Adenovirus-Mediated Transfer of a Gene Encoding Acyloxyacyl Hydrolase (AOAH) into Mice Increases Tissue and Plasma AOAH Activity

M. G. COULTHARD,[1] J. SWINDLE,[2] R. S. MUNFORD,[2,4]* R. D. GERARD,[3] AND R. S. MEIDELL[2]

Departments of Pediatrics,[1] Internal Medicine,[2] Biochemistry,[3] and Microbiology,[4] University of Texas Southwestern Medical Center, Dallas, Texas 75235

Although the host response to gram-negative bacterial infection follows largely from the interactions of bacterial lipopolysaccharides (LPS or endotoxin) with host cells, little information is available concerning the mechanisms by which the host eliminates or detoxifies LPS. Acyloxyacyl hydrolase (AOAH) is an enzyme, found in phagocytic cells, that catalyzes the enzymatic deacylation of the lipid A moiety of LPS. Enzymatically deacylated LPS is much less potent than LPS at inducing responses in human cells, and it can antagonize the ability of LPS to activate human macrophages, neutrophils, and endothelial cells. Despite these observations, the physiologic role of LPS deacylation remains undefined. To investigate the ability of AOAH to carry out LPS deacylation in vivo, we produced a recombinant adenovirus carrying a gene encoding AOAH (Ad.CMV-AOAH) and employed this vector to elicit transient overexpression of AOAH in mice. Mice infected with Ad.CMV-AOAH expressed high levels of the enzyme in plasma, liver, spleen, and kidney. Although adenovirus-induced hepatitis reduced hepatic uptake of intravenously injected [^{3}H]LPS, animals expressing the transgene deacylated a larger fraction of the [^{3}H]LPS taken up by their livers than did mice infected with a control adenovirus. These studies indicate that AOAH can catalyze the deacylation of LPS in vivo, and they provide evidence that the rates of hepatic LPS uptake and deacylation are not closely linked.

The capacity of host phagocytic cells to activate in response to bacterial lipopolysaccharide (LPS or endotoxin) is an important defense against gram-negative bacterial infection. This activation is triggered largely by interactions of the lipid A moiety of LPS with critical host cells, mediated by binding LPS to cellular CD14. Although much has been learned recently about host systems for recognizing and responding to LPS, relatively little is known about the mechanisms by which LPS is detoxified in vivo. Since an excessive response to LPS may have deleterious effects culminating in the syndrome of septic shock, there is substantial medical interest in these mechanisms and in the development of strategies to limit the host response to LPS.

Previous studies from this laboratory have explored the potential endotoxin-detoxifying role of acyloxyacyl hydrolase (AOAH), a phagocytic cell enzyme that removes secondary acyl chains from the lipid A moiety of LPS (11). Enzymatically deacylated LPS (dLPS) is 100-fold less potent than LPS at eliciting the dermal Shwartzman reaction in rabbits (22). In vitro, dLPS is also substantially less active than LPS at stimulating human monocytes, neutrophils, and endothelial cells (25). Moreover, it competitively inhibits binding of LPS to CD14 at concentrations near its binding K_d (14) and potently antagonizes the ability of LPS to stimulate NF-κB binding activity and interleukin 1 and interleukin 8 release from human cells (14, 15). Enzymatic deacylation of LPS by AOAH might thus function both to detoxify LPS and to generate an LPS antagonist in humans.

While the activity of AOAH and the effects of dLPS have been characterized in cultured cells, the function(s) of AOAH in vivo has not been examined previously. One approach to evaluating the biological role of a molecule is to examine the consequences of increasing its abundance. This may be accomplished by directly administering the molecule to animals, by creating transgenic animals that overexpress it, or by transiently overexpressing a gene that encodes it following in vivo gene transfer. To investigate the role of AOAH, we constructed a recombinant adenovirus carrying a gene in which a cDNA coding for human AOAH is expressed from the human cytomegalovirus immediate-early promoter-enhancer (Ad.-CMV-AOAH). On the basis of our prior observations of foreign gene expression following systemic administration of recombinant adenoviruses (12, 17, 26), we anticipated that intravenous administration of this recombinant adenovirus to mice would result in transient, constitutive overexpression of the human enzyme in the liver. Since the liver is the principal site of uptake of LPS from the circulation, we reasoned that hepatic overexpression of AOAH might accelerate LPS deacylation. In addition, if hepatically synthesized AOAH were released into the circulation, interaction of AOAH with circulating LPS might alter LPS clearance from the plasma space. Since both dLPS (22) and other tetraacyl lipid A analogs (8, 18) retain much of the potency of LPS in mice, on the other hand, AOAH overproduction would not be expected to protect mice against LPS challenge.

We now describe experiments demonstrating that intravenous administration of Ad.CMV-AOAH to mice results in striking increases in AOAH activity in the liver, spleen, and kidney and that overexpression of AOAH in the liver produces a proportional increase in the rate at which intravenously administered [^{3}H]LPS undergoes hepatic deacylation. Overexpression of AOAH also increased enzyme activity in plasma, yet the deacylation of [^{3}H]LPS in the plasma space was not enhanced and the rates of clearance of rough- and smooth-form LPS from the plasma space were not altered.

* Corresponding author. Mailing address: Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9113. Phone: (214) 648-3480. Fax: (214) 648-9478.

MATERIALS AND METHODS

Reagents. Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise stated. All solutions were prepared in baked glassware with pyrogen-free water (Baxter Healthcare, Deerfield, Ill.), and tissue culture media used in virus preparation were prepared by use of low-endotoxin fetal bovine serum (HyClone, Logan, Utah). [^{3}H, ^{14}C]LPS, used as substrate for enzyme assays, was prepared from *Salmonella typhimurium* as described previously (21). Rough [^{3}H]LPS was prepared from *Escherichia coli* LCD25 (20); its specific activity was 2×10^6 dpm/μg. Smooth (O-antigen-containing) [^{3}H]LPS was prepared from *S. typhimurium* G-30, grown in the presence of (i) [^{3}H]acetate and 1.0 mM D-galactose (11) or (ii) [^{3}H]galactose and 0.05 mM nonradioactive D-galactose (23). The smooth LPS were isolated by standard phenol-water extraction followed by extensive ether washes to remove contaminating phospholipids (21). Their specific activities were 2,000 and 75,000 dpm/μg, respectively.

Preparation of recombinant adenoviruses. The recombinant adenovirus Ad.CMV-AOAH was generated by homologous recombination between subgenomic fragments of a modified adenovirus genome in 293 cells as described previously (7). Briefly, a full-length cDNA encoding human AOAH (10) was subcloned into the plasmid pACCMV.pLpA (7), and the resulting plasmid (10 μg) was cotransfected into 293 cells with 5 μg of *Xba*I-digested ADdl309 DNA by calcium phosphate coprecipitation with a glycerol shock to boost transfection efficiency. Homologous recombination between the pAC plasmid and the right 91% fragment of the viral genome resulted in the formation of a recombinant viral genome of packageable size in which the CMV-AOAH fusion gene replaces the native adenovirus early region 1.

Clonally derived recombinant viruses were isolated and propagated as described previously (17) and characterized by restriction analysis and Southern blotting of viral DNA prepared from productively infected 293 cells. Recombinant viruses were subcloned twice to ensure purity. Generation of the recombinant adenoviruses Ad.CMV-Luc, carrying a gene encoding firefly luciferase, and Ad.CMV-βgal, carrying a gene encoding a nuclear-localizing variant of *E. coli* β-galactosidase, have been described elsewhere (12).

Large-scale virus preparation. Large-scale adenovirus production was performed following infection of 293 cells grown to confluence in Dulbecco modified Eagle medium containing 10% fetal bovine serum. Infected cells were lysed with 0.1% Nonidet P-40, and the cellular debris was removed by centrifugation at $12,000 \times g$ for 10 min at 4°C. Virus particles were precipitated by adding 0.5 volume of a solution containing 20% (wt/vol) polyethylene glycol 8000 and 2.5 M NaCl and then incubated on ice for 1 h. The virus was collected by centrifugation ($12,000 \times g$, 20 min, 4°C), resuspended in 1.1 g of CsCl per ml in 20 mM Tris-HCl (pH 7.4), and further purified by ultracentrifugation over a CsCl step gradient. Banded virus was collected and desalted by chromatography over a Sepharose CL-4B column in an isotonic saline buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 20 mM Tris-HCl [pH 7.4]) as described previously (7). The virus was collected after elution from the column, and low-endotoxin bovine serum albumin (BSA) was added (final concentration, 100 μg/ml) prior to storage at −70°C. Viral titer was determined by plaque assay on confluent monolayers of 293 cells (9).

Animal procedures. Male ICR and BALB/c mice (20 to 24 g) were purchased from Harlan (Indianapolis, Ind.) and housed and fed ad libitum in the Animal Resources Center. All animal procedures and experiments were conducted in accordance with National Institutes of Health guidelines and under protocols approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center at Dallas. All procedures were performed while the mice were under anesthesia obtained by intraperitoneal injection of sodium pentobarbital (100 μg/g of body weight). Adenoviruses were injected into the tail vein in a volume of 250 μl. Blood samples were taken by puncture of the retroorbital vein. Mice were euthanized by cervical dislocation following sedation by intraperitoneal injection of sodium pentobarbital (100 μg/g of body weight). Organs or tissue slices were weighed and homogenized (Tissue Tearor; Biospec Products, Bartlesville, Okla.) in lysis buffer (phosphate-buffered saline, 0.2% Triton X-100, 2.5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml, 0.5 μg of aprotinin per ml, 1 mg of BSA per ml) at 4°C and stored at −20°C.

Assays. AOAH activity was measured as described previously (21). Samples were incubated overnight at 37°C with 0.5 μg of [^{3}H, ^{14}C]LPS in a reaction mixture that contained 0.1% Triton X-100, 20 mM Tris-citrate (pH 4.8), 150 mM NaCl, and 1 mg of BSA per ml in a volume of 0.5 ml (21). Following ethanol precipitation of the LPS, the ^{3}H-fatty acids remaining in the supernatant were counted in a Minaxi Tricarb 4000 beta scintillation counter (Packard Instruments, Laguna Hills, Calif.). The specific activity of the acyl chains in the LPS substrate was approximately 50,000 ^{3}H dpm/nmol. Luciferase activity in liver tissue was determined as described previously (1, 27).

Determination of LPS deacylation, clearance, and distribution. Plasma clearance of rough [^{3}H]LPS was determined by intravenous injection of 10 μg of *E. coli* LCD25 [^{3}H]LPS (or a mixture that contained 1 μg of rough [^{3}H]LPS and 9 μg of nonradioactive LCD25 LPS) 3 or 4 days after mice had been infected with 1×10^9 to 2×10^9 PFU of Ad.CMV-AOAH, Ad.CMV-βGal, or saline. Mice were euthanized at 5 min, 30 min, or 6 h after LPS administration. Approximately 0.5 ml of blood was collected into microcentrifuge tubes containing 5 μl of 0.27 M EDTA, and the ^{3}H in the aliquots of plasma was counted following the
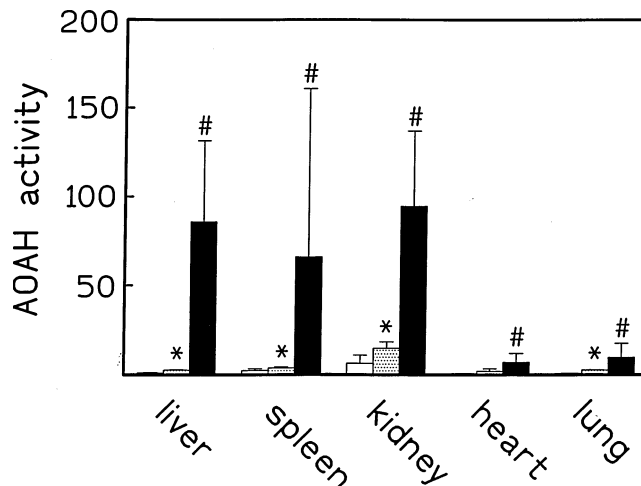

FIG. 1. AOAH activity in tissues. Mice (four per group) were injected with 250 μl of saline (open bars) or an equal volume of saline containing 2.5×10^8 PFU of Ad.CMV-βGal (stippled bars) or Ad.CMV-AOAH (shaded bars). After 4 days, samples of the indicated tissues were harvested and assayed for AOAH activity. Activity is expressed as ^{3}H dpm (10^3) released from the labeled LPS substrate during 18 h of incubation at 37°C (per milligram of tissue). Data are expressed as mean ± standard error of the mean for each treatment. Symbols: *, $P < 0.05$ versus saline-injected animals; #, $P < 0.05$ versus saline- and Ad.CMV-βGal-infected animals.

addition of sodium dodecyl sulfate-EDTA (8) and scintillant. The theoretical initial (t_0) plasma concentration of [^{3}H]LPS was determined by dividing the total dose administered by the plasma volume (in milliliters), calculated as [animal weight (grams)] × [blood volume (0.08 ml/g)] × [1 − hematocrit, or 0.45]. The percentage of the initial ^{3}H remaining in the plasma at the various time points was then calculated. Organs were weighed and homogenized as described above.

Smooth LPS clearance from plasma was determined 4 days following injection of 1×10^9 to 2×10^9 PFU of Ad.CMV-AOAH, Ad.CMV-βGal, or saline. Mice were bled at 5 min, 60 min, or 6 h after intravenous injection of either 100 μg of smooth [^{3}H]acetate-labeled LPS (two experiments) or 5 μg of [^{3}H]galactose-labeled LPS (one experiment), and the plasma LPS concentrations were expressed as a percentage of the initial plasma [^{3}H]LPS concentration as described above.

In the rough and the [^{3}H]acetate-labeled smooth LPS, essentially all of the ^{3}H disintegrations per minute are in fatty acyl chains (20). [^{3}H]LPS deacylation in liver or plasma was determined by measuring the total and ethanol-soluble ^{3}H. Thirty minutes after intravenous injection of [^{3}H]LPS, aliquots of liver homogenates in lysis buffer were mixed with ethanol (1 volume of lysate to 3 volumes of ethanol), held at −20°C for 2 h, and then centrifuged ($12,000 \times g$, 10 min, 4°C). The ^{3}H remaining in the supernatant (i.e., no longer part of the LPS, which is ethanol insoluble) was counted. The total ^{3}H in the samples was also counted, and the fraction of ^{3}H in the ethanol supernatant was expressed as a percentage. Under optimal conditions, AOAH can remove approximately 30% of the ^{3}H from [^{3}H]LPS (two of the six fatty acyl chains in the LPS).

Data analysis. Group data were analyzed by Analysis of Variance (Kwikstat Version 4; TexaSoft, Dallas, Tex.) and the Newman-Keuls multiple comparison test. Differences at the level of P of <0.05 were considered significant. Relationships between liver and plasma enzyme activities were examined by linear regression.

RESULTS

Mice injected intravenously with Ad.CMV-AOAH demonstrated significant increases in AOAH activity in the liver, spleen, and kidney 3 to 4 days after infection (Fig. 1). Enzyme activity in liver tissue harvested from Ad.CMV-AOAH-infected animals was more than 40-fold greater than that in tissue harvested from uninfected control animals and more than 10-fold greater than that in liver from animals infected with the irrelevant virus Ad.CMV-βGal. Notably, animals infected with Ad.CMV-βGal showed a small but significant increase in tissue AOAH activity over that of uninfected control animals (Fig. 1).
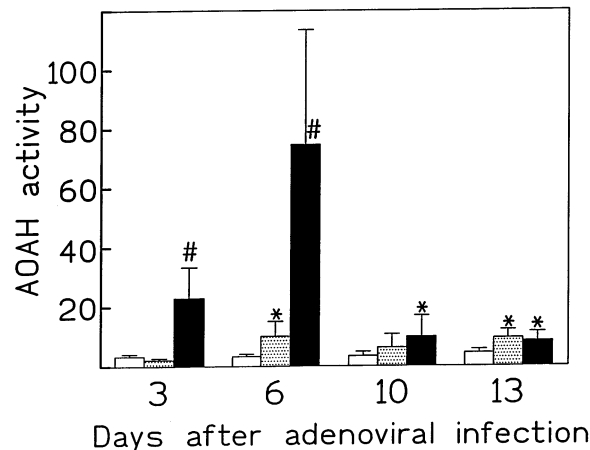
FIG. 2. Time course of plasma AOAH activity in Ad.CMV-AOAH-infected and control mice. Mice were injected with 250 μl of saline ($n = 5$; open bars) or an equal volume of saline containing 2×10^9 PFU of Ad.CMV-βGal ($n = 5$; stippled bars) or Ad.CMV-AOAH ($n = 9$; shaded bars). Blood samples for determination of plasma AOAH activity were obtained 3, 6, 10, and 13 days after injection. See legend to Fig. 1 for definitions of AOAH activity and symbols.

FIG. 3. In vivo deacylation of [³H]rough LPS in Ad.CMV-AOAH-infected and control mice. (A) Mice were injected intravenously with *E. coli* LCD25 [³H]LPS 3 days after injection of 250 μl of saline ($n = 5$) or an equal volume of saline containing 2×10^9 PFU of Ad.CMV-βGal ($n = 7$) or Ad.CMV-AOAH ($n = 13$). After 30 min, livers were harvested and total and ethanol-soluble ³H radioactivities were determined. Data are expressed as the fraction (percent) of total ³H dpm in the ethanol-soluble fraction (mean ± standard error of the mean). Symbols: ∗, $P < 0.05$ versus saline-injected and Ad.CMV-βGal-infected animals. Results from a similar experiment are shown in Table 1. (B) Correlation between in vivo deacylation of ³H-labeled LCD25 LPS and hepatic AOAH activity (determined in hepatic tissue harvested from the Ad.CMV-AOAH-infected animals shown in panel A after 500-fold dilution to eliminate assay interference by [³H]LPS in the tissue). AOAH activity is expressed as described in the legend to Fig. 1.

Overexpression of AOAH in infected mice was associated with a significant but transient increase in plasma AOAH activity (Fig. 2 and data not shown). Three days after infection, plasma enzyme activity in Ad.CMV-AOAH-infected animals was 5- to 10-fold greater than that in uninfected or Ad.CMV-βGal-infected controls. Plasma AOAH activity peaked 4 to 6 days after infection at levels more than 20-fold greater than those in uninfected controls. At 10 and 13 days after infection, plasma AOAH activities were not significantly different from levels in animals infected with the control recombinant adenovirus. There was a strong correlation between plasma and liver AOAH activities in individual animals following infection with Ad.CMV-AOAH ($n = 13$ mice; $r = 0.86$, $P = 0.0001$).

LPS exists in two forms, S or smooth (O-antigen containing) and R or rough (lacking O antigen), which are cleared from plasma in different ways. While the former (like the LPS in intravenously injected bacteria [6]) is initially cleared almost exclusively by phagocytic cells (Kupffer cells and intravascular granulocytes), the latter is cleared principally by hepatocytes (3, 13). Because systemic administration of recombinant adenovirus is associated with preferential infection of hepatocytes (12), we examined the rate of deacylation of rough [³H]LPS to determine whether overexpression of AOAH would increase the rate of intracellular LPS deacylation in vivo. The LPS deacylation rate was determined by measuring the fraction of the radiolabel that appeared in an ethanol-soluble form in hepatic tissue harvested from saline, Ad.CMV-βGal-, and Ad. CMV-AOAH-injected animals 30 min following intravenous administration of rough-form [³H]LPS. As shown in Fig. 3A, infection of mice with Ad.CMV-AOAH resulted in a significantly increased (approximately sixfold, $P < 0.05$) rate of hepatic LPS deacylation in comparison with that of control animals. Similar results were found in another experiment (Table 1). In view of the fact that AOAH removes only two of the six acyl chains from the lipid A moiety of LPS, the observed release of 4 to 6% of the radiolabel from rough LPS over the 30-min study period is consistent with enzymatic deacylation of approximately 12 to 18% of the liver-associated LPS within this time period. There was a positive correlation between the rate of in vivo hepatic deacylation of rough LPS and the assayed hepatic AOAH activity (Fig. 3B).
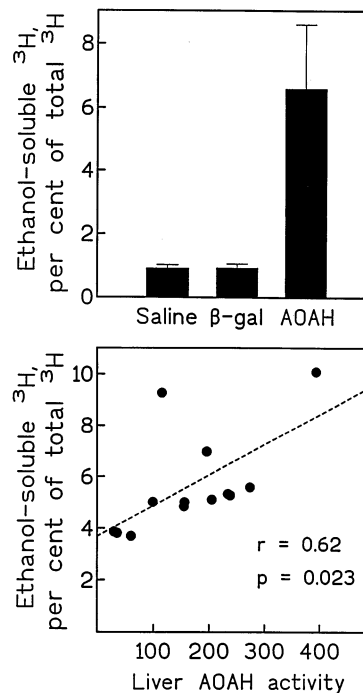
In contrast to the increased rate of LPS deacylation produced by overexpressing AOAH in the liver, the accumulation of AOAH activity in plasma following infection of mice with Ad.CMV-AOAH was not associated with detectable in vivo deacylation of rough-form [³H]LPS in the plasma space; i.e., ethanol-soluble ³H in plasma 30 min following administration of [³H]LPS was not increased in Ad.CMV-AOAH-infected mice, despite the presence of increased levels of assayable AOAH in plasma (data not shown). Although the rapid clearance of rough-form LPS from the circulation allowed a relatively brief time period for intravascular deacylation to occur, accumulation of AOAH in plasma also did not significantly alter the rate of clearance of smooth-form [³H]LPS from the plasma space (Fig. 4) or enhance its deacylation in plasma (data not shown). The unavailability of smooth LPS radiolabeled to high specific activity in the acyl chains precluded a sensitive assessment of its deacylation in vivo, however. Another potential explanation for low plasma deacylation rates could be the uptake of LPS-derived ³H-fatty acids from plasma by various tissues; the lack of correlation between plasma and liver ethanol-soluble ³H argues against this possibility.

While clearance of a small or large amount of smooth-form LPS was not altered by overexpression of AOAH, we did observe that infection of mice with recombinant adenovirus per se altered the uptake of rough-form [³H]LPS into the liver

TABLE 1. Impact of viral infection on the tissue uptake and deacylation of intravenously injected rough [³H]LPS[a]

Group	n	% Injected [³H]LPS in:			Spleen weight (mg)	% [³H]LPS deacylation[b]
		Plasma	Liver	Spleen		
Saline injected	5	4.6 ± 1.4	50.1 ± 8.4	1.8 ± 0.3	110 ± 17	1.3 ± 0.1
Ad.CMV-βGal infected	7	13.2 ± 4.4*	23.0 ± 3.2*	14.3 ± 2.3*	184 ± 27*	1.5 ± 0.1
Ad.CMV-AOAH infected	13	13.7 ± 4.5*	24.8 ± 2.7*	11.3 ± 4.0*	170 ± 27*	4.3 ± 0.7**

[a] Three days after injection with saline or 1.5×10^9 PFU of one of the indicated adenoviruses, each mouse received a 10-μg intravenous injection of [³H]LPS. Tissues were weighed and homogenized 30 min later, and aliquots were counted to determine ³H content. Data indicate means ± standard deviations. *, significantly different from saline-injected group ($P < 0.05$); **, significantly different from the other groups ($P < 0.05$).
[b] Percent deacylation = $100 \times$ [(ethanol-soluble ³H dpm)/(total ³H dpm)] in liver homogenates.

and spleen and, in some experiments, modestly delayed its clearance from plasma (Table 1). When compared with uninfected control animals, animals infected with either Ad.CMV-AOAH or Ad.CMV-βGal showed a fractional reduction in hepatic uptake of rough-form [³H]LPS and an increase in fractional splenic uptake. Infection of mice with first-generation recombinant adenovirus vectors is associated with an acute lymphocytic hepatitis (28). Consistent with this phenomenon, we observed an increase in serum alanine aminotransferase levels 6 days following infection with 2.0×10^9 PFU of recombinant adenovirus in comparison with that of saline-injected control animals (404 ± 447 IU/ml [$n = 8$] versus 34 ± 4 IU/ml [$n = 4$], $P = 0.05$). It is possible that the delayed plasma clearance and altered tissue distribution of intravenously injected rough-form [³H]LPS resulted from this viral vector-associated hepatocellular injury. In addition, animals infected with either Ad.CMV-AOAH or Ad.CMV-βGal demonstrated noticeable splenic enlargement 3 to 4 days after infection, and spleen weights in both groups were significantly greater (by approximately 50%) than those in saline-injected controls (Table 1).

In the course of these experiments, several groups of animals were infected with Ad.CMV-AOAH. We observed considerable mouse-to-mouse variability in the levels of hepatic and plasma AOAH activities, despite careful efforts to standardize the procedure for administration of virus (injections in any group were performed within 1 h by an experienced technician with a single virus preparation and no observed loss of inoculum). To investigate the cause of this interanimal variation, we performed several additional experiments. First, we measured hepatic AOAH expression in groups ($n = 11$ to 13 per group) of male ICR (outbred) and BALB/c (inbred) mice. Both strains demonstrated similar variability in hepatic AOAH activity (ranging from 3- to 21-fold between the highest and lowest producers in individual groups). Second, we infected ICR mice with an inoculum containing both Ad.CMV-AOAH (1.5×10^9 PFU) and Ad.CMV-Luc (a recombinant adenovirus carrying a gene encoding firefly luciferase; 0.25×10^9 PFU) and determined the activities of both enzymes in hepatic tissue 3 days after infection. We observed a strong correlation between the levels of AOAH and luciferase in hepatic tissue harvested from these animals (Fig. 5). Finally, repeated experiments with different batches of recombinant virus demonstrated similar ranges of interanimal variation in the levels of expressed AOAH activity. These experiments suggest that the variation in the levels of AOAH activity observed in the liver following administration of Ad.CMV-AOAH resulted from variability in the efficiency of hepatic infection rather than from virus-, batch-, recombinant protein-, or mouse strain-related factors. Adenoviral infection might be affected by diet,
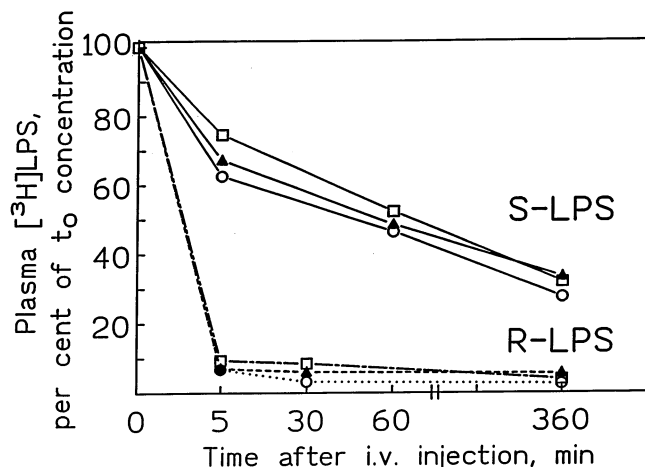

FIG. 4. Clearance of rough and smooth [³H]LPS from plasma. Groups of five or six mice were injected with labeled rough LPS (R-LPS) or smooth LPS (S-LPS) 3 days after injection with 250 μl of saline (open circles) or an equal volume of saline containing 10^9 PFU of Ad.CMV-βGal (closed triangles) or Ad.CMV-AOAH (open squares). At intervals following injection of radiolabeled LPS, blood samples were obtained for determination of the amount of the tracer remaining in the circulation. Data are expressed as the mean fraction (percent) of the tracer remaining in blood at the time indicated. The data shown for smooth LPS are from one of three experiments with similar results. i.v., intravenous.
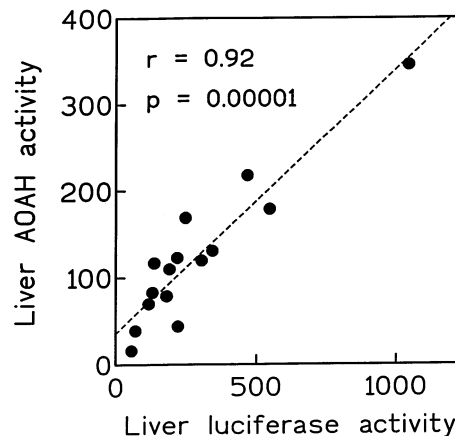

FIG. 5. Correlation between hepatic AOAH and luciferase activities in mice infected with both Ad.CMV-AOAH and Ad.CMV-Luc. ICR mice ($n = 15$) were injected with 250 μl of saline containing 1.5×10^9 PFU of Ad.CMV-AOAH and 0.25×10^9 PFU of Ad.CMV-Luc. After 3 days, liver tissue was harvested and hepatic AOAH and luciferase activities were determined as described in Materials and Methods. AOAH activity is expressed as described in the legend to Fig. 1. Luciferase activity is expressed as light units (10^3) per milligram of tissue. Each point represents results from a single animal.

endotoxin absorption from the gut, subclinical infection, or other unmeasured variables.

DISCUSSION

The experiments described here demonstrate that human AOAH catalyzes the enzymatic deacylation of bacterial LPS in vivo and that introduction of a constitutive gene encoding this enzyme into the liver in mice substantially enhances the rate at which rough-form LPS undergoes hepatic deacylation. With prior studies demonstrating that dLPS both competitively inhibits binding of LPS to CD14 (14) and interferes with other steps in the LPS signal transduction pathway to antagonize LPS-induced activation of human cells (14, 15), overexpression of AOAH might be expected to exert a protective effect against an excessive endotoxin response. In this regard, the present observations in mice are more cautionary than enthusiastic.

Our results suggest that the rates of tissue uptake and deacylation of LPS are not tightly linked. Deacylation of intravenously injected LPS normally occurs slowly in rodent livers (4), consistent with the low deacylation rates seen in control animals in this study (Fig. 3 and Table 1). Although AOAH has not been demonstrated in hepatocytes, LPS-deacylating activity has been described in these cells (5). Overexpression of AOAH increased the rate of LPS deacylation in the liver severalfold, suggesting that enzymatic activity, rather than substrate availability (i.e., the rate of LPS internalization), is rate limiting for deacylation. In fact, hepatic deacylation increased despite a fall in the fractional hepatic uptake of the tracer (and thus an absolute reduction in [^{3}H]LPS uptake), again suggesting that, at least in hepatocytes, low enzymatic activity normally limits the rate of LPS deacylation. A similar relationship may pertain to human neutrophils, which internalize LPS much more rapidly than they deacylate it (19). Enhancing intracellular LPS deacylation in hepatocytes or neutrophils should thus not interfere with LPS signals that are transduced from the cell surface, but it might diminish the ability of LPS to signal at intracellular sites or to stimulate other cells if exocytosed. Hepatic LPS degradation in vivo normally occurs very slowly over time (16), and the longer-term consequences of accelerating this process have not been explored.

When challenged intravenously with LPS, rabbits rapidly accumulate AOAH activity in plasma (2). If AOAH deacylates LPS in plasma, the enzyme might be a host mechanism for avoiding the deleterious effects of endotoxemia. Although AOAH has substantial (approximately one-third of maximal) activity at pH 7.4 when assayed in a detergent-containing buffer system in vitro (11), it has not been possible to demonstrate deacylation of LPS in neat plasma or serum (2). In the present experiments, we found that adenovirus-mediated transfer of the AOAH gene generated high levels of recombinant AOAH in plasma. This accumulation of plasma AOAH, detected by its activity in the in vitro assay system, was not associated with demonstrable deacylation of LPS in plasma, however, suggesting that AOAH is not catalytically active toward LPS in vivo in the plasma space. Similarly, the lack of a discernible effect on LPS clearance suggests that AOAH may not bind LPS in plasma. Highly purified LPS may not be the appropriate substrate for AOAH in vivo, however, where LPS is released from bacteria in membrane fragments that include protein and phospholipid and where numerous other potential substrates for AOAH (which is also a phospholipase A [24]) are present.

Although the adenoviral vector used for these experiments provided a convenient and effective mechanism for overproducing AOAH in mice, it also imposed several important lim-itations. First, since the virus predominantly infects hepatocytes, it did not augment AOAH activity selectively in the phagocytic cells (neutrophils and monocytes/macrophages) that normally express the enzyme. (For example, fractional LPS deacylation was not enhanced in the spleens of mice that received Ad.CMV-AOAH [data not shown], despite greatly increased splenic AOAH activity [Fig. 1].) Although we did not identify the adenovirus-infected cells in the spleen, they may not be the phagocytic cells that take up LPS). It is possible that overexpression of AOAH in phagocytic cells could influence phenomena such as the persistence of the in vivo response to LPS, LPS adjuvanticity, endotoxin tolerance, and chronic LPS-induced inflammation. Second, adenoviral infection induces an inflammatory response that, as shown here, produces hepatitis and splenomegaly, alters the tissue distribution of intravenously injected LPS, and induces low-level increases in AOAH activity. Third, substantial heterogeneity in adenovirus-mediated recombinant protein expression was found even in inbred mice. Such variability, while anticipated in any biological system, greatly increases the numbers of animals needed to study the impact of recombinant protein expression on a phenomenon, such as LPS-induced inflammation, that is itself highly variable in its severity.

While the activity of AOAH and the effects of dLPS have been characterized in cultured cells, potential function(s) of LPS deacylation in vivo have not been examined previously. These studies showed that mice infected with a recombinant adenovirus carrying a constitutive AOAH gene can transiently express high levels of AOAH activity in several tissues without overt toxicity from the recombinant protein. Overexpression of AOAH in the liver was associated with a substantial increase in the rate of hepatic deacylation of radiolabeled rough-form LPS in vivo without a discernible impact on LPS plasma clearance. In addition, plasma levels of AOAH activity were significantly increased, yet increased LPS deacylation in plasma was not detected. Although interpretation of these data is complicated by several technical limitations, as noted above, the studies represent an initial step toward determining the roles of AOAH and LPS deacylation in animals. Efforts to augment AOAH expression in phagocytic cells and to eliminate AOAH expression by targeted disruption of the murine AOAH gene should provide more definitive insights regarding the role of this enzyme, and its products, in vivo.

REFERENCES

1. **Brasier, A. R., and D. Ron.** 1992. Luciferase reporter gene assay in mammalian cells. Methods Enzymol. **216:**386–414.
2. **Erwin, A. L., and R. S. Munford.** 1991. Plasma lipopolysaccharide-deacylating activity (acyloxyacyl hydrolase) increases following lipopolysaccharide administration to rabbits. Lab. Invest. **65:**138–144.
3. **Freudenberg, M. A., N. Freudenberg, and C. Galanos.** 1982. Time course of cellular distribution of endotoxin in liver, lungs and kidneys of rats. Br. J. Exp. Pathol. **63:**56–65.
4. **Freudenberg, M. A., and C. Galanos.** 1985. Alterations in rats in vivo of the chemical structure of lipopolysaccharide from *Salmonella abortus equi*. Eur. J. Biochem. **152:**353–359.
5. **Fukuda, I., K. Tanamoto, S. Kanegasaki, Y. Yajima, and Y. Goto.** 1989. Deacylation of bacterial lipopolysaccharide in rat hepatocytes *in vitro*. Br. J. Exp. Pathol. **70:**267–274.
6. **Ge, Y., R. M. Ezzell, R. G. Tompkins, and H. S. Warren.** 1994. Cellular

distribution of endotoxin after injection of chemically purified lipopolysac-charide differs from that after injection of live bacteria. J. Infect. Dis. **169:** 95–104.

7. **Gerard, R. D., and R. S. Meidell.** 1995. Adenoviral vectors, p. 285–306. *In* B. D. Hames and D. M. Glover (ed.), DNA cloning: a practical approach. Oxford University Press, Oxford.

8. **Golenbock, D. T., R. Y. Hampton, and C. R. H. Raetz.** 1990. Lipopolysac-charide antagonism by lipid A precursor lipid IV$_A$ is species dependent. FASEB J. **4:**A2055.

9. **Green, M. W.** 1979. Human adenoviruses: growth, purification, and trans-fection assay. Methods Enzymol. **58:**425–435.

10. **Hagen, F. S., F. J. Grant, J. L. Kuijper, C. A. Slaughter, C. R. Moomaw, K. Orth, P. J. O'Hara, and R. S. Munford.** 1991. Expression and characteriza-tion of recombinant human acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides. Biochemistry **30:**8415–8423.

11. **Hall, C. L., and R. S. Munford.** 1983. Enzymatic deacylation of the lipid A moiety of *Salmonella typhimurium* lipopolysaccharides by human neutro-phils. Proc. Natl. Acad. Sci. USA **80:**6671–6675.

12. **Herz, J., and R. D. Gerard.** 1993. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. Proc. Natl. Acad. Sci. USA **90:**2812–2816.

13. **Hopf, U., G. Ramadori, B. Möller, and C. Galanos.** 1983. Hepatocellular clearance function of bacterial lipopolysaccharides and free lipid A in mice with endotoxic shock. Am. J. Emerg. Med. **2:**13–19.

14. **Kitchens, R. L., and R. S. Munford.** 1995. Enzymatically deacylated lipo-polysaccharide (LPS) can antagonize LPS at multiple sites in the LPS rec-ognition pathway. J. Biol. Chem. **270:**9904–9910.

15. **Kitchens, R. L., R. J. Ulevitch, and R. S. Munford.** 1992. Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. J. Exp. Med. **1760:**485–494.

16. **Kleine, B., M. A. Freudenberg, and C. Galanos.** 1985. Excretion of radioac-tivity in faeces and urine of rats injected with ^{3}H,^{14}C-lipopolysaccharide. Br. J. Exp. Pathol. **66:**303–308.

17. **Kopfler, W. P., M. Willard, T. Betz, J. E. Willard, R. D. Gerard, and R. S. Meidell.** 1994. Adenovirus mediated transfer of a gene encoding human apolipoprotein A-1 into normal mice increases circulating high density li-poprotein cholesterol. Circulation **90:**1319–1327.

18. **Kotani, S., and H. Takada.** 1990. Structural requirements of lipid A for endotoxicity and other biological activities—an overview. Adv. Exp. Med. Biol. **256:**13–44.

19. **Luchi, M., and R. S. Munford.** 1993. Binding, internalization, and deacyla-tion of bacterial lipopolysaccharides by human neutrophils. J. Immunol. **151:**959–969.

20. **Munford, R. S., L. C. DeVeaux, J. E. Cronan, Jr., and P. D. Rick.** 1992. Biosynthetic radiolabeling of bacterial lipopolysaccharide to high specific activity. J. Immunol. Methods **148:**115–120.

21. **Munford, R. S., and A. L. Erwin.** 1992. Eucaryotic lipopolysaccharide deacy-lating enzyme. Methods Enzymol. **209:**485–492.

22. **Munford, R. S., and C. L. Hall.** 1986. Detoxification of bacterial lipopoly-saccharides (endotoxins) by a human neutrophil enzyme. Science **234:**203–205.

23. **Munford, R. S., C. L. Hall, J. M. Lipton, and J. M. Dietschy.** 1982. Biological activity, lipoprotein-binding behavior, and in vivo disposition of extracted and native forms of *Salmonella typhimurium* lipopolysaccharides. J. Clin. Invest. **70:**877–888.

24. **Munford, R. S., and J. P. Hunter.** 1992. Acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides, has phospholipase, lysophospholipase, diacylglycerollipase, and acyltransferase activities in vitro. J. Biol. Chem. **267:**10116–10121.

25. **Riedo, F. X., R. S. Munford, W. B. Campbell, J. S. Reisch, K. R. Chien, and R. D. Gerard.** 1990. Deacylated lipopolysaccharide inhibits plasminogen activator inhibitor-1, prostacyclin, and prostaglandin E2 induction by lipo-polysaccharide but not by tumor necrosis factor-alpha. J. Immunol. **144:** 3506–3512.

26. **Spady, D. K., J. A. Cuthbert, M. W. Willard, and R. S. Meidell.** 1995. Adenovirus-mediated transfer of a gene encoding cholesterol 7-α hydroxy-lase into hamsters increases hepatic enzyme activity and reduces plasma total and low density lipoprotein cholesterol. J. Clin. Invest. **96:**700–709.

27. **Varley, A. W., M. G. Coulthard, R. S. Meidell, R. D. Gerard, and R. S. Munford.** 1995. Inflammation-induced recombinant protein expression *in vivo* using promoters from acute phase protein genes. Proc. Natl. Acad. Sci. USA **92:**5346–5350.

28. **Yang, Y., F. A. Nunes, K. Berencsi, E. E. Furth, E. Gönczöl, and J. M. Wilson.** 1994. Cellular immunity to viral antigens limits E1-deleted adeno-viruses for gene therapy. Proc. Natl. Acad. Sci. USA **91:**4407–4411.

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