# Attenuation of the Obesity Syndrome of *ob/ob* Mice by the Loss of Neuropeptide Y

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The obesity syndrome of *ob/ob* mice results from lack of leptin, a hormone released by fat cells that acts in the brain to suppress feeding and stimulate metabolism. Neuropeptide Y (NPY) is a neuromodulator implicated in the control of energy balance and is overproduced in the hypothalamus of *ob/ob* mice. To determine the role of NPY in the response to leptin deficiency, *ob/ob* mice deficient for NPY were generated. In the absence of NPY, *ob/ob* mice are less obese because of reduced food intake and increased energy expenditure, and are less severely affected by diabetes, sterility, and somatotropic defects. These results suggest that NPY is a central effector of leptin deficiency.

The circulating hormone leptin informs the brain about the abundance of body fat, thereby allowing feeding behavior, metabolism, and endocrine physiology to be coupled to the nutritional state of the organism. Leptin promotes weight loss by suppressing appetite and stimulating metabolism, and is required for normal endocrine function (*1*–*3*). Consequently, mutant mice that lack leptin or functional leptin receptors, such as *ob/ob* and *db/db* mice, respectively, are profoundly hyperphagic, massively obese, hypometabolic, hypothermic, diabetic, and infertile (*4*, *5*).

The mechanisms by which alterations in circulating leptin levels trigger changes in feeding behavior, metabolism, and endocrine function are unknown. Neuropeptide Y (NPY), a neuromodulator abundant in regions of the hypothalamus that participate in energy balance and neuroendocrine signaling, has been implicated as a mediator of the response to leptin deficiency (*3*, *6*, *7*). The expression and release of hypothalamic NPY are inhibited by leptin (*6*, *7*); consequently, NPY signaling is elevated in the hypothalamus of leptin-deficient rodents (*6*, *8*). In addition, chronic administration of NPY into the hypothalamus of normal animals mimics the phenotype of leptin deficiency, including obesity, hyperphagia, reduced thermogenesis, decreased fertility, and inhibition of growth hormone production (*9*). In light of these findings, our observation that mice deficient in NPY have normal body weight, body adiposity, and food intake was unexpected (*10*).

To investigate the role of NPY in the obesity syndrome resulting from the absence of leptin, we generated mice deficient in both leptin and NPY by breeding the mutant NPY allele onto the *ob/ob* background (*11*). The double mutants (NPY$^{-/-}$ *ob/ob* mice) could be distinguished from *ob/ob* mice by their narrower bodies and improved grooming (Fig. 1A). This physical difference was evident at ~6 weeks and became more pronounced with increasing age. However, double mutants were clearly larger than normal mice at all ages after weaning.

As expected from differences in body shape, NPY$^{-/-}$ *ob/ob* mice weighed less than *ob/ob* mice but more than normal mice (Fig. 1B). The difference in body weight between double mutants and *ob/ob* mice was significant after 10 weeks of age in males and 6 weeks of age in females. At 16 weeks of age, double mutant males weighed 13% less than *ob/ob* males ($P < 0.002$) and double mutant females weighed 27% less than *ob/ob* females ($P < 0.001$), reflecting a ~25% and ~40% correction of the excess weight of *ob/ob* mice, respectively.

Double mutants were not as obese as *ob/ob* mice. Whole-mouse magnetic resonance imaging (*12*) revealed that NPY$^{-/-}$ *ob/ob* mice had less fat throughout their bodies than *ob/ob* mice, although they were more obese than normal mice (Fig. 2A). In addition, the lipid:water ratio, indicative of percent body fat, for 12- to 14-week-old double mutants was ~40% less than that for *ob/ob* mice (Fig. 2B). We confirmed the lower fat content of NPY$^{-/-}$ *ob/ob* mice at 16 weeks of age by weighing discrete fat pads. The weights of inguinal, reproductive (epididymal in males, parametrial in females), retroperitoneal, and scapular fat pads were lower in double mutant mice than in *ob/ob* mice. The fat depots showing the biggest weight differences were the scapular fat pad [0.28 ± 0.05 g in normal, 7.5 ± 0.5 g in *ob/ob*, and 2.3 ± 0.4 g in double mutant mice ($P < 0.001$ compared to *ob/ob*)], and the inguinal fat pads [0.38 ± 0.05 g in normal, 7.5 ± 0.4 g in *ob/ob*, and 3.2 ± 0.3 g in double mutant mice ($P < 0.001$ compared to *ob/ob*)]. The average combined weight of the four fat pads was 51% less in NPY$^{-/-}$ *ob/ob* mice than in *ob/ob* mice (Fig. 2C).

The obesity of *ob/ob* mice is accompa-

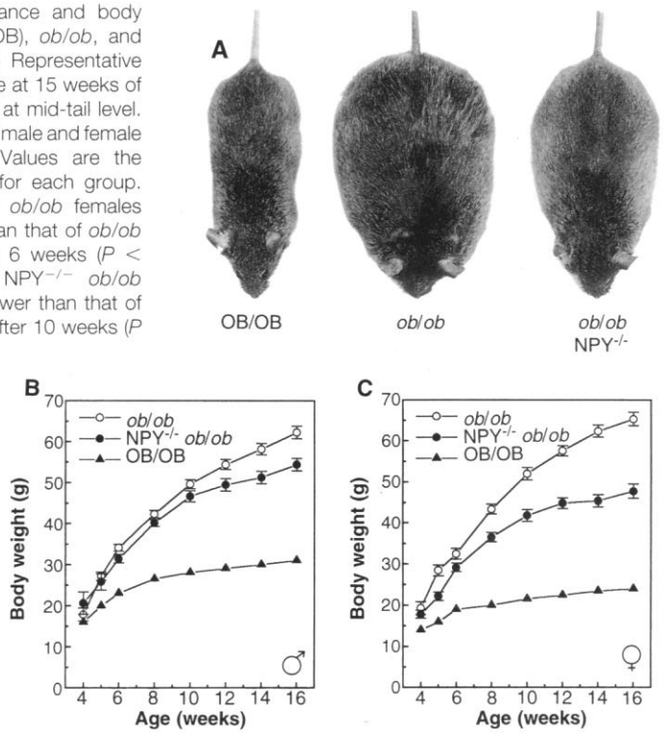


**Fig. 1.** Physical appearance and body weights of normal (OB/OB), *ob/ob*, and NPY$^{-/-}$ *ob/ob* mice. (**A**) Representative body shapes of male mice at 15 weeks of age. Photo was cropped at mid-tail level. (**B** and **C**) Body weights of male and female mice at various ages. Values are the mean ± SEM; $n > 10$ for each group. Body weight of NPY$^{-/-}$ *ob/ob* females was significantly lower than that of *ob/ob* females at all ages after 6 weeks ($P < 0.01$). Body weight of NPY$^{-/-}$ *ob/ob* males was significantly lower than that of *ob/ob* males at all ages after 10 weeks ($P < 0.02$).

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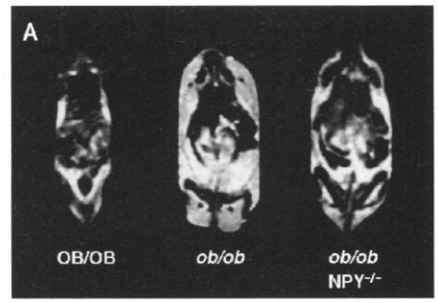
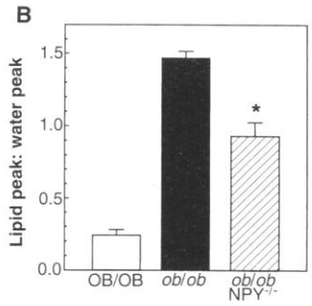
nied by extensive deposition of fat in liver, resulting in hepatomegaly. Histological examination revealed that double mutants had fatty livers, but the hepatic lipid deposits were smaller and fewer in number than in *ob/ob* mice. The average liver weight was 1.32 ± 0.05 g for normal mice, 3.72 ± 0.18 g for *ob/ob* mice, and 2.71 ± 0.15 g for NPY$^{-/-}$ *ob/ob* mice ($P < 0.001$ compared to *ob/ob*).

Body weight and adiposity are influenced by the rate of food consumption and the rate at which energy is expended metabolically. To determine whether reduced food intake or increased energy expenditure was responsible for the decreased obesity of NPY$^{-/-}$ *ob/ob* mice relative to *ob/ob* mice, we compared their rates of food and oxygen consumption. Whereas *ob/ob* mice ate 62% more food than normal mice, double mutants ate only 35% more, corresponding to a ~40% suppression of hyperphagia (Fig. 3A). In addition, basal oxygen consumption was significantly higher in double mutant mice than in *ob/ob* mice (Fig. 3B). Thus, both reduced food intake and increased metabolic rate help to normalize energy balance in NPY$^{-/-}$ *ob/ob* mice. The increased oxygen consumption of NPY$^{-/-}$ *ob/ob* mice was due in part to the maintenance of a higher body temperature (Fig. 3C). Energy expenditure of double mutants was further enhanced by increased physical activity as indicated by 24-hour ambulation (Fig. 3D). No significant differences in any of these metabolic parameters were detected between wild-type mice and NPY$^{-/-}$ mice.

The *ob/ob* mice develop a form of diabetes similar to human type-II diabetes (*5*), a condition commonly associated with obesity. To determine whether development of diabetes was delayed in NPY$^{-/-}$ *ob/ob* mice, we monitored urine glucose. Between 4 and 6 weeks of age, 42% of *ob/ob* mice, but only 20% of double mutants, had detectable levels of glucose in their urine. Between 8 and 14 weeks of age, the fraction of *ob/ob* mice with glucosuria increased to 70%, whereas the fraction of double mutants with glucosuria was only 23% (Table 1). We also measured serum glucose and insulin levels in mice at 16 weeks of age. Serum glucose levels were close to normal in double mutants but were elevated in *ob/ob* mice (Table 1). Although serum insulin levels were high in NPY$^{-/-}$ *ob/ob* mice, they were ~50% lower than the levels in *ob/ob* mice (Table 1). The decreased incidence and severity of diabetes in NPY$^{-/-}$ *ob/ob* mice is most likely related to the reduced obesity.

The hypothalamic-pituitary axis is also impaired in *ob/ob* mice as manifested by sexual immaturity and reduced secretion of growth hormone (*2*, *13*). The somatotropic axis of double mutant mice was more active than that of *ob/ob* mice, as suggested by higher liver levels of insulin-like growth factor–I (IGF-I) mRNA and longer body length, parameters that positively correlate with growth hormone production (Table 1). In addition, the absolute difference in combined fat pad weights of *ob/ob* males and double mutant males (10.1 g) and the difference in their total body fat mass estimated from MRI analysis (15 g) exceeded the difference in their body weights (7.9 g), indicating that



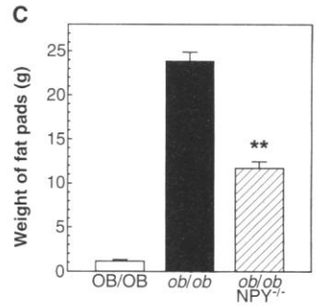
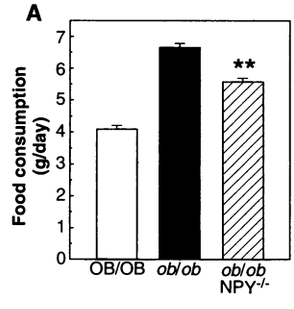
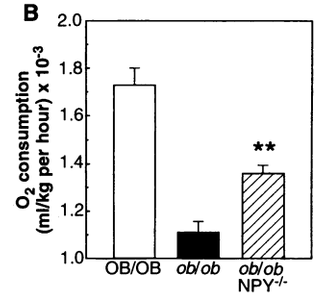
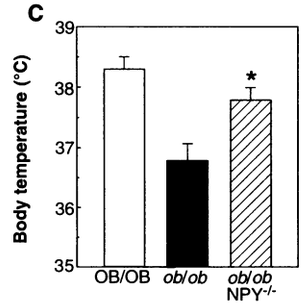




**Fig. 2.** Adiposity of normal, *ob/ob*, and NPY$^{-/-}$ *ob/ob* mice. (**A**) Fat-selective magnetic resonance images (MRIs) of male mice at 14 weeks of age (*12*). Images are 3-mm thick, body length, horizontal sections. Adipose tissue appears white. Images are oriented such that the head of each mouse is at the top. The sides of the *ob/ob* image are straight because the mouse was pressed against the walls of the MR tube. (**B**) Average lipid:water ratios of 12- to 15-week-old mice obtained from MR spectra (*12*). Values are the mean ± SEM. Each group consisted of four males and three females. *$P < 0.001$ compared to *ob/ob* mice; unpaired *t*-test. Some *ob/ob* mice, but not double mutants, could not be analyzed by this technique because they were too large to fit into the 4.2-cm-diameter coil. Consequently, the adiposity of *ob/ob* mice was slightly underestimated. (**C**) Combined weights of inguinal, retroperitoneal, scapular, and reproductive pads, measured when mice were 16 weeks of age. Values are the mean ± SEM. The *ob/ob* group consisted of 19 males and 15 females; the double mutant group consisted of 12 males and 10 females. **$P < 0.001$ compared to *ob/ob* mice, unpaired *t*-test.



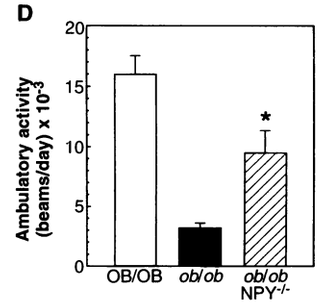




**Fig. 3.** Energy balance in normal, *ob/ob*, and NPY$^{-/-}$ *ob/ob* mice. (**A**) Average daily food consumption. Food intake was determined by measuring 5-day intake in mice at 6, 8, 12, and 14 weeks of age; $n > 25$ for each genotype. **$P < 0.001$ compared to *ob/ob* mice. (**B**) Basal oxygen consumption of 12- to 14-week-old mice as measured by indirect calorimetry (*20*); $n = 12$ for each genotype. **$P < 0.001$ compared to *ob/ob* mice. (**C**) Body temperature of 14- to 16-week-old mice. Core temperature was measured with a rectal thermister (Yellow Springs Instruments, Yellow Springs, Ohio). $n \geq 14$ for each genotype. *$P < 0.01$ compared to *ob/ob* mice. (**D**) Ambulatory activity of 10- to 14-week-old mice (*21*); $n > 10$ for each genotype. *$P < 0.01$ compared to *ob/ob* mice. All values are the mean ± SEM. Approximately equal numbers of males and females were included in all measurements. No significant differences were detected between NPY$^{-/-}$ and wild-type mice, so their values were combined and used as the values for normal mice.

double mutant mice have greater lean body mass than *ob/ob* mice.

The fertility of *ob/ob* mice was also improved by the absence of NPY (Table 1). One-third of double mutant males, but only 5% of *ob/ob* males, were fertile. Surprisingly, 2 of 10 double mutant females were fertile whereas *ob/ob* females were always infertile (Table 1) (*2*, *5*). The two fertile NPY$^{-/-}$ *ob/ob* females had litters of normal size and successfully nurtured their pups to weaning. In addition, the seminal vesicles in males and the uteri in females weighed more in double mutant mice than in *ob/ob* mice, suggesting that there was greater stimulation by sex steroids in the former (Table 1). The improved reproductive function of NPY$^{-/-}$ *ob/ob* mice, especially females, is likely due to enhanced pituitary hormone release, as *ob/ob* mice are pituitary-insufficient and exogenous NPY suppresses gonadotropin secretion (*2*, *5*, *9*). Increased fertility of double mutant males may also be a secondary effect of reduced adiposity, as thinning of *ob/ob* males by food restriction improves their reproductive capacity (*14*).

These results demonstrate that NPY is required for full manifestation of the *ob/ob* phenotype and thereby implicate NPY-containing neural pathways as mediators of the hyperphagia, hypometabolism, and endocrine alterations resulting from chronic leptin deficiency. Although NPY is found throughout the mammalian nervous system, its actions in mediating the effects of leptin deficiency probably occur in the hypothalamus, where NPY is synthesized by neurons of the arcuate nucleus and secreted from their terminals in the paraventricular nucleus and ventromedial hypothalamus (*15*). The leptin receptor is expressed by arcuate neurons (*7*, *16*) and leptin inhibits both the expression and release of hypothalamic NPY (*6*, *7*). Leptin also decreases sensitivity to NPY's appetite-stimulating effect in the brain (*17*). Our study provides evidence that antagonism of the hypothalamic NPY system is a critical element of leptin's regulatory actions on body weight.

Why does the loss of NPY attenuate the obesity syndrome of *ob/ob* mice without any discernible effect on feeding, body weight, or the response to fasting of normal mice (*10*)? The requirement of NPY in the response to leptin deficiency most likely reflects the striking elevation in hypothalamic NPY activity in this condition. Although compensatory mechanisms may substitute for the loss of NPY in normal mice, these mechanisms may be inadequate in *ob/ob* mice. Furthermore, although both *ob/ob* mice and fasted mice exhibit an increased drive to eat and a decreased metabolic rate, the loss of energy reserves in the latter is a significant threat to survival and may recruit signaling pathways in addition to those activated by leptin deficiency alone.

The involvement of other neuromodulators in the hypothalamic response to leptin deficiency is suggested by the observation that elimination of NPY did not completely reverse the phenotype of *ob/ob* mice. Localization of leptin receptor expression to brain regions outside the arcuate nucleus (*7*, *16*) supports this proposition. Melanin-concentrating hormone and corticotropin-releasing hormone are strong candidates for additional leptin-sensitive regulators of energy balance (*7*, *18*).

The high levels of leptin detected in obese humans and rodents suggests that leptin "resistance" may underlie obesity (*19*). Because NPY mediates some of the hyperphagia and hypometabolism resulting from complete absence of leptin action in *ob/ob* mice, it might also contribute to forms of obesity associated with impaired leptin signaling.

**Table 1.** Indices of endocrine function in normal, *ob/ob*, and NPY$^{-/-}$ *ob/ob* mice. The presence of glucose in urine (glucosuria) was tested in mice between 8 and 14 weeks of age on three separate occasions using Chemstrip urine test strips (Boehringer Mannheim). Insulin and glucose concentrations were measured in serum from ~16-week-old mice ($n$ = 7 OB/OB, $n$ = 11 *ob/ob*, $n$ = 9 NPY$^{-/-}$ *ob/ob*) by radioimmunoassay using a human insulin standard and the glucose oxidase method (Glucose Analyzer II, Beckman), respectively. IGF-I mRNA was quantitated in the livers of four males and four females of each group by solution hybridization (*22*). Fertility was tested by housing each mouse with a proven breeder of the opposite sex for 3 weeks beginning at 8 weeks of age. The presence of copulatory plugs was checked daily and animals were considered fertile if a litter was subsequently born. Seminal vesicle weight was measured in ~16-week-old males ($n$ = 19 OB/OB, $n$ = 19 *ob/ob*, $n$ = 12 NPY$^{-/-}$ *ob/ob*). Uterine weight was measured in ~16-week-old females ($n$ = 12 OB/OB, $n$ = 15 *ob/ob*, $n$ = 10 NPY$^{-/-}$ *ob/ob*). Values are the mean ± SEM. Two-tailed Fisher exact test and $\chi^2$ analysis were used for statistical comparison of fertility rates and incidence of glucosuria, respectively. Unpaired $t$-test was used for statistical comparison of all other measurements.

| Endocrine parameter | OB/OB | *ob/ob* | NPY$^{-/-}$ *ob/ob* | $P$ value* |
|---|---|---|---|---|
| Diabetes | | | | |
| Glucosuria (%) | 0/6 (0%) | 12/17 (70%) | 6/26 (23%) | <0.01 |
| Serum glucose (mg/dl) | 205 ± 17 | 382 ± 38 | 261 ± 12 | <0.01 |
| Serum insulin (μU/ml) | 40 ± 18 | 2820 ± 392 | 1460 ± 372 | <0.05 |
| Somatotropic axis | | | | |
| Liver IGF-I mRNA (molecules per cell) | 399 ± 6.3 | 238 ± 9.6 | 343 ± 14 | <0.001 |
| Nasal–anal length (cm) | | | | |
| Males | 10.30 ± 0.07 | 10.02 ± 0.09 | 10.49 ± 0.10 | <0.002 |
| Females | 9.95 ± 0.05 | 9.85 ± 0.09 | 10.09 ± 0.11 | <0.10 |
| Reproductive axis | | | | |
| Fertility (%) | | | | |
| Males | ND† | 1/20 (5%) | 5/15 (33%) | <0.05 |
| Females | ND | 0/15 (0%) | 2/10 (20%) | <0.15 |
| Seminal vesicle weight (mg) | 279 ± 15 | 195 ± 24 | 325 ± 33 | <0.005 |
| Uterine weight (mg) | 131 ± 16 | 30 ± 3 | 62 ± 24 | <0.2 |

*Refers to the difference between *ob/ob* mice and NPY$^{-/-}$ *ob/ob* mice. †ND, not determined.

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11. Because the NPY and *ob* genes are genetically linked on mouse chromosome 6, three crosses were required to generate *ob/ob* mice homozygous for the inactivated NPY allele. First, NPY$^{+/-}$ males (129Sv strain) were mated with *ob/+* females (C57Bl strain, Jackson Laboratory) to generate compound heterozygotes. Next, compound heterozygotes were mated and NPY$^{+/+}$ *ob/+* and NPY$^{-/-}$ *ob/+* recombinant offspring were identified. Finally, NPY$^{-/-}$ *ob/+* mice and NPY$^{+/+}$ *ob/+* mice were inbred to produce NPY$^{-/-}$ *ob/ob* mice and NPY$^{+/+}$ OB/OB mice, respectively. Approximately 5 males and 10 females were used as breeders for each group to ensure diversity of the hybrid genetic background. The genotyping strategy was based on the fact that the *ob* mutation generates a Dde I restriction site. A ~160-bp region spanning the site of the *ob* mutation was amplified by the polymerase chain reaction using oligonucleotides 5′-TGTCCAAGATGGACCAGACTC-3′ and 5′-ACTGGTCT-

GAGGCAGGGAGCA-3′, digested with Dde I, and the products resolved on a 2% NuSeive agarose gel. Animals were weaned between 3 and 4 weeks of age and housed individually. Mice had free access to standard rodent chow (Teklad) and were treated in accordance with University of Washington guidelines.
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20. We measured oxygen consumption of individual mice with an Oxymax apparatus (Columbus Instruments, Columbus, OH). Each mouse was placed in a sealed chamber with an air flow of 1 liter per minute for ~4 hours. The $O_2$ levels in air going into and out of the chamber were measured every minute. Basal oxygen consumption was the average at low plateaus, which coincided with periods of inactivity.
21. We measured ambulatory activity of individual mice in their transparent plastic, home cage (28 cm × 17 cm × 11.5 cm) over 24 hours with an Opto-varimex mini activity meter (Columbus Instruments). The distance between infrared beams was 2.65 cm.
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23. Special thanks to E. Shankland and M. Kushmerick for MRIs and spectra; G. Froelick for histology; the laboratory of S. Woods for help with indirect calorimetry; the Metabolism Laboratory of the Puget Sound Veterans Affairs Medical Center for serum insulin and glucose determinations; and M. Schwartz and D. Cummings for discussion and suggestions. J.C.E. is a Merck fellow.

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# Induction of Autoimmune Diabetes by Oral Administration of Autoantigen

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An antigen administered orally can induce immunological tolerance to a subsequent challenge with the same antigen. Evidence has been provided for the efficacy of this approach in the treatment of human autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. However, oral administration of autoantigen in mice was found to induce a cytotoxic T lymphocyte response that could lead to the onset of autoimmune diabetes. Thus, feeding autoantigen can cause autoimmunity, which suggests that caution should be used when applying this approach to the treatment of human autoimmune diseases.

Feeding antigen can cause an antigen-specific reduction in many types of immune responses, including antibody production; delayed type hypersensitivity; T cell proliferation; and recently, the generation of cytotoxic T lymphocytes (CTLs) (*1*–*5*). This approach has been examined as a potential treatment for autoimmune diseases and has been reported to be beneficial in some cases (*6*–*7*). In contrast to the reported tolerogenicity of the oral route, we found that feeding C57Bl/6 (B6) mice 20 mg of ovalbumin (OVA) resulted in the generation of CTL responses (Fig. 1A). Not all mice responded, but, based on several experiments outlined below (Fig. 2), 14 out of 22 mice produced OVA-specific CTLs when fed 20 mg of OVA and analyzed 14 days later. Of 16 unfed mice, only 1 mouse responded and this response was just above background. OVA-specific cytotoxicity induced by feeding was mediated by $CD8^+$ T cells (Fig. 1B), and these cells expressed αβ T cell receptors (TCRs) (Fig. 1C).

When mice were fed 20 mg of OVA and then challenged with an immunogenic form of OVA, their CTL responses were reduced relative to those of unfed controls (Fig. 1D). The immunogenic form of OVA consisted of irradiated B6 spleen cells intracytoplasmically loaded with OVA by osmotic shock (OVA-loaded spleen cells) (*8*). At present, it is unclear why feeding OVA reduced the CTL response induced by OVA-loaded spleen cells but on its own was able to induce OVA-specific CTLs. The relatively weak CTL responses induced by feeding alone (Fig. 1A), however, were approximately equivalent to the reduced CTL responses seen in mice that were fed OVA and then primed by OVA-loaded spleen cells (Fig. 1D). Thus, feeding appears to prevent the induction of the stronger responses induced by OVA-loaded spleen cells but does itself induce a weak CTL response. This may be related to the CD4 T cell dependence of the OVA-loaded spleen cell response (*9*) and the apparent deletion of $CD4^+$ T cells after feeding (*10*).

To analyze the kinetics of oral induction of CTLs, B6 mice fed 20 mg of OVA were examined at various time points (Fig. 2A). CTLs were most reliably detected in the spleen on day 14 but were still present 3 months after feeding. To examine the effect of antigen dose, mice were fed different doses of OVA and then tested 14 days later for the induction of CTLs (Fig. 2B). This indicated that the relatively large dose of 60 mg of OVA was the most effective dose for inducing CTLs. To further understand the mechanism of oral CTL induction, we examined this response for its dependence on $CD4^+$ T cells (Fig. 2C). No response was seen in the absence of $CD4^+$ T cells, which indicates a requirement for this T cell subset. To determine whether CTLs were generated as a result of the degradation of whole protein by digestive enzymes into antigenic peptide determinants, mice were fed a large dose of peptide antigen and then examined for CTL induction (Fig. 2D). Peptide feeding did not induce a CTL response, which suggests that processing of whole protein by host antigen-presenting cells may be necessary for priming to occur.

The above results raised the question of whether oral administration of antigen could induce autoreactive CTLs capable of causing autoimmune disease. To test this, transgenic mice expressing OVA under the control of the rat insulin promoter were generated (RIP-OVA mice). OVA expression could not be directly detected in the islet β cells of the pancreas of these mice when they were analyzed by immunohistochemistry. OVA expression was demonstrated indirectly, however, when RIP-OVA mice were crossed to a second transgenic line of mice (OT-I mice) that produce class I–restricted OVA-specific $CD8^+$ T cells that express $V_\alpha2^+$ and $V_\beta5^+$ transgenes (*11*). Double transgenic mice showed an early onset of diabetes associated with islet infiltration (Fig. 3). The induction of diabetes implied that the β cells expressed OVA. The reason why $CD8^+$ T cells destroyed β cells in this model but ignored them in others (*12*–*15*) is under investigation.

Initial experiments revealed that feeding OVA to RIP-OVA mice did not result in diabetes (*16*). Thus, a bone marrow chimera model was used. RIP-OVA mice were lethally irradiated and their bone marrow was then reconstituted with a 1:4 mixture of bone marrow from OT-I transgenic mice and Thy-1.1 congenic B6 mice. The low ratio of OT-I to B6 cells was required because of the efficient selection of the trans-

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