Development and Application of an Isolated Perfused Rat Liver Model to Study the Stimulation and Inhibition of Tumor Necrosis Factor-α Production *ex Vivo*

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Purpose. To develop an isolated perfused rat liver (IPRL) model with low baseline levels of tumor necrosis factor (TNF)-α in the outlet perfusate to study the effects of immunostimulants and immunosuppressants on the release of TNF-α from this organ.

Methods. Isolated rat livers were perfused with a buffer containing no albumin or three different bovine serum albumin (BSA) preparations. Using the no-albumin perfusate, the inhibitory effects of methylprednisolone (MP) on lipopolysaccharide (LPS)-stimulated release of TNF-α were studied in livers isolated 1 or 5 h after the intravenous administration (5 mg/kg) of MP. The concentrations of TNF-α in the outlet perfusates were measured using enzyme-linked immunosorbent assay.

Results. In the absence of albumin, the perfusate levels of TNF-α were close to zero. However, when the perfusate contained BSA, the TNF-α levels in the perfusate reached as high as 1200 pg/ml at steady state. An injection of LPS into IPRLs perfused with a no-albumin perfusate resulted in mean (± SD) TNF-α steady-state concentrations of 825 ± 125 pg/ml. The pretreatment of rats with MP before liver harvest attenuated the LPS-induced TNF-α release in the livers. However, the attenuation was substantial (>60%) and was statistically significant only 5 h after pretreatment with MP.

Conclusions. Perfusates containing BSA may result in nonphysiologically high levels of TNF-α. An IPRL with a no-albumin perfusate is more suitable for studies of the stimulation and inhibition of TNF-α production by this organ.

KEY WORDS: bovine serum albumin; endotoxin; isolated perfused rat liver; methylprednisolone; tumor necrosis factor.

INTRODUCTION

Tumor necrosis factor (TNF)-α is a cytokine that plays an important role in host defense and immune response (1). Additionally, the overproduction of TNF-α is involved in many pathophysiologic conditions such as infections and inflammation, resulting in serious consequences, including death (1).

The liver is one of the most important organs with regard to the production and effects of TNF-α. First, liver Kupffer cells or macrophages are major sources of production of TNF-α in the body (2) in response to specific and nonspecific immunostimulants and inflammatory mediators, such as bacterial lipopolysaccharide (LPS). Second, TNF-α and other cytokines released because of these stimulants may alter the liver functions, resulting in changes in the integrity of liver vasculature (3) or the regulation of liver cytochrome P450 contents and metabolic capacity (4). Therefore, studies using the liver, such as those with isolated perfused rat liver (IPRL), are attractive because they can significantly improve our understanding of the effects of TNF-α on various liver functions under physiologic and pathophysiologic conditions. However, perfusates used for IPRL may contain ingredients, such as human or bovine red blood cells and albumin, that may potentially stimulate the release of TNF-α in the perfusate of otherwise normal, unstimulated livers. Therefore, the development of an IPRL model with low baseline levels of TNF-α is a prerequisite for the use of IPRL in studies of the kinetics and dynamics of TNF-α production and release. The first aim of the present study was to develop such a model.

As for the role of TNF-α in immune response to transplanted organs, it has been suggested that TNF-α and other proinflammatory cytokines play an important role in rejecting allografts by a local, rather than systemic effect (5). Therefore, local inhibition of the production of TNF-α and other proinflammatory cytokines within the graft could reduce the possibility of rejection. In agreement with this, the beneficial effects of a liposomal tacrolimus formulation in a liver transplant model were attributed to a more effective inhibition of cytokine release in the liver resulting from high intragraft concentrations of the liposomal drug (5). Therefore, experimental evidence of local immunosuppression in the graft is important. However, it is difficult, if not impossible, to determine the local effects of systemically administered immunosuppressants in the whole animal. Instead, a suitable IPRL model may be used for studies of the production and release of these cytokines, identifying the extent of local immunosuppression in this organ after systemic administration of drugs. The second aim of the present study was, therefore, to apply an IPRL model developed using the first aim to study the effects of immunostimulants (e.g., LPS) and immunosuppressants (e.g. corticosteroids) on the release of TNF-α from this organ.

MATERIALS AND METHODS

Chemicals

Various preparations of bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). The characteristics of BSA preparations used in this study are listed in Table I. 6α-methylprednisolone (MP), bovine γ-globulin, *Escherichia coli* (serotype 0111:B4) LPS, and transaminase kits for the measurement of glutamic-oxaloacetic transaminase and glutamic-pyrovic transaminase were also obtained from Sigma. All other reagents were analytical grade and available through commercial sources.

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ABBREVIATIONS: AUC, area under the perfusate concentration time curve; BSA, bovine serum albumin; C_{ss}, steady-state concentration; Ig, immunoglobulin; IPRL, isolated perfused rat liver; LPS, lipopolysaccharide; MP, methylprednisolone; TNF, tumor necrosis factor.

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Table I. Characteristics of BSA Preparations (Fraction V) Used in the Perfusate

Product number	Description	Lot no.	Abbreviation
A-3912	Albumin ≥96%	128H0649	BSA-96
A-3675	Albumin ≥98%; low endotoxin	129H0973	BSA-low LPS
A-3059	Albumin ≥99%; γ-globulin free	129H0885	BSA-no globulin

Isolated Rat Liver Perfusion

The procedures involving animals used in this study were consistent with the guidelines set by the National Institutes of Health (publication 85-23, revised 1985) and approved by our Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (mean ± SD body weight, 233 ± 20 g) were used as liver donors. The techniques used for isolation and cannulation of the livers have been reported by us before (6–9). Briefly, rats were anesthetized by an intraperitoneal injection (2 ml/kg) of a mixture of ketamine/xylazine (60:8 mg/ml), and the bile duct, the hepatic portal vein (inlet), and the thoracic inferior vena cava (outlet) were cannulated. The liver was then excised and transferred to a temperature-controlled perfusion tray.

The isolated livers were perfused using a water-jacketed glass apparatus in a single-pass manner. To avoid contamination of the apparatus by endotoxin from bacterial growth or other sources, the apparatus was cleaned after each day of use with a 10% bleach solution (Clorox, The Clorox Company, Oakland, California), followed by an extensive rinse with deionized water.

The control perfusate, which was used in the second part of the study for the determination of the effects of MP, was Krebs-Henseleit bicarbonate buffer (pH 7.4) containing glucose (2 g/l) and was prepared in deionized water. Other tested perfusates contained various preparations of albumin in the buffer solution. The perfusate was continuously oxygenated with an O_2/CO_2 mixture (95:5) for at least 15 min before entering the liver and was delivered at a flow rate of 30 ml/min (3–4 ml/min/g liver weight).

The viability of the liver was confirmed through the overall macroscopic appearance of the liver, wet liver weights of ≤4% of body weight at the end of perfusion, low transaminase levels in the outlet perfusate during the perfusion period (0 and 120 min), and relatively stable bile flow over the entire perfusion period (120 min).

Experimental Groups: Model Development

A total of 19 livers were used in this section of the study. Livers were perfused using one of the four perfusates containing no albumin (no BSA) or 1% (w/v) BSA with ≥96% purity (BSA–96), low-endotoxin BSA (BSA-low LPS), or γ-globulin-free BSA (BSA–no globulin) (Table I). The number of livers included in each group was five except for the BSA-low LPS group, which had four livers included.

Experimental Groups: Model Application

A total of 17 livers were used in four different groups in this section of the study. Based on the results of the first section (Model Development), all the livers in this section were perfused with the no-albumin perfusate. Livers isolated from untreated rats were divided into control (n = 5) and LPS (n = 4) groups. The control (no LPS) livers were perfused with the perfusate only. The LPS group was infused with an LPS solution (300 μg/ml) at a rate of 50 μl/min for the first 20 min of the perfusion (a total of 300 μg LPS). The third and fourth groups of rats (n = 4 for each group) were injected with a single 5-mg/kg dose of MP via the tail vein at 1 h (LPS + MP-1 h) or 5 h (LPS + MP-5 h) before the liver isolation. After the liver isolation, the MP-injected groups received LPS using the same protocol described for the LPS group.

Sample Collection

After isolation, all livers were allowed to stabilize for ~10 min before the start of the experiments. Samples (1 ml) were taken from the outlet at 0, 15, 30, 45, 60, 80, 100, and 120 min and stored at –80°C for further analysis of the TNF-α levels. Additional samples were taken from the outlet at the beginning (time zero) and end (120 min) of perfusion and were stored at 4°C for the measurement of transaminase levels within 1 week. Bile samples were also collected into preweighed microcentrifuge tubes at 30-min intervals.

Sample Analysis

The concentrations of TNF-α in the outlet perfusates were measured using a commercial enzyme-linked immunosorbent assay kit (Biosource International, Camarillo, CA). Using a 50-μl sample, the assay has a linear range of 0–1000 pg/ml, a minimum detectable concentration limit of 4 pg/ml, and intra-assay and interassay precision coefficient of variation (CV) of <4%.

The concentrations of LPS in the water and albumin samples were analyzed using a semiquantitative assay (E-Toxate Kit, Sigma) based on horseshoe crab lysate, which forms a hard gel (positive test) in the presence of endotoxin. The sensitivity of the assay is determined in each analysis by identification of the lowest concentration of endotoxin standard that results in a positive test (usually ~0.01 ng/ml). If the unknown sample shows a positive response, it is further diluted until there is no gel formation. The concentration of endotoxin in the sample is then estimated from the assay sensitivity and the highest dilution of the sample found positive.

The transaminase levels were quantitated based on a colorimetric method using a commercial kit from Sigma (procedure No. 505).

Data Analysis

The steady-state concentration (C_{ss}) values of TNF-α in the outlet perfusate were estimated from the average values at 80, 100, and 120 min. The area under the perfusate concentration time curve (AUC) of TNF from time zero to 120 min was estimated using linear trapezoidal rule. The differences among the groups in terms of C_{ss} values or AUC of TNF-α were determined using one-factor analysis of variance, whereas a two-factor analysis of variance was used to determine the effects of treatments and collection times on the bile flow. In the presence of a significant result, pairwise comparison of means was conducted using Scheffe's *F* test. A simple regression analysis was used to test the relationship between

the AUC of TNF and the endotoxin content of the perfusate. All tests were performed at a significance (α) level of 0.05. The data are presented as mean ± SD.

RESULTS

Model Development

The time courses of the concentrations of TNF-α in the outlet perfusate of different groups of livers are presented in Fig. 1. When no albumin was added to the perfusate (no BSA), the perfusate concentrations of TNF-α were zero or close to zero during the entire period of perfusion. However, when the perfusate contained 1% BSA-96, the concentrations of TNF-α started to rise at ~20 min after the start of perfusion and reached a plateau at 80-120 min. A similar profile was observed when the perfusate contained 1% of a γ-globulin-free perfusate (Fig. 1). However, the TNF-α levels using the γ-globulin-free perfusate were less than the corresponding values using the 96% albumin perfusate at all the time points (Fig. 1). In contrast to the significant rise in the perfusate concentrations of TNF-α in the presence of either BSA-96 or BSA-no globulin, the perfusate concentrations of TNF-α were close to zero when the perfusate contained a low endotoxin albumin. Indeed, the TNF-α perfusate concentration-time profiles were superimposable for the no BSA and BSA-low LPS perfusates (Fig. 1).

The average C_{SS} and AUC values of TNF-α using four different perfusates used here are presented in Table II. Although the C_{SS} values were very low for the no-albumin and low-endotoxin perfusates, the TNF concentrations were significantly higher in the other two albumin-containing perfusates. Similar trends could also be observed using the AUC values of TNF-α (Table II).

Analysis of different preparations of BSA used in this study revealed endotoxin concentrations of 0.01, 5, and 8 ng/mg for the BSA-low LPS, BSA-96, and BSA-no globulin groups, respectively. Additionally, a different lot (109H0981) of BSA-no globulin was tested that contained 4 ng/mg endotoxin. This latter lot was initially used for the perfusion of two livers (data not shown). However, because of lack of availability of this lot for completion of this group, a new lot of this BSA (Table I) had to be used.

Table II. The C_{SS} and AUC of TNF-α in Rat Livers Perfused with Different Perfusates[a]

Variable	n	C_{SS}	AUC
Perfusate			
BSA-96	5	1160 ± 215[b]	7.10 ± 1.18[c]
BSA-no globulin	5	794 ± 267[b]	4.94 ± 1.38[c]
BSA-low LPS	4	54.0 ± 21.7[b]	0.242 ± 0.127[c]
No BSA	5	16.0 ± 18.9[b]	0.124 ± 0.109[c]
Statistical comparison[d]			
BSA-96 vs. BSA-no globulin		0.0378	0.0212
BSA-96 vs. BSA-low LPS		<0.0001	<0.0001
BSA-96 vs. no. BSA		<0.0001	<0.0001
BSA-no Globulin vs. BSA-low LPS		0.0002	<0.0001
BSA-no globulin vs. no BSA		<0.0001	<0.0001
BSA-low LPS vs. no BSA		0.9912	0.9982

[a] Values given as mean ± SD or *P* value.
[b] Values given as pg/ml.
[c] Values given as ng.h/ml
[d] Statistical comparison among different groups based on one-factor analysis of variance and subsequent Scheffe's F test.

The relationship between the AUC of TNF-α in the perfusate and the endotoxin content of the perfusate albumin is explored in Fig. 2 for all the four perfusate groups and the two additional liver perfusions using the lot 109H0981 (endotoxin level of 4 ng/mg) of BSA-no globulin. A regression analysis of data revealed a statistically significant ($P < 0.0001$) relationship between the two parameters. Additionally, the regression suggests that the endotoxin content of the perfusate may account for ~65% of the changes in the TNF-α concentrations of the perfusate ($r^2 = 0.66$).

The bile flow rates during the four 0.5-h collection intervals are depicted in Fig. 3 for the four groups of perfusates. As expected, the bile flow rates showed a modest decline with an increase in the perfusion time for all the groups. Generally, bile flow rates were similar for all the groups except for the γ-globulin-free perfusate, which showed a significantly higher flow rates compared with the other three perfusates (Fig. 3).

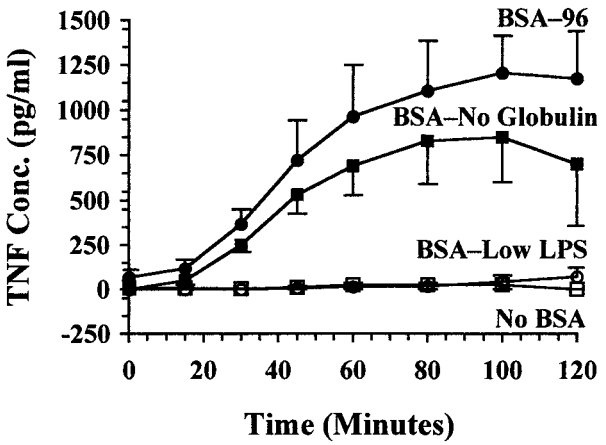

Fig. 1. The concentration-time courses of TNF-α in the outlet samples from isolated rat livers perfused with a perfusate containing no BSA (n = 5) or perfusates containing BSA-96 (n = 5), BSA-no globulin (n = 5), or BSA-low LPS (n = 4). Symbols and bars represent the average and SD values, respectively.

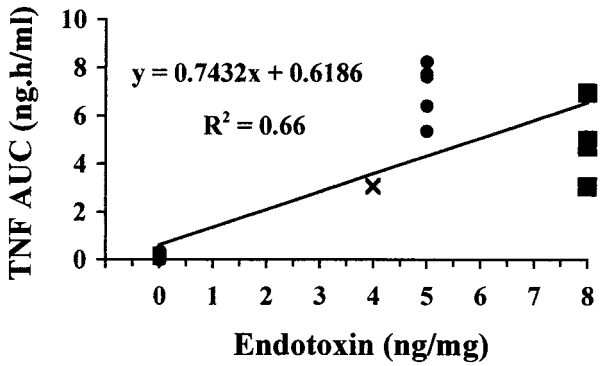

Fig. 2. The relationship between the AUC of TNF-α in the perfusate and the endotoxin content of BSA preparations used in different perfusates. Symbols and line represent the individual experimental data and the regression line, respectively. (□), no albumin; (○), BSA-low LPS; (●), BSA-96; (■), BSA-no globulin lot 129H0973; ×, BSA-no globulin lot 109H0981.

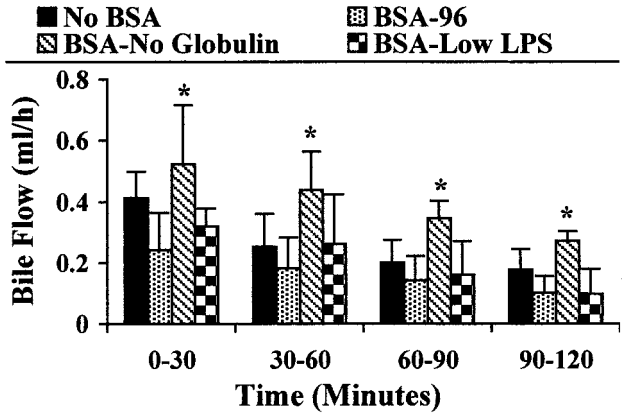

Fig. 3. Bile flow rates at different collection intervals in four groups of livers perfused with a perfusate containing no BSA (n = 5) or perfusates containing BSA-96 (n = 5), BSA-no globulin (n = 5), or BSA-low LPS (n = 4). *Significantly different than the corresponding values for the other three groups.

In terms of other viability parameters, the glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase levels for all rats were low during the entire perfusion period with no significant differences among the perfusate groups. The mean ± SD values of the wet liver weight (as a percentage of body weight) at the end of perfusion were 3.08 ± 0.27, 3.10 ± 0.25, 3.62 ± 0.29, and 3.70 ± 0.32 for the no-BSA, BSA-96, BSA-no globulin, and BSA-low LPS groups, respectively; there was no significant differences ($P > 0.05$) for pairwise comparison of the means.

Model Application

Figure 4 demonstrates the time courses of TNF-α in the perfusate of livers obtained from untreated rats in the absence (no LPS) or presence (LPS) of LPS stimulation of the liver. Additionally demonstrated in this figure are LPS-stimulated time courses of TNF-α in the livers obtained from rats treated with MP at 1 h (LPS + MP-1 h) or 5 h (LPS + MP-5 h) prior to liver harvest. The injection of 300 μg LPS, over the first 20 min of perfusion, in the group without MP pretreatment (LPS) resulted in a rise in the perfusate concentrations of TNF-α starting at 45 min and reaching a maximum at 80 min. Thereafter, the concentrations of TNF-α remained relatively constant until the end of perfusion (120 min). In contrast, in the no-LPS group, the TNF-α concentrations remained close to zero for the entire sampling period (Fig. 4). Pretreatment of rats with MP before liver harvest resulted in an attenuation of the LPS-induced TNF-α release in the livers (Fig. 4). However, the attenuation was substantial (>60%) and statistically significant only for the LPS + MP-5 h group (Fig. 4).

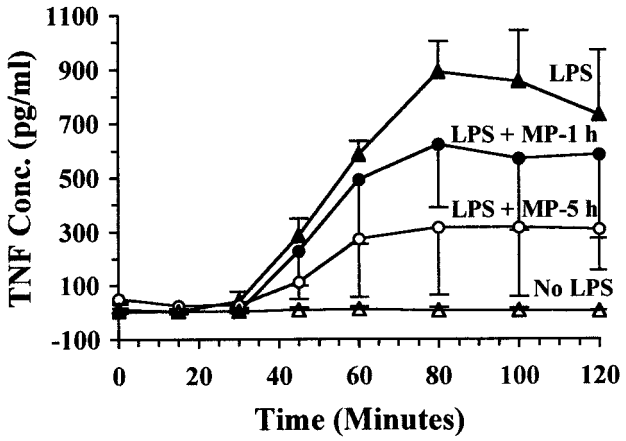

Fig. 4. The concentration-time courses of TNF-α in the outlet samples of livers obtained from control rat livers (no LPS) and LPS-perfused livers obtained from untreated rats (LPS) or rats treated with MP at 1 h (LPS + MP-1 h) or 5 h (LPS + MP-5 h) before the liver harvest. LPS (300 μg) was infused during the first 20 min of perfusion in the LPS groups. Symbols and bars represent the average and SD values, respectively.

The C_{SS} (80–120 min) values of TNF-α and the AUC of TNF-α during the entire perfusion period (0–120 min) are presented in Table III for all of the four studied groups. The infusion of LPS to untreated rats caused a significant ($P < 0.0001$) rise in the C_{SS} of TNF-α from a value close to zero in no-LPS group livers to 825 ± 125 pg/ml in the LPS group (Table III). Pretreatment with MP at 1 h before the liver harvest reduced the LPS-induced C_{SS} values of TNF to 621 ±257 pg/ml. However, this reduction did not reach statistical significance. On the other hand, the pretreatment of rats with MP at 5 h before the harvest significantly reduced the LPS-induced C_{SS} levels to <40% of the values in the MP-untreated livers (Table III). Similar conclusions could also be made based on the total AUC of TNF-α in the perfusate of the studied livers (Table III).

The bile flow rates for the four groups of livers in the Method Application section are demonstrated in Fig. 5. With regard to collection time, there was a modest decline in the bile flow rate with time, which is expected. Specifically, there was a significant difference between the flow rates at the 0–30-min collection period and those at the 60–90-min and 90–120-min collection periods. In terms of differences among the four groups, the MP-treated groups showed lower bile flow rates, which were statistically significant when compared with the no-LPS group (Fig. 5). However, the differences

Table III. The LPS-Induced C_{SS} and AUC of TNF-α in Livers Isolated from Rats 1 or 5 h after the Intravenous Administration of a Single 5-mg/kg Dose of MP[a]

Variables	n	C_{SS}	AUC
Group			
No LPS	5	5.2 ± 7.49[b]	0.049 ± 0.062[c]
LPS	4	825 ± 125[b]	4.59 ± 0.53[c]
LPS + MP-1 h	4	621 ± 257[b]	3.08 ± 0.93[c]
LPS + MP-5 h	4	313 ± 215[b]	1.81 ± 1.29[c]
Statistical comparison[d]			
No LPS vs. LPS		<0.0001	<0.0001
No LPS vs. LPS + MP-1		0.0014	0.0009
No. LPS vs. LPS + MP-5		0.1170	0.0461
LPS vs. LPS + MP-1		0.4478	0.1221
LPS vs. LPS + MP-5		0.0089	0.0030
LPS + MP-1 vs. LPS + MP-5		0.1441	0.2260

[a] Values given as mean ± SD or *P* value.
[b] Values given as pg/ml.
[c] Values given as ng.h/ml.
[d] Statistical comparisons among different groups based on one-factor ANOVA and subsequent Scheffe's F test.

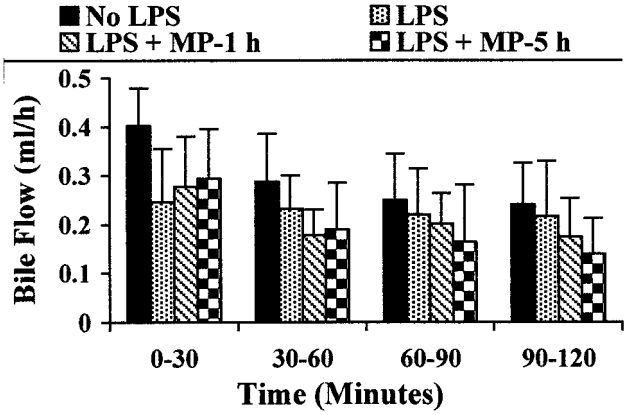

Fig. 5. Bile flow rates at different collection intervals in control rat livers (no LPS) and LPS-perfused livers obtained from untreated rats (LPS) or rats treated with MP at 1 h (LPS + MP-1 h) or 5 h (LPS + MP-5 h) before the liver harvest. LPS (300 μg) was infused during the first 20 min of perfusion in the LPS groups. Error bars represent SD values.

between the bile flow rates in the LPS and control livers (Fig. 5) did not reach statistical significance. This lack of statistical significance may have been due to the fact that a substantial (~40%) reduction in the bile flow rate of LPS-treated livers, compared with controls, was seen only during the 0–30-min collection period (Fig. 5) that overlaps with the LPS injection (0–20 min); the lower bile flow rates in the LPS-treated livers, compared with those in control livers, at the subsequent collection times were less dramatic (Fig. 5).

As for other viability indices, the wet liver weights of all groups at the end of perfusion were <4% of the body weight (no-LPS group livers, 3.42 ± 0.36%; LPS group livers, 3.10 ± 0.11%; LPS + MP-1 h group livers, 3.15 ± 0.32, and LPS + MP-5 h group livers, 3.69 ± 0.16% [as percentage of body weight]); pairwise comparison of the means revealed no significant differences among the groups for their liver weights. Additionally, the transaminase levels were low and relatively stable during the perfusion period.

DISCUSSION

Despite numerous reports on the levels of TNF-α in whole animals and isolated Kupffer cells, studies measuring TNF-α levels in IPRLs (10–12) are scarce. We became interested in using IPRL to investigate the local immunosuppressant activities of drugs, such as corticosteroids, in the liver after their systemic administration. Our initial perfusate, routinely used by us and many other investigators for drug metabolism studies, contained bovine red blood cells and albumin in a Krebs-Henseleit bicarbonate buffer. This perfusate resulted in very high outlet concentrations of TNF-α (data not shown). This was unexpected because under physiologic conditions and in the absence of Kupffer cell activation the release of cytokines, such as TNF-α, from the liver Kupffer cells is expected to be minimal. The omission of red blood cells from this perfusate, which contained BSA-96 (Table I), did not reduce the high levels of TNF-α (Fig. 1). However, when albumin was eliminated from the perfusate (no-BSA perfusate; Fig. 1), the perfusate TNF-α levels were close to zero for all the sampling points, indicating that the BSA preparation was responsible for the excessive TNF-α release.

Subsequent studies were then carried out to determine the role of different components of BSA preparations on the TNF-α release. Previous works (10–13) using isolated Kupffer cells have clearly shown that LPS would stimulate the release of TNF-α from these cells. Additionally, a study (14) using resident peritoneal macrophages of mice showed that both LPS and immunoglobulin (Ig) G, which are present in most commercially available BSA preparations, could induce the release of TNF-α in the cell culture. Therefore, in our subsequent studies we concentrated on the effects of IgG and LPS contents of albumin preparations on the TNF-α levels.

Because the main impurity in the commercial sources of BSA-96 is globulins, a γ-globulin-free albumin with ≥99% purity (BSA-no globulin; Table I) was tested. As demonstrated in Fig. 1 and listed in Table II, the perfusate containing BSA-no globulin resulted in significantly lower C_{SS} and AUC values for TNF-α, compared with the BSA-96 containing perfusate. Nevertheless, the steady-state outlet concentrations of TNF-α were still very high, suggesting a major role for other impurities in the albumin preparations.

Because LPS is a known stimulator of Kupffer cells, a low endotoxin preparation of BSA (BSA-low LPS; Table I), which contained 0.01 ng/mg LPS, was tested. The TNF-α levels in the outlet perfusate of these IPRLs were not significantly different from the low values for IPRLs with no BSA (Table II and Fig. 1), suggesting that the LPS content of BSA is mostly responsible for the induction of TNF-α release. This is in agreement with the positive correlation between the LPS content of albumin and the AUC of TNF-α in the perfusate (Fig. 2).

The relationship shown in Fig. 2, although statistically significant, indicates that the endotoxin content of albumin cannot completely explain the rises in the TNF-α AUC, suggesting that other factors may also be involved. For example, although the endotoxin level of BSA-no globulin with lot 129H0973 (8 ng/mg) was higher than that of BSA-96 (5 ng/mg), the TNF-α concentrations were higher for BSA-96 (Fig. 2), indicating that the γ-globulin content of BSA-96 can also contribute to the observed higher levels of TNF-α. Indeed, a different lot of BSA-no globulin (109H0981) with a lower (4 ng/mg) endotoxin levels showed lower TNF-α AUCs, compared with BSA-96 with similar endotoxin levels (Fig. 2), confirming the role of IgG in stimulation of TNF-α release.

Searching the literature for studies measuring cytokines in IPRL perfusates, three reports (10–12) were identified. Tran-Thi *et al.* (10) used a single-pass IPRL model to study the effects of LPS on the release of TNF-α, IL-1, and IL-6 from the rat liver. These investigators used an RPMI medium containing 1% newborn-calf serum as a perfusate and showed relatively low levels of cytokines in the outlet perfusate of control (untreated) livers. The low cytokine levels in this study (10) may be due to the use of 1% newborn calf serum instead of BSA preparations that are normally used in IPRLs. However, in agreement with our results, these authors demonstrated that the injection of LPS into the liver significantly increased the levels of TNF-α in the outlet perfusate.

Two other reports, by Liao *et al.* (11,12), used a perfusate containing 1% albumin in Krebs-Henseleit buffer, which is similar to the perfusates used here. However, the species or

other information about the albumin preparation was not reported. These authors (11,12) reported mean outlet concentrations of ≤ 250 pg/ml for TNF-α in the outlet perfusate of control livers during a 0–80-min perfusion period. This apparently lower value of TNF-α, compared with the concentrations observed in our study (Fig. 1), may be due to several factors. First, our studies demonstrate that the concentrations of TNF-α in the perfusate gradually increase, starting at ~20 min after the perfusion, and then rise to a maximum at ~100 min (Fig. 1). Therefore, an average concentration during the 0–80-min period used by Liao *et al.* (11,12) may be an underestimation of the C_{SS} values for TNF-α. Second, it is possible that the albumin preparation that these authors used may have contained lesser LPS content than the BSA-96 or BSA-no globulin used in our studies. Nevertheless, the studies presented here clearly show that, depending on the type of albumin preparations, different time courses of TNF-α release may be expected.

We selected the IPRL model with no albumin in the perfusate for our next studies because of its low levels of TNF-α (Fig. 1). The use of an alternative model with BSA-low LPS perfusate was prohibitive because of the very high cost of such a preparation. In the application studies, it was demonstrated that the selected model may be used for the determination of the effects of systemically administered MP on the LPS-induced stimulation of TNF-α release (Fig. 4). Based on our data, the maximum effect of LPS on the release of TNF-α appeared at ~80 min after the start of perfusion (or ~60 min after the end of LPS infusion) (Fig. 4). This is consistent with the literature data showing that the maximum TNF-α concentrations are achieved at ~1 h after the injection of LPS in isolated perfused livers (10,11), isolated Kupffer cells (10), or *in vivo* (15).

Corticosteroids are known to inhibit the production of cytokines such as TNF-α *in vivo* (16). Similar observations were made in our IPRL model after the *in vivo* administration of MP. Additionally, our data showed that the time of administration of MP affects the degree of inhibition of TNF-α release (Fig. 4), a phenomenon that is in agreement with the literature data on dexamethasone (15). Waage (15) demonstrated that dexamethasone dose-dependently decreased the LPS-induced TNF-α serum concentrations in rats with a maximum of 70–90% reduction at a dose of 2 mg/kg. Interestingly, the maximum inhibitory effect of dexamethasone was also observed when the steroid was administered ≥5 h before the *in vivo* injection of LPS (15).

Although not studied here, our data may have some implications for drug metabolism investigations using IPRLs. This is because the proinflammatory cytokines, such as TNF-α, can significantly impact the metabolism of various drugs (4). Additionally, BSA preparations have been used routinely in IPRL studies either as oncotic agents (17) and/or for studies of the effects of protein binding on drug metabolism or uptake by the liver (8). Our results indicate that the livers producing high TNF-α levels were otherwise normal in terms of usual viability parameters (e.g., macroscopic appearance, bile flow rate, liver enzyme levels, and wet liver weight at the end of perfusion). Normal viability parameters were also reported (18) by others after the injection of TNF-α into IPRLs. Therefore, high and variable levels of TNF-α in the perfusates containing various concentrations and preparations of BSA could potentially alter the metabolism of drugs. This postulate, however, remains to be proven in further studies concentrating on the levels of different cytochrome P450s and the rate of metabolism of model drugs.

In conclusion, our data demonstrate that some BSA preparations used in the perfusates of IPRLs may stimulate the release of TNF-α from the liver. This stimulating effect is mostly related to the endotoxin content of the albumin preparation. An IPRL model with a Krebs-Henseleit perfusate without albumin, however, results in low baseline levels of TNF-α. The model was successfully used for detection of the inhibitory effect of systemically administered MP on the release of TNF-α from LPS-stimulated isolated livers.

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REFERENCES

1. Z. Szatmary. Tumor necrosis factor-alpha: molecular-biological aspects. *Neoplasma* **46:**257–266 (1999).
2. U. Karck, T. Peters, and K. Decker. The release of tumor necrosis factor from endotoxin-stimulated rat Kupffer cells is regulated by prostaglandin E2 and dexamethasone. *J. Hepatol.* **7:**352–361. (1988).
3. L. H. Toledo-Pereyra and S. Suzuki. Neutrophils, cytokines, and adhesion molecules in hepatic ischemia and reperfusion injury. *J. Am. Coll. Surg.* **179:**758–762 (1994).
4. E. T. Morgan. Regulation of cytochromes P450 during inflammation and infection. *Drug Metab. Rev.* **29:**1129–1188. (1997).
5. S. Ko, Y. Nakajima, H. Kanehiro, M. Horikawa, A. Yoshimura, K. Kido, J. Taki, Y. Aomatsu, T. Kin, K. Yagura, T. Ohyama, K. Ohashi, T. Yamada, and H. Nakano. Different immunosuppressive effects of liposomal FK506 in liver and kidney transplantation. *Transplant Proc.* **28:**1066–1068 (1996).
6. R. Mehvar, J. M. Reynolds, M. A. Robinson, and J. A. Longstreth. Enantioselective kinetics of verapamil and norverapamil in isolated perfused rat livers. *Pharm. Res.* **11:**1815–1819 (1994).
7. R. Mehvar and J. M. Reynolds. Input rate-dependent stereoselective pharmacokinetics: experimental evidence in verapamil-infused isolated rat livers. *Drug Metab. Dispos.* **23:** 637-641 (1995).
8. R. Mehvar and J. Reynolds. Reversal of stereoselectivity in the hepatic availability of verapamil in isolated perfused rat livers - Role of protein binding. *Drug Metab. Dispos.* **24:**1088–1094 (1996).
9. R. Mehvar. Kinetics of hepatic accumulation of dextrans in isolated perfused rat livers. *Drug Metab. Dispos.* **25:**552–556 (1997).
10. T-A. Tran-Thi, L. Weinhold, C. Weinstock, R. Hoffmann, A. Schulze-Specking, H. Northoff, and K. Decker. Production of tumor necrosis factor-α, interleukin-1 and interleukin-6 in the perfused rat liver. *Eur. Cytokine Netw.* **4:**363–370 (1993).
11. J. Liao, J. A. Keiser, A. Keiser, W. E. Scales, S. L. Kunkel, and M. J. Kluger. Role of corticosterone in TNF and IL-6 production in isolated perfused rat liver. *Am. J. Physiol.* **268:**R639–R706 (1995).
12. J. Liao, J. A. Keiser, A. Keiser, W. E. Scales, S. L. Kunkel, and M. J. Kluger. Role of epinephrine in TNF and IL-6 production from isolated perfused rat liver. *Am. J. Physiol.* **268:**R896–R901 (1995).
13. M. Yoshioka, T. Ito, S. Miyazaki, and Y. Nakajima. The release of tumor necrosis factor-alpha, interleukin-1, interleukin-6 and prostaglandin E2 in bovine Kupffer cells stimulated with bacterial lipopolysaccharide. *Vet. Immunol. Immunopathol.* **66:**301–307 (1998).
14. Z. M. Zheng, S. C. Specter, and G. Lancz. Bovine serum albumin

preparations enhance in vitro production of tumor necrosis factor α by murine macrophages. *Immunol. Invest.* **24:**737–756 (1995).
15. A. Waage. Production and clearance of tumor necrosis factor in rats exposed to endotoxin and dexamethasone. *Clin. Immunol. Immunopathol.* **45:**348–355 (1987).
16. D. G. Remick, R. M. Strieter, J. P. Lynch III, D. Nguyen, M. Eskandari, and S. L. Kunkel. *In vivo* dynamics of murine tumor necrosis factor-alpha gene expression: kinetics of dexamethasone-induced suppression. *Lab. Invest.* **60:**766–771 (1989).
17. K. S. Pang. Liver perfusion studies in drug metabolism and drug toxicity. In J. R. Mitchell, M. G. Horning (eds.), *Drug Metabolism and Drug Toxicity,* Raven Press, New York, 1984 pp. 331–352.
18. K. E. Shattuck, C. D. Grinnell, A. S. Goldman, and D. K. Rassin. The acute effects of TNF-alpha on the isolated perfused rat liver. *J. Investig. Med.* **44:**64–69 (1996).