Old Herborn University Seminar Monograph 5:
Effective and ineffective defence mechanisms in the gastrointestinal tract.
Editors: Hans Cottier, Peter J. Heidt, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 94-105 (1994).

NATURAL AND ARTIFICIAL LIPOPROTEINS MODULATE LIPOPOLYSACCHARIDE-INDUCED TUMOUR NECROSIS FACTOR PRODUCTION *IN VITRO* AND *IN VIVO*

JAN EVA DORAN, PETER LERCH, and ALPHONSE P. HUBSCH

Central Laboratory, Swiss Red Cross Blood Transfusion Service, Bern, Switzerland

SUMMARY

The role of serum and serum proteins in lipopolysaccharide (LPS) binding and detoxification has been studied extensively during the course of the last decade. It is now well established that lipoproteins are able to react with and modify LPS functional activity. We have prepared and tested a spectrum of natural as well as artificial lipoproteins for their ability to modulate LPS-induced tumour necrosis factor (TNF) production *in vitro*. Our experiments show that the natural lipoproteins had a pronounced influence on TNF production, decreasing it by as much as 33%. However, these effects were easily surpassed through the use of artificially constructed lipoproteins. Lipoproteins prepared by the admixture of apolipoprotein A-I (the major protein component of high density lipoprotein) and phosphatidylcholine (designated ApoLipo) were far more efficacious in reducing TNF production *in vitro* than were the natural lipoproteins (TNF reductions >85% were achieved). The supplemental addition of triglycerides or cholesterol to the artificial lipoprotein mixture further diminished TNF production in a dose dependent manner. ApoLipo's influence on TNF production *in vitro* did not require precomplexation with LPS, and was observable even after LPS stimulation had begun.

We have also tested ApoLipo (apoA-I: Lipid at a 1:200 molar ratio) in an *in vivo* rabbit endotoxin shock model. ApoLipo dose dependently reduced LPS-induced TNF production, and attenuated the metabolic and cellular changes associated with endotoxin shock.

INTRODUCTION

The experimental infusion of purified bacterial lipopolysaccharide (LPS) into animals in sufficient doses results in the development of pathophysiological changes, collectively known as endotoxin shock. Manifestations of endotoxin shock include fever, hypotension, alterations in cellular adhesiveness and activation leading to leukopenia and thrombocytopenia as well as metabolic disturbances (e.g., acidosis) and disseminated intravascular coagulation (DIC). If sufficient in magnitude and duration, these pathophysiological changes may lead to multiple organ failure and ultimately to death. In small doses, the injection of LPS stimulates immune and phagocytic system function, resulting in an increased resistance to subsequent challenge.

Although LPS can interact directly with host systems, it mediates most of

its toxic and also its beneficial effects by stimulating the release of mediators, principally tumour necrosis factor (TNF or cachectin) from host cells. The kinetics of LPS induced TNF in plasma are virtually identical between species: Plasma TNF levels peak at 90 - 120 minutes post infusion of LPS, returning to baseline by 4 to 6 h (*Mathison* et al., 1988; *Beutler* et al., 1985; *Hinshaw* et al., 1990; *Feuerstein* et al., 1990; *Michie* et al., 1988). The evidence that TNF is one of the major mediators in the pathogenesis of endotoxin shock comes from two lines of investigation. The first is that the infusion of recombinant human TNF into research animals produces many of the same sequelae as seen in endotoxin shock (*Tracey* et al., 1986; *Lehmann* et al., 1987; *Natanson* et al., 1979; *Remick* et al., 1987). Second, monoclonal antibodies against TNF are protective in model systems of endotoxin shock and gram negative bacterial sepsis (*Beutler* et al., 1985a; *Tracey* et al., 1987; *Mathison* et al., 1988; *Sanchez-Cantu*, 1989; *Hinshaw* et al., 1990; *Silva* et al., 1990). This is not to say that TNF is the only mediator in endotoxin shock, but it clearly plays a central role in the pathogenesis of endotoxin and septic shock.

Methods of reducing or neutralising LPS activity *in vivo* have long been sought. When LPS is exposed to plasma or serum, a number of its biological activities are lost or diminished (*Rall* et al., 1957; *Ulevitch* and *Johnston*, 1978; *Ulevitch* et al., 1979; *Freudenberg* et al., 1980). LPS has been shown to interact with a multitude of plasma proteins [reviewed in: *Doran*, 1991); however, it is only the interactions of LPS's with lipoproteins that will be discussed in this report. Their role in LPS binding and detoxification has been extensively studied in the last decade. *Ulevitch* et al. (1979, 1981), *Mathison* and *Ulevitch* (1979), *Freudenberg* et al. (1980), *Munford* et al. (1981) and their respective co-workers showed that LPS forms complexes predominantly with high density lipoprotein (HDL) in normal animals. Other lipoprotein fractions including low density lipoprotein (LDL), very low density lipoproteins (VLDL) and chylomicrons have all been examined for their interactions with LPS (*Ulevitch* et al, 1979; *van Lenten* et al., 1986; *Harris* et. al., 1990). The binding of LPS by HDL or other lipoproteins has been shown to modify the biological activity of LPS *in vitro* and *in vivo*. Lipoprotein binding decreases LPS induced Interleukin 1 (IL-1) (*Warren* et al., 1988; *Flegel* et al., 1989; *Cavaillon* et al., 1990), Interleukin 6 (IL-6), and TNF production by mononuclear cells (*Cavaillon*, 1990). LPS-lipoprotein complexes are also less reactive *in vivo*: pyrogenicity, leukopenia and thrombocytopenia induced by HDL-LPS complexes is reduced in comparison to LPS alone (*Ulevitch* and *Tobias*, 1988). Although a protective capacity of lipoproteins has been demonstrated *in vitro* and *in vivo*, benefits are usually only seen when the LPS has been precomplexed with lipoproteins *ex vivo* (*Harris* et al., 1990). The extent and significance of lipoprotein-mediated LPS detoxification in the natural setting *in vivo* is not known.

We have investigated the ability of an artificial lipoprotein designated ApoLipo to modulate cytokine production *in vitro*. We further investigated if the prophylactic infusion of ApoLipo prevents TNF production *in vivo* and the pathophysiological manifestations of endotoxin shock in a rabbit endotoxaemia model. One critical difference between these studies and those found in the literature is that the *in vitro* and *in vivo* studies were performed without prior complexation of our artificial lipoprotein with LPS.

MATERIALS AND METHODS

Lipoproteins and apoA-I

Chylomicrons and the lipoprotein fractions VLDL, LDL, and HDL were prepared from normal human plasma by ultracentrifugation (*Schumaker* and *Puppione*, 1986). Apolipoprotein A-I (apoA-I), the major protein component of HDL was purified from human HDL by delipidation and size exclusion chromatography or was purified from precipitates obtained by cold ethanol fractionation of human plasma (*Lerch* et al., 1989). ApoLipo was prepared by a method similar to that of *Chen* and *Albers* (1982). Briefly, apoA-I was mixed with phosphatidylcholine (PC) (Molar ratio of apoA-I to PC was 1:200) and sodium cholate, followed by extensive dialysis against buffer (1 mM $NaHCO_3$, 10% Saccharose). Additional artificial lipoproteins were prepared in which other components (triglycerides and/or cholesterol were added to the apoA-I:PC mixture prior to dialysis (see results section for ratios used). Total protein (*Markwell* et al., 1978), phospholipid (*Chen* et al., 1956), triglyceride (Merkotest Triglycerides #14354), and cholesterol content (Boehringer Mannheim Monotest Cholesterin # 290319) of each of the natural lipoprotein preparations were also measured. The apoA-I and ApoLipo preparations used in these studies were shown to be sterile and nonpyrogenic.

Tumour Necrosis Factor Production and Assay

The whole blood assay introduced by *Desch* and colleagues (1989) was adapted for use in these studies. Whole heparinised blood or ACD blood from single donors (200 μl) was incubated with LPS (25 μl of 100 μg LPS/ml) in the presence of various concentrations of apoA-I, ApoLipo or other synthetic lipoproteins, natural lipoprotein fractions, saline, or other controls (25 μl) at 37°C. After 6 h, 750 μl of pyrogen free saline was added to the reaction mixture; TNF containing supernatants were harvested by centrifugation and tested for immunoreactive TNF (Medigenix, Belgium).

Bioassayable TNF was measured in samples of rabbit serum by the L929 cytotoxicity assay (*Ruff* and *Gifford*,1981). Care was taken to avoid any microbial contamination of the serum samples. It was shown that the addition of ApoLipo to standard concentrations of recombinant human TNF does not influence the L929-cytotoxicity assay. Recombinant mouse TNF (Genzyme, Boston, MA, USA) was used as a standard.

LPS Preparations

A mixture of 13 smooth strain LPS (all Difco: including *E. coli*, *Salmonella*, *Serratia*, *Klebsiella*, and *Pseudomonas* variants) was used as the stimulant in all of the *in vitro* assays. *E. coli* O111:B5 (a generous gift of Prof. B. Urbaschek) was used throughout the *in vivo* rabbit studies.

Animals

In these preliminary studies, outbred rabbits of both sexes with an average weight of 3.2 ± 0.4 kg (mean ± SD) were used. These studies were approved by the Animal Protection Committee of the Canton of Bern, Bern, Switzerland. In brief, arterial and venous catheters were implanted in 4 groups of anaesthetised rabbits for measurements of physiologic parameters, blood sampling and infusions. All animals remained under anaesthesia during the entire course of the experiment. Animals received either a prophylactic infusion of ApoLipo [250 mg/kg (n=3) or 75 mg/kg (n=6)] or control protein

[Physiogel 250 mg/kg, n=4], followed by a 6 h continuous infusion of *E. coli* LPS (4.17 μg/kg/h). These groups are designated as ApoLipo250-LPS ApoLipo75-LPS, and Control-LPS respectively. The fourth group [ApoLipo75-Control (n=4)] represents the non-endotoxin treated control. Here ApoLipo was administered at a dose of 75 mg/kg, the endotoxin infusion was replaced with pyrogen free saline.

Blood samples were withdrawn at specified intervals for the measurement of bioreactive TNF, blood gas and haematological analyses (leukocyte and platelet count, haematocrit, and haemoglobin determinations), simultaneous with the recording of physiologic parameters (e.g., temperature, heart rate, blood pressure, respiratory parameters). Only the results from the TNF and blood gas analyses, and from the leukocyte and platelet determinations will be presented in this report.

Data Presentation

The *in vitro* data presented in Figures 1 - 6 are from single experiments but are representative of multiple experiments.

Spearman rank correlation analysis was also performed. The *in vivo* results presented are as mean ± standard error of the mean (SEM), unless otherwise specified. Where appropriate, comparison with baseline values were made by Student's t test for paired samples; comparisons between groups was made by Student's t test for unpaired samples. TNF levels were compared with the Mann-Whitney U test. In all instances, probabilities less than 0.05 are considered significant.

RESULTS

In Vitro Studies

Preliminary studies were performed which showed that our adaptation of the whole human blood assay for TNF was both LPS dose and time dependent (*Doran* et al., 1991). In the absence of exogenously added LPS, immunoreactive TNF levels were routinely less than 50 pg/ml. Following LPS stimulation, TNF production was shown to be time dependent, demonstrating an almost linear increase over the 6 h test period (*Doran* et al., 1991). TNF levels at the end of 6 h rose to levels between 2.5 and 12.5 ng/ml depending on individual donor, and LPS concentration used for stimulation (*Doran* et al. 1991).

Table I provides the total protein, triglyceride, phospholipid, and cholesterol contents of plasma, dialysed

Table 1: Protein, triglyceride, phospholipid and cholesterol content of plasma, dialyzed plasma, lipoprotein free plasma (LFP), HDL, LDL, VLDL, and chylomicrons

	Protein g/l	Triglyceride mM	Phospholipid mM	Cholesterol mM
Plasma	78.5	1.4	6.2	5.4
Dialysed Plasma	69.3	1.0	4.7	4.1
LFP	124.5	0.0	0.8	0.2
HDL	12.1	1.3	8.0	9.4
LDL	5.3	1.6	8.2	24.9
VLDL	1.0	4.8	2.1	4.8
Chylomicrons	70.0	1.1	2.5	4.1

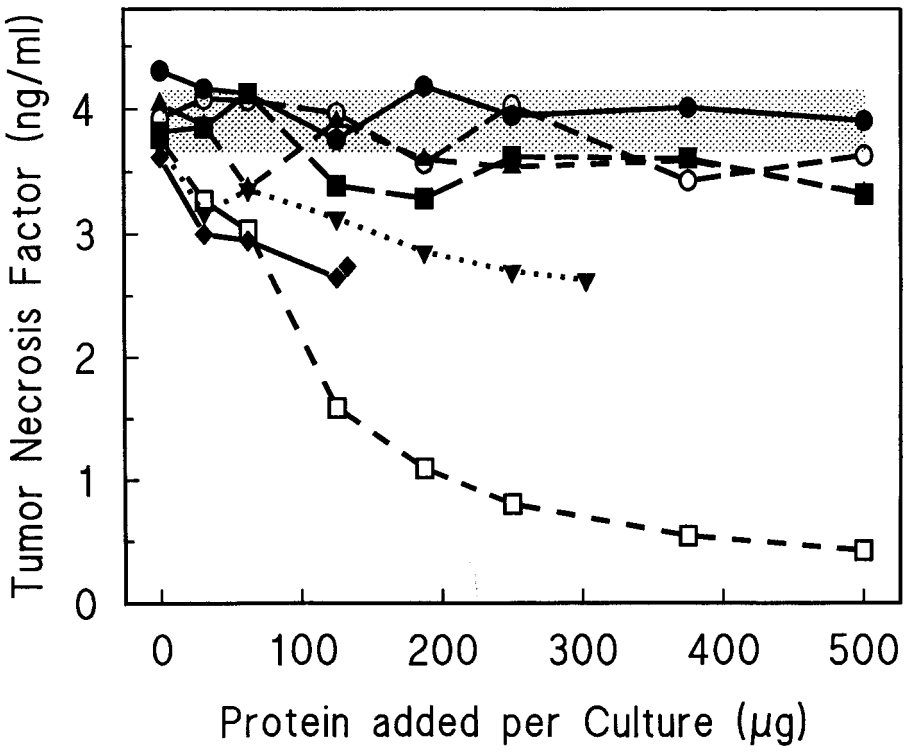

Figure 1: Inhibition of TNF production by natural and artificial lipoproteins. Whole blood cultures were incubated concomitantly with LPS and plasma (J), dialysed plasma (B) lipoprotein free plasma (LPF plasma; E), natural lipoproteins [HDL (P) , LDL (F)], chylomicrons (H), or ApoLipo [apoA-I:PC molar ratio 1:200; (G)] for 6 h at 37°C. The supernatants were tested for TNF activity. The hatched bar represents the mean ± SD of TNF produced by whole blood cultures in the presence of saline and LPS (n=8).

plasma, lipoprotein free plasma, and purified HDL, LDL and VLDL and chylomicron fractions. These preparations as well as our artificial lipoprotein ApoLipo (apoA-I:PC at a 1:200 molar ratio) were tested for their ability to modulate LPS induced TNF production. All preparations were added to the culture concomitantly with LPS and incubated for 6 h at 37°C. In Figure 1, TNF measured in the culture supernatants is plotted versus the total protein added per culture for each of the preparations tested. As seen in this figure, plasma, dialysed plasma, chylomicrons and lipoprotein free plasma had virtually no inhibitory effect on LPS stimulated TNF production, whereas LDL, HDL, and ApoLipo each demonstrated inhibitory effects. Because of the low protein content of the VLDL, it could only be tested at one protein concentration, and was not included in Figure 1. TNF production in the presence of LPS and 24 µg VLDL protein was 3 ng/ml. Clearly, phospholipid rich (e.g., ApoLipo), cholesterol rich (LDL, HDL), or triglyceride rich preparations (VLDL) all can decrease LPS-induced TNF production. Using data from all of these preparations, correlation analysis showed a significant negative association ($r = -0.899$, $p < 0.001$, $n = 32$) between TNF levels and lipid content.

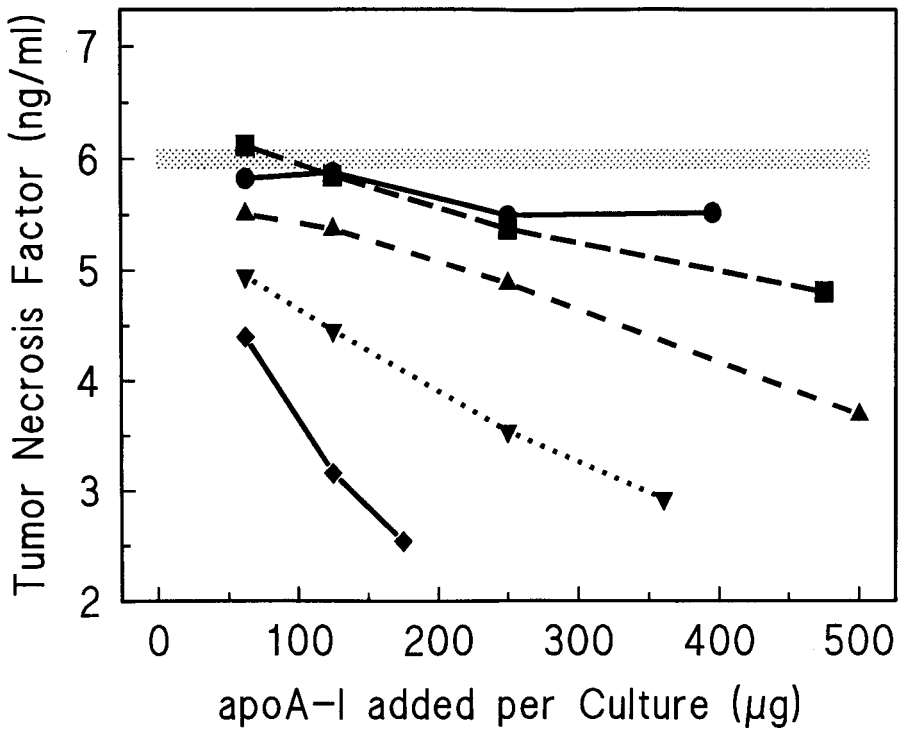

Figure 2: Influence of apoA-I:lipid ratio on TNF production. Artificial lipoproteins were prepared at apoA-I to phosphatidyl choline ratios of 1:20 (J), 1:50 (B), 1:100 (H), 1:200 (P) and 1:250 (F). Each of these preparations were then tested in the whole blood assay at different protein concentrations in the presence of LPS. TNF was measured in the supernatants collected at 6 h. The hatched band represents mean ± SD TNF production (n=3) in the presence of saline and LPS.

When the data from the ApoLipo preparation were removed from the analysis, negative correlations between TNF and cholesterol and triglyceride content of the preparations could also be discerned ($r = -0.54$, $p < 0.01$, and $r = -0.71$, $p < 0.001$ respectively). Clearly, triglycerides and cholesterol are not required for TNF inhibition, as ApoLipo is inhibitory in their absence, however they may still contribute to the inhibitory activity of the natural lipoproteins.

In order to more clearly show the importance and influence of lipids, cholesterol and triglycerides in this model system, artificial lipoprotein combinations with various compositions were prepared. The influence of lipid on TNF production and release is shown in Figure 2. A spectrum of apoA-I:PC ratios between 1:20 and 1:250 were tested for TNF inhibiting activity in the whole blood assay. These results clearly demonstrate a greater TNF inhibiting effect in preparations having more lipid. Supernatant TNF is further diminished by the addition of either triglycerides or cholesterol to the apoA-I: PC mixtures (shown in Figures 3 and 4 respectively). Analogous to the situation found with natural lipoproteins, the more triglyceride (in this case trimyristin) that is present (Figure 3), or the more cholesterol that is present (Figure 4), the greater the TNF inhibition. The influence of triglycerides is not triglyceride specific, as similar dose response curves were obtained when

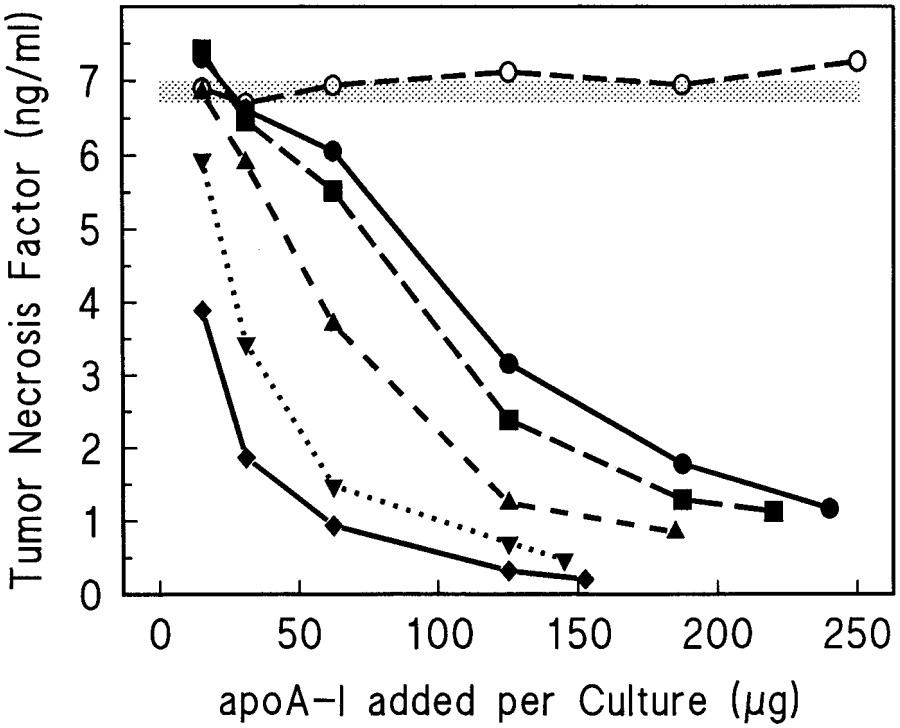

Figure 3: Effect of apoA-I:PC:triglyceride preparations on TNF production. Artificial lipoproteins at apoA-I : PC : trimyristin ratios of 1:100:0 (J), 1:100:10 (B), 1:100:20 (H), 1:100:50 (P) and 1:100:100 (F) were compared with apoA-I alone (1:0:0) (E) for TNF production inhibition.

triolein was used. It should be noted that none of these preparations required precomplexation with LPS to show TNF modulating effects.

The importance of lipid in these interactions must also be carefully considered. The evidence thus far presented points to a non-specific lipid-LPS interaction as the cause of the TNF inhibition. However, tests performed with apoA-I-PC mixtures prepared in the absence of cholate (Figure 5) or by mechanical means (Microfluidiser; data not shown) show that these preparations have markedly reduced TNF inhibiting activity. It is apparent from these studies that the configuration of the components involved may be critical for their biological activity.

We also wished to determine the temporal requirements for ApoLipo to mediate a beneficial effect. Using the whole blood assay system, TNF production inhibition was still evident when ApoLipo was given up to 1 h after LPS exposure (Figure 6). The inhibition is ApoLipo dose dependent (maximal inhibition shown with 500 μg added per culture). ApoLipo is able to diminish cellular TNF production/release even after stimulation has occurred. Data from additional studies suggest that ApoLipo may also have a direct effect on cells. Cells pulsed with ApoLipo and then washed, are refractory to LPS stimulation as assessed by TNF production (data not shown). This effect is clearly not attributable to early LPS tolerance.

In Vivo Studies

The ApoLipo preparation was also

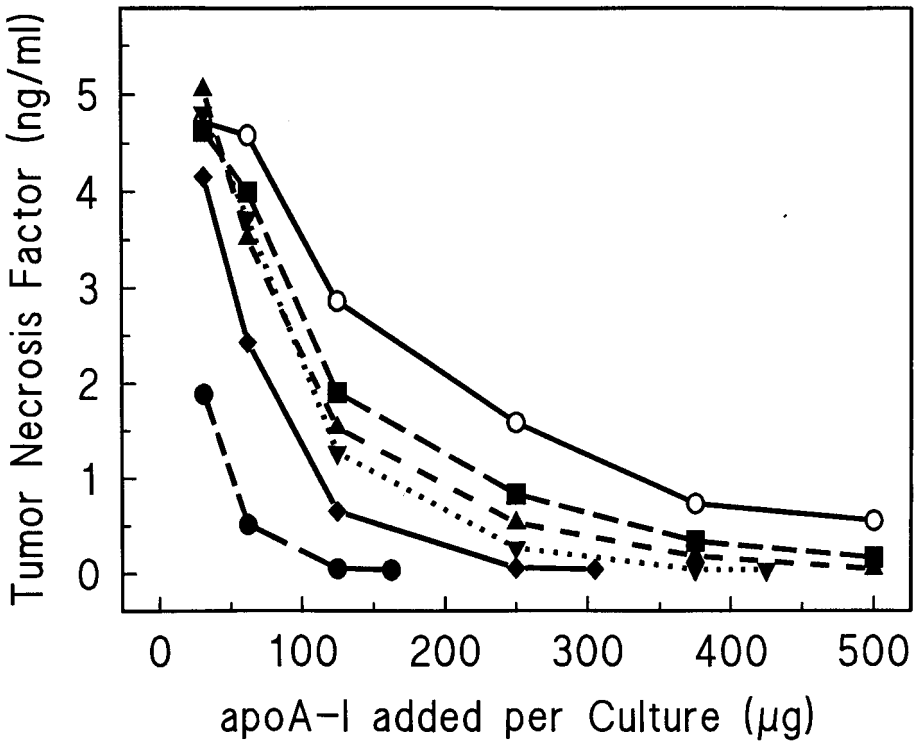

Figure 4: Effect of apoA-I:PC:cholesterol preparations on TNF production. Artificial lipoproteins at apoA-I:PC:Cholesterol ratios of 1:200:0 (E), 1:200:2 (B), 1:200:5 (H), 1:200:10 (P), 1:200:15 (F), and 1:200:20 (J) were tested for their ability to reduce TNF production by whole blood cultures in the presence of LPS.

used prophylactically in an *in vivo* rabbit endotoxaemia model system (described in detail by *Hubsch* et al., 1991). Control-LPS rabbits exhibit many but not all of the manifestations of endotoxin shock within the 6 h LPS infusion period. The Control-LPS group developed a marked leukopenia within 15 minutes of the beginning of LPS infusion (76% baseline). After 1 h of LPS infusion, leukocyte count continued to decrease (43% baseline), reaching its nadir (24% baseline) after 4 h of LPS infusion. Plasma TNF levels rose significantly above baseline levels after 1 h of LPS infusion, reaching their maximum (29.4 ng/ml) at 2 h. Metabolic acidosis as assessed by arterial base excess was clear-cut and statistically significant at 2 h (-5.3 ± 0.8), worsening as LPS continued to be infused. Thrombocytopenia began to appear at 30 minutes of LPS infusion (92% baseline), and progressed steadily during the period of LPS infusion.

Doses of ApoLipo approximating that used in the *in vitro* studies (75 mg/kg body weight) or above (250 mg/kg) significantly attenuated TNF production *in vivo*, and many of the manifestations of shock. At 2 h, TNF levels in LPS treated controls were 29.4 ± 6.5 ng/ml (n=4), as compared to 1.2 ± 0.2 ng/ml (n=6) in ApoLipo75-LPS treated animals and 0.7 ± 0.3 in ApoLipo250-LPS treated animals (n=3). Metabolic acidosis was also significantly attenuated by ApoLipo. At

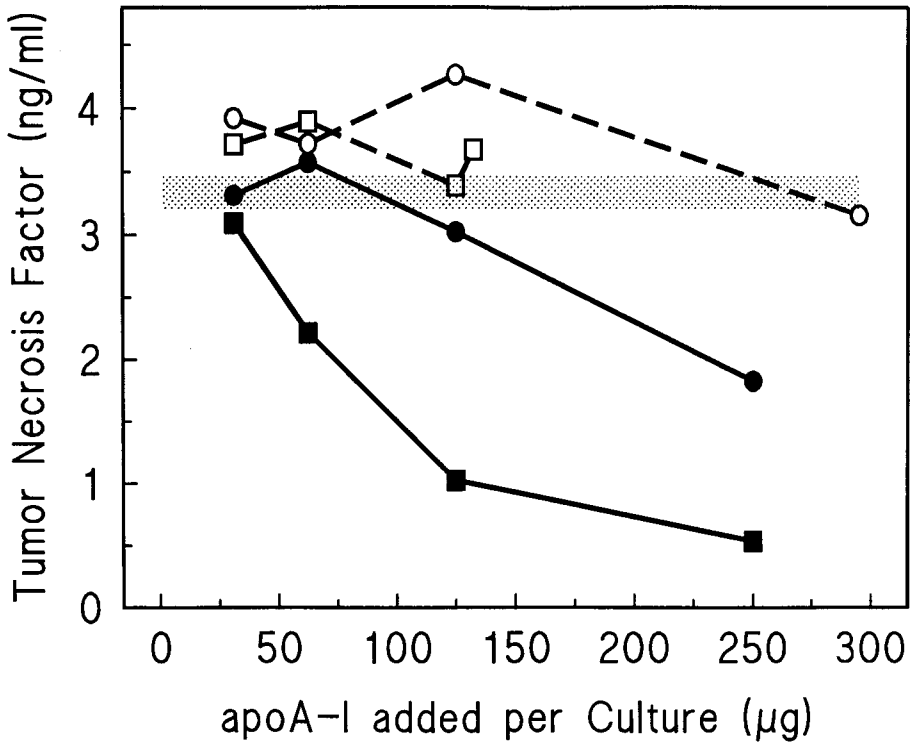

Figure 5: Importance of lipoprotein configuration for activity. ApoA-I:PC mixtures with ratios of 1:100 (squares) and 1:200 (circles) were prepared in the presence (solid symbols, solid line) and absence (open symbols, dotted line) of cholate. These preparations were incubated concomitantly with LPS in the whole blood assay. Supernatants were collected at 6 h and TNF determined. The hatched bar represents the mean ± SD (n=3) of TNF produced in the presence of saline and LPS.

5 h, the base excess levels reached their nadir (-9.0 ± 2.1) in LPS treated controls, whereas nadir levels were -4.1 ± 0.6 and -2.8 ± 0.2 in ApoLipo75-LPS and ApoLipo250-LPS treated animals respectively (also at 5 h). These base excesses correspond to arterial blood pH values of 7.22 ± 0.05 for LPS control animals and 7.33 ± 0.01 and 7.28 ± 0.01 for ApoLipo75 and ApoLipo250 groups respectively. The complete base excess and TNF data for the Control-LPS, ApoLipo75-LPS and ApoLipo250-LPS groups are shown in Figure 7. TNF values in the ApoLipo75-Control and ApoLipo250-LPS groups never statistically exceeded their background controls. Base excess for the ApoLipo75-Control group remained above -2.2 throughout the experimental course. The ApoLipo250-LPS group maintained their metabolic acidosis between that of the ApoLipo75-Control group and that of ApoLipo75-LPS. The cellular aberrations associated with endotoxin shock were also significantly attenuated (platelet decrease; Figure 8) or delayed (leukocyte drop; Figure 9) by ApoLipo infusion. It is only with the high doses of ApoLipo that these cellular changes associated with endotoxin shock are clearly attenuated.

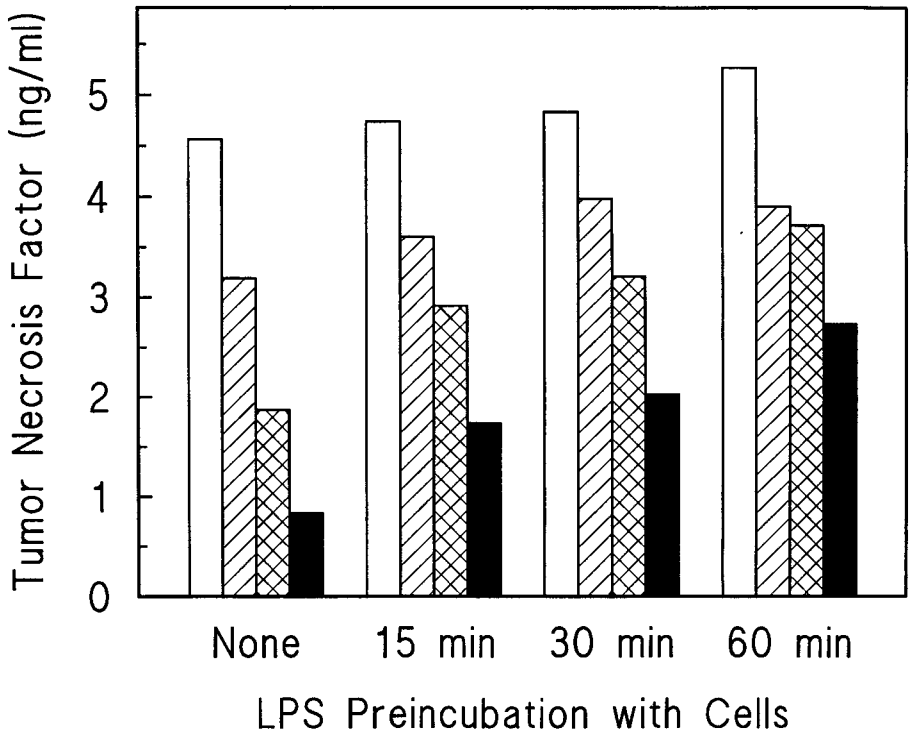

Figure 6: Influence of ApoLipo on TNF production after LPS Stimulation. Whole blood cultures were preincubated with LPS for 0, 15, 30, or 60 minutes, before ApoLipo or saline was added to the cultures. The effects of saline (open) and ApoLipo at three different protein concentrations (125, 250, and 500 μg; hatched, crosshatched and solid, respectively) on TNF production were assessed.

DISCUSSION

It is well established that the binding of LPS by HDL results in altered/diminished LPS functional activity. However, LPS's functional activities are not always reduced to the same extent. *Ulevitch* and *Tobias* (1988) reported that Re595 LPS complexation with HDL reduces LPS induced complement activation and leukopenia 1000 fold; pyrogenicity and thrombocytopenia were reduced to a lesser extent (100 and 50 fold respectively). In their hands, the development of shock and DIC in rabbits was diminished, but mitogenicity of murine B cells was virtually unaffected by LPS's complexing with HDL. It is quite likely that lipoprotein binding to LPS effects the systemic manifestations of LPS through the regulation or modulation of cytokine production. Lipoprotein binding to LPS reduces LPS induced IL-1 (*Warren* et al., 1988; *Flegel* et al., 1989; *Cavaillon* et al., 1990), IL-6, and TNF production by mononuclear cells (*Cavaillon* et al., 1990; Figure 1).

Other lipoprotein fractions have also been examined for LPS binding and modulating activities, including LDL, VLDL and chylomicrons (*Ulevitch* et al., 1979; *van Lenten*, 1986; *Harris* et al., 1990). In contrast to the earlier reports that LPS bound only to HDL, *van Lenten* et al. (1986) reported that all

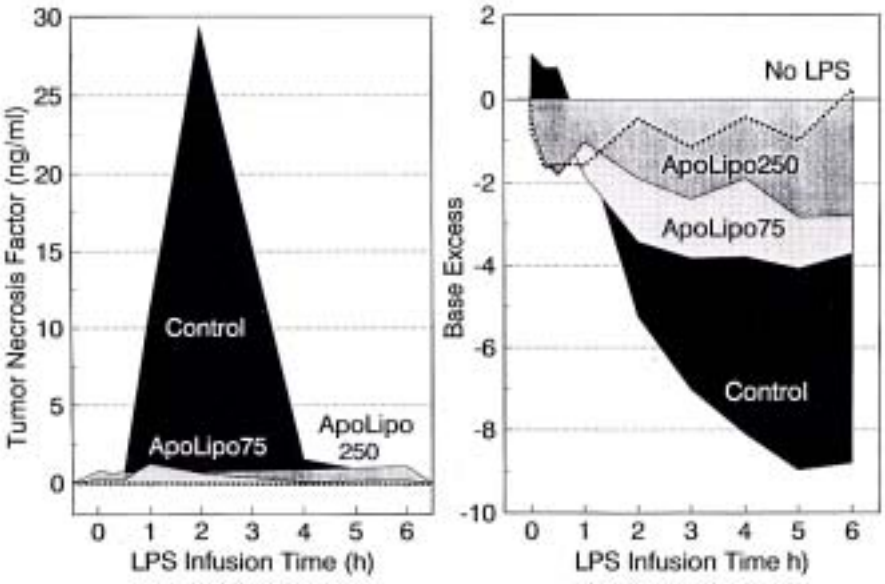

Figure 7: Influence of ApoLipo on rabbit TNF production *in vivo* and metabolic acidosis (Base Excess) in a rabbit endotoxin shock model. Bioreactive TNF and base excess are displayed for the 4 rabbit groups. The control-LPS group is shown in black, ApoLipo75-LPS as dotted, and ApoLipo250-LPS group as grey. The ApoLipo75-Contol is shown as a dotted line. At 1 and 2 h of LPS infusion, TNF in the control-LPS treated group was significantly increased over the ApoLipo treated groups ($p<0.05$). Statistically significant ($p<0.05$) differences in Base Excess between control-LPS and one or both ApoLipo-LPS treated groups were demonstrated at 4, 5, and 6 h.

lipoprotein classes were able to bind LPS; binding was in direct proportion to their cholesterol concentration. Binding was also shown to lead to endotoxin detoxification. Using an *ex vivo* incubation system, *Harris* et al. (1990) demonstrated that cholesterol rich LDL and HDL, triglyceride rich VLDL and chylomicrons, and a commercial lipid emulsion (Soyacal) were all able to protect mice against endotoxin induced lethality, albeit at vastly different concentrations. Interestingly, protection required small amounts of lipoprotein free plasma, and was dependent on the incubation time and lipoprotein lipid concentration. These data would support our observations on the importance of lipid for ApoLipo functionality (Figure 2). Although *Harris* and colleagues (1990) demonstrated a protective capacity of all tested lipoprotein fractions, the systems they used required a 6 h pre-incubation of LPS with lipoproteins *ex vivo* for efficacy to be demonstrated. Judging by the requirement for precomplexation in these systems, one might hypothesise that the natural lipoprotein system may only be able to detoxify small amounts of LPS (e.g. that naturally crossing the intestine). The significance and extent of lipoprotein mediated LPS detoxification in bacteraemia and sepsis remains unknown.

In previous studies (*Doran* et al., 1991), we have demonstrated that ApoA-I, the major protein component of HDL does not appear, by itself, to neutralise or substantially modulate endotoxin activity. However, in association with lipids (as ApoLipo), it clearly

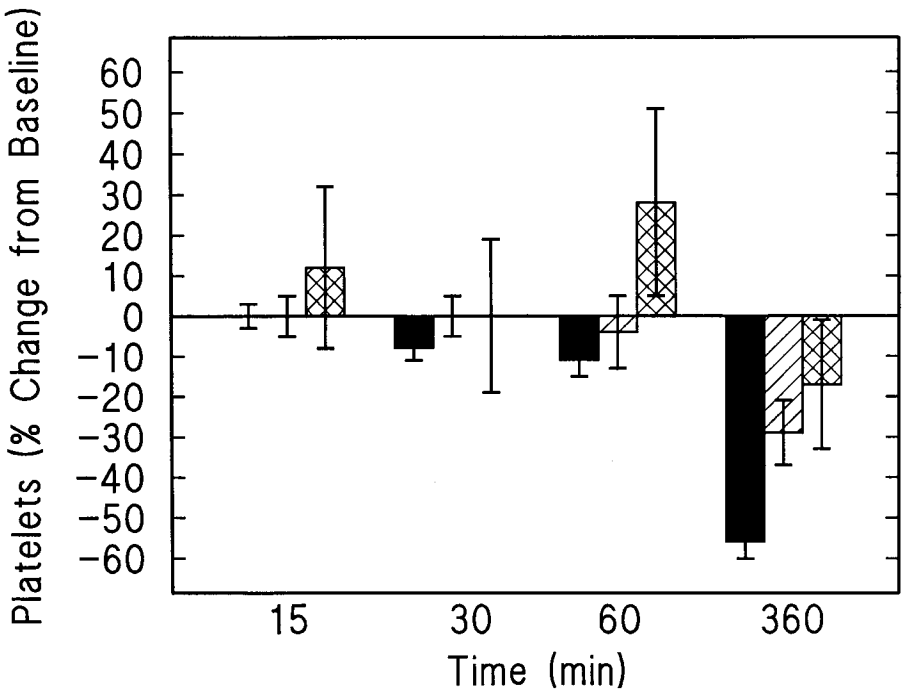

Figure 8: Change in platelet count with LPS administration. The percent change of platelet count from baseline for each of the LPS treated groups are shown. Control-LPS, ApoLipo75-LPS, and ApoLipo250-LPS are shown as solid, hatched and crosshatched bars respectively. The platelet decrease in the Control-LPS group is significantly different ($p<0.05$) from that of the ApoLipo75-LPS and ApoLipo250-LPS groups only at 4h (data not shown) and at 6 h (last set of bars) .

diminishes TNF production by monocytic cells in response to LPS exposure. The *in vitro* studies presented in this report have focused on TNF, but other cytokines (IL-1, IL-6) are similarly diminished (data not shown). In contradistinction to what is reported in the literature for the natural lipoproteins, precomplexation with LPS is not required for ApoLipo to modulate LPS's biolological activity. The influence of lipids, triglycerides, and cholesterol are clearly shown in Figures 2-4. Caution, however, must be exercised in the interpretation of these results. High concentrations of artificial lipoproteins at Apo:PC ratios above 1:200 caused demonstrable erythrocyte lysis. Lysis was increased by the addition of triglycerides or cholesterol, and was demonstrable at lower lipoprotein concentrations as the cholesterol or triglyceride concentrations increased. The role which cholate plays in the formation of efficacious preparations still needs to be elucidated. Clearly, particle size and/or protein-lipid conformation is important for TNF modulating activity to be present. Experiments are in progress to better delineate the particle size and composition necessary for maximal protective effects.

What is perhaps most important, is that the effects mediated by ApoLipo *in vitro* were also realised *in vivo*. TNF production *in vivo*, as well as some of the pathophysiological changes associated with endotoxin infusion were attenuated in our model system by the prophylactic administration of ApoLipo

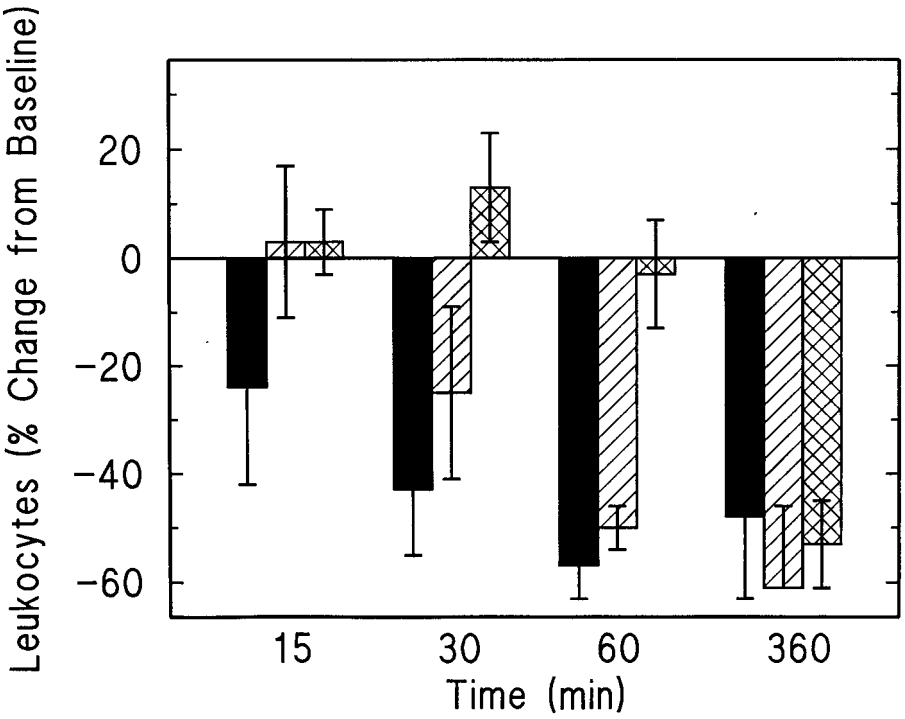

Figure 9: Change in leukocyte count with LPS administration. The percent change of leukocyte count from baseline for each of the LPS treated groups are shown. Control-LPS, ApoLipo75-LPS, and ApoLipo250-LPS are shown as solid, hatched and crosshatched bars respectively. The leukocyte drop of the Control-LPS and ApoLipo75-LPS groups are significantly different from that of the ApoLipo250-LPS group at 60 min ($p<0.05$).

(*Hubsch* et al., 1991; Figures 7-9). In our rabbit model, LPS induces high levels of TNF 2 h after the start of its infusion (Figure 7), a severe metabolic acidosis and significant decreases in leukocyte and platelet counts (Figures 7-9). Except for the leukopenia, all of these derangements were significantly attenuated by the prophylactic infusion of ApoLipo. The TNF levels observed in this study are consistent with the findings by *Mathison* et al. (1988), who reported peak TNF levels of 20 to 30 ng/ml, from 45 to 100 min after a bolus injection of 10 μg of LPS in rabbits (comparable to our Control-LPS group). In this same model system, the injection of plasma precomplexed LPS induced only a minimal (< 2.5 ng/ml) TNF response, comparable to our ApoLipo-LPS groups. One can argue that the TNF produced acts in concert with other biological mediators to alter metabolic status and cellular adhesiveness. Thus, in our system, ApoLipo most likely modulates metabolic status (acidosis) as a consequence of its action on TNF production. The fact that other sequelae are not affected to the same extent by ApoLipo (e.g., leukopenia), suggest that these sequelae are due to the direct effects of LPS as opposed to a dependence on TNF. Although high doses of ApoLipo were able to reduce and delay leukopenia, it should be noted that the leukocyte drop occurred very early in the course of LPS infusion, at a time when TNF was not measurable in

the circulation. The leukocytes may be reacting with LPS directly via their LPS receptors (*Wright* et al., 1989).

The mechanisms by which ApoLipo mediates its effects *in vivo* require further clarification. At present, we speculate that ApoLipo may function through two vastly different mechanisms of action: the formation of ApoLipo-LPS complexes and a down regulation of macrophage and perhaps other cells' reactivity. Our cell pulse experiments and the lack of requirement for precomplexation with LPS supports the second hypothesis. In each case, ApoLipo treatment is consistent with decreased TNF production. It is still too early to speculate on the clinical utility of ApoLipo. A greater understanding of the mechanisms of protection are clearly needed and experiments to this end are in progress.

ACKNOWLEDGEMENTS

The authors wish to thank and acknowledge the following technicians for their attention to detail and the quality of their work. The lipoprotein analyses, and production of the experimental lipoprotein preparations were performed by Ms. M. Illi and Ms. E. Frei. The TNF measurements *in vitro* and *in vivo* were conducted by Ms. B. Reist, Ms. V. Balmer, and Mrs. E. Ehrengruber. Technical assistance in the animal experiments was provided by Ms. V. Gafner.

LITERATURE

Beutler, B.A., Milsark, I.W., and Cerami, A.: Cachectin/Tumor Necrosis Factor: production, distribution, and metabolic fate *in vivo*. J. Immunol. 135, 3972-3977 (1985).

Beutler, B., Milsark, I.W., and Cerami, A.C.: Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 229, 869-871 (1985).

Cavaillon, J-M., Fitting, C., Haeffner-Cavaillon, N., Kirsch, and S.J., Warren, H.S.: Cytokine response by monocytes and macrophages to free and lipoprotein-bound lipopolysaccharide. Infect. Immun. 58, 2375-2382 (1990).

Chen, P.S., Toribara, T.Y., and Warner, H.: Microdetermination of phosphorous. Anal. Chem. 28, 1756-1758 (1956).

Chen, C.H., and Albers J.J.: Characterization of proteoliposomes containing apoprotein A-I: a new substrate for the measurement of lecithin:cholesterol acyltransferase activity. J. Lip. Res. 23, 680-691 (1982).

Desch, C.E., Kovach, N.L., Present, W., Broyles, C., and Harlan, J.M.: Production of human tumor necrosis factor from whole blood *ex vivo*. Lymphokine Res. 8,141-146 (1989).

Doran, J.E.: Biological effects of endotoxin. In: Enterogenic Infectious-Toxic Shock Syndrome. Pathogenesis and immunoprophylactic and therapeutic possibilities. (Eds: Cottier, H., and Kraft, R..). Karger Verlag, Basel, 1991.

Doran, J.E., Lerch, P.G., Hubsch, A.P., Hodler, G., Morgenthaler, J-J., and Heiniger, H.J.: *In vitro* modulation of tumor necrosis factor alpha production by apolipoprotein a-I proteoliposomes but not by apolipoprotein A-I alone. 2nd International Congress on the Immune Consequences of Trauma, Shock and Sepsis: Mechanisms and Therapeutic Approaches; Munich, 6-9 March 1991 (1991).

Flegel, W.A., Wölpl, A., Männel, D.A., and Northoff, H.: Inhibition of endotoxin-induced activation of human monocytes by human lipoproteins. Infect Immun 57, 2237-2245 (1989).

Feuerstein, G., Hallenbeck, J.M., Vanatta. B., Rabinovici, R., Perera, P.Y., and Vogel, S.N.: Effect of gram-negative endotoxin on levels of serum corticosterone, TNF-alpha, circulating blood cells, and the survival of

rats. Circ. Shock 30, 265-278 (1990).
Freudenberg, M.A., Bøg-hansen, T.C., Back, U., and Galanos, C.: Interaction of lipopolysaccharides with plasma high-density lipoprotein in rats. Infect. Immun. 28, 373-380 (1980).
Harris, H.W., Grunfeld, C., Feingold, K.R., Rapp, J.H.: Human very low density lipoproteins and chylomicrons can protect against endotoxin-induced death in mice. J. Clin. Invest. 86, 696-702 (1990).
Hinshaw, L.B., Tekamp-Olson, P., Chang, A.C.K., Lee, P.A., Taylor, F.B., Murray, C.K., Peer, G.T., Emerson, T.E., Passey, R.B., and Kuo, G.C.: Survival of primates in LD 100 septic shock following therapy with antibody to tumor necrosis factor (TNF alpha). Circ. Shock 30, 279-292 (1990).
Hubsch, A.P., Doran, J.E., Lerch, P.G., Hodler, G., Morgenthaler, J-J., and Heiniger, H.J.: Apolipoprotein A-I enriched lipoprotein attenuates TNF production and shock in an anesthesized rabbit endotoxemia model. Second International Congress on the Immune Consequences of Trauma, Shock, Sepsis : Mechanisms and therapeutic approaches. Munich, Germany. (1991).
Lehmann, V., Freudenberg, M.A., and Galanos, C.: Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamin-treated mice. J. Exp. Med. 165, 657-663 (1987).
Lerch, P.G., Morgenthaler, J-J., Peitsch, M.C., Friedli, H., Hodler, G., Doran, J.E., Isliker, H., and Heiniger, H-J.: Isolation and properties of Apolipoprotein A for therapeutic use. Protides of the Biological Fluids 36, 409-416 (1989).
Markwell, M.A.K., Haas, S.M., Bieber, L.L., and Tolbert, N.E.: A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87, 206-210 (1978).
Mathison, J.C., and Ulevitch, R.J.: The clearance, tissue distribution, and cellular localization of intravenously injected lipopolysaccharide in rabbits. J. Immunol. 123, 2133-2143 (1979).
Mathison, J.C., Wolfson, E., and Ulevitch, R.J.: Participation of tumor necrosis factor in the mediation of gram negative bacterial lipopolysaccharide-induced injury in rabbits. J. Clin. Invest. 81, 1925-1937 (1988).
Michie, H.R., Manogue, K.R., Spriggs, D.R., Revaug, A., O'Dwyer, S., Dinarello, C., Cerami, A., Wolff, S.M., and Wilmore, D.W.: Detection of circulating tumor necrosis factor after endotoxin administration. N. Engl. J. Med. 318, 1481-1486 (1988).
Munford, R.S., Hall, C.L., and Dietschy, J.M.: Binding of *Salmonella typhimurium* lipopolysaccharides to rat high-density lipoproteins. Infect. Immun. 34, 835-843 (1981).
Natanson, C., Eichenholz, P.W., Danner, R.L., Eichacker, P.Q., Hoffman, W.D., Kuo, G.C., Banks, S.M., MacVittie. T.J., and Parrillo, J.E.: Endotoxin and tumor necrosis factor challenges in dog simulate the cardiovascular profile of human septic shock. J. Exp. Med. 169, 823-832 (1979).
Rall, D.P., Gaskins, J.R., and Kelley, M.G.: Reduction of febrile response to bacterial polysaccharide following incubation with serum. Am. J. Physiol. 188, 559-562 (1957).
Remick, D.G., Kunkel, R.G., Larrick, J.W., and Kunkel, S.L.: Acute *in vivo* effects of human recombinant tumor necrosis factor. Lab. Invest. 56, 583-590 (1987).
Ruff, M.R., and Gifford, G.E.: Tumor Necrosis Factor. Lymphokines 2, 235-272 (1981).
Sanchez-Cantu, L., Rode, H.N., and Christou, N.V.: Endotoxin tolerance is associated with reduced secretion of tumor necrosis factor. Arch. Surg. 124, 1432-1436 (1989).
Silva, A.T., Bayston, K.F., and Cohen, J.: Prophylactic and therapeutic effects of a monoclonal antibody to tumor necrosis factor-alpha in experimental gram-negative shock. J. Infect. Dis. 162, 421-427 (1990).
Schumaker, V.N., and Puppione, D.L.: Sequential flotation ultracentrifugation. Methods in Enzymology 128, 155-170 (1986).
Tracey, K.J., Beutler, B., Lowry, S.F., Merryweather, J., Wolpe, S., Milsark, I.W., Hariri, R.J., Fahey III, T.J., Zentella, A., Albert, J.D., Shires, G.T., and Cerami, A.: Shock and tissue injury induced by recombinant human cachectin. Science 234, 470-474 (1986).
Tracey, K.J., Fong, Y., Hesse, D.G., Manogue, K.R., Lee, A.T., Kuo, G.C., Lowry, S.F., and Cerami, A.: Anti-

cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature 330, 662-664 (1987).

Ulevitch, R.J., and Johnston, A.R.: The modification of biophysical and endotoxic properties of bacterial lipopolysaccharide by serum. J. Clin. Invest. 62, 1313-1324 (1978).

Ulevitch, R.J., Johnston, A.R., and Weinstein, D.B.: New function for high density lipoproteins. Their participation in intravascular reactions of bacterial lipopolysaccharides. J. Clin. Invest. 64, 1516-1524 (1979).

Ulevitch, R.J., Johnston, A.R., and Weinstein, D.B.: New function for high density lipoproteins: Isolation and characterization of a bacterial lipopolysaccharide-high density lipoprotein complex formed in rabbit plasma. J. Clin. Invest. 67, 827-837 (1981).

Ulevitch, R.J., and Tobias, P.S.: Interactions of bacterial lipopolysaccharides with serum proteins. In Bacterial Endotoxins: Pathophysiological Effects, Clinical Significance, and Pharmacological Control, (Eds.: Levin, J., ten Cate, J.W., Büller, H.R., van Deventer, S.J.H., and Sturk, A.). Alan R Liss, New York, 309-318 (1988).

van Lenten, B.J., Fogelman, A.M., Haberland, M.E., and Edwards, P.A.: The role of lipoproteins and receptor-mediated endocytosis in the transport of bacterial lipopolysaccharide. Proc. Natl. Acad. Sci. USA 83, 2704-2708 (1986).

Warren, H.S., Riveau, G.R., deDeckker, F.A., and Chedid, L.A.: Control of endotoxin activity and interleukin-1 production through regulation of lipopolysaccharide-lipoprotein binding by a macrophage factor. Infect. Immun. 56, 204-212 (1988).

Wright, S.D., Levin, S.M., Jong, M.T., Chad, Z., and Kabbash, L.G.: CR3 (CD11b/CD18) expresses one binding site for Arg-Gly-Asp containing peptides and a second site for bacterial lipopolysaccharides. J. Exp. Med. 169, 175-183 (1989).