Anesthesiology 2005; 102:768–73

Endotoxemia-induced Lymphocyte Apoptosis Is Augmented by a Hyperinsulinemic–Euglycemic Clamp

Jeppe Sylvest Nielsen, M.B., Anders Larsson, D.M.Sc.,† Vibeke Brix-Christensen, M.D., Ph.D.,‡ Jens Randel Nyengaard, D.M.Sc.,§ Thomas Ledet, D.M.Sc.,‖ Else Tønnesen, D.M.Sc.#*

Background: **Sepsis and endotoxemia are associated with lymphocyte apoptosis. This has been regarded as harmful, contributing to further immune suppression in already immune-compromised patients. Because normalization of blood glucose improves outcome in critically ill patients, the authors hypothesized that one of the effects of insulin and normoglycemia would be inhibition of lymphocyte apoptosis. Therefore, in this experimental study in pigs, the authors examined the separate and combined effects of acute endotoxemia and a hyperinsulinemic–euglycemic clamp (HEC) on lymphocyte apoptosis.**

Methods: **After 60 min of stabilization, 38 anesthetized and mechanically ventilated pigs (weight, 35–40 kg) were divided (by randomization performed before the experiment) into four groups and were then studied for 570 min. Group 1 received no intervention. Group 2 received a HEC (5 mM p-glucose, insulin infusion rate of 0.6 mU · kg^{-1} · min^{-1}) for 570 min. Group 3 received a lipopolysaccharide infusion for 180 min. Group 4 was given a combination of a HEC and a lipopolysaccharide infusion. After the 570-min study period, the pigs were killed, and tissue was sampled from the spleen and frozen. In four sections of each sample, the apoptosis of B and T lymphocytes were analyzed using stereologic methods: The number of apoptotic B and T cells was estimated by fluorescence immunohistochemistry with anti–active caspase-3 and either anti-CD21 (B lymphocytes) or anti-CD3ε (T lymphocytes). The number of apoptotic B and T lymphocytes was then compared using two-way analysis of variance, and the interaction between endotoxemia and the clamp (hyperinsulinemia and euglycemia) was investigated.**

Results: **Endotoxemia induced apoptosis of B ($P < 0.001$) and T lymphocytes ($P = 0.016$) in the spleen, and this effect was independent of the clamp. The ratios of apoptotic cells in the spleen tissue of pigs with and without endotoxemia were 2.4 (confidence interval, 1.7–3.4) and 1.6 (confidence interval, 1.1–2.2) for B and T lymphocytes, respectively. Independent of endotoxin infusion, HEC increased the number of apoptotic lymphocytes ($P = 0.029$ and $P = 0.038$ for B and T lymphocytes, respectively). The ratios of the number of apoptotic spleen cells in pigs treated and not treated with HEC were 1.5 (confidence interval, 1.0–2.1) and 1.5 (confidence interval, 1.0–2.1) for B and T lymphocytes, respectively.**

Conclusion: **In this porcine model, both endotoxemia and a HEC increased the number of apoptotic B and T lymphocytes in the spleen. Contrary to our hypothesis, lymphocyte apoptosis during acute endotoxemia was augmented by a HEC.**

INCREASED lymphocyte apoptosis has been proposed to be an important cause of immunodepression and mortality in critical illness.[1] An increased number of apoptotic lymphocytes has been observed in experimental endotoxemia and was later also found in autopsy studies of septic patients.[2–4] A reversal of lymphocyte apoptosis with caspase-3 inhibitors showed increased survival among septic mice.[5] Furthermore, apoptotic cells have shown excessive immune-modulating mechanisms. Fadok *et al.*[6] revealed that macrophages digesting apoptotic cells inhibited the production of proinflammatory cytokines, and Sauter *et al.*[7] demonstrated that dendritic cells consuming apoptotic cells did not exhibit costimulatory factors and were unable to activate T cells.

We have previously found that a hyperinsulinemic–euglycemic clamp (HEC) attenuates plasma concentrations of tumor necrosis factor α, glucagon, and free fatty acids in an acute endotoxemic porcine model.[8] It has also been shown that the use of insulin for normalization of blood glucose concentrations decreases mortality and morbidity in critically ill patients.[9] Although it has been suggested that the metabolic control, as reflected by normoglycemia, rather than the infused insulin dose *per se*, was related to the beneficial effects of intensive insulin therapy,[10,11] we hypothesized that the positive effects of a HEC could in part be ascribed to an insulin-induced inhibition of lymphocyte apoptosis. We tried to elicit this question by studying the effects of a HEC on splenic lymphocyte apoptosis in a porcine model of acute endotoxemia.

Materials and Methods

The model has been presented previously.[8] The National Committee on Animal Research Ethics (Copenhagen, Denmark) approved the protocol, and the work was conducted according to the guidelines in the *Guide for the Care and Use of Laboratory Animals*.[12]

Thirty-eight female Landrace pigs (weight, 35–40 kg) were fasted overnight but were allowed free access to water. They were premedicated with 10 mg/kg ketamine and 0.25 mg/kg midazolam intramuscularly. Further ketamine (10 mg/kg) was given intravenously before intubation and mechanical ventilation. Anesthesia was maintained with a continuous intravenous infusion of 60 μg · kg^{-1} · h^{-1} fentanyl and 6 mg · kg^{-1} · h^{-1}

* Department of Anesthesiology and Intensive Care, Clinical Institute, and Research Laboratory for Biochemical Pathology, ‡ Department of Anesthesiology and Intensive Care and Clinical Institute, # Professor, Department of Anesthesiology and Intensive Care and Clinical Institute, § Associate Professor, Stereological Research and Electron Microscopy Laboratory, ‖ Professor, Research Laboratory for Biochemical Pathology, Aarhus University Hospital. † Professor, Clinical Institute, Aarhus University Hospital, and Aalborg University Hospital, Aalborg, Denmark.

Received from the Research Laboratory for Biochemical Pathology and the Clinical Institute, Aarhus University Hospital, Aarhus, Denmark. Submitted for publication July 20, 2004. Accepted for publication November 24, 2004. Supported by the Danish Research Council, grant Nos. 22-2-0010 and 22-02-0174, Aarhus University Research Foundation, Aarhus, Denmark; The A. P. Møller Foundation for the Advancement of Medical Science, Copenhagen, Denmark; and Police Inspector J.P.N. Colind and Wife Asmine Colinds Memorial Foundation, Aarhus, Denmark.

Address reprint requests to Dr. Nielsen: Department of Anesthesiology and Intensive Care Medicine, Aarhus University Hospital, Nørrebrogade 44, DK-8000 Århus C, Denmark. Address electronic mail to: sylvest@ki.au.dk.

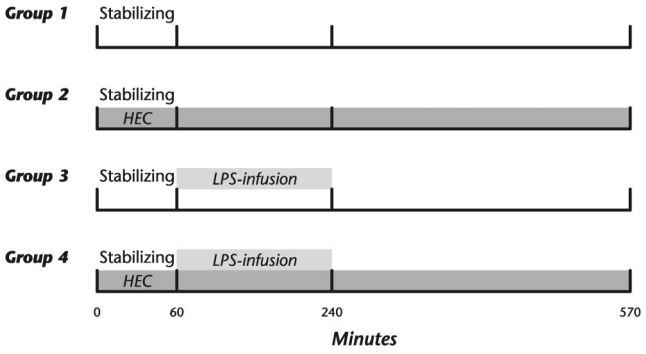

Fig. 1. Illustration of the four groups and their individual treatments. HEC = hyperinsulinemic–euglycemic clamp; LPS = lipopolysaccharide.

midazolam. Neuromuscular blocking drugs were not used. Blood samples were collected from the arterial catheter every 30 min and more frequently in relation to lipopolysaccharide infusion for analysis of blood gases and acid–base status.

Hemodynamic Monitoring

Continuous observations were performed of arterial blood pressure, heart rate (from the electrocardiogram), and pulmonary artery pressure. Pulmonary capillary wedge pressure was measured intermittently. Cardiac output was monitored using an Edwards Vigilance Monitor (Edwards Lifescience Corp., Irvine, CA).

Experimental Design

Thirty-eight pigs were allocated into four groups (fig. 1). The animals in group 1 (anesthesia, n = 10) were subjected to general anesthesia solely for 570 min. In group 2 (HEC, n = 9), the animals were in addition exposed to a HEC for 570 min. In group 3 (lipopolysaccharide, n = 10), the animals were stabilized for 1 h before they were given a 180-min infusion of lipopolysaccharide. Finally, the animals in group 4 (lipopolysaccharide–HEC, n = 9) were subjected to a combination of HEC and lipopolysaccharide infusion. During the 570-min study period, the animals in group 1 (anesthesia) and group 2 (HEC) received 10 ml · kg^{-1} · h^{-1} isotonic saline, whereas animals in the two other groups received 20 ml · kg^{-1} · h^{-1} to maintain adequate blood pressure during and after lipopolysaccharide infusion. At study termination, the animals were killed by evisceration of the heart preceded by a fentanyl bolus of 1 mg.

Lipopolysaccharide Infusion *Escherichia coli*. Lipopolysaccharide endotoxin, (*Escherichia coli* 026:β6, Bacto Lipopolysaccharides; Difco Laboratories, Detroit, MI) was dissolved in saline 120 min before each experiment to dissolve any precipitate. After a stabilization period, lipopolysaccharide infusion was started at baseline at a rate of 2.5 μg · kg^{-1} · h^{-1}, and it was increased stepwise to 15 μg · kg^{-1} · min^{-1} during 30 min. After this, the fusion was kept at a rate of 2.5 μg · kg^{-1} · h^{-1} during 150 min and was thereafter discontinued.

Hyperinsulinemic–Euglycemic Clamp. Insulin (Insulin Actrapid; Novo-Nordisk, Copenhagen, Denmark) was infused intravenously at a constant rate of 0.6 mU · kg^{-1} · min^{-1} for 570 min. Plasma glucose was clamped at 5 mM/l by infusion of 20% glucose.

Measurement Sequence and Laboratory Analyses

Blood for determination of insulin was drawn every half hour. Plasma glucose concentrations were measured every 5–10 min. They were determined in duplicate immediately after sampling (Beckman Instruments, Pale Alto, CA). Serum insulin concentrations were measured in duplicate by a two-site immunospecific insulin enzyme-linked immunosorbent assay (DAKO Diagnostics, Cambridgeshire, United Kingdom).

Preparation of the Sections. Immediately after evisceration of the heart, tissue samples were taken from the spleen (0.5 × 0.5 × 0.5 cm). These were covered in Tissue-Tek® (Sakura Finetek, Europe, B.V., The Netherlands), transferred first to liquid nitrogen (−180°C) and then to dry ice (−80°C), after which they were stored in a freezer at −80°C.

Four arbitrary sections (two for each of the B- and T-lymphocyte investigations) were sliced 5 μm thick by a freeze microtome. The sections were washed in phosphate buffered saline (PBS), fixed in acetone (−20°C for 5 min), and air-dried for 20 min. Hereafter, they were stored in a −80°C freezer. Sections were thawed in pairs in a 37°C incubator for 10 min, after which they were rehydrated in PBS. Endogen peroxidases were blocked with a 3% hydrogen peroxide solution dissolved in methanol. The frozen sections were again blocked with a PBS-BB (1% bovine serum albumin, 0.3 Triton × 100, 0.2% powdered milk) for 30 min. Incubation with first primary antibody anti-CD21 (purified mouse anti-human monoclonal anti-CD21 immunoglobulin [Ig] G1, κ-R; BD Pharmingen, Broenby, Denmark; 0.5 μg/ml) or anti-CD3ε (rat anti-human CD3; Serotec, Kidlington, Oxford, United Kingdom; 10 μg/ml) was performed for 60 min in a moisturizing chamber. Antibodies to human CD21 was chosen because of previous workshop findings.[13] Human CD3ε antibody was used because of empirical experience with higher morphologic and fluorescence quality. The sections were washed in PBS and incubated with the first secondary antibody (goat anti-rat IgG-rhodamin 4 μg/ml or goat anti-mouse IgG-rhodamine, 2.3 μg/ml; both Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min in a moisturizing chamber. Hereafter, the sections were incubated with the second primary antibody for 60 min (anti–active caspase-3; R&D Systems, Abingdon, Oxon, United Kingdom; 2.5 μg/ml). They were washed in PBS and incubated with the second secondary antibody for 60 min (swine anti-rabbit IgG-horseradish peroxidase; DAKO A/S, Glostrup, Denmark; 2.8 μg/ml). The frozen sections were again washed in PBS before incubation with tyramide signal amplification

(NEN Life Science, PerkinElmer, Boston, MA) for 10 min. The sections were rewashed in PBS and rinsed in distilled water. They were mounted with Vectorshield (Vector Laboratories, Inc., Burlingame, CA) and sealed with nail polish. They were kept cool overnight (4°C), and fluorescence microscopy was performed the following day.

Fluorescence Microscopy and Stereology

The stereologic analyses were preformed using an Olympus BX 50 microscope (Olympus, Copenhagen, Denmark). Pictures were taken with a 3-CCD (charged-coupled device) video camera (KY-F55; JVC, Copenhagen, Denmark). Fluorescence light was delivered by a reflected light fluorescence attachment (BX-FLA; Olympus) and powered by a U-RFL-T power supply unit (Olympus). Using the software CAST 2.0 (Visiopharm, Hoersholm, Denmark), a motorized section stage (Marzhauser, Wetzlar-Steindorf, Germany) was controlled, and counting grids were superimposed onto the digital images. All stereologic examinations were preformed by an examiner blinded to the treatment.

B Lymphocytes. The number of double positive B lymphocytes was counted using CAST 2.0. (Visiopharm). The screen was divided into a certain amount of tests points with a given area per point, a(p). All germinal centers (GCs) found in the section were examined. Each GC was examined by a shift in the fluorescence light looking for both fluorescin isothiocyanate (showing active caspase-3) and rhodamin (CD3ϵ or CD21) with a 100× objective. The total amount of points, P(GC), hitting the GC and the number of double positive cells, Q(B-pos), were noted. The number of double positive B lymphocytes per GC area, Q_A(B-pos/GC), could therefore be estimated:

$$Q_A(\text{B-pos/GC}) = \frac{\sum Q(\text{B-pos})}{a(p) \cdot \sum P(GC)}. \quad (1)$$

T Lymphocytes. The total number of double positive T lymphocytes in the counting frame (Q(T-pos)) was counted by a systematic uniformly random sampling process. Using the motorized section stage, the computer was programmed to a certain step-distance in x and y coordinates, thereby visualizing approximately 120 two-dimensional unbiased counting frames per pig. Each counting frame was examined with a shift in the fluorescence light, as done with the B lymphocytes. Furthermore, the number of counting frames corners, (c), hitting the spleen was noted. Each corner was calculated to correspond to one fourth of the area of the entire frame, a(c). The total amount of double positive cells per spleen area, Q_A(T-pos/spleen), was estimated:

$$Q_A(\text{T-pos/spleen}) = \frac{\sum Q(\text{T-pos})}{a(c) \cdot \sum c(\text{spleen})}. \quad (2)$$

An average from the two sections was calculated, and the amount of double positive cells per volume, N_v, was estimated[14]:

$$N_v(\text{B-pos/GC}) = \frac{Q_A(\text{B-pos/GC})}{\bar{d} + t}, \quad (3)$$

$$N_v(\text{T-pos/spleen}) = \frac{Q_A(\text{T-pos/spleen})}{\bar{d} + t}, \quad (4)$$

where $\bar{d}$ is the average diameter of approximately 50 counted positive profiles, and t is the thickness of the section. N_v is thereby a parameter estimating the number of apoptotic B lymphocytes per μm^3 GC and the number of apoptotic T lymphocytes per μm^3 spleen.

Statistical Analysis

Two-way analysis of variance (ANOVA) was used to analyze data for interaction between HEC and endotoxemia and was further used to compare the effect of HEC and endotoxemia on the group's number of apoptotic lymphocytes per μm^3 GC (N_v(B-pos/GC)) or spleen (N_v(T-pos/spleen)). Probability values less than 0.05 were considered as significant. After assessment of probability plots and ln(mean)-ln(SD) plots, all data were ln-transformed. All statistical analyses were made using STATA 8.0 (StataCorp, College Station, TX).

Results

Plasma glucose and insulin concentrations in the four groups are shown in figures 2A and B.

B Lymphocytes

The number of apoptotic B lymphocytes in the GC of the spleen tissue is shown in figure 3. Both endotoxemia and the HEC significantly increased the number of apoptotic B lymphocytes, but there was no significant interaction of the ln-transformed data between the two factors (two-way ANOVA, $P = 0.55$).

Independent of the clamp, endotoxemia significantly increased the number of apoptotic B lymphocytes per μm^3 GC (two-way ANOVA, $P < 0.001$). The ratio between the number of apoptotic B lymphocytes/μm^3 GC in the spleen tissue of the endotoxin-treated and non–endotoxin-treated pigs was 2.4 (confidence interval, 1.7–3.4).

Independent of endotoxemia, HEC significantly increased the number of apoptotic B lymphocytes (two-way ANOVA, $P = 0.029$). The ratio between the number of apoptotic B lymphocytes/μm^3 GC in the spleen tissue of clamped and nonclamped pigs was 1.5 (confidence interval, 1.0–2.1)

T Lymphocytes

The number of apoptotic T lymphocytes in spleen tissue is shown in figure 4. Both endotoxemia and the

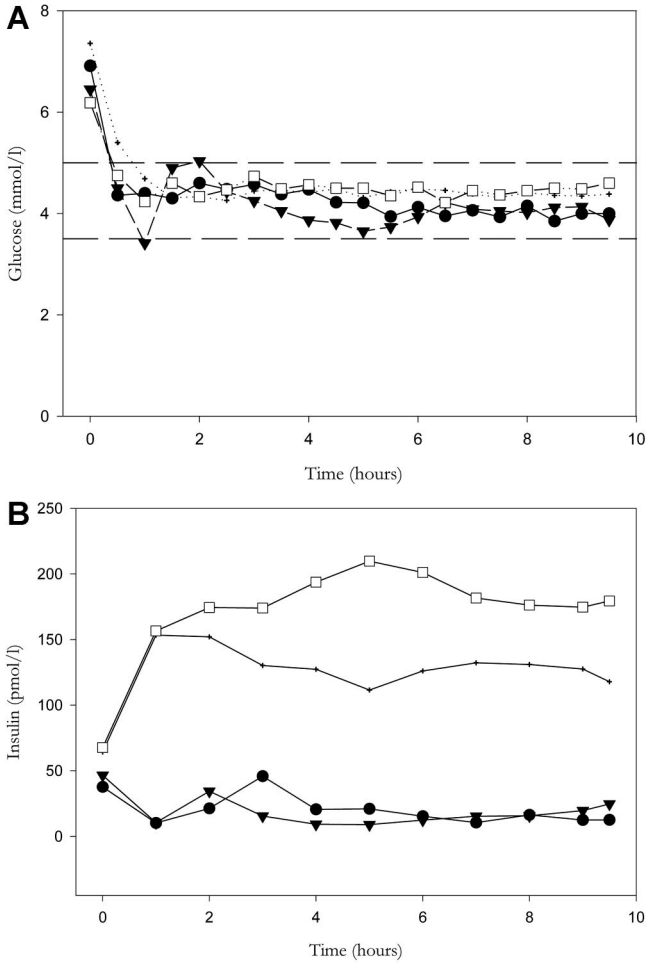

Fig. 2. Glucose concentrations (*A*) and insulin concentrations (*B*) in the four groups. ● = group 1 (anesthesia); + = group 2 (hyperinsulinemic–euglycemic clamp); ▼ = group 3 (endotoxemia); □ = group 4 (hyperinsulinemic–euglycemic clamp + endotoxemia). The two *dotted lines* indicate blood glucose concentrations at 3.5 and 5.0 mM.

HEC significantly increased the number of apoptotic T lymphocytes, but there was no significant interaction of the ln-transformed data between the two factors (two-way ANOVA, $P = 0.29$).

Independent of the clamp, endotoxemia increased the number of apoptotic T lymphocytes per μm^3 spleen (two-way ANOVA, $P = 0.016$). The ratio between the number of apoptotic T lymphocytes/μm^3 spleen tissue in endotoxin-treated and non–endotoxin-treated animals was 1.6 (confidence interval, 1.1–2.2).

Independently of endotoxemia, HEC also increased the number of apoptotic T lymphocytes (two-way ANOVA, $P = 0.038$). The ratio between the number of apoptotic T lymphocytes/μm^3 spleen in the clamped and non-clamped pigs' spleens was 1.5 (confidence interval, 1.0–2.1).

Nine tissue samples from the B-lymphocyte investigation and 13 from the T-lymphocyte investigation had to be discarded. Seven tissue samples were discarded because of human errors, whereas initially 3 from the B-lymphocyte investigation and 8 from the T-lymphocyte investigation were regarded as unusable because of tissue damage. These 3 and 8 samples were recut, restained, and recounted. Two samples from the B-lymphocyte investigation and 6 samples from the T-lymphocyte investigation still had to be discarded because of bad quality.

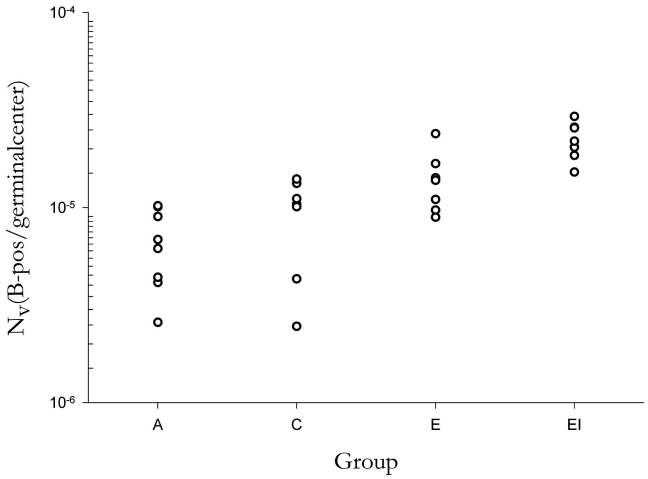

Fig. 3. Graph of N_v(B-pos/GC) showing the number of apoptotic B lymphocytes/μm^3 germinal center on log^{10} scale. The x-axis shows the four groups: A (anesthesia), C (hyperinsulinemic–euglycemic clamp), E (endotoxin), and EI (hyperinsulinemic–euglycemic clamp + endotoxin).

Discussion

This study showed that acute endotoxemia in pigs induced apoptosis of splenic B and T lymphocytes. This observation is in accord with previous porcine studies of endotoxemia showing an increased number of apoptotic

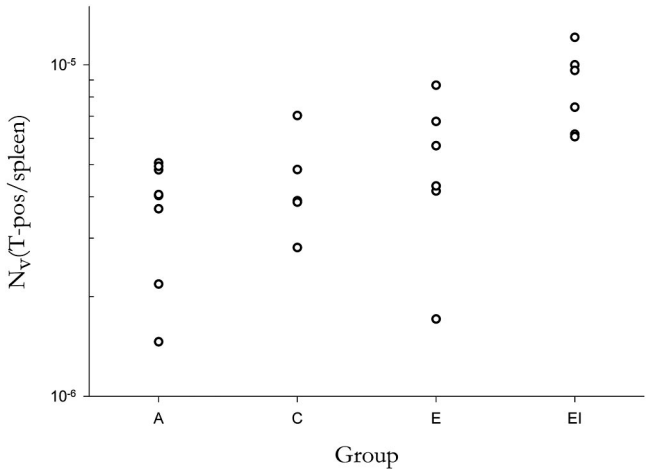

Fig. 4. Graph of N_v(T-pos/spleen) showing the number of apoptotic T lymphocytes/μm^3 spleen tissue on log^{10} scale. The x-axis shows the four groups: A (anesthesia), C (hyperinsulinemic–euglycemic clamp), E (endotoxin), and EI (hyperinsulinemic–euglycemic clamp + endotoxin).

spleen B memory cells[3] and unspecified lymphocytes.[2] The other major finding was that the HEC independently of endotoxemia increased the number of apoptotic lymphocytes in spleen tissue. Furthermore, the HEC augmented lymphocyte apoptosis during acute endotoxemia. This was in contrast to our hypothesis and, to our knowledge, has not been reported previously.

The incentive for our project was first the study by Van den Berghe *et al.*[9] showing that tight glucose control by insulin infusion improved outcome in critically ill patients. Second, it was hypothesized by Hotchkiss and Karl[1] that apoptotic cells may trigger sepsis-induced anergy and thereby contribute to the severe immunosuppression observed during critical illness. Hotchkiss and Karl based this hypothesis on the fact that large numbers of lymphocytes and gastrointestinal epithelial cells become apoptotic during sepsis in critically ill patients[4,15,16] and that inhibition of apoptosis decreases mortality rates of septic mice.[5,17] The results of this study could not support our hypothesis, *i.e.*, that some of the beneficial effects of insulin could be mediated by decreased lymphocyte apoptosis. In contrast, we found that insulin therapy, in the form of HEC, was associated with increased apoptosis of splenic lymphocytes. This effect of insulin was unexpected because it has been regarded as an antiapoptotic molecule in relation to other cell types.[18–20]

However, the question remains, why do insulin treatment and metabolic control decrease morbidity and mortality in critical illness when HEC exhibited proapoptotic features in our porcine study and apoptosis is associated with increased mortality rates in other animal experiments?

First, results from animal studies are only indicative and not conclusive regarding pathophysiology and the effects of interventions in humans. Second, our model is an endotoxemia model and does not accurately reflect all aspects of critical illness or sepsis. Our model is especially different from classic sepsis in respect to time, where we focus on the acute response contrary to long-term critically ill sepsis patients. Third, based on a multivariate logistic regression analysis, Van den Berghe *et al.*[11] concluded that blood glucose control rather than the absolute amounts of infused insulin was related to the beneficial effect of intensive insulin therapy. In accordance with this finding, Finney *et al.*[10] showed in a prospective, observational study that increased insulin administration was positively associated with death in the intensive care unit regardless of the prevailing blood glucose concentration. Therefore, in critical illness, it is not clear whether insulin *per se* contributes to the improved outcome. Finally, we have previously found in the same model of endotoxemia that HEC inhibited a tumor necrosis factor-α response in plasma, indicating an antiinflammatory effect. This down-regulated proinflammatory response by insulin has been demonstrated in other human and animal studies. Because apoptosis has known antiinflammatory mechanisms, this is in accord with the increased lymphocyte apoptosis found in the current study.

We have previously demonstrated that a HEC decreased the glucagon and free fatty acid response to acute endotoxemia.[8] This correlates with the conclusions of Weekers *et al.*,[21] who suggest that the prophylactic treatment with insulin to achieve normoglycemia prevents excessive inflammation, catabolism, acidosis, and impaired innate immune function in critically ill patients.

Consequently, insulin could be considered an antiinflammatory molecule with, dependent on the underlying condition, both beneficial and harmful effects. In addition, the positive effect of insulin by normalizing blood glucose and decreasing glucagon and free fatty acid concentrations may in some conditions override the possible negative effects of antiinflammation. In addition, because an antiinflammatory effect might be considered positive in the early phase of sepsis, timing might be pivotal for the end result of the inflammatory modulation by insulin and normoglycemia in this condition.

This study has several limitations. (1) The HEC model ensures high insulin concentrations and normoglycemia. This method is not equivalent to the clinical intensive insulin treatment, but it has several advantages: It maintains high insulin availability and fixates blood glucose concentrations while avoiding both hyperglycemia and hypoglycemia. Because increased glucose availability has not, to our knowledge, been shown as proapoptotic, the HEC should not interfere with our results. (2) The study relies on estimation of the number of double positive cells per volume as outcome. If the volume of the spleen is altered in the different groups, the data could be incorrect. However, if the spleen changes volume during acute endotoxemia, it would probably increase because of inflammation. That would produce less cells per volume spleen, and our results become even more significant. On the other hand, the spleen might shrink when releasing erythrocytes as a compensatory mechanism to increase a possible reduced blood volume induced by endotoxemia. However, the animals were adequately fluid resuscitated and did not show any major signs of hypovolemia. (3) Cells changing in shape or size could influence the results. We assumed that the cell size was the same in all groups, but cells undergoing apoptosis experience a phenomenon called *blebbing*. Blebbing is a process in which the internal structures of the cell are externalized with intact cell membrane. These vesicles may contain active caspase-3, which can be colored and counted as multiple apoptotic cells. N_v is calculated from the average diameter of 50 positive cells. If there is a significant difference in the average size of the cells between the four groups, the estimates may be incorrect. However, we later studied approximately 100 cells per group and found no significant difference in cell

profile diameter among the four groups. The use of a disector-sampling technique[22] could have eliminated the bias concerning cell size and shape. (4) Because it is hard to explain why a HEC itself should increase the number of apoptotic lymphocytes, it is intriguing whether a larger number of test animals would disclose a significant interaction between HEC and endotoxin, thereby indicating that a HEC had an effect only during endotoxemia and not on the healthy anesthetized pigs. Finally, it was not always possible to analyze the section because of bad quality. In total, 34% of the samples from the T-lymphocyte examinations and 24% of the samples from the B-lymphocyte examinations had to be discarded. Nevertheless, we had sufficient samples to perform an adequate analysis.

In conclusion, this experimental porcine study showed that both endotoxemia and a HEC increased the number of apoptotic B and T lymphocytes in spleen tissue. Further we showed that a HEC augmented lymphocyte apoptosis during acute endotoxemia. The reasons and consequences of these results remain unclear, and further studies are needed to resolve these issues.

The biostatistical skills of Niels Trolle, Ph.D. (Department of Biostatistics, Aarhus University, Aarhus, Denmark), have been indispensable. The skilled technical assistance of the technicians Lene Vestergaard (Department of Anesthesiology and Intensive Care, Aarhus University Hospital), Anette Mengel (Clinical Institute, Aarhus University Hospital), and Henrik Sørensen (Clinical Institute, Aarhus University Hospital) is also gratefully acknowledged.

References

1. Hotchkiss RS, Karl IE: The pathophysiology and treatment of sepsis. N Engl J Med 2003; 348:138–50

2. Norimatsu M, Ono T, Aoki A, Ohishi K, Takahashi T, Watanabe G, Taya K, Sasamoto S, Tamura Y: Lipopolysaccharide-induced apoptosis in swine lymphocytes in vivo. Infect Immunol 1995; 63:1122–6

3. Yokochi T, Kato Y, Sugiyama T, Koide N, Morikawa A, Jiang GZ, Kawai M, Yoshida T, Fukada M, Takahashi K: Lipopolysaccharide induces apoptotic cell death of B memory cells and regulates B cell memory in antigen-nonspecific manner. FEMS Immunol Med Microbiol 1996; 15:1–8

4. Hotchkiss RS, Tinsley KW, Swanson PE, Schmieg RE Jr, Hui JJ, Chang KC, Osborne DF, Freeman BD, Cobb JP, Buchman TG, Karl IE: Sepsis-induced apoptosis causes progressive profound depletion of B and CD4+ T lymphocytes in humans. J Immunol 2001; 166:6952–63

5. Hotchkiss RS, Chang KC, Swanson PE, Tinsley KW, Hui JJ, Klender P, Xanthoudakis S, Roy S, Black C, Grimm E, Aspiotis R, Han Y, Nicholson DW, Karl IE: Caspase inhibitors improve survival in sepsis: A critical role of the lymphocyte. Nat Immunol 2000; 1:496–501

6. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM: Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest 1998; 101:890–8

7. Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N: Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med 2000; 191:423–34

8. Christensen V, Andersen S, Andersen R, Orskov H, Mengel A, Larsson A, Andersen N, Schmitz O, Tonnesen EK: Acute hyperinsulinemia restrains endotoxin-induced systemic inflammatory response: An experimental study in a porcine model. ANESTHESIOLOGY 2004; 100:861–70

9. van den Berghe G, Wouters P, Weekers F, Verwaest C, Bruyninckx F, Schetz M, Vlasselaers D, Ferdinande P, Lauwers P, Bouillon R: Intensive insulin therapy in the critically ill patients. N Engl J Med 2001; 345:1359–67

10. Finney SJ, Zekveld C, Elia A, Evans TW: Glucose control and mortality in critically ill patients. JAMA 2003; 290:2041–7

11. Van den Berghe G, Wouters PJ, Bouillon R, Weekers F, Verwaest C, Schetz M, Vlasselaers D, Ferdinande P, Lauwers P: Outcome benefit of intensive insulin therapy in the critically ill: Insulin dose versus glycemic control. Crit Care Med 2003; 31:359–66

12. Institute of Laboratory Animal Resources: Guide for the Care and Use of Laboratory Animals, 7th edition. Washington, DC, National Academy Press, 1996

13. Boersma WJ, Zwart RJ, Sinkora J, Rehakova Z, Haverson K, Bianchi AT: Summary of workshop findings for porcine B-cell markers. Vet Immunol Immunopathol 2001; 80:63–78

14. Cruz-Orive, LM. Distribution-free estimation of sphere size distribution from slabs showing overprojection and truncation, with review of previous methods. J Microsc 1983; 145:265–290

15. Hotchkiss RS, Tinsley KW, Swanson PE, Grayson MH, Osborne DF, Wagner TH, Cobb JP, Coopersmith C, Karl IE: Depletion of dendritic cells, but not macrophages, in patients with sepsis. J Immunol 2002; 168:2493–500

16. Hotchkiss RS, Swanson PE, Freeman BD, Tinsley KW, Cobb JP, Matuschak GM, Buchman TG, Karl IE: Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. Crit Care Med 1999; 27:1230–51

17. Hotchkiss RS, Tinsley KW, Swanson PE, Chang KC, Cobb JP, Buchman TG, Korsmeyer SJ, Karl IE: Prevention of lymphocyte cell death in sepsis improves survival in mice. Proc Natl Acad Sci U S A 1999; 96:14541–6

18. Iida KT, Suzuki H, Sone H, Shimano H, Toyoshima H, Yatoh S, Asano T, Okuda Y, Yamada N: Insulin inhibits apoptosis of macrophage cell line, THP-1 cells, via phosphatidylinositol-3-kinase-dependent pathway. Arterioscler Thromb Vasc Biol 2002; 22:380–6

19. Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, Ma XL: Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: The roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. Circulation 2002; 105:1497–502

20. Goetze S, Blaschke F, Stawowy P, Bruemmer D, Spencer C, Graf K, Grafe M, Law RE, Fleck E: TNF-alpha inhibits insulin's antiapoptotic signaling in vascular smooth muscle cells. Biochem Biophys Res Commun 2001; 287:662–70

21. Weekers F, Giulietti AP, Michalaki M, Coopmans W, Van Herck E, Mathieu C, Van den Berghe G: Metabolic, endocrine, and immune effects of stress hyperglycemia in a rabbit model of prolonged critical illness. Endocrinology 2003; 144:5329–38

22. Sterio DC: The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 1984; 134:127–36