Chloroquine Attenuates Hemorrhagic Shock-Induced Suppression of Kupffer Cell Antigen Presentation and Major Histocompatibility Complex Class II Antigen Expression Through Blockade of Tumor Necrosis Factor and Prostaglandin Release

By Wolfgang Ertel, Mary H. Morrison, Alfred Ayala, and Irshad H. Chaudry

Hemorrhagic shock suppresses the ability of Kupffer cells (KC) to present antigen and express the major histocompatibility complex class II antigen (Ia). These alterations are concomitant with an enhanced release of cytokines (tumor necrosis factor [TNF], interleukin-1 [IL-1], IL-6) and prostaglandin E2 (PGE2) by KC after hemorrhagic shock. The aim of this study was to determine whether chloroquine (CQ) administration in vivo before or after hemorrhage affects the altered cytokine and PGE2 release by KC as well as the capacity of KC to present antigen and express Ia. To study this, C3H/HeN mice were bled to and maintained at a mean arterial blood pressure of 35 mm Hg for 60 minutes, followed by fluid resuscitation. Chloroquine (10 mg/kg body weight) was injected intramuscularly 2 hours before or during resuscitation following shock. The administration of CQ led to a significant reduction in the hemorrhage-induced elevation of TNF, IL-6, and PGE2 release by KC; however, IL-1 secretion was not affected by CQ. In addition, CQ treatment abolished the hemorrhage-induced increase in circulating TNF and IL-6. These changes in cytokine and PGE2 release following CQ administration correlated with a significant enhancement of the antigen-presenting capacity of KC. No differences were observed between pretreatment and posttreatment with CQ. Our data indicate that CQ selectively inhibits the release of TNF, IL-6, and PGE2 by KC, while IL-1 secretion was unaffected. Because the reduction of these inflammatory mediators was concomitant with a significant improvement of KC capacity to present antigen and express Ia, we propose that TNF, IL-6, and PGE2 play a pivotal role in the induction of posthemorrhage immunosuppression. Furthermore, the data suggest that the suppression of KC functions occurs during or after resuscitation, because posttreatment with CQ was as effective as pretreatment. Additional studies indicated that the survival of animals after hemorrhage and sepsis was significantly increased by posttreatment of hemorrhaged mice with CQ. Thus, CQ, because of its unique ability to selectively inhibit the release of inflammatory cytokines and prostaglandins, represents a potent immunomodulating agent in the treatment of conditions associated with increased cytokine release and for decreasing the mortality from sepsis after hemorrhage.

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TRAUMATIC INJURY due to burn and soft tissue damage induces a profound suppression of both cell-mediated and humoral immunity. However, until recently, little was known about the contribution of hemorrhage, a commonly encountered complication of traumatic and/or surgical injury, in the depression of the host’s immune responsiveness. Studies using a nonlethal murine hemorrhage model have demonstrated that macrophage (peritoneal, splenic, and Kupffer cell [KC]) functions such as antigen presentation and major histocompatibility complex (MHC) class II antigen (Ia) expression are significantly depressed after hemorrhage. Furthermore, splenocyte functions including proliferation and lymphokine synthesis were markedly inhibited after hemorrhage. The severe suppression of different immune functions correlated with increased plasma levels of tumor necrosis factor (cachectin, TNF), interleukin-6 (IL-6), and prostaglandin E2 (PGE2). KC, which represent the largest pool of macrophages in the body, were found to produce significantly higher amounts of TNF, IL-6, and IL-1 after hemorrhage. These changes in cytokine release correlated with increased cytokine plasma levels. However, such alterations were also associated with a depression in KC ability to present antigen or express the MHC class II (Ia) antigen.

Chloroquine is an antimalarial agent that has been used extensively in the treatment of rheumatoid arthritis. It has been suggested that chloroquine exerts its therapeutic effect in the treatment of arthritis by inhibiting the inflammatory events underlying the disease process. In fact, in vitro studies have shown that chloroquine inhibits thymidine incorporation in lymphocytes in a dose-dependent manner by interfering with the accessory functions of monocytes. Furthermore, Auth and Traggor described an inhibitory effect of chloroquine on phospholipase A activity leading to decreased production of prostaglandins. Although Salmeron and Lipsky suggested that chloroquine might inhibit the release of lymphocyte activating factors, such as IL-1 by macrophages, experimental evidence to demonstrate this was lacking in their studies. Our in vitro studies showed that chloroquine selectively inhibits TNF and IL-6 release without influencing the secretion of IL-1 by normal macrophages. However, it remains to be determined whether or not chloroquine has any effect on the altered cytokine release and PGE2 production after hemorrhage in vivo. Because KC are the predominant macrophage population that releases significant amounts of TNF, IL-1, and IL-6 after hemorrhage, the effect of chloroquine administration on these cells was studied. If chloroquine blocks the release of these inflammatory mediators by KC, the investigation of KC antigen presentation and Ia expression after hemorrhage and treatment with chloroquine

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might allow us to determine whether or not the above mediators are involved in the depression of KC functions after hemorrhage.

Therefore, the aim of this study is to investigate the effects of in vivo chloroquine administration on the release of cytokines and PGE₂ synthesis by KC after hemorrhagic shock. Furthermore, the effect of chloroquine treatment on KC dysfunctions after hemorrhage was determined.

MATERIALS AND METHODS

Animals. Inbred C3H/HeN (Charles River Labs, Portage, MI) male mice, 6 to 8 weeks old, weighing 20 to 25 g, were fasted for 18 hours before the experiment. Food and water ad libitum were allowed following the experiment.

Hemorrhage model. Hemorrhage was induced as described by Stephan et al. Briefly, mice were lightly anesthetized with methoxyflurane and one femoral artery cannulated with PE-10 tubing (Clay Adams, Parsippany, NJ) under aseptic conditions. Mice were heparinized (80 U/kg body weight [BW] heparin; Upjohn Labs, Kalamazoo, MI), restrained in a supine position, and allowed to awaken. The implanted catheter was used for blood pressure (BP) monitoring and bleeding through a three-way stopcock. An average blood volume of 0.75 ± 0.03 mL in the vehicle-treated group, 0.76 ± 0.04 mL in the hemorrhaged group with chloroquine pretreatment, and 0.76 ± 0.03 mL in the hemorrhaged group with chloroquine posttreatment was removed to achieve a mean BP of 35 mm Hg within 10 minutes (BP prehemorrhage was 105.5 ± 1.7 mm Hg [vehicle-treated group], 107.2 ± 1.5 mm Hg [chloroquine pretreated group], and 107.8 ± 1.9 mm Hg [chloroquine post-treated group]). This BP was maintained for 60 minutes followed by reinfusion of the shed blood and Ringer’s lactate solution (twice the shed blood volume) to provide adequate fluid resuscitation. Hemorrhaged mice responded to resuscitation with a rapid increase of BP close to prehemorrhage values (97.8 ± 3.2 mm Hg [vehicle-treated group], 98.3 ± 2.5 mm Hg [chloroquine pretreated group], and 101.2 ± 2.5 mm Hg [chloroquine posttreated group]). Chloroquine (10 mg/kg BW), which was dissolved in a total volume of 0.1 mL saline, was injected intramuscularly either 2 hours before hemorrhage (pretreatment) or immediately after resuscitation (posttreatment). The vehicle-treated hemorrhaged group received an intramuscular injection of 0.1 mL saline. After resuscitation, the catheters were removed and the groin incisions closed. Control (sham) animals underwent the same anesthetic and surgical procedures, which included ligation of one femoral artery, but the pellets from these two centrifugations were pooled and resuspended in Click’s medium containing 10% FBS. The cell suspension (2 × 5 mL) was layered over 2 × 5 mL metrizamide (16%; Accurate Chemical Corp, Westbury, NY) in HBSS (5.0 mL) and centrifuged at 1,300g for 45 minutes at 4°C. This technique separated the KC, which form a band at the metrizamide-cushion interphase, from the remaining parenchymal cells in the pellet. After removing the KC from the interphase with a Pasteur pipette, the cells were washed twice by centrifugation (500g, 15 minutes, 4°C). The pellet was then dispersed and resuspended in Click’s medium containing 10% FBS. Cell viability was determined by Trypan Blue exclusion. No differences in cell yields and viability were observed between the four groups. KC were resuspended at a final concentration of 1 × 10⁶ KC/mL in Click’s medium containing 10% FBS.

Antigen presentation assay. KC were allowed to adhere onto 60-mm plastic Petri dishes at 37°C for 5 hours. Nonadherent cells were removed by washing the Petri dishes four to five times with Click’s medium. The monolayer of KC was then incubated for 20 minutes (37°C, 5% CO₂, in the dark) with 30 μg mitomycin C/mL (Sigma Chemical Co, St Louis, MO) in phosphate-buffered saline (PBS). The plates were extensively washed with Click’s medium and the cells scraped off the plate using 3 mL of Click’s medium plus 10% FBS. Viability was determined and KC concentration adjusted to 2.5 × 10⁶ KC/mL in Click’s medium containing 10% FBS. This procedure provided cell cultures that contained ≥90% KC using the macrophage-specific monoclonal antibody (MoAb) F4/80.

The capacity of KC to present the specific antigen conalbumin was assessed by coculturing KC with the mouse Tₐ-cells D10.G4.1. The concentrations of KC ranged between 2.5 × 10⁴ to 3.2 × 10⁵ cells/well, while D10.G4.1 cells were kept constant at a concentration of 2 × 10⁹ cells/well. The D10.G4.1 cell clone (kindly provided by Dr Charles Janeway, New Haven, CT) solely proliferates in the presence of conalbumin presented by MHC class II antigen compatible macrophages. The degree of the D10.G4.1 proliferation, determined by [H]-thymidine incorporation, directly correlates with the capacity of KC to present conalbumin.

Determination of MHC class II antigen expression. MHC class II antigen (Ia) expression was determined using a microscopic technique as described by Ayala and Kierszenbaum. KC were labeled with fluorescein-conjugated mouse Ia⁺ alloantisera (Accurate Scientific, Westbury, NY). Background was controlled by using a fluorescein isothiocyanate (FITC)-conjugated murine MoAb against HLA-DR (1:10) (Olympus Corp, Lake Success, NY) as control. Similarly, before staining, the cells were preincubated with mouse IgG to block nonspecific binding of mouse IgGs. Not fewer than 200 cells were screened in each culture, recording both the number of fluorescent cells (Ia positive) and nonfluorescent cells. From these values, the percent Ia positive cells was calculated.

Assessment of monokine release. KC (1 × 10⁶ KC/mL/well) were allowed to adhere onto a 24-well plate for 5 hours. Nonadherent and dead cells were removed by washing the cells five times followed by a 24-hour incubation (37°C, 5% CO₂, with 10 μg lipopolysaccharide W (LPS, from Escherichia coli 055:B5; Difco Labs, Detroit, MI) per milliliter of Click’s medium with 10% FBS.

The liver tissue was finely minced, incubated at 37°C for 15 minutes, and then further dissociated by pipetting it through a wide-bore 10 mL plastic pipette followed by a standard-bore pipette. The cell suspension was passed through a sterile 150 mesh stainless steel screen into a 150-mL beaker containing 10 mL of cold Click’s medium (Irvine Scientific, Santa Ana, CA) plus 10% fetal bovine serum (FBS; Biologos Inc, Naperville, IL) and centrifuged at 300g for 15 minutes at 4°C. The supernatant was removed and recentrifuged as before to collect additional cells in the supernatant. The pellets from these two centrifugations were stored at -70°C until assayed.

For harvesting the liver, the abdomen was opened in addition to the thorax. The peritoneal cavity was packed with sterile gauze. The liver was blanched by a retrograde perfusion with 40 to 50 mL ice-cold Hank’s balanced salt solution (HBSS; GIBCO Labs, Grand Island, NY), free of Ca⁺⁺ and Mg⁺⁺, through the right ventricle of the heart. This was immediately followed by perfusion with 10 mL of 0.05% collagenase class IV (Worthington Biochemical Corp, Freehold, NJ) in HBSS without Ca⁺⁺ and Mg⁺⁺ at 37°C. The liver was then removed en bloc and transferred to 100-mm Petri dishes containing 5 mL warm (37°C) HBSS with 0.05% collagenase.
The KC supernatants were harvested, filtered, aliquoted, and stored at −70°C until assayed for IL-1, IL-6, and TNF.

The IL-1 activity in these cell cultures was determined by adding serial dilutions of the supernatant to D10.G4.1 cells (2 × 10⁴ cells/well) in the presence of Concanavalin A (2.5 μg/mL; Pharmacia Fine Chemicals, Piscataway, NJ), as previously described.⁴ Proliferation of the D10.G4.1 cells was measured by ³H-thymidine incorporation. Previous studies⁴,¹⁸ clearly demonstrated that the D10.G4.1 cells do not respond to murine IL-6, which is present in high amounts in KC supernatants. To further confirm these findings, IL-6 in KC supernatants that contained high amounts of IL-6 was neutralized with a monoclonal IL-6 antibody (IgG₂) (final antibody concentration 1:10), which was prepared from the D6906.B4.M hybridoma (kindly provided by Dr J. Van Snick, Brussels, Belgium). IL-1 activity in the antibody-treated supernatants was not statistically different from the IL-1 activity found in untreated supernatants.

IL-6 activity was determined by the amount of proliferation of the murine hybridoma cell line 7TD1 (kindly provided by Dr J. Van Snick) which only grows in the presence of IL-6.²¹ ²² 7TD1 cells/well, 4 × 10³, stimulated with serial dilutions of plasma or KC supernatants, were grown for 72 hours. For the last 4 hours of incubation, MTT-tetrazolium (5 mg/mL; Sigma) was added. The amount of dark blue formazan crystals produced during this time was measured spectrophotometrically. To further confirm that 7TD1 cells solely react to IL-6, the monoclonal antimurine IL-6 antibody (D6906.B4.M hybridoma) was added for control measurements.

TNF in KC supernatants was determined by its cytotoxic effect on the fibrosarcoma cell line WEHI 164 subclone 13²³ ²⁴ (kindly provided by Dr S. Kunkel, Ann Arbor, MI). The proliferation of unlysed WEHI cells was measured, as described previously, using the MTT assay. Positive samples, in which TNF was neutralized with a polyclonal rabbit antimurine TNF antiserum (Genzyme, Cambridge, MA) (final antibody concentration 1:20), were assayed in parallel.

All samples in the previously described assays were tested in duplicate. The unit(s) of monokine activity per milliliter of supernatant or plasma were determined by comparison of the curves produced from the dilutions of a purified human IL-1β standard (20 U/mL; Genzyme), a recombinant human IL-6 standard (200 U/mL; Amgen Corp, Thousand Oaks, CA), or a murine TNF standard (200 U/mL; Amgen) according to the methods of MizeLZ3.

**Determination of TNF plasma levels.** For detection of TNF in plasma, a mouse TNF enzyme-linked immunosorbent assay (ELISA) kit (Genzyme), employing the multiple antibody sandwich principle, was used. The assay was performed according to the manufacturer’s directions.

**Assessment of PGE₂ production.** KC were prepared in the same way as described for monokine release measurements. For determination of PGE₂, KC supernatants, KC were stimulated with 1.0 μg/mL LPS in Click’s medium with 10% FBS for 24 hours. At the end of the incubation time, 10 μL ibuprofen (1 mg/mL; Sigma) was added to the KC cultures, and the supernatants were harvested, filtered, and stored at −70°C until assayed for PGE₂.

The amounts of PGE₂ present in the KC supernatants were determined with the radioimmunoassay (RIA) technique (NEN DuPont, Boston, MA; cross-reactivity with PGE₁: 3.7%, DHKPGE₂: 0.04%, PGF₂α: 0.04%, other prostaglandins and arachidonic acid: <0.01%).

**Sepsis model.** In an additional group of mice, sepsis was produced as described in detail previously.⁶ Briefly, on the third day after hemorrhage with or without chloroquine posttreatment or sham operation, respectively, mice were anesthetized and a midline laparotomy performed. The cecum was isolated and a 5-0 silk ligature was placed around it, thus ligating the cecum just below the ileocecal valve. The ligated cecum was then punctured twice with a 22-gauge needle, returned to the peritoneal cavity, and the abdominal incision was closed. Normal saline (4 mL/100 g BW) was administered subcutaneously at this time. Survival was determined every 24 hours over a 3-day period after cecal ligation and puncture (CLP).

**Statistics.** Results are presented as mean ± SEM. Data are analyzed by means of a split plot analysis of variance (ANOVA) according to the model: yᵢⱼ = Mean + Aᵢ + Sᵢ + Bᵢ + (AB)ᵢⱼ + εᵢⱼ, where A represents the fixed effect of the four groups, B the fixed effect of the two time points, S the whole plot error, and E the split-plot error. Post-hoc analysis was performed using Bonferroni's t-test for multiple comparisons. The data with percent values (antigen presentation) were analyzed using the Wilcoxon rank sum test. Significance was predetermined as P < 0.05.²⁴ Statistical analysis of the survival study was performed using Chi-square analysis.

**RESULTS**

In normal animals, administration of chloroquine did not produce any significant differences in KC cytokine release, PGE₂ synthesis, or the antigen presentation process when compared with untreated normal animals. Furthermore, chloroquine administration did not cause any hypotension or mortality in hemorrhaged animals.

**TNF release.** TNF release by KC from hemorrhaged animals (0.51 ± 0.04 U/mL) was increased sixfold (P < 0.01) at 2 hours and fourfold at 24 hours (0.53 ± 0.04 U/mL) (P < 0.01) when compared with sham-operated animals (0.09 ± 0.05 U/mL at 2 hours; 0.10 ± 0.06 U/mL at 24 hours) (Fig 1A). Administration of chloroquine, whether injected before hemorrhage or immediately after hemorrhage, significantly (P < 0.01) inhibited the secretion of biologically active TNF (Fig 1A). The amounts of TNF released by KC of hemorrhaged animals with chloroquine treatment were in the range of sham-operated animals. To confirm that the bioactivity measured in the WEHI bioassay was due to TNF, a polyclonal rabbit anti-TNF antibody was added to selected samples containing high amounts of TNF. The addition of the TNF antibody completely abolished the TNF activity in TNF-positive samples.

**IL-6 release.** The release of IL-6 by KC from hemorrhaged animals (0.15 ± 0.02 U/mL) was significantly (P < 0.01) elevated at 2 hours compared with sham-operated animals (0.07 ± 0.02 U/mL) (Fig 1B). Pretreatment (0.06 ± 0.02 U/mL) and posttreatment (0.05 ± 0.01 U/mL) with chloroquine significantly (P < 0.01) inhibited the secretion of IL-6 by KC after hemorrhage (Fig 1B). At 24 hours no significant difference between the four groups was observed (Fig 1B). The neutralization of IL-6 in these supernatants with a monoclonal anti–IL-6 antibody inhibited 7TD1 cells and indicates the specific response of this cell line to IL-6.

**IL-1 release.** KC from hemorrhaged animals released significantly (P < 0.05) higher amounts of IL-1 (1.14 ± 0.19 U/mL) at 2 hours compared with sham-operated animals (0.51 ± 0.05 U/mL), while no difference was found at 24 hours between these two groups (Fig 2A). Chloroquine treatment did not affect the release of IL-1 by KC from the
hemorrhaged group at 2 hours (Fig 2A). At 24 hours, IL-1 release by KC from hemorrhaged animals with chloroquine pretreatment (1.03 ± 0.22 U/mL) and posttreatment (1.37 ± 0.28 U/mL) was enhanced by 78% and 136%, respectively, compared with the vehicle-treated hemorrhaged group (0.58 ± 0.11 U/mL) (Fig 2A). However, these differences were not found to be statistically significant. The neutralization of IL-6 in these supernatants with the anti-IL-6 antibody did not significantly reduce the proliferative response to D10.G4.1 cells. This indicates that D10.G4.1 cells do not respond to murine IL-6, which is in line with the observations of Suda et al17 and Hopkins and Humphrey's.18

PGE₂ production. PGE₂ production by KC from hemorrhaged animals (58.0 ± 8.3 pg/mL) was significantly (P < .05) elevated at 2 hours compared with sham-operated animals (30.8 ± 10.6 pg/mL) (Fig 2B). Chloroquine, administered as pretreatment or posttreatment, resulted in a 56% reduction (P < .05) of PGE₂ production (25.5 ± 8.5 pg/mL with pretreatment; 25.3 ± 8.7 pg/mL with posttreatment) compared with the vehicle-treated hemorrhaged group (Fig 2B). At 24 hours after hemorrhage, PGE₂ production by KC was similar in all four groups.

Plasma levels of TNF and IL-6. Circulating TNF was elevated at 2 hours (32.4 ± 4.0 U/mL) (P < .01) and 24 hours (13.8 ± 1.7 U/mL) compared with sham-operated animals (11.5 ± 2.9 U/mL at 2 hours; 3.2 ± 2.3 U/mL at 24 hours) (Fig 3A). Chloroquine pretreatment or posttreatment abolished the hemorrhage induced increase of TNF at 2 hours (P < .01) and 24 hours (Fig 3A).

Hemorrhage also induced a significant (P < .05) increase of circulating IL-6 at 2 hours (1.68 ± 0.12 U/mL) and an increase of 67% at 24 hours (0.70 ± 0.05 U/mL) in
immunomodulatory effects of chloroquine

Fig 3. Circulating TNF (U/mL) (A) and IL-6 (U/mL) (B) plasma levels. Plasma was obtained from sham-operated, hemorrhaged, and hemorrhaged animals with chloroquine pretreatment or posttreatment 2 and 24 hours after hemorrhage. TNF plasma levels were measured with ELISA and IL-6 plasma levels with a bioassay using the IL-6 specific cell line 7TD1. The data are presented as mean ± SEM. *P < .05/**P < .01 hem versus sham; ###P < .01 hem versus hem + chloroquine treatment.

Fig 4. Capacity of KC (5 x 10^4 KC/well) to present antigen (percent of sham animals) (A) and express the MHC class II (Ia) antigen (percent of Ia positive KC) (B). Measurements were performed in sham-operated, hemorrhaged, and hemorrhaged animals with chloroquine pretreatment or posttreatment 2 and 24 hours after hemorrhage. The antigen presentation capacity was determined using the mouse T cell clone D10.G4.1. The Ia expression was measured with a fluorescein conjugated mouse Ia^a antisera. The data are presented as mean ± SEM. *P < .05/**P < .01 hem versus sham; #P < .05/##P < .01 hem versus hem + chloroquine treatment.

comparison with sham-operated animals (1.01 ± 0.11 U/mL at 2 hours; 0.42 ± 0.07 U/mL at 24 hours) (Fig 3B). Administration of chloroquine before or immediately after hemorrhage attenuated (P < .01) the increase in plasma IL-6 at 2 hours, while no effect was seen at 24 hours (Fig 3B). The addition of the anti-IL-6 antibody to plasma samples completely inhibited the proliferative response of 7TD1 cells induced by the tested samples without the antibody. This indicates the highly specific response of 7TD1 cells to IL-6 present in plasma samples.

Antigen presentation. The capacity of KC to present the antigen conalbumin to D10.G4.1 cells was significantly (P < .01) decreased in the hemorrhaged group (67.2% ± 6.7% at 2 hours; 57.2% ± 5.9% at 24 hours) compared with sham animals (Fig 4A). However, treatment with chloroquine restored KC antigen presentation at 2 and 24 hours after hemorrhage to values observed in sham-operated animals (Fig 4A). Chloroquine posttreatment even elevated (P < .05) antigen presentation capacity of KC by 77% at 24 hours compared with the sham group (Fig 4A).

MHC class II antigen (Ia) expression. In parallel with antigen presentation, the capacity of KC to express the MHC class II (Ia) antigen was reduced (P < .01) by 60% at 2 hours and 47% at 24 hours following hemorrhage compared with the sham-operated group (Fig 4B). However, pretreatment or posttreatment with chloroquine resulted in a restoration (P < .01) of Ia expression at both 2 and 24 hours (Fig 4B).

Effect of chloroquine posttreatment on survival after hemorrhage and sepsis. All animals in the vehicle-treated hemorrhaged group died within 3 days after CLP, while 44% in
the sham-operated group survived through day 3 \( (P = .011) \) (Table 1). The administration of chloroquine after hemorrhage significantly increased \( (P = .011) \) the survival of hemorrhaged animals (44% after 3 days) compared with vehicle-treated hemorrhaged animals (Table 1).

### DISCUSSION

The results presented in this study demonstrate that chloroquine administration before or after hemorrhagic shock selectively inhibits the release of TNF, IL-6, and PGE\(_2\), without decreasing the secretion of IL-1 by KC. This was associated with the restoration of hemorrhage-induced suppression of KC functions such as antigen presentation capacity and MHC class II antigen expression. It should be pointed out that the mouse T-cell clone D10.G4.1 was used in this study for measuring antigen presentation. Although this T-cell clone has been used by a large number of investigators for different purposes, including antigen presentation, it is possible that if other T-cell clones were used the results may not have differed. Nonetheless, the results clearly demonstrate that whereas none of the posthemorrhage mice subjected to sepsis survived on day 3, the survival rate in posthemorrhaged mice treated with chloroquine was comparable with that of the sham-operated mice. Thus, posttreatment of hemorrhaged mice with chloroquine also decreases the mortality from sepsis.

Hemorrhagic shock induces a severe suppression of macrophage, \(^6\) T-lymphocyte, \(^6\) and B-lymphocyte \(^2\) functions. Besides the depression of peritoneal macrophage functions, \(^4\) a decreased capacity of KC to present antigen and express the MHC class II antigen was detected. \(^5\) The depression of these KC functions may be deleterious for the host, because KC play a major role in clearing bacteria released into the circulation due to translocation from the gut during hemorrhagic shock. \(^2\) \(^2\) Therefore, the suppression of KC functions might be responsible for the increased susceptibility to sepsis observed after hemorrhage. \(^4\) The precise mechanisms causing the observed suppression of KC after hemorrhage and their potential inducers are, however, not known. Previous studies from our laboratory \(^2\) \(^2\) have shown that the depression of the antigen-presenting process occurred in parallel with an increased TNF release by KC and significantly increased circulating TNF levels at 2 hours after hemorrhage. \(^7\) Although TNF may in itself be an inducer of immunosuppression after hemorrhage and resuscitation, this cytokine also enhances the synthesis and release of arachidonic metabolites such as PGE\(_2\). Therefore, it is possible that the increased PGE\(_2\) synthesis induced by TNF rather than TNF itself may be pivotal for the induction of posthemorrhage immunosuppression. This suggestion is based on the observations that PGE\(_2\) profoundly inhibits various immune responses. \(^8\) This includes an inhibitory effect of PGE\(_2\) on antigen presentation, \(^9\) MHC class II antigen expression, \(^2\) lyphokine synthesis, \(^1\) B-lymphocyte proliferation, \(^9\) and Ig synthesis. \(^10\) Because we previously demonstrated a persistent increase in PGE\(_2\) plasma levels up to 24 hours after hemorrhage, \(^4\) it is likely that PGE\(_2\) plays a central role in suppressing KC functions.

The results described previously suggest that hemorrhage induces an elevation of TNF leading to increased PGE\(_2\) release, which may be involved in the depression of KC functions. Our in vitro investigations \(^1\) showed a significant inhibition of TNF and IL-6 release by macrophages after incubation with chloroquine. This, together with data from Authi and Tragnor, \(^11\) who described an inhibitory effect of chloroquine on prostaglandin synthesis in vitro, raised the issue of whether or not administration of chloroquine in a murine hemorrhagic shock model would alter cytokine and prostaglandin synthesis as well as the depressed KC functions, such as antigen presentation and IA expression. Because significant changes in cytokine release occur during and after shock, \(^7\) chloroquine was administered following hemorrhage in one set of experiments, while in additional experiments it was administered 2 hours before the onset of hemorrhage.

Hemorrhagic shock significantly enhanced KC capacity to release TNF, IL-6, and IL-1 at 2 hours after hemorrhage. Plasma levels of TNF and IL-6 were also elevated at 2 hours, and increased TNF plasma levels persisted up to 24 hours. In addition, a significant increase of PGE\(_2\) release by KC was detected at 2 hours. These alterations in the profiles of cytokine and PGE\(_2\) were associated with a significant decrease in antigen presentation and IA expression by KC at 2 and 24 hours.

The pretreatment or posttreatment of hemorrhaged animals with chloroquine (10 mg/kg IM) significantly inhibited TNF, IL-6, and PGE\(_2\) release by KC at 2 hours after hemorrhage. The inhibition of TNF release by chloroquine persisted up to 24 hours. These alterations in TNF and IL-6 release were associated with a significant reduction of plasma TNF and IL-6 levels.

It should be noted that functional studies on KC were performed in vitro. Because this involved isolation of these cells followed by several steps of purification, the studies presented here of KC function after hemorrhage with and without chloroquine treatment more accurately reflect the capacity/capability of these cells to perform various functions in vitro. Nonetheless, the measurement of plasma cytokine levels as performed in this study, and as described in another study, \(^1\) clearly demonstrate a correlation between altered cytokine release by KC in vitro and changes in plasma cytokine levels. Further support for this sugges-

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**Table 1. Effect of Chloroquine on Survival After Hemorrhage and Sepsis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Time After CLP (h)</th>
<th>24</th>
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<tr>
<td>Hemorrhage + chloroquine</td>
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<td>56</td>
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Three days after hemorrhage and resuscitation with and without chloroquine treatment, mice were subjected to cecal ligation and puncture as described in Materials and Methods, and survival was measured over a period of 3 days. There were 16 animals in each group.

* \( P = .011 \) hemorrhage + vehicle \( vs \) sham.
* \( P = .011 \) hemorrhage + chloroquine \( vs \) hemorrhage.
tion comes from the present study, which showed that after chloroquine treatment the decrease in KC cytokine secretion in vitro correlated with decreased cytokine levels.

The present results are in agreement with our previous in vitro investigation and demonstrate that chloroquine, not only in vitro but also in vivo in normal animals, selectively blocks KC TNF, IL-6, and PGE, release without inhibiting IL-1 secretion. However, the mechanism by which chloroquine inhibits the release of TNF and IL-6 is not known. It has been reported that chloroquine interferes with the lysosomal function of macrophages and reduces the release of proteases. It is also possible that chloroquine exerts an inhibitory effect at the transcriptional or translational level of these cytokines. However, if chloroquine produces these effects through blockade of cytokine secretion itself rather than at the transcriptional and/or translational level, then in situ hybridization, as well as immunohistochemistry, may not provide any further elucidation of the exact in vivo mechanism of chloroquine’s action. Additional in vitro and in vivo studies are needed to determine the precise mechanism by which chloroquine selectively affects macrophage cytokine synthesis and release. Concerning the inhibitory effect of chloroquine on prostaglandin production seen in our in vivo experiments, there is evidence from in vitro studies that chloroquine exerts its suppressive effect on PGE, synthesis through the blockade of phospholipase A. The administration of chloroquine to hemorrhaged animals resulted in a restoration of KC ability to present antigen and express the Ia antigen. Because the improvement of KC antigen presentation and Ia expression was concomitant with an inhibition of TNF, IL-6, and PGE, in the local environment and in the blood, it can be hypothesized that these factors are associated with the suppression of KC functions after hemorrhage. However, our data do not allow us to distinguish whether any of the previous factors, singularly or collectively, are responsible for producing KC dysfunctions. Further studies using agents that inhibit single mediators in a more specific manner, such as cyclooxygenase inhibitors or MoAbs against cytokines, may answer this question.

The alterations of KC cytokine and PGE, release were not different whether the hemorrhaged animals received chloroquine before or after hemorrhage. This finding suggests that the mechanisms which are responsible for the suppression of KC antigen presentation and Ia expression do not occur during the shock state, but during and after resuscitation. This hypothesis is supported by the studies of Nakanishi et al., who showed that tissue damage did not occur during low flow conditions, but during reperfusion and reoxygenation. The fact that posttreatment with chloroquine also beneficially affected the immune responses after hemorrhage in the same manner as pretreatment suggests that chloroquine might be a useful immunomodulatory agent in patients after trauma.

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