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Tolerance to Appetite Suppression Induced by Peptidoglycan

KARLA J. BIBERSTINE, DAVID S. DARR, AND RAOUL S. ROSENTHAL*

Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana 46202

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Physiologically realistic peptidoglycan (PG) fragments, derived from *Neisseria gonorrhoeae*, were shown previously to dose-dependently suppress food consumption and body weight gain in rats following single intraperitoneal injections. The present study, examining the effects of repeated daily injection of PG, provides additional support to our underlying hypothesis, i.e., that soluble PG fragments contribute to the loss of appetite commonly associated with bacterial infections. An initial intraperitoneal injection of purified, soluble, macromolecular, extensively *O*-acetylated PG fragments (S-*O*-PG) (240 μg/kg of body weight) decreased overnight food consumption in male Lewis rats (150 g) by approximately 35% relative to animals receiving diluent alone ($P < 0.05$). However, subsequent daily injections of S-*O*-PG resulted in progressively smaller effects on food consumption until, by the fourth day, rats were completely nonresponsive (tolerant) to S-*O*-PG-induced hypophagia. Rats that developed tolerance to the effects of S-*O*-PG on appetite were also tolerant to three other known hypophagic agents, lipopolysaccharide (LPS), muramyl dipeptide, and interleukin-1, when challenged one day after establishment of S-*O*-PG tolerance. Similarly, rats developed tolerance to repeated injections of muramyl dipeptide or LPS and were cross-tolerant to S-*O*-PG when challenged 1 day later. However, 30 days after establishment of S-*O*-PG tolerance, rats remained nonresponsive to S-*O*-PG but regained full responsiveness to LPS-mediated hypophagia. Thus, at least two mechanisms of tolerance to S-*O*-PG hypophagia exist: an early tolerance which is nonspecific and a late tolerance which is specific for S-*O*-PG. Late, but not early, tolerance to S-*O*-PG-mediated suppression of appetite was associated with an increase in specific anti-PG antibody activity as measured in an enzyme-linked immunosorbent assay.

Peptidoglycan (PG), a heteropolymer unique to bacterial cell walls, was once believed to be merely a biologically inert corset that determined cell shape and maintained bacterial integrity. However, it is now clear that PG fragments are potent biological effectors which modulate a remarkably diverse set of inflammatory and immune reactions in vivo and in vitro (reviewed in reference 16). Curiously, several biological properties of PG, e.g., pyrogenicity (26) and somnogenicity (17), apparently involve the central nervous system, since body temperature and sleep are normally regulated by the brain. Recently, we (3) and others (18) reported that naturally occurring and synthetic soluble PG fragments decreased food intake and body weight gain in rats following a single intraperitoneal (i.p.) injection, thus possibly providing additional evidence of the impact of PG on central nervous system function. On the basis of these studies, we speculated that PG contributes to the fever, sleepiness, and loss of appetite that are universally recognized symptoms of bacterial illness.

It has been documented that lipopolysaccharide (LPS), a unique component of gram-negative outer membranes, and muramyl dipeptide (MDP), an adjuvant peptide which is the minimal immunologically active structure in Freund's adjuvant (11) and a synthetic component derived from the PG of bacterial cell walls, both decrease food intake in rats following a single administration (18). Repeated injections of LPS ultimately result in loss of responsiveness to LPS hypophagia, a condition described as tolerance. Tolerance to other biological effects of LPS, notably fever, is well recognized and thought to be due to both cellular and humoral mechanisms (reviewed in reference 13). Interestingly, it has been reported that MDP, administered i.p. every other day for 4 days, does not result in tolerance to MDP-induced hypophagia (18).

Herein, we report that repeated daily administration of soluble, macromolecular, extensively *O*-acetylated PG (S-*O*-PG) results in physiological tolerance to S-*O*-PG hypophagia which is initially cross-reactive with LPS, MDP, and interleukin-1 (IL-1). While cross-tolerance to LPS is not evident after 30 days, specific tolerance to S-*O*-PG persists for at least 30 days and is associated with elevated levels of anti-PG antibodies (Abs).

MATERIALS AND METHODS

Animals and housing conditions. Male Lewis rats (125 to 149 g) obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.) were housed individually in metabolic cages in a temperature-controlled room (72 ± 5°F) and kept on a 12:12 h light-dark cycle with lights on from 0700 to 1900 h.

Rat appetite model. Food consumption and body weight gain of rats were determined as described previously (3). Overnight food consumption was measured during the 12-h period beginning at the time of injection, approximately 1900 h. Rats were divided into experimental and control groups of typically nine or more per group and injected i.p. with a 500-μg bolus of saline alone (controls) or of S-*O*-PG (240 μg/kg), MDP (1.6 mg/kg; Sigma Chemical Company, St. Louis, Mo.), LPS (10 μg/kg) from *Escherichia coli* 055:B5 (Sigma), or rhIL-1β produced in *E. coli* (5.6 μg/kg; 2.3×10^6 U/mg; gift from S. Garcia, Indiana University School of Medicine, Indianapolis). All of the reagents were suspended in saline. S-*O*-PG was treated with polymyxin B-agarose to reduce endotoxin contamination as described previously (3). We determined previously (3) that contaminating LPS did not account for the hypophagic qualities of PG preparations. In fact, the level of LPS was less than 1/1,000th of the amount necessary for LPS alone to produce the hypophagic response. The amount of endotoxin contamination in the rhIL-1 was 12.6 endotoxin units per mg of protein.

Data are presented as the mean food consumed or body weight gained (± standard deviation) for at least nine rats. Group means were compared by using a one-way analysis of variance and, when statistically significant differences were detected, multiple comparisons were performed by using either the Student-Newman-Keuls test or the Dunnett's test; a P of < 0.05 was considered significant. All statistical functions were performed by using Sigmastat software (Jandel Scientific, San Rafael, Calif.).

At the culmination of some experiments, rats were sacrificed by inhalation of

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Indiana University School of Medicine, 635 Barnhill Dr., Indianapolis, IN 46202. Phone: (317) 274-4398. Fax: (317) 274-4090. Electronic mail address: rrosenth@indycms.iupui.edu.

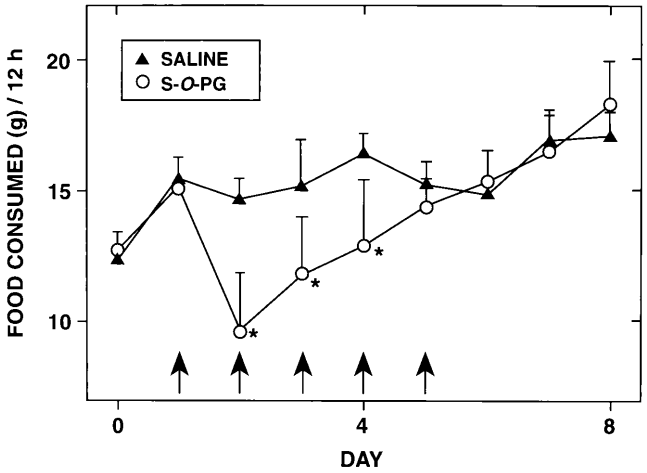

FIG. 1. Rats become tolerant to S-*O*-PG (240 μg/kg)-induced hypophagia. Error bars indicate standard deviations for nine rats. Animals received daily injections as indicated by the arrows. Asterisks denote a P of < 0.05 versus saline controls.

CO_2 or Halothane (Halocarbon Laboratories, North Augusta, S.C.) and bled by cardiac puncture. Blood samples collected in Vacutainer tubes (Becton Dickinson and Company, Rutherford, N.J.) were refrigerated for at least 1 h and then centrifuged (600 × *g*) at 4°C for 10 min and serum was stored at −70°C until use.

ELISA to measure anti-PG antibodies. An enzyme-linked immunosorbent assay (ELISA) was developed to measure anti-PG Ab activity in rat serum. Briefly, 96-well polyvinyl tissue culture plates (Costar, Cambridge, Mass.) were coated with 20 μg of S-*O*-PG suspended in 100 μl of 50 mM sodium carbonate buffer, pH 9.7. After overnight incubation at 4°C, wells were washed with phosphate-buffered saline (PBS) plus 0.05% Tween-20, pH 7.4, incubated in PBS plus 1.0% ovalbumin (Sigma) for 30 min at room temperature, and then rewashed with PBS plus Tween-20. Test rat sera, diluted in PBS to a total volume of 100 μl, were added to the plates and incubated for 100 min, after which the plates were washed as above and 100 μl of alkaline phosphatase-conjugated goat anti-rat immunoglobulin G (IgG) and IgM (1:5,000 dilution in PBS plus 1.0% ovalbumin; HyClone Laboratories, Inc., Logan, Utah) was added, and the mixture was incubated for 80 min prior to the addition of the substrate, *p*-nitrophenyl phosphate, disodium salt (final concentration, 1 mg/ml; Pierce, Rockford, Ill.) dissolved in diethanolamine buffer (Pierce). After a 30-min incubation at 25°C, NaOH was added to a final concentration of 0.67 N to stop the reaction, and the optical density at 405 nm (OD_{405}) was measured by using an automated plate reader (Molecular Devices, Menlo Park, Calif.). PG-specific binding was defined as total OD minus the OD obtained from corresponding wells that were not coated with PG. Typically, the mean OD_{405} for each independent variable and for each control was determined from triplicate wells.

RESULTS

Repeated injections result in physiological tolerance to the hypophagic effect of S-*O*-PG. Groups of rats received either daily injections of S-*O*-PG (240 μg/kg) or saline, and food intake was measured. The initial S-*O*-PG injection reduced food consumption by about 35% ($P < 0.05$) compared with saline controls (Fig. 1), confirming our previous report (3) that PG induces hypophagia in rats. However, each successive injection resulted in progressively smaller decreases in food consumption, until by the fourth injection, rats were completely nonresponsive, i.e., physiologically tolerant, to S-*O*-PG-induced hypophagia. Data presented (Fig. 1) are from one experiment; however, the pattern of tolerance to repeated injections of S-*O*-PG was observed in eight of eight additional experiments.

S-*O*-PG-tolerant rats are also nonresponsive to other hypophagic agents. To determine if the acquired tolerance to S-*O*-PG-induced hypophagia was specific for macromolecular PG, S-*O*-PG tolerant rats were challenged with MDP, a synthetic component of PG previously documented to decrease food intake in rats (18). As before, rats received four daily injections of either S-*O*-PG or saline, and food consumption was measured. On the fifth day, both groups of rats were challenged with single i.p. injections of MDP (1.6 mg/kg). The S-*O*-PG tolerant rats exhibited little or no decrease in food intake in response to MDP, whereas, as expected, MDP did decrease food intake (by about 20%) ($P < 0.05$) in the nontolerant saline control group (Fig. 2A). The reverse experiment was performed on other sets of rats which initially received a series of injections of either MDP or saline followed by a challenge injection of S-*O*-PG. Daily administration of MDP resulted in hypophagic tolerance to MDP and cross-tolerance to S-*O*-PG (Fig. 2B).

Other experiments tested whether hypophagic tolerance to S-*O*-PG could be overcome by administration of LPS, a potent appetite suppressor (19). Rats received four daily injections of S-*O*-PG or saline prior to challenge with LPS (10 μmg/kg). Rats that were made tolerant to the hypophagic effect of S-*O*-PG exhibited little or no response to LPS-induced suppression of appetite (Fig. 3A) 1 day after tolerance to S-*O*-PG was achieved and, conversely, LPS tolerant rats were also cross-

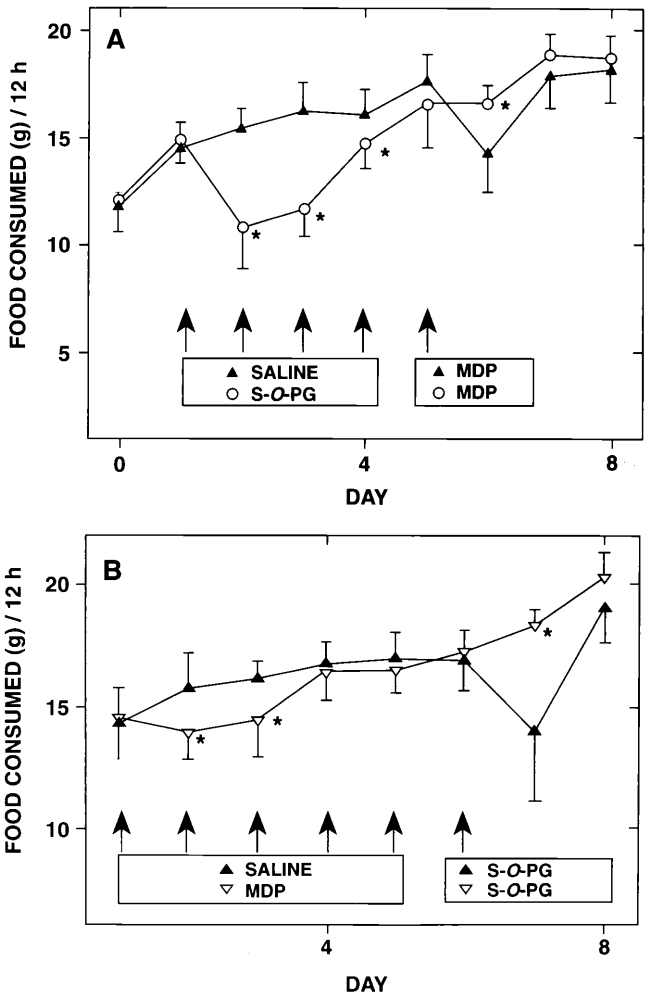

FIG. 2. Rats that exhibit early tolerance to S-*O*-PG-induced hypophagia are also nonresponsive to MDP (A). Conversely, rats exhibiting early tolerance to MDP-induced hypophagia are also nonresponsive to S-*O*-PG (B). Error bars indicate standard deviations for nine rats. Animals received daily injections as indicated by the arrows. Asterisks denote a P of < 0.05 versus saline controls.

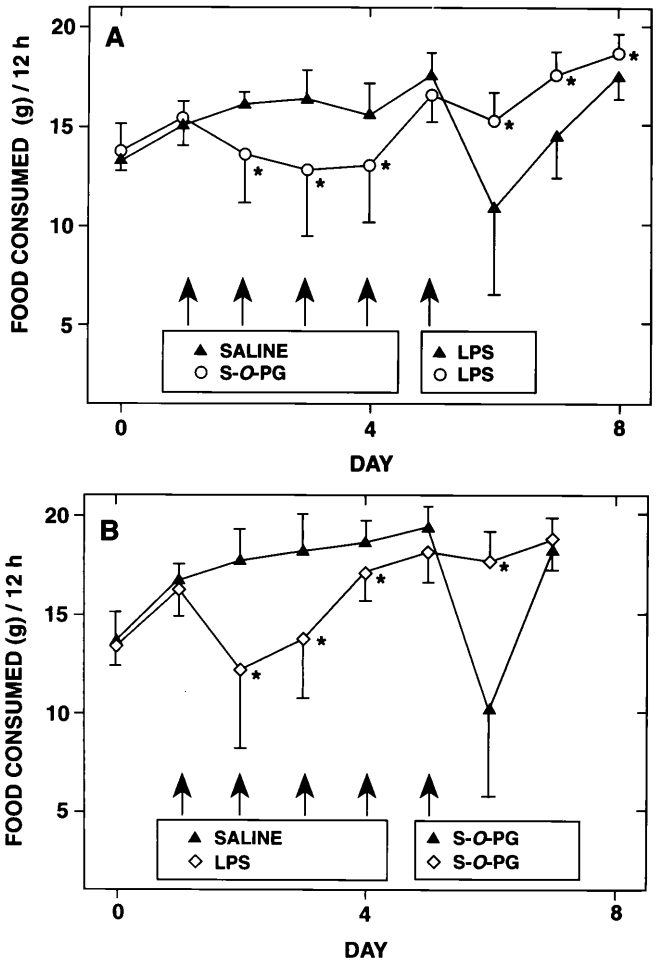

FIG. 3. Rats that exhibit early tolerance to S-*O*-PG-induced hypophagia are also nonresponsive to LPS (A). Conversely, rats exhibiting early tolerance to LPS-induced hypophagia are also nonresponsive to S-*O*-PG (B). Error bars indicate standard deviations for nine rats. Animals received daily injections as indicated by the arrows. Asterisks denote a P of < 0.05 versus saline controls.

tolerant to S-*O*-PG (Fig. 3B). However, S-*O*-PG tolerant rats regained responsiveness to LPS hypophagia when challenged with LPS 3 days after tolerance to S-*O*-PG was achieved (data not shown), suggesting that cross-tolerance between S-*O*-PG and LPS was transient.

Another group of S-*O*-PG tolerant rats was challenged with IL-1 (5.6 μg/kg) 1 day after complete tolerance to S-*O*-PG was established. S-*O*-PG-pretreated rats were nonresponsive to the hypophagic effect of IL-1, whereas nontolerant, saline-pretreated rats exhibited a decrease in food consumption of about 20% ($P < 0.05$) following a single injection of IL-1, as expected (Table 1).

Tolerance to S-*O*-PG-induced hypophagia persists for at least 30 days and is specific for S-*O*-PG. S-*O*-PG was administered daily to several groups of rats, and groups were challenged with S-*O*-PG at various times after hypophagic tolerance was achieved to determine the length of time that tolerance persisted. Saline was administered in parallel to control groups. PG tolerant rats that were challenged on day 34 (30 days after tolerance was established) remained completely nonresponsive to S-*O*-PG (Fig. 4), as did PG tolerant animals that were challenged at intermediate time points, i.e., 1, 4, 7, and 16 days posttolerance (data not shown). Upon completion

TABLE 1. Rats exhibiting early tolerance to the hypophagic activity of S-*O*-PG are also nonresponsive to IL-1[a]

Rat	Food consumption (g/12 h) following administration of:		
	Saline	S-*O*-PG	IL-1
Control	18.0 ± 1.9	14.9 ± 3.5[b]	14.8 ± 3.1[b]
S-*O*-PG tolerant	ND[c]	16.5 ± 2.0	16.7 ± 3.1

[a] Rats were rendered tolerant to S-*O*-PG hypophagia following four daily injections of S-*O*-PG; control rats received four daily injections of saline. One day after tolerance was established, the S-*O*-PG tolerant (or control) rats were administered either saline, S-*O*-PG (240 μg/kg), or IL-1 (5.6 μg/kg). Data are expressed as means ± standard deviations for nine rats.
[b] $P < 0.05$ versus saline-injected control rats.
[c] ND, not determined.

of the experiment (at day 31 posttolerance) the saline-treated rats, now serving as positive controls, exhibited about a 30% reduction in food intake ($P < 0.05$; data not shown) following a single i.p. injection of S-*O*-PG, as expected; hence, lack of a hypophagic response in the experimental groups was dependent upon S-*O*-PG pretreatment. Interestingly, rats that developed persistent tolerance to S-*O*-PG-induced hypophagia were fully responsive to a single injection of LPS 30 days after tolerance to S-*O*-PG was established (Table 2). Thus, late hypophagic tolerance to PG, evident 30 days posttolerance, was specific for PG.

Late, but not early, tolerance to S-*O*-PG is associated with increased levels of anti-PG Ab activity. Pooled sera obtained from nine rats 31 days after achievement of tolerance to S-*O*-PG exhibited approximately eight-fold higher Ab activity in the ELISA than pooled sera obtained from control rats ($P < 0.05$) (Fig. 5). Therefore, late tolerance was associated with an increase in specific anti-PG Ab which, in other experiments, did not cross-react with LPS (data not shown). However, similar to control sera, early sera obtained from S-*O*-PG-treated rats had little or no anti-PG activity, suggesting that Ab to S-*O*-PG is not responsible for early tolerance to S-*O*-PG hypophagia (Fig. 5). Figure 5 depicts data from one experiment comparing the four variables indicated; similar data were obtained in other experiments with two additional, independently generated sets of sera. In experiments such as that shown in

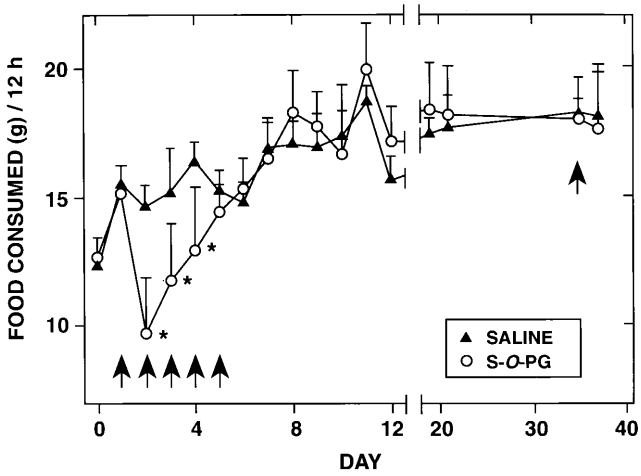

FIG. 4. Tolerance to S-*O*-PG-induced hypophagia persists for at least 30 days. Error bars indicate standard deviations for nine rats. Arrows indicate the days of injections. Asterisks denote a P of < 0.05 versus saline controls.

TABLE 2. Late hypophagic tolerance to S-*O*-PG is specific for S-*O*-PG[a]

Rat	Food consumption (g/12 h) following administration of:		
	Saline	S-*O*-PG	LPS
Control	19.2 ± 1.8	15.4 ± 3.2[b]	11.3 ± 4.3[b]
S-*O*-PG tolerant	ND[c]	18.0 ± 2.1	12.7 ± 3.9[b]

[a] Rats were rendered tolerant to S-*O*-PG hypophagia following four daily injections of S-*O*-PG; control rats received four daily injections of saline. Thirty days after tolerance was established, the S-*O*-PG tolerant (or control) rats were administered either saline, S-*O*-PG (240 μg/kg), or LPS (10 μg/kg). Data are expressed as means ± standard deviations for nine rats.
[b] $P < 0.05$ versus saline-injected control rats.
[c] ND, not determined.

Fig. 5, the net (PG-specific) OD_{405} of 1% sera from PG tolerant rats (representing the highest OD) was about 0.58 and background was about 0.10.

DISCUSSION

PG fragments constitute a unique family of structurally conserved molecules that are emerging as versatile biological effectors in vivo, perhaps rivalled only by the endotoxin component of LPS in the diversity of reactions they are capable of mediating. Recent studies have revealed that PG fragments, like LPS (4, 5), stimulate production of numerous inflammatory mediators, i.e., IL-1 (7), IL-6 (9), and tumor necrosis factor (8), by various host target cells, primarily macrophages. Indeed, activation of the vast cytokine network may be a common mechanism underlying many of the biological activities of PG derivatives and LPS, and thus, the biological impact of PG and LPS on immune and inflammatory reactions is strikingly similar. We have focused particularly on PG and LPS as bacterial signals which trigger fever, enhanced sleep, and decreased appetite, all of which are universally recognized features of infection that are under the joint control of the immune and central nervous systems.

The seminal observation of the present studies is that repeated administration of soluble PG fragments, i.e., S-*O*-PG, induces a state of nonresponsiveness to the hypophagic effects of PG. Physiological tolerance to the effects of PG fragments upon repeated administration is not restricted to appetite, as tolerance to fever (30) and induction of colony-stimulating factor (31) mediated by PG fragments has been reported previously. The tolerance to PG-induced hypophagia which we observed is reminiscent of the tolerance induced by repeated injections of endotoxin (19), IL-1 (15, 20, 21, 24), or TNF (20, 29) in appetite models. We have found that tolerance to macromolecular S-*O*-PG, similar to endotoxin (13), occurs in separate stages with each stage apparently due to a distinct mechanism. The first or early stage is evident immediately after a short course of tolerance-inducing injections, and the late stage persists for a month or longer after tolerance is first achieved and correlates with development of specific antibody to the tolerance-inducing substance.

The pro-inflammatory properties of LPS generally, and its hypophagic properties specifically, are regulated in part by the hypothalamic-pituitary-adrenal (HPA) axis, which acts as a negative-feedback loop to dampen the host response through intervention by glucocorticosteroids (2, 27). Activation of the HPA axis is thought to be initiated at the level of the paraventricular nucleus by endotoxin-induced IL-1 which stimulates production of corticotropin-releasing hormone (1, 28, 32). Corticotropin-releasing hormone, in turn, enhances synthesis of adrenocorticotropic hormone by the pituitary gland, thus leading to increased levels of corticosteroids. The glucocorticosteroids exert their anti-inflammatory action at several levels but are thought to act predominantly by down-regulating transcription of diverse mediators (including IL-1) considered key links between the immune system and centers of appetite, sleep, and temperature regulation in the hypothalamus (22). Because early tolerance to LPS and PG hypophagia are mutually cross-reactive (Fig. 3), and because the actions of LPS and PG likely involve IL-1, it is reasonable to hypothesize that a common mechanism, perhaps involving the HPA axis, contributes to the development of early tolerance to both bacterial macromolecules. There is precedence for cross-tolerance between PG and LPS in that pretreatment with lipid A renders mice nonsusceptible to the PG-mediated enhancement of colony-stimulating factor (31).

However, if tolerance to PG does involve feedback inhibition through the HPA axis, the mechanism would seem to involve down-regulation of existing IL-1 activity (and not just suppression of de novo IL-1 synthesis), since administration of exogenous rIL-1 does not overcome early tolerance to PG hypophagia (Table 1). Net inhibition of IL-1 activity could conceivably involve, among other mechanisms, a decrease in IL-1 receptors, generation of IL-1 "decoys" and receptor antagonists, or modulation of the cysteine protease responsible for activation of IL-1 (6, 10, 12, 14).

Although we observed early tolerance to daily injections of both high- and low-molecular-weight PG fragments, others have reported that repeated administration of MDP (as a bolus injection every 48 h) fails to evoke hypophagic tolerance (18). Also, tolerance to MDP-induced fever has been documented upon daily injection in rabbits (30). Perhaps the development of early tolerance to natural and synthetic PG fragments requires daily administration. In contrast to early tolerance, which probably involves modulation of the production and function of a complex network of effectors, it is conceivable that late tolerance to PG hypophagia is due solely to the

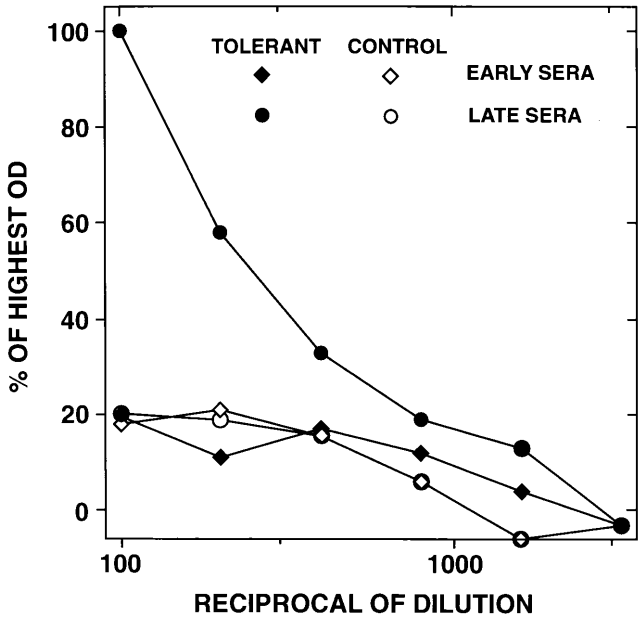

FIG. 5. Late tolerance to S-*O*-PG-induced hypophagia correlates with development of anti-PG Ab as measured by ELISA.

development of specific antibody capable of neutralizing the bacterial initiator. While late tolerance to LPS hypophagia has not been reported, it is clear that late tolerance to LPS fever is mediated by antibodies in serum (13) which persist for weeks. Certainly, there is evidence that antibody to the D-alanyl-D-alanine terminus of gram-positive PG is detectable in normal individuals and is stimulated during certain disease states (25). However, we are aware of only one report in animals (23) of antibody to gram-negative PG, which is deficient in the D-alanyl-D-alanine end, and we are not aware of any such reports in humans.

Anecdotally, the development of anti-PG Ab in rats following daily i.p. administration of gonococcal S-*O*-PG in aqueous buffer was itself unexpected, especially since we have failed for years to generate antibody to gram-negative PG using many of the "optimal" and universally accepted animal species, conjugated antigens, adjuvants, and routes of inoculation. We speculate that most natural and artificial means to introduce gram-negative PG do not result in formation of specific antibody, and we doubt that antibody-mediated (late) tolerance to the hypophagic effects of gram-negative PG plays a significant role in vivo. Thus, it would be surprising if nonimmunogenic derivatives, i.e., MDP, or nonimmunogenic means of administration would induce late tolerance to PG.

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