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# Hyperlipoproteinemia Enhances Susceptibility to Acute Disseminated *Candida albicans* Infection in Low-Density-Lipoprotein-Receptor-Deficient Mice

MIHAI G. NETEA,<sup>1</sup> PIERRE N. M. DEMACKER,<sup>1</sup> NATASJA DE BONT,<sup>1</sup> OTTO C. BOERMAN,<sup>2</sup> ANTON F. H. STALENHOF,<sup>1</sup> JOS W. M. VAN DER MEER,<sup>1</sup> AND BART JAN KULLBERG<sup>1\*</sup>

Division of General Internal Medicine, Department of Medicine,<sup>1</sup> and Department of Nuclear Medicine,<sup>2</sup> University Hospital Nijmegen, 6500 HB Nijmegen, The Netherlands

Received 16 December 1996/Returned for modification 28 February 1997/Accepted 29 March 1997

Recent studies have suggested the use of lipoproteins as an adjuvant treatment of lethal gram-negative infections. However, other important microorganisms for the etiology of sepsis, such as *Candida* species, grow better in lipid-rich environments. We investigated the effect of hyperlipoproteinemia on systemic candidiasis in low-density-lipoprotein-receptor-deficient (LDLR<sup>-/-</sup>) mice, in which the loss of the receptor results in a seven- to ninefold-higher plasma LDL level than that in their wild-type littermates (C57BL/6J). LDLR<sup>-/-</sup> mice died earlier, and the outgrowth of *Candida albicans* in the kidneys and livers of LDLR<sup>-/-</sup> mice was significantly higher compared with that of controls. After infection, circulating cytokine concentrations were significantly higher in LDLR<sup>-/-</sup> mice. In vitro, *C. albicans* grew better in plasma samples of LDLR<sup>-/-</sup> mice than in control plasma samples and peritoneal macrophages of LDLR<sup>-/-</sup> mice challenged with heat-killed *C. albicans* produced more cytokines than did those of controls. This latter phenomenon was probably due to increased binding of yeast cells to macrophages of LDLR<sup>-/-</sup> mice. These data suggest that hyperlipoproteinemia is deleterious in systemic candidiasis.

Acute disseminated candidiasis is a life-threatening condition that occurs predominantly in immunocompromised hosts. The mortality rate associated with disseminated candidiasis is high (31), and the incidence of this disease has increased in recent years (1, 3). *Candida albicans* ranks fourth among the organisms most frequently isolated from blood cultures in the United States (1). Clinically, systemic candidiasis sometimes mimics gram-negative sepsis. Viable *Candida* cells and cell wall constituents are able to induce the synthesis of proinflammatory cytokines in vitro (13, 14, 18), similar to gram-negative bacteria and their lipopolysaccharide (LPS) component (23). However, the role of these cytokines in systemic candidiasis is probably beneficial rather than deleterious (20, 26). In contrast, the induction of proinflammatory cytokines, such as interleukin-1 $\alpha$  (IL-1 $\alpha$ ) and IL-1 $\beta$  and tumor necrosis factor alpha (TNF- $\alpha$ ), is merely a deleterious event in gram-negative sepsis (4) and treatment aimed at blocking cytokine action has proved beneficial in various experimental models (2, 27, 30). In sepsis in humans, anticytokine strategies have not been successful so far. This is the reason why the focus of several recent studies has been on the capacity of lipoproteins in the circulation to bind and neutralize LPS and subsequently inhibit cytokine production. Preincubation of LPS with lipoproteins prior to injection into animals decreased mortality after LPS injection (28). Moreover, in vivo infusion of lipoproteins markedly reduced cytokinemia and mortality after LPS administration (11). Transgenic mice that express human apolipoprotein A-1 at high levels and have elevated plasma high-density-lipoprotein concentrations are protected against LPS challenge (17). Furthermore, low-density-lipoprotein-receptor-deficient (LDLR<sup>-/-</sup>) mice, in which the plasma LDL concentration is

increased seven to nine times (12), were significantly protected not only against lethal endotoxemia but also against lethal *Klebsiella pneumoniae* infection (21). In addition, an infusion of lipoproteins in rats protected the animals against mortality due to gram-negative bacterial sepsis in a model of cecal ligation and puncture (24). Taken together, these experiments demonstrate the capacity of lipoproteins to neutralize LPS and support their potential use as adjuvants in the therapy of sepsis.

On the other hand, earlier in vitro studies of lipid-containing parenteral solutions introduced for clinical use suggested that certain microorganisms, such as *C. albicans* and *Staphylococcus aureus*, grew better in a lipid-rich environment (5, 9, 16). Therefore, it is important to know the effect of hyperlipoproteinemia on the outcome of a systemic infection with these organisms before suggesting the application of infusion with lipoproteins as an adjuvant therapy in sepsis. In the present study, we assessed the influence of hyperlipoproteinemia on experimental systemic candidiasis in LDLR<sup>-/-</sup> mice.

## MATERIALS AND METHODS

**Animals.** Homozygous C57BL/6J LDLR<sup>-/-</sup> mice and wild-type littermates were obtained from Jackson Laboratory (Bar Harbor, Maine) as mating pairs and bred in our local facility. For experiments, 6- to 8-week-old mice, weighing 20 to 25 g, were used. The animals were fed standard laboratory chow (Hope Farms, Woerden, The Netherlands) and housed under specific-pathogen-free conditions. The experiments were approved by the ethical committee for animal experiments at the Catholic University Nijmegen.

***C. albicans* infection.** *C. albicans* (strain UC820), maintained on agar slants at 4°C, was inoculated into 100 ml of Sabouraud broth and cultured for 24 h at 37°C. After three washes with pyrogen-free saline by centrifugation at 1,500  $\times$  g, the number of yeast cells was counted in a hemacytometer; occasional strings of two or more yeast cells were counted as 1 *C. albicans* CFU. The suspension was diluted to the appropriate concentration with pyrogen-free saline. The viability of yeast cells was at least 98%, as confirmed by plating serial dilutions on Sabouraud dextrose agar plates. Mice were injected intravenously (i.v.) in the retro-orbital plexus with 10<sup>6</sup> or 10<sup>7</sup> CFU of *C. albicans*. Survival was assessed daily for 14 days in groups of at least 15 animals. In separate groups, after 4 h and 1 and 3 days, subgroups of five mice were killed by cervical dislocation and blood samples were collected for the measurement of plasma cytokine concentrations.

\* Corresponding author. Mailing address: Dept. of Medicine (541), University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Phone: 31-24-3614763. Fax: 31-24-3541734. E-mail: B.Kullberg@aig.AZN.nl.

The outgrowth of microorganisms from the livers, spleens, and kidneys of animals was quantified on days 1 and 3 after *C. albicans* injection. For this purpose, the organs were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. The number of viable *C. albicans* cells in tissue samples was determined by plating serial dilutions on Sabouraud dextrose agar plates as described previously (15), and CFU were counted after overnight incubation at 37°C. The results were expressed as the log CFU per gram of tissue.

**Ex vivo cytokine production.** Resident peritoneal macrophages were harvested by rinsing the peritoneal cavity aseptically with cold phosphate-buffered saline containing 0.38% (wt/vol) sodium citrate. After centrifugation, cells were resuspended in RPMI 1640 (Dutch modification; Flow Laboratories, Irvine, United Kingdom) containing 1 mM pyruvate, 2 mM L-glutamine, and 100 µg of gentamicin per ml and incubated for 1 h at 37°C (10<sup>5</sup>/well) in 96-well microtiter plates (Costar Corporation, Cambridge, Mass.). Nonadherent cells were discarded, and the remaining cell population consisted of more than 90% macrophages, as assessed by light microscopy. Heat-killed (30 min, 100°C) *C. albicans* cells were washed three times in RPMI 1640 (final concentration, 2 × 10<sup>7</sup> CFU/ml). Two hundred microliters of suspension containing 10<sup>6</sup> microorganisms in RPMI 1640 was added to peritoneal macrophages and incubated at 37°C. After 24 h, supernatants were collected and stored at -70°C until assayed. To the remaining macrophages, 200 µl of RPMI 1640 was added, and cells were disrupted by three freeze-thaw cycles to determine the cell-associated cytokine contents. The samples were stored at -70°C until assayed.

**Cytokine measurements.** TNF-α, IL-1α, and IL-1β concentrations were determined by specific radioimmunoassays developed in our laboratory, as previously described (21).

***C. albicans* binding studies.** *C. albicans* cells were radioiodinated by the iodogen method (8) with minor modifications. Briefly, 10<sup>8</sup> *C. albicans* cells in 50 mM phosphate buffer (pH 7.2) were incubated in an iodogen-coated vial (20 µg/100 µl) in the presence of 300 µCi of Na<sup>125</sup>I (specific activity, 15 mCi/µg; Amersham) in a total volume of 100 µl. After 15 min of incubation, the suspension was washed three times with phosphate-buffered saline and resuspended in RPMI 1640 containing 1 mM of pyruvate, 2 mM of L-glutamine, and 100 µg of gentamicin per ml (labelling efficiency, 45%). Peritoneal macrophages from LDLR<sup>-/-</sup> and control mice were collected as described above. Macrophages (2 × 10<sup>5</sup>) were incubated with 10<sup>6</sup> CFU of radioiodinated *C. albicans* in a total volume of 200 µl of RPMI 1640 at 37°C in 96-well microtiter plates. After 5, 15, and 30 min, the supernatant was discarded and the radioactivity associated with adherent cells was measured with a gamma counter. Nonspecific binding was determined in wells in which *C. albicans* cells were incubated in the absence of macrophages. Total binding of *C. albicans* cells to macrophages (including both specific binding to receptors and nonspecific binding through membrane hydrophobicity or other nonspecific interactions) was determined by subtracting the binding of *C. albicans* cells to plastic from total counts.

**Growth of *C. albicans* in vitro.** To investigate whether the growth of *C. albicans* in the plasma of LDLR<sup>-/-</sup> mice is similar to that in plasma of control mice, 2 × 10<sup>4</sup> CFU of *C. albicans* were incubated in plasma samples obtained from LDLR<sup>-/-</sup> and control mice diluted 50% with Sabouraud broth in 10-ml glass tubes (Hospidex, Nieuwkoop, The Netherlands) in a final volume of 2 ml. After 12 and 24 h of incubation at 37°C, aliquots of 0.1 ml were removed, serial dilutions were plated on Sabouraud agar, and CFU were counted after overnight incubation at 37°C. The adherence of *C. albicans* cells to tubes was checked and found to be less than 1% of total counts.

**Determination of plasma lipids.** Cholesterol and triglycerides were determined by enzymatic methods with a Hitachi 747 analyzer.

**Statistical analysis.** The survival curves for control and LDLR<sup>-/-</sup> mice were compared by the Kaplan-Meier log rank test. Differences in concentrations of cytokines and in organ counts of microorganisms were analyzed by the Mann-Whitney U test. Differences were considered significant at *P* < 0.05. All experiments were performed at least twice.

## RESULTS

***C. albicans* infection.** Plasma cholesterol concentrations decreased significantly during *C. albicans* infection in both mouse strains (*P* < 0.05) but remained three to four times higher in LDLR<sup>-/-</sup> mice than in control mice (*P* < 0.01) (Table 1). The initial plasma triglyceride levels in LDLR<sup>-/-</sup> mice were two times higher than those of controls (*P* < 0.01). Four hours after infection, triglyceride levels decreased significantly in LDLR<sup>-/-</sup> mice (*P* < 0.05) and only marginally in control animals (*P* > 0.05) (Table 1). The triglyceride levels 4 h after *C. albicans* infection did not differ significantly between the two mouse strains (*P* > 0.05) (Table 1). After i.v. injection of either 10<sup>6</sup> or 10<sup>7</sup> CFU of *C. albicans*, LDLR<sup>-/-</sup> mice died significantly earlier than did control animals (*P* < 0.05) (Fig. 1).

One day after infection with 10<sup>6</sup> CFU of *C. albicans*, yeast

TABLE 1. Plasma cholesterol and triglyceride concentrations before and 4 h after *C. albicans* infection in LDLR<sup>-/-</sup> and C57BL/6J mice<sup>a</sup>

Mouse strain	Time	Mean concn (mmol/liter) ± SD <sup>b</sup>	
		Cholesterol	Triglycerides
LDLR <sup>-/-</sup>	Before	9.55 ± 1.11*	1.25 ± 0.33*
	After	6.76 ± 0.92*,**	0.58 ± 0.05**
C57BL/6J	Before	2.25 ± 0.45	0.65 ± 0.12
	After	1.84 ± 0.09**	0.49 ± 0.11

<sup>a</sup> Mice were infected i.v. with 10<sup>6</sup> CFU of *C. albicans*. Each group consisted of five mice.

<sup>b</sup> \*, *P* < 0.01 for comparison between results for LDLR<sup>-/-</sup> and control mice; \*\*, *P* < 0.05 for comparison between results before and after *C. albicans* infection.

outgrowth in the kidneys of LDLR<sup>-/-</sup> mice was significantly higher compared with that of controls; 3 days after infection, outgrowth of *C. albicans* was increased in both the kidneys and livers of LDLR<sup>-/-</sup> mice (Table 2). No difference in the outgrowth of *C. albicans* in the spleen was detected between the two mouse strains (Table 2).

Four hours after infection, plasma TNF-α concentrations were below the detection limit and IL-1α concentrations were higher in LDLR<sup>-/-</sup> mice than in controls (140 ± 22 versus 78 ± 48 pg/ml, respectively; *P* < 0.05). Circulating IL-1β concentrations were similar in both strains (56 ± 29 versus 37 ± 28 pg/ml, respectively; *P* > 0.05). Plasma TNF-α concentrations were significantly higher in LDLR<sup>-/-</sup> mice compared with those of control animals at both 24 (42 ± 5 versus 18 ± 4 pg/ml, respectively; *P* < 0.01) and 72 (302 ± 237 versus 98 ± 58 pg/ml, respectively; *P* < 0.02) h after infection (Fig. 2). No differences in plasma IL-1α and IL-1β concentrations were observed at these time points (Fig. 2).

**In vitro cytokine production.** We investigated the capacity of peritoneal macrophages of both mouse strains to produce cytokines when stimulated in vitro with heat-killed *C. albicans*. Compared with those of controls, the TNF-α concentrations in supernatants from macrophages of LDLR<sup>-/-</sup> mice were significantly higher (Fig. 3a). The IL-1α and IL-1β concentrations were only marginally increased (Fig. 3a). The cell-associated IL-1α concentration was significantly increased in macrophages of LDLR<sup>-/-</sup> mice, but the cell-associated TNF-α and

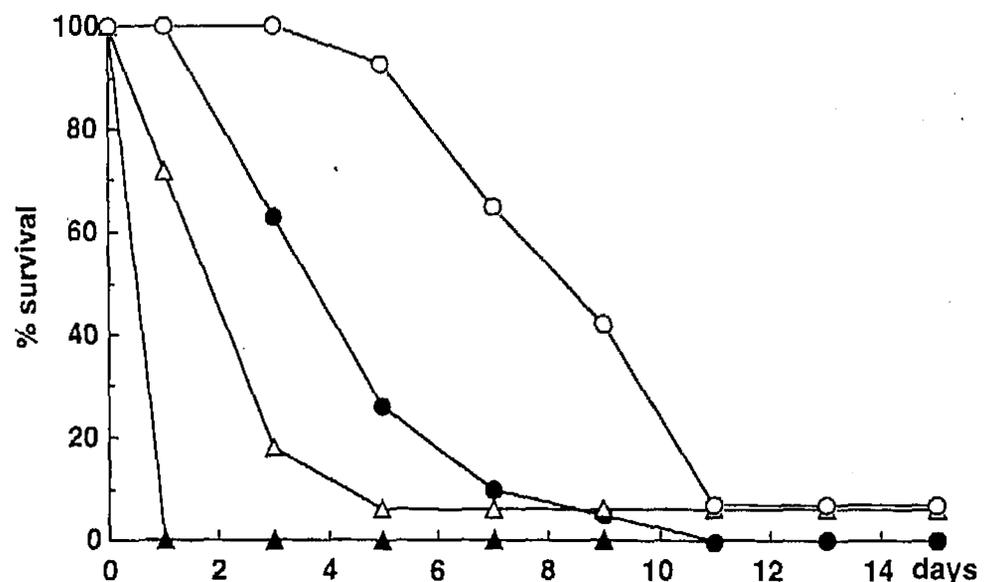


FIG. 1. Survival during *C. albicans* infection. LDLR<sup>-/-</sup> mice (closed symbols) infected with either 10<sup>7</sup> (triangles) or 10<sup>6</sup> (circles) CFU of *C. albicans* died significantly earlier than did C57BL/6J mice (open symbols) (*P* < 0.05; Kaplan-Meier log rank test). The data are pooled results of two experiments with at least 15 animals per group.

TABLE 2. Outgrowth of *C. albicans* in the organs of LDLR<sup>-/-</sup> and C57BL/6J mice after infection with 10<sup>6</sup> CFU of *C. albicans*

Mouse strain	Organ	Day	Log CFU/g of tissue <sup>a</sup>
LDLR <sup>-/-</sup>	Kidney	1	6.3 ± 0.5*
		3	7.4 ± 0.3**
	Liver	1	4.8 ± 0.3
		3	5.2 ± 0.2**
	Spleen	1	4.9 ± 0.2
		3	5.1 ± 0.5
C57BL/6J	Kidney	1	5.9 ± 0.2
		3	6.8 ± 0.3
	Liver	1	4.8 ± 0.1
		3	4.9 ± 0.1
	Spleen	1	5.1 ± 0.2
		3	5.0 ± 0.2

<sup>a</sup> Data are means ± standard deviations of pooled data from two experiments with 10 animals per group. \*, *P* < 0.01; \*\*, *P* < 0.05.

IL-1β concentrations did not differ between the two mouse strains (Fig. 3b).

***C. albicans* binding.** To investigate the total binding of *C. albicans* to macrophages from the two mouse strains, we incubated radioiodinated *C. albicans* cells with macrophages (5:1 ratio) and determined the amounts bound to macrophages after 5, 10, and 30 min of incubation. The binding of yeast cells to macrophages of LDLR<sup>-/-</sup> mice was increased and more rapid compared to that of control macrophages (Fig. 4).

**Growth of *C. albicans* in vitro.** To investigate whether the outgrowth of *C. albicans* in the organs of LDLR<sup>-/-</sup> mice is due to enhanced growth of yeast cells in a lipoprotein-rich environment, we compared the in vitro growth of 10<sup>4</sup> CFU of *C. albicans* in plasma samples (diluted 1:1 with Sabouraud medium) of LDLR<sup>-/-</sup> mice and controls. The capacity of LDLR<sup>-/-</sup> plasma to inhibit the outgrowth of *C. albicans* was decreased compared with that of control plasma, as shown by the growth of *C. albicans* in the two types of plasma samples after 12 [(7.2 ± 2.4) × 10<sup>3</sup> versus (2.4 ± 1.5) × 10<sup>3</sup> CFU/ml, respectively; *P* < 0.05] and 24 [(4.2 ± 1.6) × 10<sup>3</sup> versus (1.5 ± 1.0) × 10<sup>3</sup> CFU/ml, respectively; *P* < 0.05] h of incubation.

## DISCUSSION

The main conclusion from the present study is that hyperlipoproteinemia has deleterious effects on the outcome of severe *C. albicans* infection, in contrast to gram-negative bacterial infections. We have shown that LDLR<sup>-/-</sup> mice, with seven- to nine-times-higher LDL levels, are more susceptible to *C. albicans* infection than are their wild-type littermates. The earlier mortality of LDLR<sup>-/-</sup> mice was associated with increased outgrowth of *C. albicans* in their organs, and these mice produced significantly more proinflammatory cytokines than did control mice.

In general, mortality after infection may be due to lethal cytokinemia or to functional impairment by the growth of microorganisms in the organs of an animal. The increased susceptibility of LDLR<sup>-/-</sup> mice to *C. albicans* is likely to be due to the latter mechanism, because *C. albicans* did not induce an impressive cytokinemia. The hypothesis that cytokines are not responsible for the deaths of animals during *C. albicans* infection is in agreement with studies showing that the stimulation of neutrophils and macrophages by TNF and IL-1 enhanced their capability to kill *C. albicans* cells (6, 29), whereas anti-TNF antibodies (26) or pharmacologic inhibition of proinflammatory cytokines proved to be deleterious during severe *C. albicans* infection (20). Thus, proinflammatory cytokines seem to play a beneficial rather than a deleterious role in the defense against *C. albicans*. It should be noted that despite the greater cytokine response, LDLR<sup>-/-</sup> mice were not protected against *C. albicans* infection, probably due to the overwhelming outgrowth of yeast cells in their organs.

It may be hypothesized that the enhanced outgrowth of *C. albicans* in the organs of LDLR<sup>-/-</sup> mice is due to elevated lipoprotein concentrations. Normal serum has a candidicidal effect (22), and as shown by in vitro growth experiments, this property was significantly decreased in plasma samples from LDLR<sup>-/-</sup> mice. This effect may be due to the use of lipoproteins as a nutrition factor by *C. albicans*, as has been suggested by earlier studies showing increased growth of *C. albicans* in lipid-containing parenteral solutions compared with that in formulations without lipid contents (5, 9, 16). Another possible mechanism by which lipoproteins could influence *C. albicans* growth is interaction with other plasma factors. We cannot

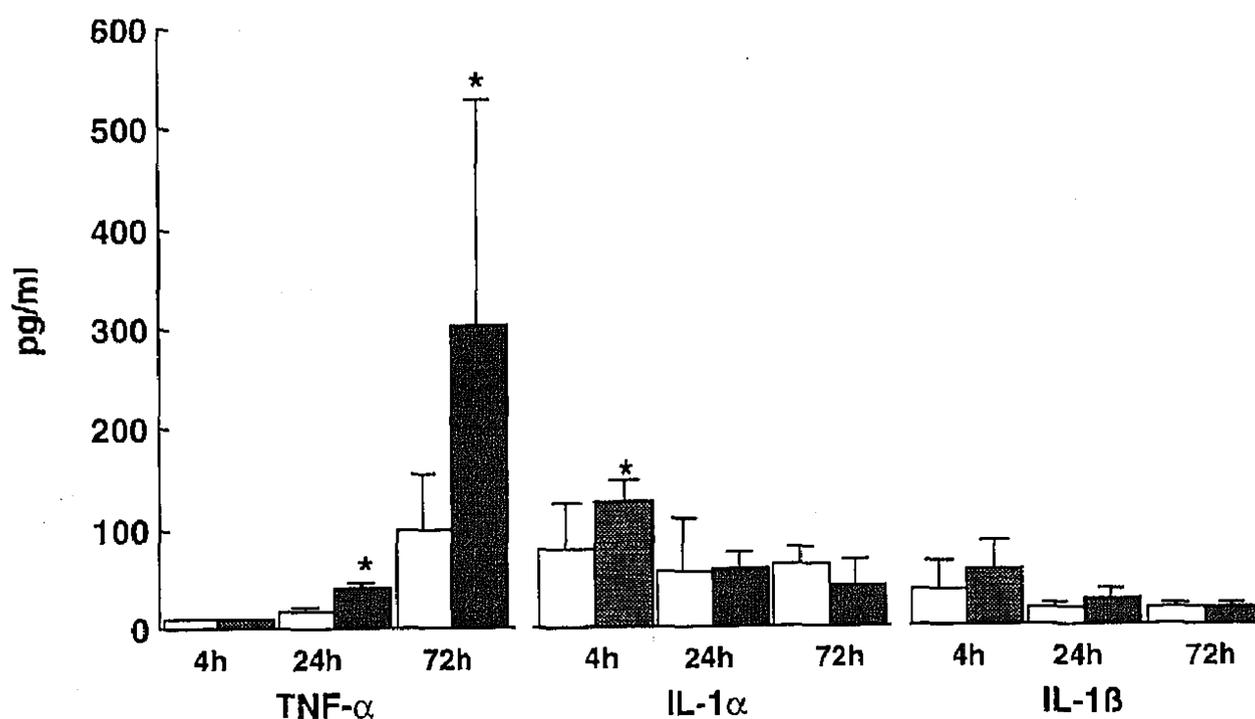


FIG. 2. In vivo cytokine concentrations during *C. albicans* infection. Shown are circulating concentrations of proinflammatory cytokines in control (open bars) and LDLR<sup>-/-</sup> (shaded bars) mice at 4, 24, and 72 h after the injection of 10<sup>6</sup> CFU of *C. albicans*. \*, *P* < 0.05.

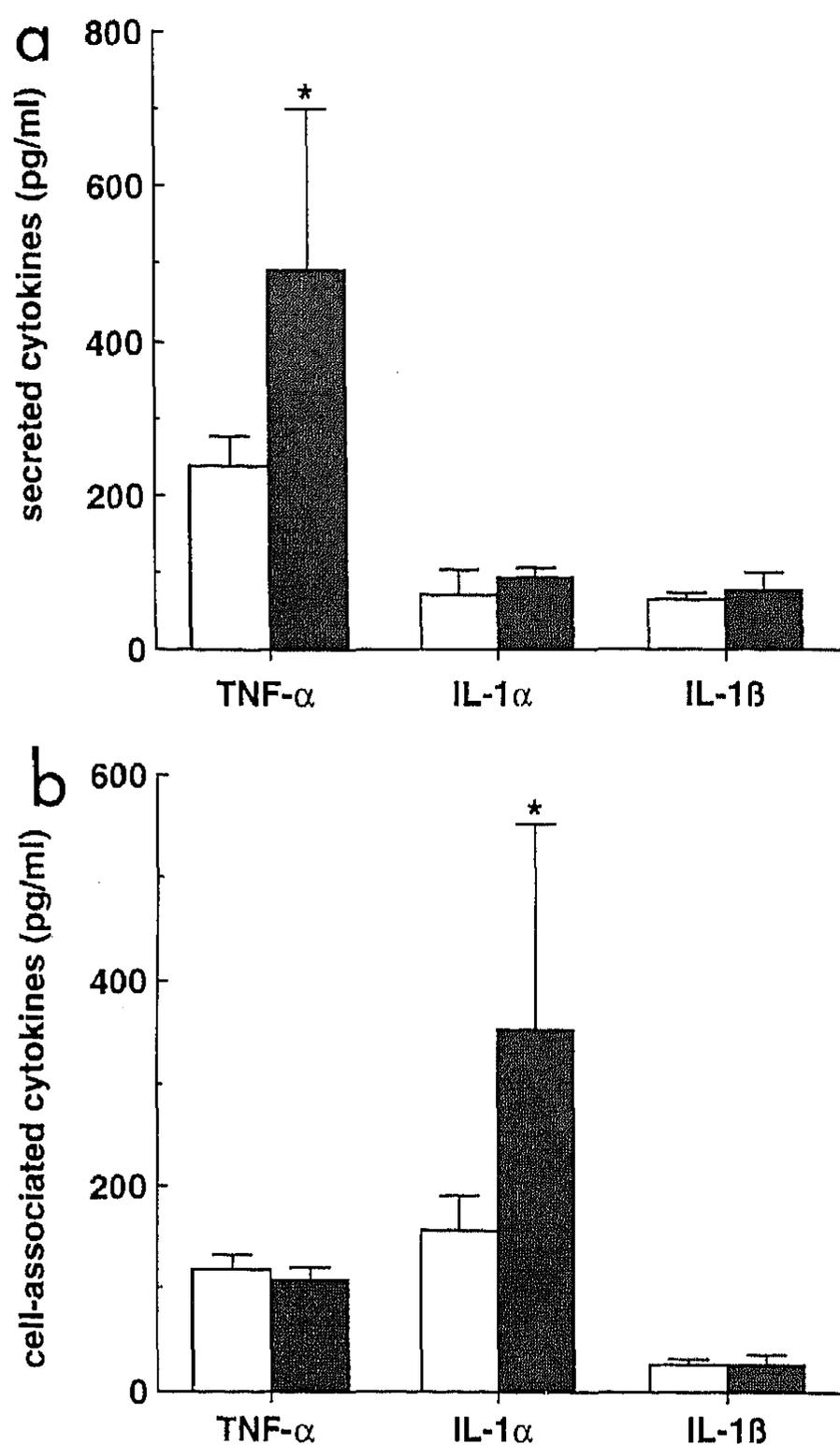


FIG. 3. In vitro cytokine production capacity of peritoneal macrophages. In vitro production of secreted (a) and cell-associated (b) cytokines by peritoneal macrophages of control (open bars) and LDLR<sup>-/-</sup> (shaded bars) mice was stimulated with heat-killed *C. albicans* ( $2 \times 10^7$  CFU/ml). \*,  $P < 0.05$ .

exclude the possibility that plasma candidicidal factors, such as platelet microbicidal protein (32) and the calprotectin complex (19), are bound and inactivated by lipoproteins.

The higher cytokine concentrations during infection in LDLR<sup>-/-</sup> mice, compared with those of controls, were probably at least in part a response of the host against enhanced *C. albicans* outgrowth in the organs of LDLR<sup>-/-</sup> mice. However, surprisingly, stimulated in vitro with heat-killed *C. albicans*, macrophages of LDLR<sup>-/-</sup> mice produced significantly more TNF-α and IL-1α than macrophages of control mice did. Most likely, this was due to the observed increased binding of *C. albicans* to macrophages of LDLR<sup>-/-</sup> mice compared with that of control macrophages. This phenomenon may be explained by the influence of constitutively increased lipoprotein concentrations in LDLR<sup>-/-</sup> mice on the *Candida*-binding proteins on macrophage. It has been shown previously that hypercholesterolemia is able to modify the number and clustering of other receptors, such as the LPS receptor CD14 (7, 25). Earlier, we observed similar higher binding of radiolabelled LPS to macrophages of LDLR<sup>-/-</sup> mice, followed by higher cytokine production (21). Thus, similar changes in the number and/or

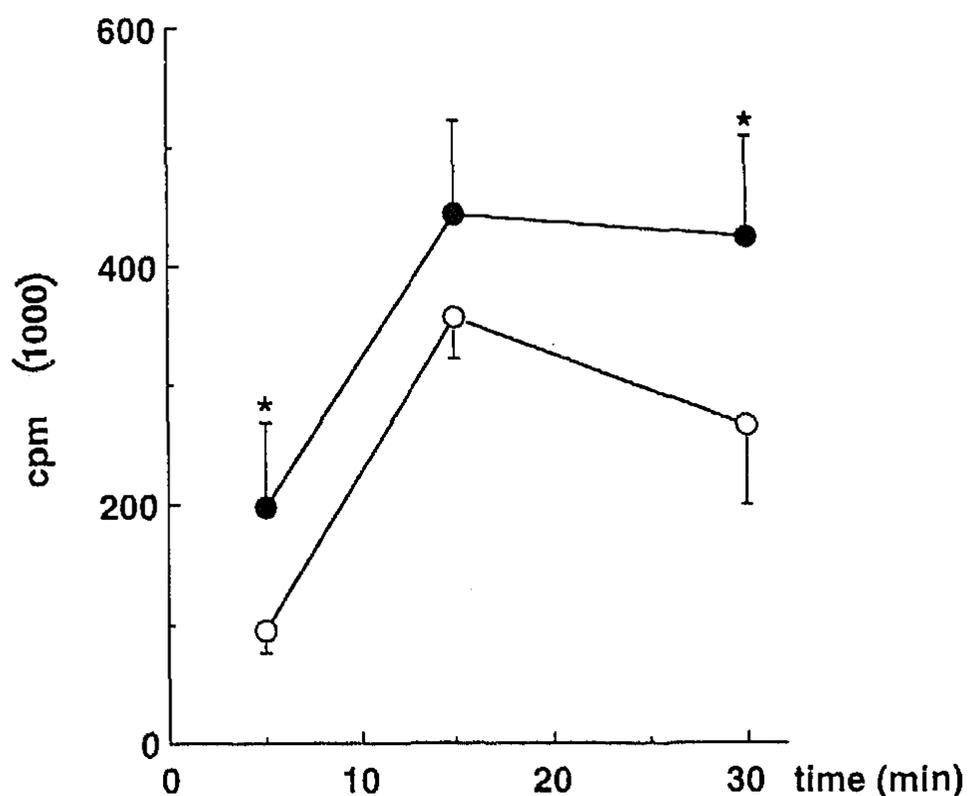


FIG. 4. *C. albicans* binding to peritoneal macrophages. Peritoneal macrophages of control (open circles) and LDLR<sup>-/-</sup> (closed circles) mice were incubated for various time intervals with radiolabelled *C. albicans* cells (1:5 ratio of macrophages to *C. albicans* cells). \*,  $P < 0.05$ .

clustering of *Candida*-binding proteins may facilitate the binding of *C. albicans* to macrophages of LDLR<sup>-/-</sup> mice, with a subsequent increase in cytokine production. Hyperlipoproteinemia could also modify the hydrophobicity of cells, which may also influence the adherence of *C. albicans* to macrophages, as has been shown for endothelial cells (10). Which of these mechanisms is responsible for the observed increase in cytokine production by macrophages of LDLR<sup>-/-</sup> mice is under study.

An alternative desirable experiment to our model in order to investigate the influence of hyperlipoproteinemia in *C. albicans* infection would have been to infuse lipoproteins into animals before and during infection. However, an infusion of lipoproteins into mice is not possible and other models of lipoprotein infusion in rabbits (11) and rats (24) are short-term models that are not suitable for sustained lipid infusion during systemic candidiasis. Therefore, the genetically modified mouse model is a good alternative for studying the in vivo effects of hyperlipoproteinemia in models of sustained infection.

In conclusion, hyperlipoproteinemia has deleterious effects on the course of acute disseminated *C. albicans* infection, in contrast to its beneficial effect in gram-negative infection. Although no epidemiological studies have been done to show a relationship between hyperlipoproteinemia and increased susceptibility to *C. albicans*, an infusion of lipoproteins into a patient with disseminated candidiasis under the presumptive diagnosis of gram-negative sepsis may prove deleterious. These divergent effects of hyperlipoproteinemia should be taken into account when the use of lipoproteins as an adjuvant treatment of sepsis is considered.

#### ACKNOWLEDGMENTS

We thank Ineke Verschuieren for performing cytokine determinations and Margo van den Brink, Monique Bakker, Theo van der Ing, and Yvette Brom for assistance with animal experiments.

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