FULL PAPER *Immunology*

The A^y allele at the agouti Locus Enhances Sensitivity to Endotoxin-Induced Lethality in Mice

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(Received 6 March 2007/Accepted 18 May 2007)

ABSTRACT. In the course of investigations on anorexia during infection, I found that B6-A^y mice had significantly increased sensitivity to lipopolysaccharide (LPS)-induced lethality as compared with isogenic B6 mice. I also found that the sensitivity to the lethal effect of LPS dramatically increased in aged mice (age effect), both B6 and B6-A^y. However, the A^y effect of enhancing sensitivity to LPS-induced lethality was still significant, suggesting that the A^y effect is independent of age. In the absence of tumor necrosis factor α (TNFα), the A^y effect was still significant, suggesting that the A^y effect is independent of TNFα toxicity. A dose of LPS of 100 μg per mouse caused 15% lethality in B6, 65% in B6-A^y (significantly higher than B6), and 100 % in leptin-deficient B6-*ob/ob* (significantly higher than B6 and B6-A^y). The results support the hypothesis that endogenous leptin has a protective role against infection, and that a part of this leptin effect is mediated by α-melanocyte-stimulating hormone (αMSH). In contrast to the results of simple blockade at the melanocortin 4 receptor (MC4R), B6-A^y suffered more severe LPS-induced anorexia than did B6; therefore, the pathway involving MC4R is not absolutely required for the LPS-induced anorexia, and the presence of pathways involving other melanocortin receptor types was suggested. Because αMSH is suggested to be an endogenous anti-inflammatory peptide, and because melanocortin 1 receptor (MC1R) is expressed in various cutaneous cell types, the A^y effect might be caused via the pathway involving MC1R. Physiologic significance of αMSH-MC1R interaction in host defense against infection is discussed.

KEY WORDS: anorexia, A^y allele, lethality, lipopolysaccharide (LPS), melanocortin receptors.

J. Vet. Med. Sci. 69(9): 931–937, 2007

Anorexia and weight loss are frequently associated with acute or chronic infections [11, 24]. Since several inflammatory cytokines, most notably TNFα and IL-1β, have been shown to induce anorexia and weight loss, it appears to be likely that these cytokines mediate metabolic changes of infection [9, 18, 23, 26, 27, 35, 37]. However, the mechanisms and mediators by which infection induces anorexia are still poorly understood. For experimental induction of an inflammatory response, lipopolysaccharide (LPS, gram-negative bacterial endotoxin) has been extensively used. LPS administration causes anorexia [5, 6, 11, 15, 24, 32]. Toxic effects exerted by LPS are largely mediated by the effects of cytokines including the above-mentioned ones; thus, LPS administration is used as a model of systemic infection [5, 6, 32].

It is now widely recognized that the central regulator of food intake and body weight is leptin [38]. Leptin expression can be induced by the inflammatory cytokines and LPS administration; therefore, it was once postulated that leptin is a mediator of anorexia during infection [11, 24]. However, subsequent studies revealed that functional leptin system was not essential in LPS-induced anorexia, because LPS caused anorexia in both leptin deficient-Lep^{ob}/Lep^{ob} (hereafter called *ob/ob* for convenience) and leptin receptor-deficient $Lepr^{db}/Lepr^{db}$ (hereafter called *db/db*) mice [5, 6, 32]. Although *ob/ob* mice were more sensitive to LPS-induced anorexia than their +/? littermates, *db/db* mice were relatively resistant to the anorectic effect of LPS [5]. Furthermore, *ob/ob* mice were revealed to be more susceptible to LPS-induced lethality than their lean littermates through the studies on anorexia [6]. In contrast to *ob/ob* mice, *db/db* mice were shown to have a similar sensitivity with their lean +/? littermates to LPS-induced lethality [6]. Recent study suggests that resistance to LPS-induced toxicity in *db/db* mice may be due to combined effects of hyperleptinemia (high levels of free leptin) and reduced TNFα secretion; however, details are mostly unknown [15]. With regard to the anorectic and lethal effect of TNFα, essentially the same results were reported [32]. Fasting enhanced sensitivity to TNFα-induced lethality, probably because of reduced circulating level of leptin [7]. Leptin treatment protected *ob/ob* mice against TNFα-induced lethality [6]. It has thus been suggested that leptin is an endogenous protective protein in the host responses against inflammation [5, 6, 32].

Despite the above-mentioned discrepancies in anorectic and lethal effect of LPS between *ob/ob* and *db/db* mice, it is highly likely that a certain part of the protective effect of leptin is mediated by α-melanocyte-stimulating hormone (αMSH), which is also suggested to have anti-inflammatory property [14, 32]. In the present study, I investigate the physiologic significance of αMSH in the mouse strain harboring the A^y allele at the agouti locus (A^y mice). In A^y mice, intact agouti peptide is overexpressed ubiquitously, because the agouti gene is placed under the control of the *Raly* promoter [4, 19, 20]. The A^y mice show obesity as a consequence of a constitutive antagonism of αMSH action at the melanocortin 4 receptor (MC4R). The ectopic agouti peptide mimics the effect of endogenous antagonist, agouti-

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related protein, therefore A^y mice result in hyperphagia [8, 13]. In addition, the A^y mice have a yellow coat color as a consequence of a constitutive antagonism of αMSH action at the melanocortin 1 (MC1R). The ectopic agouti peptide also mimics the action of endogenous antagonist, agouti peptide, thereby impedes the eumelanin (black pigments) synthesis [8, 22]. By use of the A^y mice, I addressed the question whether or not these receptor types also play roles in the lower course of leptin signaling in terms of host response to inflammation.

MATERIALS AND METHODS

Mice: C57BL/6J (hereafter called B6), C57BL/6J-A^y (hereafter called B6-A^y), and C57BL/6J-Lep^{ob}/Lep^{ob} (hereafter called B6-*ob/ob*) mice were purchased from CLEA Japan Inc. (Tokyo) and the Jackson Laboratory (Bar Harbor, ME). Tumor necrosis factor (*Tnf*) gene deficient mice (C57BL/6J-$Tnf^{-/-}$, hereafter called B6-$Tnf^{-/-}$) were a generous gift from Dr. K. Sekikawa [33]. B6-$Tnf^{-/-}$ mice carrying the A^y allele (C57BL/6J-$Tnf^{-/-}$-A^y/a, hereafter called B6-$Tnf^{-/-}$-A^y) were generated by crossing of ♀B6-$Tnf^{-/-}$ × ♂(♀B6-$Tnf^{-/-}$ × ♂ B6-A^y) F_1-A^y. Homozygous mutants ($Tnf^{-/-}$ mice) were distinguished from heterozygotes ($Tnf^{+/-}$ mice) by PCR as described previously [21]. Only females were used for the subsequent experiments. All mice were housed in a specific-pathogen-free room, with a regular cycle of 12 hr light/12 hr dark and with the temperature controlled at 22 ± 3°C. They had free access to food (rodent pellet chow CE-2, CLEA Japan Inc.) and water.

Preparation and administration of LPS: Lipopolysaccharide (*E. coli* O55:B5, Difco Laboratories, Detroit, MI) solution was freshly prepared on the day of use by being dissolved in Dulbecco's phosphate-buffered saline (GIBCO BRL, Grand Island, NY). Intraperitoneal administration of LPS was carried out with 100 μl of solution either at 12 weeks (young mice) or at 10 months (aged mice). Because feeding conditions have a profound influence on LPS-induced lethality [7], all mice were fasted for 24 hr prior to LPS administration. Twenty-four-hr fasting seems to be sufficient for measuring fasting glucose levels, and also seems to be preferable to prompt feeding [28].

LPS-induced anorexia: The degree of anorexia was evaluated by daily food intake (DFI) and daily weight gain (DWG). DFI was estimated as follows: Three mice were housed together, and food intake during 24 hr by each individual mouse was estimated by the calculation [food given (g) – leftovers (g)]/3. DWG was determined as follows: Three mice were housed together, and weight gain during 24 hr was expressed as the difference in body weight (g) between two successive days (DFI and DWG were evaluated in the same mouse). Food was weighed and replaced daily, and the absence of any massive spilled food was confirmed. In the measurements of DFI and DWG, the numbers of animals regularly were six in control groups. On the other hand, in experimental groups, some mice were removed daily for collection of blood for another purpose (usually n=6); therefore, the numbers of mice on each day are indicated in the figures. In the experimental groups, a dose of LPS of 100 μg per mouse was given.

Statistics: The statistical significance of differences between two independent groups was analyzed by Student's or Welch's *t*-test with Stat123/Win software (Shinko Trading Co. Ltd. Publication Department, Tokyo). Survival curves (Kaplan-Meyer plots) were compared by use of a Log-rank test with SPSS software (SPSS for Windows Release 7.5.1 J, SPSS Inc., Chicago, IL). In all cases, $P<0.05$ was considered to indicate significant difference.

RESULTS

LPS-induced lethality in young B6 and B6-A^y mice: For all studies, lethality was evaluated daily over seven days of LPS administration. After seven days, no further death was observed in any experiments (observations were usually discontinued within the following few days, because surviving mice seemed to have their appetite and health restored, judging from their external appearance).

At 12 weeks old, the average body weight of B6 mice (hereafter called B6-12w) was 19.6 g, and that of B6-A^y mice (hereafter called B6-A^y-12w) was 25.1 g. Although B6-A^y-12w were significantly heavier than B6-12w, it did not seem appropriate to regard them as 'obese' [this is particularly true for B6-*ob/ob*, because they weigh more than twice B6-A^y at 12 weeks (see below). Because the A^y allele also increases linear growth, a difference in body weight between B6-A^y and B6 at this age should be attributed to the difference in body length as well as to the difference in fatness]. Therefore, B6-A^y-12w and B6-12w are called 'young' in this paper [and therefore, B6-A^y at 10 months (hereafter called B6-A^y-10m) is called 'aged' rather than 'obese', as mentioned later].

For evaluation of the effect of the A^y allele on the sensitivity to LPS-induced lethality, mice were challenged with different doses of LPS. As shown in Fig. 1, introduction of the A^y allele on the B6 background markedly enhanced the sensitivity to LPS-induced lethality. A dose of LPS of 50 μg/mouse did not induce lethality in B6-12w, and induced only 10% lethality in B6-A^y-12w; therefore, there was no significant difference in survival rates between B6-12w and B6-A^y-12w (Fig. 1A). A dose of LPS of 100 μg/mouse caused 15% lethality in B6-12w and 65% lethality in B6-A^y-12w; therefore, B6-A^y-12w had a significantly lower survival rate than did B6-12w (Fig. 1B). At this dose of LPS, the survival rate was also examined in B6-*ob/ob* of the same age as reference, because leptin has been suggested to have a protective role against inflammation, and it has been suggested that part of the leptin effects may be mediated by αMSH [6, 7]. The average body weight of B6-*ob/ob* was 52.8 g, which was significantly heavier than that of B6-12w and B6-A^y-12w. Some caution will be required for the interpretation of the results for B6-*ob/ob*; that is, although twenty-four mice had been challenged with a dose of LPS of 100 μg/mouse on day 0, six surviving mice were removed for blood collection

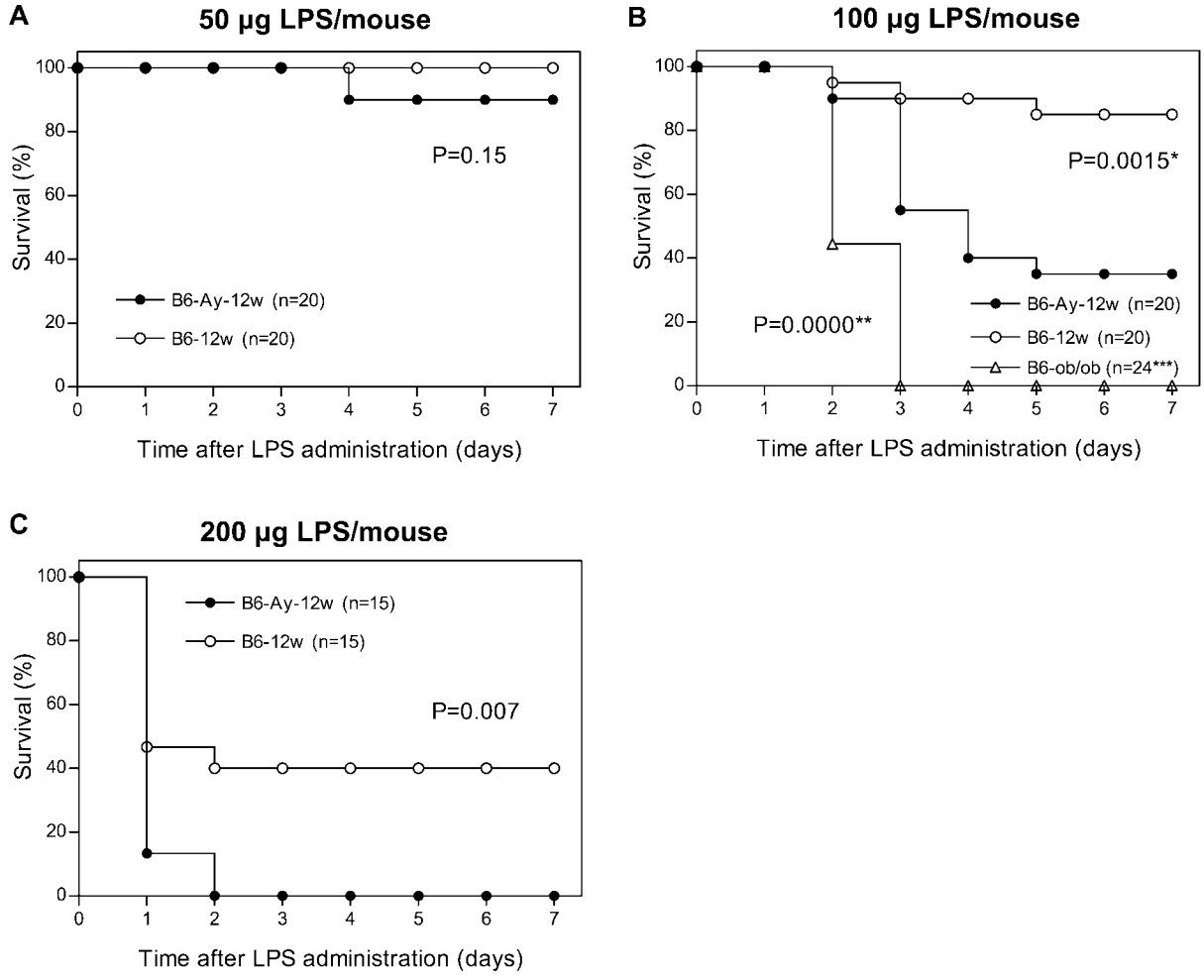

Fig. 1. Comparison of sensitivity to LPS-induced lethality among B6-12w (○), B6-A^y-12w (●), and B6-*ob/ob* (Δ). The x-axis represents time (days) after LPS administration and the y-axis represents survival rate (%). LPS was administered intraperitoneally to mice (numbers are indicated in parentheses) fasted for 24 hr. Lethality was evaluated daily over seven days of LPS administration. **A**: Administration of a dose of LPS of 50 μg per mouse. **B**: Administration of a dose of LPS of 100 μg per mouse. * B6-12w vs. B6-A^y-12w, ** B6-12w vs. B6-*ob/ob*. (P=0.0001 for B6-A^y-12w vs. B6-*ob/ob*). *** Twenty-four B6-*ob/ob* were initially given LPS. **C**: Administration of a dose of LPS of 200 μg per mouse.

on day 1. Ten out of eighteen died up to day 2, and of the remaining 8, the 2 survivors were removed for blood collection on day 2. All of the remaining six died by day 3. In consequence, B6-*ob/ob* had a significantly lower survival rate than did B6-12w and B6-A^y-12w (Fig. 1B); therefore, the overall sensitivity to LPS-induced lethality increased in the order of B6-12w (15%), B6-A^y-12w (65%), and B6-*ob/ob* (100 %) at this dose of LPS. A dose of LPS of 200 μg/mouse caused 65 % lethality in B6-12w, and 100 % lethality in B6-A^y-12w; therefore, B6-A^y-12w had a significantly lower survival rate than did B6-12w (Fig. 1C). I did not perform experiments with still higher doses of LPS, but it had been confirmed earlier that all mice (except for B6-$Tnf^{-/-}$ and B6-$Tnf^{-/-}$-A^y) died when a dose of LPS of 500 μg/mouse was given.

LPS-induced lethality in aged B6 and B6-A^y mice: Obesity itself is suggested to sensitize rats to LPS toxicity [36]. To ascertain whether the A^y effect of enhancing LPS-induced lethality is due to obesity, a secondary effect of the A^y allele, B6-A^y mice were housed for 10 months to become obese through aging.

At 10 months, the average body weight of aged B6 (hereafter called B6-10m) was 23.3 g, and that of aged B6-A^y mice (B6-A^y-10m) was 51.0 g; thus, B6-A^y-10m were significantly heavier than B6-10m.

In the same way as in the case of young mice (12 weeks old), I challenged aged mice with different doses of LPS to evaluate the effect of the A^y allele on the sensitivity to LPS-induced lethality. In aged mice, introduction of the A^y allele also enhanced the sensitivity to LPS-induced lethality (Fig. 2). However, unlike the case of young mice, a dose of LPS of 50 μg/mouse, which induced no lethality in B6-12w,

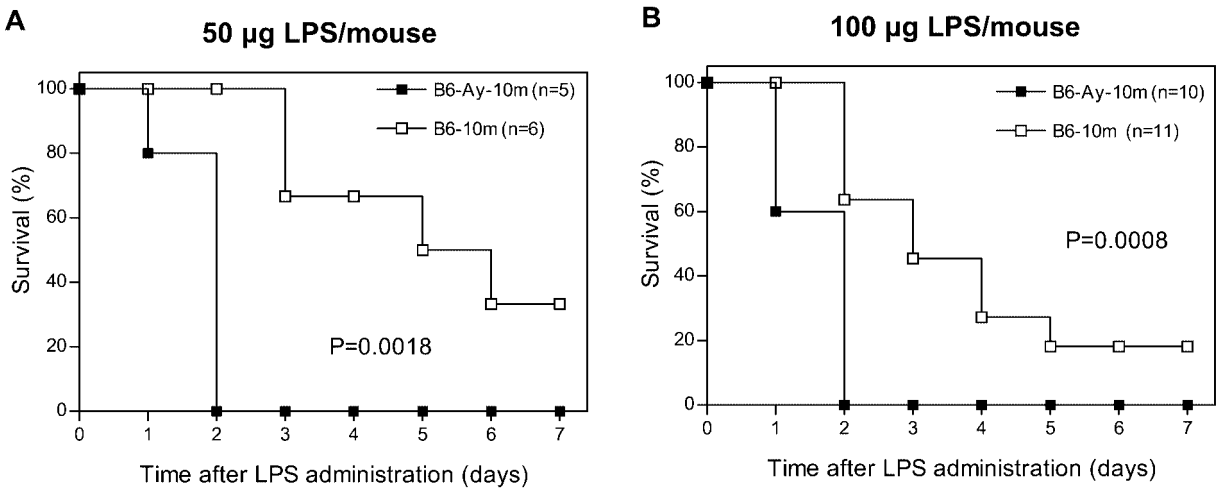

Fig. 2. Comparison of sensitivity to LPS-induced lethality between B6-10m (□) and B6-A^y-10m (■). The x-axis represents time (days) after LPS administration and the y-axis represents survival rate (%). LPS was administered intraperitoneally to mice (numbers are indicated in parentheses) fasted for 24 hr. Lethality was evaluated daily over seven days of LPS administration. **A**: Administration of a dose of LPS of 50 μg per mouse. **B**: Administration of a dose of LPS of 100 μg per mouse.

induced 67% lethality in B6-10m and 100% lethality in B6-A^y-10m; therefore, B6-A^y-10m had a significantly lower survival rate than did B6-10m (Fig. 2A). A dose of LPS of 100 μg/mouse induced 82% lethality in B6-10m and 100% lethality in B6-A^y-10m; therefore, B6-A^y-12w had a significantly lower survival rate than did B6-12w (Fig. 2B). B6-A^y-10m showed complete lethality within two days after LPS administration; however, a dose of LPS of 50 μg/mouse induced only 20% lethality within one day after LPS administration, and a dose of 100 μg/mouse induced 40% lethality within one day. Because B6-10m were not obese, I considered that aging alone dramatically enhanced the sensitivity to LPS-induced lethality in both B6 and B6-A^y. In terms of survival rate, the overall sensitivity to LPS toxicity increased in the order of B6-12w, B6-A^y-12w, B6-10m, and B6-A^y-10m, when a dose of LPS of 100 μg was administered (Figs. 1B and 2B). In accordance with similar experimental results reported previously [3, 34], I considered that age is one of the components that determine the sensitivity to LPS toxicity. Nevertheless, the A^y effect was still significant, suggesting that the A^y effect is independent of the effect of age.

LPS-induced lethality in B6 and B6-Ay mice in the absence of the Tnf gene: Because no prominent obese phenotype was observed in both B6-*Tnf*$^{-/-}$ and B6-*Tnf*$^{-/-}$-A^y at 12 weeks, and no significant body weight differences were noticed with the naked eye in the comparison between B6-*Tnf*$^{-/-}$-A^y and B6-A^y, and in the comparison between B6-*Tnf*$^{-/-}$ and B6, body weights were not determined. At 20 weeks, there were no significant difference in body weight between B6-*Tnf*$^{-/-}$ and B6 in both sexes (data not shown).

In the absence of TNFα, a central mediator of LPS action, a dose of LPS of 200 μg/mouse induced no lethality in B6-*Tnf*$^{-/-}$, but induced 36% lethality in B6-*Tnf*$^{-/-}$-A^y; therefore, B6-*Tnf*$^{-/-}$-A^y had a significantly lower survival rate than did B6-*Tnf*$^{-/-}$ at 12 weeks (Fig. 3). Apparently, absence of TNFα exerted a protective effect against LPS toxicity, but an A^y effect of enhancing the sensitivity to LPS-induced lethality was still observed, suggesting that the A^y effect was independent of TNFα toxicity.

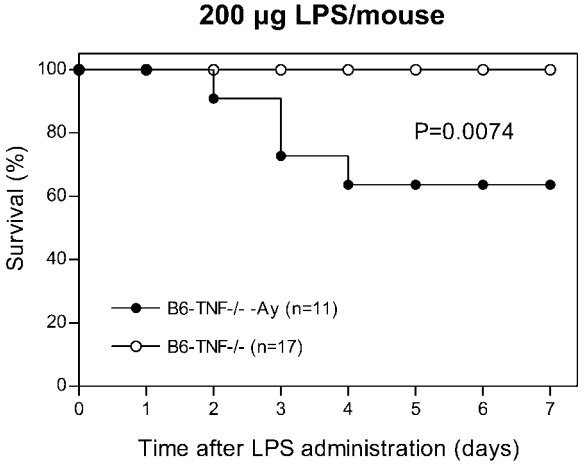

Fig. 3. Comparison of sensitivity to LPS (200 μg per mouse)-induced lethality between B6-*Tnf*$^{-/-}$ (○) and B6-*Tnf*$^{-/-}$-A^y (●). The x-axis represents time (days) after LPS administration and the y-axis represents survival rate (%). LPS was administered intraperitoneally to mice (numbers are indicated in parentheses) fasted for 24 hr. Lethality was evaluated daily over seven days of LPS administration.

LPS-induced anorexia: Food intake (DFI) and body weight gain (DWG) were evaluated daily for five days of LPS administration on B6-12w and B6-A^y-12w mice for comparison of the sensitivity to LPS-induced anorexia. Anorexia is one of the most remarkable systemic changes

during infection and/or inflammation, and is known to be associated with the development of cachexia [5]. In control groups (without LPS), B6-A^y-12w showed significantly higher DFI than did B6-12w on and after day 2 (Fig. 4A). The result was in accordance with the fact that B6-A^y are hyperphagic. In contrast, in experimental groups, B6-A^y-12w showed significantly lower DFI than did B6-12w on and after day 3 (There was no significant difference on the day 2. Statistical comparison was not applicable at day 5, because only two B6-A^y-12w were available.) (Fig. 4A). At variance with the results for the control groups, B6-12w showed significantly higher DFI at day 1 than did B6-A^y-12w in LPS groups. Probably the discrepancy was caused by the difference in the number of mice used for statistical analysis. Indeed, the number of B6 and B6-A^y was six in the control group, whereas the number of B6 and B6-A^y was thirty in the LPS group. DWG data clearly substantiated the data on DFI; that is, B6-A^y-12 had significantly lower DWG than did B6-12 on days 1, 3, and 4, and there was no significant difference on day 2 (Fig. 4B). The results clearly showed that B6-A^y-12w were more sensitive to LPS-induced anorexia than were B6-12w.

DISCUSSION

Although precise mechanisms and mediators of anorexia during infections are far from established, leptin appeared as one convincing mediator of anorexia during the last decade, because leptin decreases food intake (therefore body weight) [12], because leptin expression is induced by the administration of LPS and TNFα [11, 24], and because leptin expression decreases during fasting [1]. However, because LPS can induce profound anorexia in the absence of a functional leptin-leptin receptor system in *ob/ob* and *db/db* mice, it is unlikely that leptin is a mediator of LPS-induced anorexia [5]. Rather, leptin is suggested to have other physiologic roles in addition to food intake. Indeed, several lines of evidence suggest that leptin is an endogenous protein that is protective against inflammation [6, 7, 32]. Faggioni *et al.* [6, 7], Takahashi *et al.* [32], and this study reported that the leptin deficiency enhances sensitivity to LPS- and/or TNFα-induced lethality.

The observation that B6-A^y mice are more sensitive to LPS-induced lethality than B6 mice suggests a potential role of melanocortin receptors, MC1R and MC4R, in the host response cascade against inflammation. The result that B6-A^y mice are relatively resistant to the lethal effect of LPS when compared to B6-*ob/ob* mice supports the hypothesis that a certain part of the anti-inflammatory effect of leptin is mediated by αMSH, which has also been suggested to have anti-inflammatory activity [5, 14]. αMSH arises from proteolytic cleavage of proopiomelanocortin (POMC), and POMC neurons are known to be direct targets for leptin in the hypothalamus, because leptin receptors are located on POMC neurons [2]. αMSH exerts its influence on the host both centrally and peripherally [14]. Among five melano-

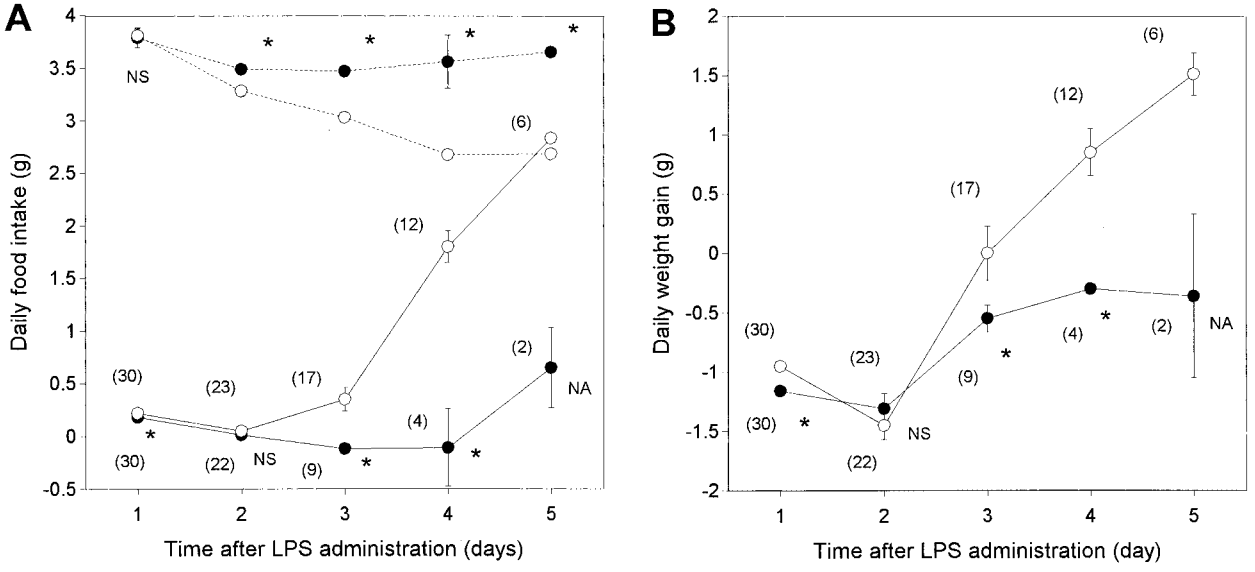

Fig. 4. LPS-induced anorexia. **A**: Comparison of daily food intake in mice with (experimental groups marked by solid lines) or without (control groups marked by broken lines) LPS between B6-A^y-12w (●) and B6-12w (○). In control groups, each time point represents mean ± S.E. of six measurements (mice). In experimental groups, each time point represents mean ± S.E. of several measurements (indicated in parentheses). *: Significant difference between B6-A^y-12w (●) and B6-12w (○). NS: not significant. NA: not applicable (due to too few surviving B6-A^y-12w). **B**: Comparison of daily weight gain in mice with LPS administration between B6-A^y-12w (●) and B6-12w (○). In control groups, each time point represents mean ± S.E. of six measurements (mice). In experimental groups, each time point represents mean ± S.E. of several measurements (indicated in parentheses). *: Significant difference between B6-A^y-12w (●) and B6-12w (○). NS: not significant. NA: not applicable (due to too few surviving B6-A^y-12w).

cortin receptors (MC1R-MC5R) identified to date [10], MC1R is expressed in various cutaneous cell types, and was shown to have role in pigmentation (therefore, A^y mice are yellow). On the other hand, MC4R, which is expressed in the hypothalamus, are shown to have a role in feeding (therefore A^y mice are obese) downstream of the leptin signaling [6, 7, 32]. As postulated by Takahashi *et al.* [32], peripheral anti-inflammatory effects of αMSH may be mediated by MC1R, and central effects may be mediated by MC4R, although the involvement of MC4R in the anti-inflammatory property of the leptin is still unclear. Furthermore, because B6-A^y suffered more severe anorexia than did B6, MC4R is not absolutely required for the LPS-induced anorexia. Along with the results that leptin alone is not required for LPS-induced anorexia [5], the leptin-MC4R pathway does not play a crucial role in LPS-induced lethality. Shimomura *et al.* [25] also reported that exogenous IL-1β suppresses food intake in mice, and that the sensitivity to IL-1β-induced anorexia is enhanced in A^{vy} (viable yellow) mice. In contrast, Marks *et al.* [17] reported that anorexia induced by LPS is ameliorated by a central MC4R blockade. Apparently the discrepancy between these studies suggests that augmented anorexia in A^y (and probably in A^{vy}) mice is not simply due to antagonism of MC4R, and suggests that other melanocortin receptor types may be involved in the enhanced LPS-induced anorexia of A^y mice.

I demonstrated that the sensitivity to the lethal effect of LPS dramatically increased in aged mice (age effect), in both B6 and B6-A^y. However, the A^y effect of enhancing sensitivity to LPS-induced lethality was still significant, suggesting that the A^y effect is independent of the age effect. The age effect is probably associated with various systemic changes; among these, the most likely age effect will be related to augmentation of pro-inflammatory cytokine production. However, the A^y effect was still significant in the absence of TNFα, a principal mediator of LPS toxicity; therefore, it is suggested that the A^y effect is independent of TNFα toxicity. This result is consistent with the postulation by Takahashi *et al.* [32]. They speculate that TNFα and leptin control the body weight independently, because TNFα can induce weight loss in the absence of a functional leptin system.

Taken together, because αMSH is likely to be an endogenous anti-inflammatory peptide, because MC1R is expressed in various cutaneous cell types including neutrophils and macrophages, and because αMSH has a strong affinity to MC1R, the A^y effect might be caused by way of a pathway involving MC1R. For characterizing the mechanism of the in A^y effect further, it is crucially important to identify genes that are involved in the interaction between αMSH and MC1R. I adopted quantitative trait locus (QTL) mapping analysis as one of the most suitable and practicable methods for this purpose, and identified five statistically significant QTLs [29–31]. It is interest to note that 2 of these QTLs include *Slc11a1* (formerly *Nramp1*) and *Slc11a2* (formerly *Nramp2*) in their confidence intervals as plausible candidate genes [16]. Although it is not achieved yet, identification of causative genes underlying these loci will promise better understanding of molecular nature of the A^y effect. Thus, αMSH-MC1R interaction is suggested to have a more important role in innate immunity than thought previously.

ACKNOWLEDGEMENT. The author thanks Dr. K. Sekikawa for providing *Tnf* gene deficient mice.

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