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Increased lipopolysaccharide-induced tumour necrosis factor levels and death in hypercholesterolaemic rabbits

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SUMMARY

Nutritional-induced hypercholesterolaemia in New Zealand rabbits causes increased susceptibility to experimental infections. Rabbits fed cholesterol (0·5 g%) for 8 weeks were injected intravenously with varying doses of *Escherichia coli* 0127 : B8 lipopolysaccharide (LPS; 3–100 μg/kg). The levels of cholesterol, triglycerides, tumour necrosis factor (TNF), and the survival rates of treated rabbits were then measured. Rabbits fed either normal chow or chow impregnated with sesame oil were used as controls. LPS induced higher serum TNF levels in hypercholesterolaemic rabbits than in normal rabbits or rabbits fed with chow containing sesame oil. TNF levels rose faster in hypercholesterolaemic rabbits than in normal rabbits, reaching maximum levels at 60 min and 120 min, respectively, after LPS injection. The survival rate of hypercholesterolaemic rabbits (1/11) was lower than in normal rabbits (6/7) or rabbits fed with the sesame oil chow (4/4) at the higher LPS doses. No death occurred at lower doses. One possible interpretation of these results, also supported by neutralization experiments, is that increased TNF secretion in hypercholesterolaemic rabbits raises the host's susceptibility to experimental endotoxaemia and possibly to Gram-negative infection.

Keywords endotoxin experimental infection hypercholesterolaemia susceptibility tumour necrosis factor

INTRODUCTION

Lipopolysaccharide (LPS) induces the release of several products such as tumour necrosis factor (TNF) that mediate the host immune response during Gram-negative sepsis [1,2]. TNF is an important mediator of biological activities caused by endotoxin in Gram-negative bacterial infections, such as endotoxic shock, cachexia [3] and of several chronic inflammatory disease states [4]. TNF acts either directly on the cells through specific receptors [5] or indirectly via the induction of other mediators and cytokines such as IL-1 [6], IL-6 and prostaglandins [7]. TNF is involved in the primary changes that lead to the cascade of events that culminates in lethal hypotensive shock and disseminated intravascular coagulation and death during Gram-negative sepsis [8].

While TNF and other cytokines are essential to host defences, the inflammatory response, the most primitive immune mechanism of all, may produce injury or death if it is sufficiently intense or prolonged [9]. Recently, it has been shown that in human and rabbit arterial wall, TNF could be actively involved in the inflammatory events associated with atherosclerosis [10,11]. Atherosclerotic lesions show many characteristic features of inflammation such as monocyte recruitment, accumulation and preferential retention of immunoglobulins and complement components, and *in situ* complement activation [12].

In addition, the potentiating effect of a high cholesterol diet on the susceptibility to, and severity of, viral and bacterial infections in different animal species including rabbits is well known [13–17]. Previous studies found no alterations in the phagocytic function of monocytes in hypercholesterolaemic rabbits [15,16]; also, complement system integrity (Romano and Londoño, unpublished observations) and lymphocyte physiology were not grossly affected [18]. In this study we provide evidence for a decreased resistance to LPS challenge in hypercholesterolaemic rabbits which is probably due to an earlier and greater secretion of TNF. The importance of TNF as a mediator of LPS-induced death in a dose-dependent fashion in hypercholesterolaemic rabbits is also highlighted.

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MATERIALS AND METHODS

Animals

Outbred New Zealand White rabbits weighing 2·5–3·0 kg, maintained on a normal commercial rabbit chow (NR) (Conejarina; Alimentos Protinal C.A., Valencia, Venezuela), served as control animals for the cholesterol-rich diet fed rabbits (ChR). The diet for the latter animals was prepared by the addition of 0·5 g% cholesterol (Sigma Chemical Co., St Louis, MO), dissolved in 12·5 ml% sesame oil (C. A. Facegra, Valencia, Venezuela), to the feed pellets. Feed pellets impregnated with sesame oil without cholesterol were also used as a second control diet (SR). The animals were studied 8 weeks after initiation of the diets.

LPS

Escherichia coli, strain 0127:B8 endotoxin (Sigma) was used throughout the study.

Depyrogenation

To avoid contamination with exogenously derived endotoxin, all heat-stable materials and solutions used in the study were rendered sterile and free of detectable endotoxin ($\leqslant$0·015 Endotoxin Units/ml; E-Toxate assay, Sigma) by a combination of steam autoclaving followed by dry heating at 180°C for a minimum of 4 h.

Haematologic parameters

Rabbit blood was collected aseptically from the central auricular artery and allowed to clot at 4°C. The serum was obtained the same day and maintained at −70°C until assayed. Total and differential leucocyte counts were performed according to standard methods. Serum cholesterol levels were determined according to the methods of Bowman & Wolf [19]. Serum triglycerides were determined using a colorimetric assay [20].

TNF cytolytic assay

TNF activity was measured using a cytolytic assay on WEHI 164 clone 13 cells treated with 1 μg/ml actinomycin-D as described by Espevik & Nissen-Meyer [21]. Addition of SDS 0·5% to cells was used to define complete lysis (100%), and cultures without TNF were used to define total viability. Each plate, including a human recombinant TNF-α (Genentech, San Francisco, CA) with a specific activity of $4{\cdot}03 \times 10^7$ U/mg, was used for the standard curve to calibrate the assay. Assay results were expressed as U/ml, with one unit defined as the amount of TNF resulting in lysis of 50% of the cells. The degree of lysis was quantified spectrophotometrically (560 nm) using a plate reader (EL-307C; Bio-Tek Instruments, Winooski, VT). The assay is unaffected by IL-1, IL-2 or IL-6, and there is no synergy between interferon-gamma (IFN-γ) and TNF in this assay [22]. The specificity of the TNF assay was checked by neutralization of TNF bioactivity with the fusion protein p55-sf2 (kindly donated by Centocor, Malvern, PA, through Dr J. Ghrayeb). This hybrid molecule consists of the extracellular domain of the human p55 TNF receptor fused to the J sequence and the constant domains of human IgG1. Total inhibition of rabbit TNF cytotoxicity (approximately $1{\cdot}84 \times 10^3$ U/ml) was observed when 300 pM of p55-sf2 were used in our standardized bioassay. Since the p55 binds both TNF-α and TNF-β, a contribution of TNF-β to the effects observed cannot be discounted.

In vivo *studies*

After baseline parameters were established, ChR, NR and SR animals were injected intravenously through the marginal ear vein with LPS. Fifty and 100 μg of LPS/kg were the doses that elicited differences among the rabbit groups.

Rabbit serum levels of cholesterol, triglycerides (TG) and TNF were estimated in blood samples obtained at different times. Monitoring of the core temperature (CT) and collection of blood samples through the central ear artery proceeded for 200–300 min. A 24-h follow-up time was established to define survival or death of the experimental rabbits, even though they were observed for at least 2 more weeks. Representative physiological parameters in rabbits at the beginning of the observation period were: CT, 39 ± 1·34°C; and total leucocyte count, 8867 ± 0·69 cells/μl. Since an important role of TNF as a mediator of LPS-induced injury has been described [8], 2 mg of anti-TNF construct p55-sf2 were infused to neutralize TNF and verify the involvement of this cytokine in the increased susceptibility to LPS observed in the ChR animals.

Statistical analysis

Data are expressed as mean ± s.e.m. Differences in TNF levels in the ChR, NR and SR animals were analysed for statistical significance using the non-parametric Mann–Whitney *U*-test.

RESULTS

Cholesterol and triglyceride levels

The serum levels of cholesterol and TG after 8 weeks of the experimental diets are shown in Table 1. LPS administration did not induce any gross change in serum cholesterol and TG in any rabbit group at any dose of LPS administered within the monitoring time.

Survival rates of rabbits treated with E. coli *0127:B8 LPS*

A reduced host resistance to experimental endotoxaemia was observed in the ChR: 9% of ChR survived compared with 85·7% of NR and 100% of SR. The differences between ChR, and NR and SR were statistically significant ($0{\cdot}025 < P \leqslant 0{\cdot}05$) (Table 2). No deaths occurred at lower doses (3 and 20 μg LPS/kg, data not shown). The mean survival time ±s.e.m. of ChR at 50 μg/kg was 8·6 ± 2·7 h, which was reduced to 5·2 ± 1 h at 100 μg/kg. With both doses of LPS, the NR and SR survived

Table 1. Average serum concentration (mg/dl) of cholesterol and triglycerides (TG) in the different rabbit groups after 8 weeks of feeding with the different diets

	Cholesterol	Triglycerides
NR (7)	62·8 ± 9·3	80 ± 24·1
ChR (11)	619·4 ± 11·7	294·5 ± 24
Sr (4)	111·2 ± 5·8	265 ± 32

The numbers in parentheses represent the total number of rabbits in that group.

NR, Rabbits fed normal diet; ChR, rabbits fed cholesterol/sesame oil-rich diet; SR, rabbits fed sesame oil-rich diet.

Table 2. Survival rate after injection of two doses of *Escherichia coli* 0127 : B8 lipopolysaccharide (LPS) in rabbits fed with experimental diets for 8 weeks

LPS doses (μg/kg)	Diet group (surviving/treated)		
	Nr	ChR	SR
50	3/3	0/4*	2/2
100	3/4	1/7*	2/2

The observation period was 24 h. Mean survival time ±s.e.m. of ChR at 50 μg/kg was 8·6 ± 2·7 h and at 100 μg/kg was 5·2 ± 1·1 h. In all cases NR and SR survived more than 24 h after the bolus i.v. injection of LPS 0127 : B8. NR, Rabbits fed normal diet; ChR, rabbits fed cholesterol/sesame oil-rich diet; SR, rabbits fed sesame oil-rich diet.

* $0{\cdot}025 < P \leqslant 0{\cdot}05$.

more than 24 h and remained apparently healthy during the follow-up period. It was also observed that the NR survival rate began to decrease markedly at higher doses of LPS, 200–400 μg/kg (data not shown). Upon treatment with 2 mg of p55-sf2, the survival time of a ChR after 100 μg/kg LPS injection was prolonged from 5 h to 20 h.

Leucocyte and differential counts following E. coli *0127 : B8 LPS administration*

Leucocyte and differential counts following i.v. LPS injection in rabbits showed the same tendency in all groups with different types of diet, and no statistically significant differences were found. Each of the doses of LPS employed resulted in leukopenia, followed by a gradual return to pre-injection levels (Figs 1 and 2). A mean differential count of 30% polymorphonuclear (PMN) cells, 61% lymphocytes and 9% monocytes changed at 60 min after the administration of LPS to 4% PMN, 7% monocytes and 84% lymphocytes.

Core temperature variations in rabbits following E. coli *0127 : B8 LPS administration*

The basal CT was 39 ± 1·3°C. As expected, a slight increase of CT in ChR was observed after LSP administration (Figs 1 and 2). The same effect was observed at lower LPS doses (data not shown).

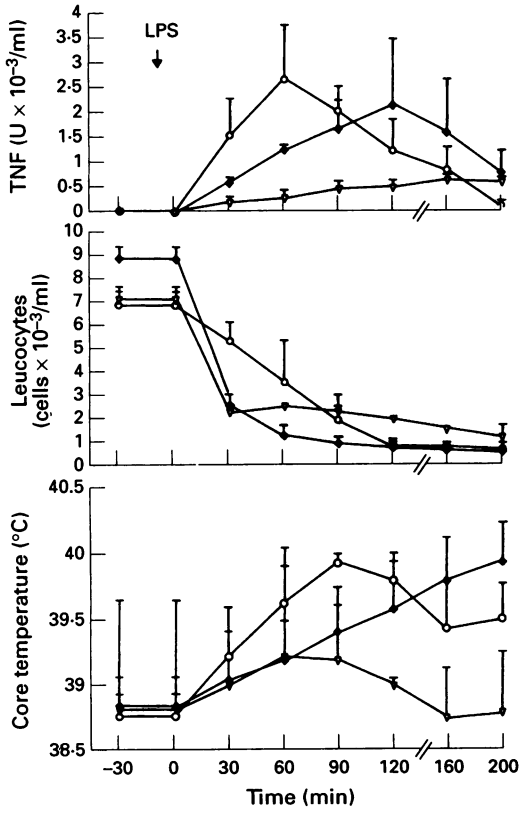

Fig. 1. Induction of serum tumour necrosis factor (TNF), mean leucocyte counts and core temperature changes following intravenous injection of 50 μg/kg *Escherichia coli* 0127 : B8 lipopolysaccharide (LPS) in rabbits. LPS was administered as a bolus injection through the marginal ear vein, and blood samples were collected at the indicated times. The data are mean ± s.e.m. The number of rabbits per group was: rabbits fed cholesterol/sesame oil-rich diet (ChR), 3 (○); rabbits fed normal diet (NR), 3 (◆); and rabbits fed sesame oil-rich diet (SR), 2 (▽).

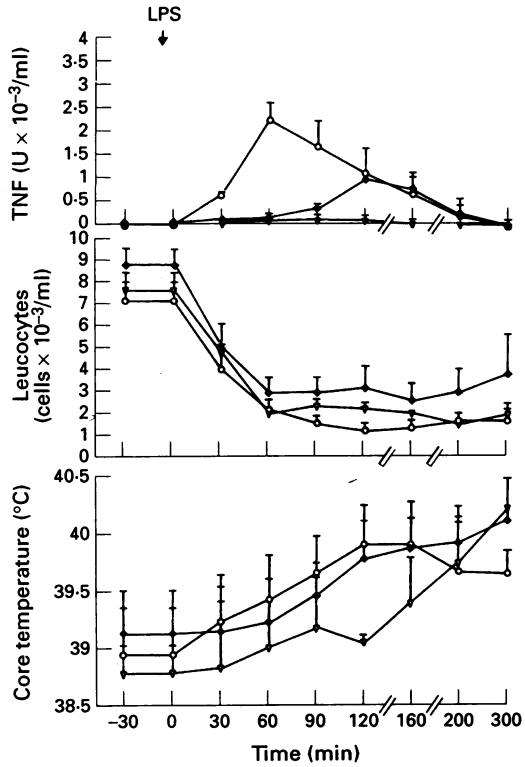

Fig. 2. Induction of serum tumour necrosis factor (TNF), mean leucocyte counts and core temperature changes following intravenous injection of 100 μg/kg *Escherichia coli* 0127 : B8 lipopolysaccharide (LPS) in rabbits. LPS was administered as a bolus injection through the marginal ear vein, and blood samples were collected at the indicated times. The data are mean ± s.e.m. The number of rabbits per group was: rabbits fed cholesterol/sesame oil-rich diet (ChR), 6 (○); rabbits fed normal diet (NR), 4 (◆); and rabbits fed sesame oil-rich diet (SR), 2 (▽).

Table 3. Highest serum tumour necrosis factor (TNF) ($U \times 10^3$/ml) levels observed after a bolus injection of varying doses of *Escherichia coli* 0127 : B8 lipopolysaccharide (LPS)

LPS dose (μg/kg)	Diet group		
	NR	ChR	SR
3	0·14 (1)	0·20 (1)	—
20	0·11 (1)	0·78 (1)	—
50	2·23 ± 1·33 (3)	2·78 ± 1·01 (3)	0·70 ± 0·1 (2)
100	1·00 ± 0·28 (4)	2·33 ± 0·48* (6)	0·10 ± 0·1 (2)

Data represent mean ± s.e.m. Numbers in parentheses represent the total number of rabbits in that group.

* $0{\cdot}025 < P \leq 0{\cdot}05$ compared with control groups. NR, rabbits fed normal diet; ChR, rabbits fed cholesterol/sesame oil-rich diet; SR, rabbits fed sesame oil-rich diet.

Induction of TNF in vivo *by* E. coli *0127 : B8 LPS*

Serum TNF levels were higher in ChR than in NR and in SR (Figs 1 and 2, Table 3). In all rabbit groups, an important animal-to-animal variation was observed. The lowest levels of TNF were found in SR. TNF levels rose faster in ChR than in NR, reaching the highest level at 60 min and 120 min, respectively, when injected intravenously with 50 and 100 μg LPS/kg. Administration of lower doses, 3 and 20 μg LPS/kg, resulted in lower serum TNF levels, again higher in ChR than in NR (Table 3). There was no detectable TNF in serum samples pre-LPS injection in any of the different rabbit groups. The injection of endotoxin-free saline instead of LPS in control rabbits did not elicit any variation in TNF, leucocyte count or CT.

An *in vivo* inhibition of TNF levels, in the order of 95%, was observed when anti-TNF construct was used before LPS administration.

DISCUSSION

In the present study it is shown that LPS treatment causes a differential effect in hypercholesterolaemic rabbits compared with control rabbits. LPS has been shown previously to be a potent *in vivo* stimulus for the secretion of TNF and other proinflammatory cytokines [6,8]. Although the correlation between TNF and mortality in septic shock is well established [7,8], the finding of a more severe effect of LPS treatment in ChR compared with NR and SR is novel.

The decreased survival rate observed in the ChR after administration of LPS (50 and 100 μg/kg) compared with NR and SR, suggests a magnification of the effects elicited by LPS. In the SR group, although leukopenia and fever were seen, these rabbits did not display signs of weakness such as prostration, difficulty in breathing and no drinking or eating, as observed in ChR and NR. This is probably related to a protective effect of polyunsaturated fatty acids present in sesame oil. Since NR and SR remained alive and apparently healthy during the following time of at least 2 weeks after LPS injection, this effect did not represent a delayed response of NR and SR to LPS. It suggests that these animals are more resistant to LPS treatment than ChR.

In 1985, Beutler *et al.* proposed TNF as the mediator of endotoxin-induced injury [23]. Later, Mathison *et al.* [8] provided experimental evidence for an important role of TNF as a mediator of septic shock. The present study shows that ChR injected with LPS produced more TNF and at a faster rate than NR and SR, suggesting a direct relation between TNF levels and Gram-negative shock and death in experimental hypercholesterolaemia in rabbits. On the other hand, TNF alone does not explain all the observations. For instance, even though the survival time in anti-TNF-treated ChR was prolonged four-fold, they died, suggesting that other factors besides TNF mediate the pathophysiologic changes that are induced by LPS in hypercholesterolaemic rabbits. As shown in Table 3, slightly higher levels of TNF were observed in rabbits treated with 50 μg LPS/kg in comparison with 100 μg LPS/kg; we have no explanation for this finding.

The cholesterol-rich diet induced hyperlipidaemia and the appearance of atherosclerotic lesions that included a series of inflammatory features such as monocyte infiltration, smooth muscle cell proliferation, foam cells containing cholesterol crystals, and the presence of proinflammatory cytokines such as TNF and IL-1 [10–12,24]. Recently an acute-phase protein able to bind LPS, lipopolysaccharide binding protein (LBP) has been isolated in rabbits [25]. Tobias *et al.* have reported that an increase in the production of TNF *in vivo* correlates with an increase in LBP levels and with a reduction in its time of appearance in plasma [26], being a possible explanation for the phenomenon observed in ChR.

Since cholesterol was dissolved in sesame oil to prepare the cholesterol-rich diet, it was important to study the effect of the diet impregnated only with sesame oil, which is high in unsaturated free fatty acids (UFA). As shown in Table 3, SR rabbits produced the lowest TNF levels. An inhibitory effect of UFA on the immune system has been described, especially of linoleic acid, the main component of sesame oil (linoleic acid 49%, oleic acid 37%, palmitic acid 10%, stearic acid 4%) [27] which is a dietary precursor of the prostaglandins (PG). It is known that PGE_2 suppresses TNF production by resident and peritoneal macrophages [28–30]. Thus, a low TNF production in SR may be explained through an increased secretion of PG. Additionally, previous studies [31–35] provide evidence of LPS interaction with other plasma constituents including different lipoproteins; increased LPS interaction with triglyceride-rich lipoproteins in SR may delay and diminish LPS biological effects. In ChR, cholesterol deposition in tissues and appearance of macrophage activators such as oxidized low density lipoproteins may counterbalance the beneficial effect of UFA.

Our findings provide evidence for an alteration of resistance against infectious agents in ChR, and a protection to LPS biological effect on SR, suggesting that nutritional status exerts an effect on the immune system, and raising the possibility that such an alteration could be useful in modulating immunity. It should be considered in the management of patients with high cholesterol levels and Gram-negative sepsis.

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