6 production, which is caused not by the absence of splenic IL-6 production, but by a decrease in nonsplenic IL-6 production. Therefore, the spleen is an important mediator in the complete activation of nonsplenic IL-6 production by endotoxin.

In gram-negative infections, endotoxins induce a complex cascade of endogenous mediators such as interleukin-6 (IL-6) and tumor necrosis factor (TNF) in the host (10, 32). These endogenous mediators are thought to mediate the changes induced by endotoxin in multiple physiological functions. The spleen is involved in the production of these cytokines, because endotoxin administration in mice induces high transcription levels of cytokines in the spleen as well as in other organs (14, 31). Moreover, the cytokine response to endotoxin is disturbed after splenectomy, resulting in decreased plasma IL-6 (32). Although these studies indicate an important contribution of the spleen to whole-body cytokine kinetics, the quantitative contribution of the spleen to whole-body IL-6 production is presently unclear.

Endotoxin is cleared from the blood mainly by the reticuloendothelial system (12). Although the liver appears to be the major site of endotoxin clearance (3, 12, 20, 26, 28), the spleen also participates in endotoxin removal from the blood (12, 28). However, the contribution of the spleen to endotoxin kinetics has not been documented in detail.

Therefore, this study was performed to quantitate the relative contribution of the spleen to whole-body IL-6 production in control (n = 7) and in splenectomized (n = 7) dogs during 4 h after injection of a single bolus of endotoxin. In addition, the effect of splenectomy on endotoxin kinetics in these dogs was evaluated. This study was performed with dogs because they enabled us to obtain consecutive measurements of splenic and hepatic blood flow and of IL-6 and TNF concentrations in different vessels. The dose of endotoxin (1.0 μg/kg of body weight) was chosen to induce a considerable cytokine response without the induction of hemodynamic shock (19).

**MATERIALS AND METHODS**

**Animals.** Fourteen male mongrel dogs (weight, 28 ± 2 kg [mean ± standard error]) were studied. Prior to the study, all dogs were observed for 2 weeks. Only dogs with normal stools, absence of febrile disease, normal physical examination, and laboratory results were included. The dogs were fed once a day with a standard diet consisting of 64% carbohydrate, 7% fat, 26% protein, and 3% fiber based on dry weight (D. B. Brok, Hope Farms, Woerden, The Netherlands). This study was approved by the Ethical Committee for Animal Experiments and was performed according to the guidelines of the Dutch Law for Animal Experiments.

**Operative procedure.** After an overnight fast of 18 h, the dogs were anesthetized, intubated, and ventilated artificially. General anesthesia was induced by intramuscular injection of xylazine (0.15 ml/kg of body weight; Rompun; Bayer), ketamine hydrochloride (20 mg/kg; Aescoket; Aesculaap BV), and atropine (0.05 mg/kg; Centrafarm Services BV, Etten-Leur, The Netherlands) and maintained by intravenous sufentanil (2 μg/kg/h; Janssen Pharmaceutica BV, Tilburg, The Netherlands) and maintained by intravenous sufentanil (2 μg/kg/h; Janssen Pharmaceutica BV, Tilburg, The Netherlands) and maintained by intravenous sufentanil (2 μg/kg/h; Janssen Pharmaceutica BV, Tilburg, The Netherlands) and maintained by intravenous sufentanil (2 μg/kg/h; Janssen Pharmaceutica BV, Tilburg, The Netherlands) and maintained by intravenous sufentanil (2 μg/kg/h; Janssen Pharmaceutica BV, Tilburg, The Netherlands) and 1% isoflurane (Forene; Abbott Laboratories, Queensborough, Kent, United Kingdom) and N2O-O2 (1:1) ventilation. A urine catheter and a rectal temperature probe were inserted. A Swan Ganz thermal dilution catheter was inserted into the pulmonary artery through the external jugular vein. A femoral artery catheter was inserted for blood sampling and continuous intra-arterial blood pressure monitoring. A catheter was inserted into the right cephalic vein for infusion of saline.

Subsequently, the abdominal cavity was opened in all dogs through a midline incision. Splenectomy was performed for 7 of the 14 dogs. The gastroduodenal artery was ligated at its junction with the portal vein. Doppler flow probes (20-MHz pulsed Doppler modules, epoxy probe; obtained from G. J. Hartley, Baylor College of Medicine, Houston, Tex.) were positioned around the hepatic artery.
portal vein, and (in the control group) splenic vein. Flexible Tygon catheters (S34-HL; Norton Company, Rubber BV, Hilversum, The Netherlands [inside diameter, 0.040 in.; outside diameter, 0.070 in.; length, 15 in.]) were inserted into the hepatic and portal veins between the left medial and lateral lobes of the liver. The splenic catheter was advanced over 2 cm into the vein, and the catheter in the portal vein was advanced retrogradely over 5 cm into the vein with the tip distal to the portal vein flow probe. In the control group, a similar catheter was inserted into the splenic vein and advanced retrogradely over 10 cm into the vein. After insertion, the catheters were kept patent by slow infusion of saline.

Experimental design. Operating time preparing the animal model took an average of 120 min. Endotoxin derived from Escherichia coli (0111:B4, lot 31H00000, Sigma Chemical Co., St. Louis, Mo.) was suspended in sterile, pyrogen-free saline. A stock solution of 100 µg/ml was made, divided into several tubes (Costar, Cambridge, Mass.), and stored at −20°C. Prior to injection, the endotoxin solution was thawed at 37°C, vortexed for 3 min, diluted, and vortexed again for 10 min. After the surgical preparation was completed, general anesthesia was maintained by sufentanil, isoflurane, and N2O-O2 (1:1). Subsequently, blood samples for basal measurements were taken, followed by a bolus injection of 1.0 µg of E. coli endotoxin per kg of body weight to the right cephalic vein of control dogs (n = 7) or splenectomized dogs (n = 7). The observation period after endotoxin administration lasted 240 min. Arterial blood samples for determination of plasma endotoxin levels were collected before and at 2, 4, 7, 10, 15, 20, 30, 40, and 60 min after endotoxin administration. Blood samples for the determination of plasma TNF and IL-6 levels were collected hourly and 30 and 90 min after endotoxin administration from the femoral artery and hepatic, and portal, and (in the control group) splenic veins. Arterial blood samples for the determination of plasma cortisol levels were collected hourly. For determination of plasma TNF, IL-6, and cortisol concentrations, arterial blood was collected in sterile heparinized tubes (50 IU of heparin/ml of whole blood) and stored at −20°C. Prior to analysis of endotoxin concentration, arterial samples for the determination of plasma TNF and IL-6 levels were collected from basal values within each group were tested by analysis of variance for randomized block design, with the Newmann-Keuls test used when appropriate. For comparison between sampling sites within each group, the Wilcoxon test for paired samples was used. A P value of <0.05 was considered to be statistically significant.

RESULTS

Clinical effects. Basal hemodynamic measurements were not different between the two groups. Although arterial blood pressure decreased transiently in both groups 60 min after endotoxin administration (not significant between groups), blood pressure was significantly decreased only in the splenectomized group (P < 0.05 versus basal). Pulmonary artery pressure, wedge pressure, and central venous pressure did not change after endotoxin administration in both groups, indicating that there was no difference in intravascular filling pressure between the groups.

Cardiac output, total hepatic blood flow, and splenic blood flow are shown in Fig. 1. Cardiac output significantly increased in both groups after endotoxin administration (P < 0.05 versus basal) and was higher in the control group than in the splenectomized group (P < 0.05). Conversely, calculated systemic vascular resistance was significantly decreased at 60 min after endotoxin administration in both groups (P < 0.05 versus basal). The difference in cardiac output was not caused by a difference in intravascular filling pressures. Prior to endotoxin administration, total hepatic blood flow was 8.6 ± 0.8 ml/kg · min in the control group versus 7.4 ± 0.3 ml/kg · min in the splenectomized group (P = 0.09). After endotoxin administration, total hepatic blood flow was higher in the control group than in the splenectomized group (P < 0.05). Splenic blood flow was slightly, but not significantly, increased after endotoxin administration.

Endotoxin kinetics. Arterial endotoxin levels prior to endotoxin administration were low (controls, 0.08 ± 0.02 EU/ml of PRP; splenectomized, 0.08 ± 0.03 EU/ml of PRP). After endotoxin administration, plasma endotoxin levels decreased rapidly in both groups of dogs (Fig. 2). Although identical doses of endotoxin were administered, the AUC of plasma endotoxin concentrations was higher in splenectomized dogs (P < 0.05 [Table 1]), although there were no differences for individual time points. This difference in AUC was caused by a decrease in F in splenectomized dogs (P < 0.05). There was no difference in elimination t1/2 between the groups (Table 1).

TNF and IL-6 concentrations. Pre-endotoxin arterial plasma TNF levels were low (0.6 ± 0.3 mg/ml in the control group versus 0.7 ± 0.4 mg/ml in the splenectomized group [nonsignificant]). After endotoxin administration, arterial plasma
TNF levels increased in both groups with a maximum response at 60 min (Fig. 3; nonsignificant between the groups; \( P < 0.05 \) versus basal in both groups). There were no differences in plasma TNF levels between the different sampling sites within each group or between the groups.

Prior to endotoxin administration, arterial plasma IL-6 levels were low and not different between the two groups (Fig. 4). After endotoxin administration, plasma IL-6 levels increased significantly at each sampling site from 90 to 240 min in both groups (\( P < 0.01 \)). However, maximal arterial IL-6 levels in the splenectomized group were only \( \approx 25\% \) of the value obtained in the control group (\( P < 0.01 \)) (Fig. 4). In the control group, plasma IL-6 levels were consistently higher (\( \approx 70\% \)) in the splenic vein than were the arterial plasma IL-6 levels (\( P < 0.05 \)).

After endotoxin administration, not only plasma concentrations of IL-6 but also the pattern of changes in IL-6 concentrations was different between the groups (Fig. 4). In splenectomized dogs, plasma IL-6 levels were maximal at 120 min after endotoxin administration, but the levels decreased subsequently (\( P < 0.05 \) between 120 and 240 min). In contrast, in control dogs, plasma IL-6 levels increased over time and remained steadily elevated during the whole observation period.

Calculated whole-body IL-6 production over the 4 h after endotoxin administration (Fig. 5) was significantly higher in the control group (median, 32.3 mg/4 h; range, 22.7 to 70.2 mg/4 h) than in the splenectomized group (median, 8.7 mg/4 h; range, 3.9 to 11.4 mg/4 h) (\( P = 0.02 \)). In the control group, splenic IL-6 production over 4 h after endotoxin administration was 0.6 \( \pm \) 0.2 mg/4 h, which represented only \( \approx 2\% \) of whole-body IL-6 production.

**Cortisol concentrations.** After surgery, but before endotoxin administration, plasma cortisol levels in arterial blood were not different between the two groups. Plasma cortisol levels did not change in response to endotoxin in the control group. However, in splenectomized dogs, plasma cortisol levels were significantly increased and were higher than those in the control group (\( P < 0.05 \) [Fig. 6]).

**DISCUSSION**

In this study, we evaluated the effects of splenectomy on endotoxin kinetics and on endotoxin-induced IL-6 production.

**TABLE 1. Pharmacokinetic parameters of endotoxin after equivalent intravenous doses (1.0 \( \mu \)g/kg) of E. coli endotoxin in control versus splenectomized dogs**

<table>
<thead>
<tr>
<th>Kinetic parameter(^a)</th>
<th>Result for group (mean ± SE)</th>
<th>( P ) value (control vs splenectomized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_0 ) (EU/ml of PRP)</td>
<td>0.08 ± 0.02</td>
<td>0.08 ± 0.03 NS</td>
</tr>
<tr>
<td>AUC (EU · min/liter)</td>
<td>68.9 ± 11.4</td>
<td>114.4 ± 20.5 &lt;0.05</td>
</tr>
<tr>
<td>( t_{1/2a} ) (min)</td>
<td>6.1 ± 1.5</td>
<td>5.7 ± 1.2 NS</td>
</tr>
<tr>
<td>( t_{1/2b} ) (min)</td>
<td>48.4 ± 12.2</td>
<td>45.7 ± 10.5 NS</td>
</tr>
<tr>
<td>CL (liters/min · kg)</td>
<td>6.1 ± 1.5</td>
<td>3.5 ± 0.7 &lt;0.05</td>
</tr>
<tr>
<td>( V_{aUC} ) (liters/kg)</td>
<td>12.6 ± 2.4</td>
<td>7.8 ± 1.8 &lt;0.05</td>
</tr>
<tr>
<td>( V_c ) (liters/kg)</td>
<td>2.9 ± 0.5</td>
<td>1.9 ± 0.4 &lt;0.05</td>
</tr>
</tbody>
</table>

\( ^a \) \( C_0 \), plasma endotoxin concentration prior to endotoxin administration; \( t_{1/2a} \), distribution-phase half-life; \( t_{1/2b} \), elimination-phase half-life; CL, volume of blood cleared of endotoxin per unit of time; \( V_{aUC} \), apparent volume of endotoxin distribution calculated by area method; \( V_c \), apparent volume of central compartment.

\( ^b \) NS, not significant.
In splenectomized dogs, plasma endotoxin concentrations were higher than those in control dogs, indicating that the spleen modulates endotoxin kinetics. In line with other studies (14, 31, 32), the spleen is a net producer of IL-6 in vivo after endotoxin administration in control dogs. However, the decrease in IL-6 concentrations after splenectomy was not caused by the absence of splenic IL-6 production. Analysis of endogenous IL-6 kinetics in control dogs revealed that the spleen was only a minor contributor to whole-body IL-6 production. Therefore, decreased endotoxin-induced IL-6 concentrations after splenectomy are due to decreased IL-6 production outside the spleen.

Endotoxin is eliminated from the blood mainly by the liver (3, 12, 20, 26, 28). In the presence of impaired liver function, endotoxin is also disposed of by macrophages within the spleen (28). In addition, our data document that the spleen is involved in endotoxin kinetics in the presence of uncompromised liver function, because arterial endotoxin concentrations were higher in splenectomized dogs than in control dogs after administration of an identical dose of endotoxin. Analysis of endotoxin kinetics revealed that the spleen contributed to endotoxin clearance by having an important effect on the $V'$ of endotoxin.

We evaluated splenic IL-6 production after endotoxin administration in two different ways. First, in control dogs, splenic IL-6 production was measured from arteriovenous differences in IL-6 concentrations and blood flow across the spleen. Second, the contribution of the spleen to whole-body IL-6 production, calculated by deconvolution, was studied by comparing control dogs and splenectomized dogs. There appeared to be a major discrepancy between the results of these two approaches: measured IL-6 production across the spleen was only a fraction of the difference in whole-body IL-6 production rates between control and splenectomized dogs. Therefore, potential sources of error have to be considered in both methods. In general, the measurement of production rates of substrates across tissues by the arteriovenous balance technique is hampered by a relatively high coefficient of variation in the measurement of blood flow compared with the measurement of substrate concentrations. However, the magnitude of the difference in whole-body IL-6 production rates between control and splenectomized dogs indicates that even if splenic blood flow would have been severalfold higher, our conclusions would not have been affected. Moreover, our data on splenic blood flow are in agreement with previous observations by others (2). We assumed the same IL-6 clearance rates in control and splenectomized dogs. Therefore, IL-6 production rates would be affected if IL-6 clearance rates were altered by splenectomy. However, the liver, not the spleen, is the major organ of IL-6 clearance (23, 29). Moreover, if there were any effect of splenectomy on IL-6 clearance, it would be expected to decrease rather than increase the clearance rates, which
would further strengthen our conclusions. From these arguments, we conclude that our conclusions are not merely explained by potential sources of error in our methods.

The effect of the spleen on systemic IL-6 production might be related to several mechanisms. First, it is possible that the spleen produces a factor that induces IL-6 production elsewhere in the body. Factors that are known to induce IL-6 after endotoxemia include TNF, IL-1, gamma interferon, and prostaglandins (5, 6, 9, 15, 18, 21, 24, 27). In the present study, we were unable to find an effect of splenectomy on plasma TNF levels, suggesting that splenectomy does not affect TNF metabolism to a considerable extent and, thereby, does not directly affect IL-6 production. However, we cannot exclude the possibility of effects of splenectomy on other mediators that stimulate IL-6 production. Second, it is possible that the spleen acts as a trigger on blood cells that traverse the spleen and that are, directly or indirectly, involved in IL-6 production outside the spleen. Third, splenectomy could be associated with increased production of inhibitors of IL-6 production, for instance, cortisol (4). Plasma cortisol concentrations were slightly increased in splenectomized dogs. Nonetheless, it seems unlikely that the relatively small difference in cortisol concentrations between the two study groups accounted for the major effects of splenectomy on IL-6 concentrations. Furthermore, the presence of inhibitors of IL-6 production in plasma of splenectomized dogs seems unlikely, because dilution of plasma samples from both groups did not result in different dose-response effects in the B9 bioassay (data not shown). Therefore, we could not find support for the assumption of the presence of an inhibitor of IL-6 production in splenectomized dogs. Finally, it is unlikely that the spleen secretes IL-6 predominantly by other routes, e.g., the lymphatic route. In that case, arterial IL-6 levels would be expected to be higher than the concentrations obtained at other catheter sites.

The striking effects of splenectomy on endotoxin-induced IL-6 response are not only reflected in decreased plasma IL-6 levels, but are also reflected by the response over time of plasma IL-6 concentrations. In the control group, IL-6 production persisted during the whole 4-h observation period, as reflected in persistently increased plasma IL-6 levels. However, in the splenectomized group, plasma IL-6 levels were maximal at 120 min after endotoxin administration and decreased subsequently, suggesting lack of continued stimulation of IL-6 production in the absence of the spleen.

To investigate whether there might be a shift in endotoxin-induced TNF production in the absence of splenic TNF production, we measured plasma TNF levels in the splenic vein of the control dogs. However, there was no significant difference between arterial and splenic vein levels of TNF, indicating that there was no net splenic production of TNF during the first 4 h after endotoxin administration in this dog model. Giroir et al. (14) measured TNF biosynthesis in mice after endotoxin administration and found increased TNF production in several organs, including the spleen. However, the increase in TNF secretion by splenic tissue 4 h after endotoxin administration was only minimal compared with basal values and compared with other organs, in accordance with our results. In addition, Ulich and coworkers (33) failed to detect endotoxin-induced TNF mRNA levels in whole-organ extracts of the spleen within the first 4 h after endotoxin administration in rats, despite marked elevation in serum TNF levels. Therefore, these data are in line with the concept that the spleen does not contribute considerably to plasma TNF concentrations during the first hours after low-dose endotoxemia.

In a previous study of postabsorptive volunteers, Fong et al. reported net splanchic TNF production in response to endotoxin in human volunteers (11). The absence of a net increase in TNF in the hepatic vein compared to the femoral artery could not merely be explained by the assay we used. All TNF analyses of each dog were done in the same run in duplicate with an intra-assay coefficient of variation of 5 to 8%. The interassay variation of the bioassay was 6 to 10%. Therefore, the discrepancy in splanchic TNF production between the present study and the study of Fong et al. must be related to other factors, such as differences between species (dogs versus human volunteers) and study designs (surgery prior to endotoxin administration versus postabsorptive state).

The question arises of whether there is cross talk between the spleen and TNF production elsewhere in the body, like we demonstrate for IL-6 production. Although there was no significant difference in TNF levels in hepatic veins between con-
trol and splenectomized animals, it is possible that this is due to a type II error. For instance, TNF concentrations in hepatic veins were 7.7 ± 2.1 versus 10.9 ± 6.2 pg/ml for control and splenectomized dogs, respectively, 60 min after endotoxin administration (Fig. 3). In addition to the relatively small number of dogs, this may be due to the relatively large interindividual variation in TNF responses to endotoxin, as has been demonstrated for humans (22). Therefore, we cannot exclude the possibility of an increase in hepatic TNF production in splenectomized dogs compared with control dogs, reflecting inhibitory cross talk between the spleen and TNF production elsewhere.

IL-6 is a known feedback inhibitor of TNF production. The lack of splenic IL-6 could lead to increased Kupffer cell production of TNF. For instance, Aderka et al. (1) found an inhibitory effect of IL-6 on TNF production. In accordance, endotoxin administration to IL-6 knockout mice (8) resulted in a three-fold increase in TNF levels in the hepatic vein between the two groups is due to a type II error (see above). Alternatively, the effects of the spleen on the activation of the cytokine network by endotoxin are probably more complicated than can be explained merely from changes in IL-6 and TNF concentrations.

In conclusion, the spleen contributes to the clearance and of endotoxin. Splenectomy results in decreased endotoxin-induced IL-6 production, which is explained not by the absence of splenic IL-6 production but by a decrease in nonsplenic IL-6 production. Therefore, the presence of the spleen is important for the complete activation of nonsplenic IL-6 production by endotoxin.

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