Response of circulating immune cells to major gunshot injury, haemorrhage, and acute surgery


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Summary

Purpose: The purpose of this study was to use an established porcine model to investigate the effects on immune function of severe gunshot injury.

Methods: Twelve pigs sustained two standardised rounds, one through right femur and one through left upper abdomen. First aid treatment and acute surgery was started immediately. Blood samples were drawn before shooting and after 75 min. Circulating neutrophils were isolated and reactive oxygen species (ROS) measured. Serum levels of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-10 were determined at 0, 75 min, as well as 2 h after incubation with 1 μg/ml endotoxin in an ex vivo whole blood model.

Results: TNF-α, IL-1β, and IL-6 significantly increased at 75 min. ROS in circulating granulocytes tended to increase (NS). Incubation with endotoxin led to a more than 100-fold increase of TNF-α pre-trauma, compared to a three-fold increase post-trauma (p < 0.0001 between groups). A similar pattern was obtained for IL-1β, and IL-6. IL-10 was below detection in all samples. The granulocytes maintained their ability to react to the protein kinase C activator phorbol myristate acetate (PMA) after trauma.

Conclusion: Severe gunshot injury and peritraumatic stress rapidly activate circulating immune cells, but reduce their capacity to react to a subsequent challenge to endotoxin.

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Introduction

Multiple organ failure is one of the leading causes of secondary death after major trauma.8 Despite intensive research interest and a vastly increased insight in triggering mechanisms, no decisive breakthrough of either prophylaxis or treatment has
materialised. Major trauma with extensive destruction of tissues exposes the organism to an overwhelming inflammatory load that may easily exceed its clearing capacity. It is generally believed that a minority of multi-traumatised patients react with an immediate and exaggerated autodestructive inflammatory response so severe that sequential organ dysfunction and early demise result. A number of those who recuperate from the initial insult will later succumb due to delayed immunosuppression and increased susceptibility to infection.

Following major trauma, a considerable load of living microorganisms and bacterial products may enter the circulation. Abdominal gunshot wounds penetrating the intestines inevitably lead to extensive soiling of the peritoneal cavity. Furthermore, shocked polytraumatised victims often develop dysfunction of the gastrointestinal mucosal barrier even without direct injury, and are later equally threatened by gut-derived virulent microbes and their toxins. Indeed, bullet wounds in any location may introduce a considerable load of microorganisms into the body. A moderate activation of the immune system is mandatory in order to eliminate these foreign invaders, and a smooth course of events hangs upon a complex and finely adjusted interplay between immune cells. Many aspects governing post-injury inflammation and anti-inflammation are still incompletely understood, but release of proximal pro-inflammatory cytokines like tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) occupies a key position.

It has long been known that animals pre-exposed to a low dose of bacterial endotoxin, react to a subsequent lethal injection with reduced mortality. Diminished serum levels of TNF-α after re-exposure are a hallmark of endotoxin tolerance or hyporeactivity. Later investigations suggest cross-reactivity with other exogenous stimuli, including trauma and surgical stress. It is not clear whether this represents a beneficial adaptive response, or rather exposes the victims to an increased risk of developing sepsis and multiple organ dysfunction. Moreover, the initial injury may even represent a trigger for an enhanced response to a second hit.

In an established and standardised porcine model of gunshot injury, we wanted to study the early influence of major trauma/haemorrhage/acute surgery on the activation of the inflammatory machinery. As an extension of the in vivo investigation, lipopolysaccharide (LPS) was added to an ex vivo whole blood model to simulate post-traumatic intrusion of endotoxin into the circulation.

Methods

Animal preparation

The study was conducted as part of a course in traumatology and war surgery, arranged by the Norwegian Armed Forces and the University of Oslo, as described previously. Norwegian landrace pigs (n = 12) weighing 41.6 ± 1.6 kg were used for the study. The animals were handled according to the Animal Welfare Act and statutes from the Norwegian Ministry of Agriculture. To avoid any form of suffering they were fully anaesthetised at the start of the experiment and remained anaesthetised to the end. Medetomidin hydrochloride (CliniPharm, Switzerland) 0.06 mg/kg and tiletamin/zolazepam (Boehringer Ingelheim, Germany) 3 mg/kg intramuscularly were used for premedication, followed by butorphanol tartrate (American Home Products Corporation, USA) 0.2 mg/kg as an analgesic. After induction of anaesthesia, the pigs were orally intubated and hand ventilated with room air. An additional precaution to assure complete freedom of pain, lidocaine 2% was given epidurally in the lumbosacral region. The dose was adjusted to body length. Adequacy of analgesia was tested with forceps pressure interdigitally. An ear vein cannula was used for infusion of fluids and injection of drugs. After sterile cut-down of the left groin, the femoral artery was isolated and cannulated with a Secalon-T subclavian catheter (Viggo, Denmark) for haemodynamic monitoring and blood sampling. Anaesthesia was continued with a mixture of fluothane, oxygen, and air.

Experimental procedure

While fully anaesthetised the animals were transported to a firing range. They were exposed to a standardised trauma: one rifle shot from a distance of 25 m hitting the right thigh, and one pistol shot from short distance against the upper abdomen. The entrance points were marked in advance. To avoid immediate demise care was taken to avoid direct injury to liver, spleen or large abdominal blood vessels. After shooting, first aid treatment was immediately started, including dressing of wounds, compression of external bleeding, control of ventilation, and intravenous fluid resuscitation. The pigs were then transported to a nearby field hospital where initial life saving surgery was performed by trained surgeons. Fluid was infused as needed to compensate for blood loss and to maintain a systolic blood pressure at or above 90 mmHg. Normal saline (0.9% NaCl) and Dextran 70/NaCl were used for resuscitation. Blood transfusion or blood substitutes
were not given. The in vivo experimental period was terminated after 75 min.

To imitate an early post-traumatic flooding of the circulation with bacterial toxins, the effect of in vivo injury on a subsequent stimulation with LPS was investigated in an ex vivo whole blood model. Blood samples of 2 ml were drawn at time points 0 and 75 min and stimulated with 1 µg/ml LPS (Escherichia coli serotype 0111:B4, Sigma, St. Louis, MO, USA) or an equivalent amount of normal saline. The injuries after shooting were evaluated as uniform. All enrolled pigs survived the first 75 min.

**Measurements**

Systolic and diastolic blood pressures, heart rate, and oxygen saturation were continually monitored. Arterial blood was sampled at 0 and 75 min. Analyses done immediately on the spot included arterial blood gases, haemoglobin, and electrolytes. Blood samples for later analysis were obtained at the same time points. EDTA was used as an anti-coagulant, and the blood was cooled on ice before processing. Plasma values of the early cytokines TNF-α, IL-1β, and IL-6, as well as the anti-inflammatory cytokine IL-10, were determined at 0 and 75 min with standard kits (R&D Systems Inc., Minneapolis, MN, USA). The measurements were repeated in the whole blood model after 2 h of incubation. The results were corrected according to leukocyte concentration in the sample.

**Cell isolation and measurement of reactive oxygen species (ROS)**

As an additional indicator of post-injury activation of the immune apparatus, production of reactive oxygen species (ROS) was determined at time points 0 and 75 min in circulating neutrophils. The cells were isolated by the standard density-gradient centrifugation method and ROS measured by means of the fluorescent probe 2,7-dichloro fluorescein diacetate (DCFH-DA). DCFH-DA is freely permeable across cell membranes. Inside cells, the acetate moiety is cleaved by esterases, leaving non-fluorescent DCFH. ROS oxidizes the DCFH to DCF(2), which is fluorescent. The cells (2.5 × 10⁶ cells/ml) were incubated with DCFH-DA (5 µM) in HEPES-buffered (20 mM) Hanks’ balanced salt solution (HBSS, pH 7.4) with glucose (5 mM) at 37 °C for 15 min. Following centrifugation, the extracellular buffer with DCFH-DA was exchanged with fresh buffer and the suspension was gently mixed. Thereafter, the cells (100 µl; final concentration 1 × 10⁶ cells/ml) were transferred to 250 µl wells (microtiter plate reader, 96 wells) containing 150 µl buffer. The intracellular DCF-fluorescence was measured every second minute for 60 min at 37 °C in a spectrometer (Luminescence Spectrometer LS50B, Perkin-Elmer, UK), using excitation wavelength 488 nm and emission wavelength 525 nm. ROS was also measured after exposure of the cells to the protein kinase C activator phorbol myristate acetate (PMA 10⁻⁷ M).

**Statistical analysis**

Data are presented as mean ± S.E.M. Paired t-test was used to evaluate differences between baseline and end values. Differences between groups were evaluated with the Student’s t-test for independent samples. A p-value less than 0.05 was considered statistically significant.

**Results**

**Trauma and survival**

The injuries after shooting were evaluated as uniform. All enrolled pigs survived the first 75 min.

**Other physiological variables**

Bleeding was generally profuse. After 75 min, haemoglobin had fallen from a mean of 11.4 ± 0.5 to 7.4 ± 0.7 g/dl (Table 1). Circulating leukocytes were reduced from 23.1 ± 2.9 to 9.0 ± 1.4 × 10⁶ cells/ml. Although a significant decrease of systolic blood pressure was noted, it was mostly relatively well preserved. The fluid administered to correct hypovolaemia amounted to 2.8 ± 0.4 l of NaCl/Ringer’s acetate plus 1.5 ± 0.2 l of Dextran/NaCl.

| Table 1 Haemodynamics and blood biochemistry (mean ± S.E.M.) |
|-----------------|-----------------|-----------------|
| **Time (min)**  | **Systolic arterial pressure** (mmHg) | **Haemoglobin** (g/dl) |
| 0               | 113 ± 7         | 11.4 ± 0.5      |
| 75              | 86 ± 10*        | 7.4 ± 0.7*      |
| **White blood cells** (10⁶ cells/ml) | **S,O₂ (%)** | **pCO₂ (kPa)** |
|                 | 23.1 ± 2.9      | 90.0 ± 1.4*     |
| **S,O₂ (%)**    | 93 ± 1          | 6.8 ± 0.2       |
| **pCO₂ (kPa)**  | 97 ± 1          | 5.9 ± 0.6       |
| **pH**          | 7.44 ± 0.02     | 7.31 ± 0.04*    |
| **Base excess (BE)** | 8.2 ± 0.6 | -4.7 ± 0.9 |

* p < 0.05 compared with baseline.
Cytokine production

Trauma increased the synthesis of TNF-α from the negligible 8.1 ± 1.6 pg/ml/10⁶ cells to 34.8 ± 8.6 pg/ml/10⁶ cells (p < 0.01, Fig. 1). Addition of 1 μg/ml LPS to the control sample led to a more than 100-fold increase of TNF-α to 971.5 ± 198.6 pg/ml/10⁶ cells, compared to the more modest three-fold increase to 108.8 ± 29.4 pg/ml/10⁶ cells after prior in vivo exposure to trauma (p < 0.0001 between groups, Fig. 2). The values for IL-1β showed a nearly identical pattern, and the same was discernible also for IL-6. Trauma alone, however, was not sufficient to evoke a measurable amount of IL-6 in plasma after 75 min. IL-10 values were below detection in all samples.

Reactive oxygen species

After 75 min, the in vivo ROS synthesis was nearly unchanged compared with baseline (2329 ± 258 versus 2649 ± 435; NS; Fig. 3). The same measurements were also done after stimulation with PMA, which induced an increase to 4314 ± 640 at baseline (p < 0.05). In the granulocytes harvested at time point 75, PMA retained its stimulatory effect (4646 ± 695).

Discussion

The present study was designed so as to mimic a common clinical scenario: a major injury, followed by early first aid treatment in the field, and swift transfer to hospital for life-saving surgery. Penetrating wounds and severe haemorrhage may rapidly lead to hypovolaemia and global ischaemia. Urgent control of bleeding, and aggressive volume resuscitation, are therefore mainstays to keep up perfusion and prevent patients from entering into a vicious circle of exaggerated immune activation and sequential multiple organ dysfunction. In our study, circulation and blood pressure were mostly
satisfactorily maintained, but despite almost instantaneous treatment we could not prevent the release of ample amounts of potent proximal pro-inflammatory cytokines like TNF-α and IL-1β. Circulating levels of both substances were significantly enhanced 75 min after trauma. IL-6 concentrations were generally below detection level, possibly because peak synthesis was only reached at a later stage. The anti-inflammatory cytokine IL-10 was below detection in all samples.

At an early stage, the trauma and immediate peritraumatic events had thus created an internal environment with increased levels of circulating pro-inflammatory cytokines. Rapid activation of the innate immune response is indispensable to efficiently clear invading organisms, and circulating granulocytes are important effector cells within this system. Synthesis and release of ROS is part of their impressive cytotoxic armamentarium. ROS may therefore function as indicators of activation. By this measure, the spontaneous granulocyte activation was limited. If control values are set to 100, injury and 75 min of peritraumatic stress did not significantly increase the average level of ROS production to 116 (p = 0.49). At this stage of post-trauma, our results thus point to an internal environment not being characterised by exaggerated levels of inflammatory effector substances.

Lipopolysaccharide is another potent activator of immune competent cells. After binding to the CD14-Toll receptor 4-MD2 surface receptor complex, LPS initiates a phosphorylation cascade leading to degradation of the inhibitor molecule IκB and release of the transcription factor NFκB. When liberated from its inhibitor, NFκB translocates from cytoplasm to nucleus and binds to the promoter regions of various inflammatory genes, including the locus coding for TNF-α. The peritraumatic events obviously induced a partial blockade of this signalling pathway. Seventy-five minutes after gunshot injury, the circulating immune cells exhibited a greatly reduced capacity to respond to the subsequent challenge with endotoxin. While previously unstrained cells enhanced their synthesis of TNF-α more than 100-fold, a mere three-fold increase was observed after prior exposure to trauma, bleeding, and intraoperative stress. The same general inhibition was measured for IL-1β and IL-6.

The down-regulation of selected signalling pathways in these cells seems to be an early phenomenon. Studies have shown that various leukocytes quickly adapt to LPS, and repetitive stimulation induces a state of endotoxin tolerance or hyporesponsiveness. However, the nature and mechanism of endotoxin hyporeactivity are still elusive. Cross-tolerance apparently exists with a host of exogenous substances and stimuli. A similar deactivation has even been described following polytrauma. Our results are in agreement with these studies and further underline the unspecific nature of the phenomenon. The results also suggest an almost globally reduced capacity of whole blood cells to react to LPS stimulation with increased cytokine production. That goes for TNF-α, IL-1β, and IL-6. From the literature, production of TNF-α is invariably reported to be reduced, and actually serves as a measure of endotoxin tolerance. Synthesis of IL-6 is likewise generally inhibited, while IL-1β may move in either direction. Differences in the primary triggering event may be partly responsible for subtle variations in the secondary responses. The anti-inflammatory cytokine IL-10 has been suggested to contribute to the hyporeactivity to LPS. We did not find detectable amounts of circulating IL-10 at any time point, but local synthesis and release are not always reflected in the circulation, a fact that may be even more pronounced when combined with severe haemorrhage. Furthermore, the concept of compartmentalisation may imply opposing states of inflammation when comparing measurements in locally inflamed tissues and plasma values.

LPS is capable of initiating an inflammatory cascade that may eventually lead to multiple organ failure and death. Granulocytes and granulocyte-derived effector substances play a vital part in this development. In granulocytes stimulated by trauma, an insignificant increase of ROS was detected compared to the control situation. Interestingly, these cells retained their ability to react to PMA even after trauma. ROS synthesis doubled after stimulation with PMA 10^{-7} M, and the intensity was independent of previous triggering events. PMA is a direct activator of protein kinase C and thus circumvents interaction with membrane receptors, which have been implicated in LPS hyporesponsiveness, e.g. in mice lacking Toll receptor 4. This may suggest a role for Toll receptors in endotoxin tolerance. The effects of PMA are in line with earlier reports, and may point to interference of cellular signalling at a level proximal to protein kinase C. In addition, differences apparently depend on the initial triggering event.

It has not been established whether the trauma-induced hyporesponsiveness to bacterial products represents a protective mechanism or, on the contrary, may pave the way for serious infections. When unabated, host-mediated tissue damage may become life-threatening. On the other hand, if too slow and feeble, bacterial invasion and replication may overwhelm the organism. Following penetrating colon injuries infectious complications are...
common. Although originally thought to be a protective mechanism, recent investigations may suggest that lower levels of whole blood LPS-stimulated cytokine release may lead to poorer clinical outcome.

Conclusions

The study demonstrates that trauma, together with a customary immediate treatment regimen, cause the release of key pro-inflammatory mediators from circulating immune cells at an early stage. However, despite almost immediate treatment, the peritraumatic stresses rapidly induce a secondary hyporesponsiveness to subsequent endotoxin challenge. As severe gunshot wounds inevitably lead to primary bacterial contamination, this combination may pave the way for infectious complications. Direct activation of protein kinase C seemed to circumvent the inhibiting effects of trauma on immune competent cells, suggesting that the hyporesponsiveness may be counteracted by pharmacological means.

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References