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Effect of Simvastatin, an Established Lipid-Lowering Drug, on Pulmonary *Chlamydia pneumoniae* Infection in Mice

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The effects of simvastatin treatment on *Chlamydia pneumoniae* lung infection, inflammation, and serum lipids in mouse model were studied. Simvastatin decreased viable chlamydial counts and increased inflammatory cell infiltrates in the lung tissue, suggesting that simvastatin treatment had both antichlamydial and immunomodulatory effects during an acute *C. pneumoniae* infection.

Numerous studies have addressed the possible role of infectious agents in the pathogenesis of atherosclerosis, and *Chlamydia pneumoniae*, a human respiratory pathogen, displays the strongest association (7). Atherosclerosis is a combination of chronic inflammation and a cholesterol overload of endothelial macrophages (32). Statins are widely used as cholesterol-lowering drugs, and recently, growing interest has also been focused on their immunomodulatory actions (11, 18, 33a, 37). Clinical studies have shown that statins decrease cardiac events in persons with average cholesterol levels and slightly elevated C-reactive protein levels, and most importantly, they reduce elevated inflammatory markers, suggesting other protective mechanisms (26, 30, 31, 33). Several animal model and in vitro studies have also reported anti-inflammatory action (20, 34, 38). Recently, in humans and in two mouse models, statins have been shown to be beneficial during bacteremia and sepsis (1, 2, 22, 25).

Statins have been shown to affect *C. pneumoniae* infection in vitro: cerivastatin slightly decreases the infection rate in human macrophages and the infection rate of vascular smooth muscle cells through *C. pneumoniae*-infected monocytes (8, 19). Our aim was to study how lipophilic simvastatin affects *C. pneumoniae* infection in a mouse model and whether a high-fat diet modulates the outcome.

The Animal Care and Use Committee of National Public Health Institute, Helsinki, Finland, approved all procedures. Eight- to nine-week-old female NIH/S mice fed a regular chow diet ($n = 138$) (Altromin) or a high-fat diet ($n = 137$) (21% total fat, 0.2% cholesterol, and 19.5% casein; Harlan Teklad) were given simvastatin (L-644; Merck & Co., Inc.) in daily intraperitoneal injections (100 μl in 1% dimethyl sulfoxide) for 24 days (days −3 to 21 postinfection [p.i.]). At day 0, the mice were intranasally inoculated with *C. pneumoniae* Kajaani 7 (5.3×10^5 inclusion-forming units in 40 μl of saccharose-phosphate-glutamate [SPG] solution) (12). Samples ($n = 6$ mice) were collected after a minimum 4-h fast. (Fig. 1). The right lung was mechanically homogenized in 2 ml of SPG solution, and the supernatant was cultured in HL cells. For inclusion detection, the Pathfinder Chlamydia Confirmation System was used (Kallestad Diagnostics). DNA was extracted using the QIAamp tissue kit (QIAGEN GmbH). *C. pneumoniae* LightCycler real-time quantitative PCR (Roche) was performed using 16S rRNA-specific primers and a hybridization probe (13, 29). *C. pneumoniae* immunoglobulin G antibodies (serum dilution, 1:100) were measured by enzyme immunoassay (Ani-Labsystems). Inflammatory changes in the lungs were determined by histology from hematoxylin and eosin–stained longitudinal cross sections and graded as no changes (histology score = 0), minimal (score = 1), slight (score = 2), moderate (score = 3), marked (score = 4), or severe (score = 5), depending on the number of mononuclear cells and the area affected. In the milder forms, the inflammatory cell infiltrates were limited to focal areas or occurred in small scattered foci, but in the severe cases, large tissue areas were affected. Serum amyloid A concentrations were measured by enzyme immunoassay (BioSource International), and lipids were measured with fully enzymatic methods (Roche Diagnostics and Wako Chemicals GmbH). For statistical analysis, the nonparametric Mann-Whitney U test was used.

Following simvastatin treatment, inclusion-forming-unit counts in the lungs at the early stages of infection (days 3 and 6 p.i., respectively) were reduced 65 to 80% from those for the vehicle-treated mice with a regular diet (Fig. 2A) and 55 to 82% for mice on a high-fat diet (Fig. 2B). Similar decreases in chlamydial genome numbers, 78 to 83% (in regular diet-fed mice, day 6 p.i.; $P = 0.002$) were also demonstrated by PCR.

Simvastatin treatment slightly increased pulmonary inflammation at each time point in mice on a regular diet (histology score, 1.5 to 3.2 versus. 1.2 to 2.7, respectively) (Fig. 2C) and also on a high-fat diet (Fig. 2D). Serum immunoglobulin G antibody responses against *C. pneumoniae* and the serum amyloid A concentrations were not affected by simvastatin treatment (data not shown).

After 2 weeks on a high-fat diet, total cholesterol levels increased by 70.9% (day −3 p.i.; $P = 0.002$) and triglyceride

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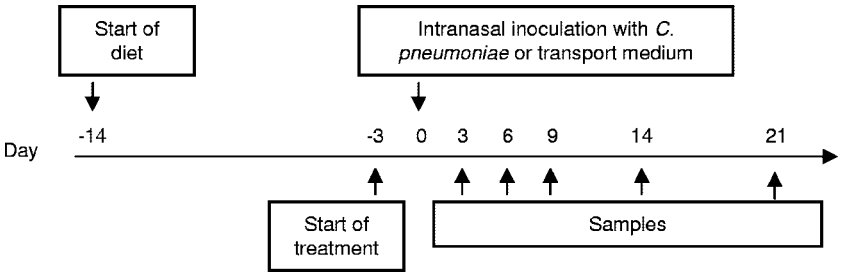

FIG. 1. Experimental design for the two separate experiments using different diets. Feeding with the high-fat diet was initiated 2 weeks, and treatment with simvastatin (0.5 mg/kg of body weight) or 1% dimethyl sulfoxide 3 days, prior to the *C. pneumoniae* challenge/SPG inoculation. Samples were taken until day 20 p.i.

levels by 37.5% (P = not significant). Simvastatin had no effects on serum lipid levels (Table 1).

The present study showed that simvastatin treatment affects the course of acute *C. pneumoniae* infection by decreasing chlamydial counts in the lungs and, surprisingly, by amplifying the pulmonary inflammatory response in infected mice. Interestingly, statins have displayed antimicrobial effects in recent studies: they reduce the intracellular growth of *Salmonella enterica* serovar Typhimurium both in vitro and in vivo and the intracellular replication of cytomegalovirus, human immunodeficiency virus, and *C. pneumoniae* in vitro (6, 9, 19, 27). Hydroxymethylglutaryl (HMG)-coenzyme A reductase is an important enzyme catalyzing the rate-limiting reaction of the mevalonate pathway, leading to biosynthesis of isoprenoids and cholesterol in eucaryotes. The enzyme is also found in some gram-positive bacteria; thus, statins, inhibitors of the HMG-coenzyme A reductase, might have a direct antibiotic effect (14). *Chlamydia* is a gram-negative bacterium and does not have HMG-coenzyme A reductase but has genes encoding nonmevalonate isoprenoid pathway enzymes (21). Further,

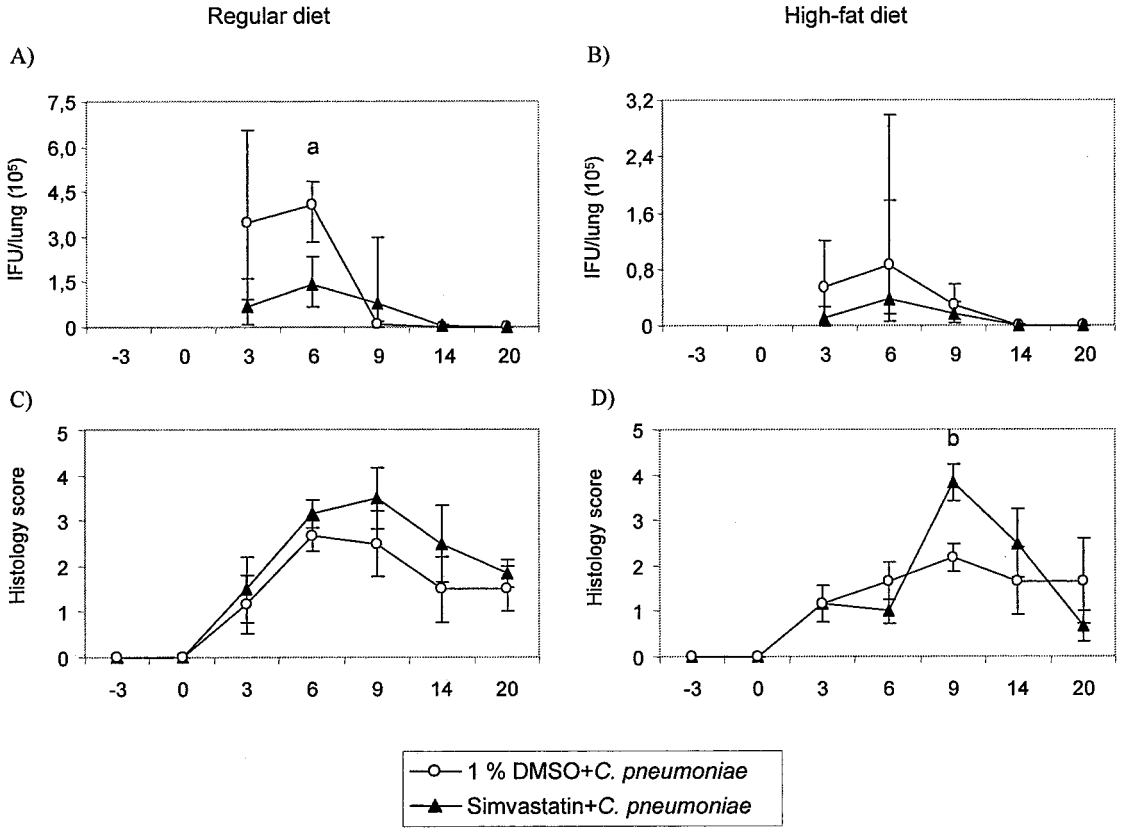

FIG. 2. Chlamydia culture and pulmonary histopathology findings of *C. pneumoniae*-infected NIH/S mice fed either a regular diet (A and C) or a high-fat diet (B and D). Mice were treated with simvastatin (0.5 mg/kg of body weight) or 1% dimethyl sulfoxide (DMSO). (A, B) Chlamydia culture results from lung tissue, median ± interquartile range (25th and 75th percentile). "a" indicates simvastatin-treated *C. pneumoniae*-infected regular-diet-fed mice compared to 1% DMSO–treated *C. pneumoniae*-infected regular-diet-fed mice; $P = 0.026$. (C, D) Pulmonary histopathology results, mean histology scores ± standard errors. "b" indicates simvastatin-treated *C. pneumoniae*-infected high-fat-diet-fed mice compared to 1% DMSO-treated *C. pneumoniae*-infected high-fat-diet-fed mice; $P = 0.015$. The nonparametric Mann-Whitney U test was used.

TABLE 1. Serum lipid levels at baseline (day −3 p.i.) and at the end point (day 20 p.i) of SPG inoculated mice treated with 0.5-mg/kg simvastatin

Diet	Amt of total cholesterol, mmol/liter, median (IQR[a])		Amt of triglycerides, mmol/liter, median (IQR[a])	
	Baseline	End point	Baseline	End point
Regular diet	3.3 (2.6–3.6)	3.3 (3.1–3.6)	1.9 (1.6–2.4)	2.0 (1.9–2.1)
Fatty diet	5.7 (5.4–6.6)	6.4 (5.9–6.9)	2.6 (2.4–3.0)	2.3 (2.2–2.4)

[a] Interquartile range, 25th to 75th percentile.

Chlamydia and *Salmonella* do not have the capacity to synthesize cholesterol, but cholesterol is required for their intracellular multiplication and is also an essential component of, e.g., chlamydial particles (39). Thus, these bacteria are dependent on the availability of host cholesterol inside the cells where they replicate (5, 6). *Chlamydia* may use cholesterol derived either from the extracellular space via low-density lipoprotein uptake or from intracellular cholesterol stores (5). Lipophilic simvastatin has easy access to all cell types in which *C. pneumoniae* multiplies, and by decreasing host cell isoprenoid and cholesterol levels or by disturbing intracellular trafficking of cholesterol, statins may affect chlamydial intracellular multiplication (8, 15, 16). Statins may also interfere with the chlamydial cell entry, as during human immunodeficiency virus infection (9). Specific plasma membrane microdomains, caveolae or lipid rafts, are rich in cholesterol and are important for the entry of several *Chlamydia* species, including *C. pneumoniae* (36).

The second interesting finding was that simvastatin amplified pulmonary inflammatory response by increasing inflammatory cell infiltration into the lungs during acute *C. pneumoniae* infection. The anti-inflammatory effect of statins with noninfectious stimuli has been reported in several in vivo studies (10, 24, 28, 34, 35, 40). However, Kiener et al. have previously pointed to the possible proinflammatory effects of statins, and they also showed that lipophilic statins increase cellular influx to the peritoneal cavity after injection of thioglycolate (17). The present study is the first one in which the effect of a statin treatment on immunological parameters after an active infection caused by an intracellularly multiplying pathogen has been studied in vivo under controlled experimental conditions.

In mice, a cholesterol-lowering effect of statins has been achieved with high doses even in mice lacking low-density lipoprotein receptors (4). In the present study, with the simvastatin dose similar to the therapeutic dose for treating hypercholesterolemia in humans, we did not see any decrease in the total cholesterol or triglyceride levels independent of the diet. The response to a high-fat diet was displayed as elevated serum cholesterol and triglycerides. Cholesterol increased especially in the high-density lipoprotein (HDL) fraction (data not shown). Since mice preferentially have HDL in circulation, it might well be that simvastatin does not affect this pool of cholesterol. Human statin trials depict only minor effects on HDL levels (23).

In conclusion, the present study confirms the previous in vitro findings that statin treatment may have an antichlamydial effect in vivo, too (19). The present data further suggest a possible proinflammatory effect in the antichlamydial process in vivo. However, probably due to the low numbers of mice in different groups, statistically significant differences between treatment and control groups were reached only at a few time points. The short follow-up of the present study does not allow any speculation on the effects of statin treatment on chronic *C. pneumoniae* infection, which is considered important in the development of atherosclerosis.

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