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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Induction of Autoimmune Diabetes by Oral Administration of Autoantigen

Effrossini Blanas, Francis R. Carbone, Janette Allison, Jacques F. A. P. Miller,* William R. Heath

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-

E. Blanas, J. Allison, J. F. A. P. Miller, W. R. Heath, Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia.
F. R. Carbone, Monash Medical School, Prahran, Victoria 3181, Australia.
*To whom correspondence should be addressed.

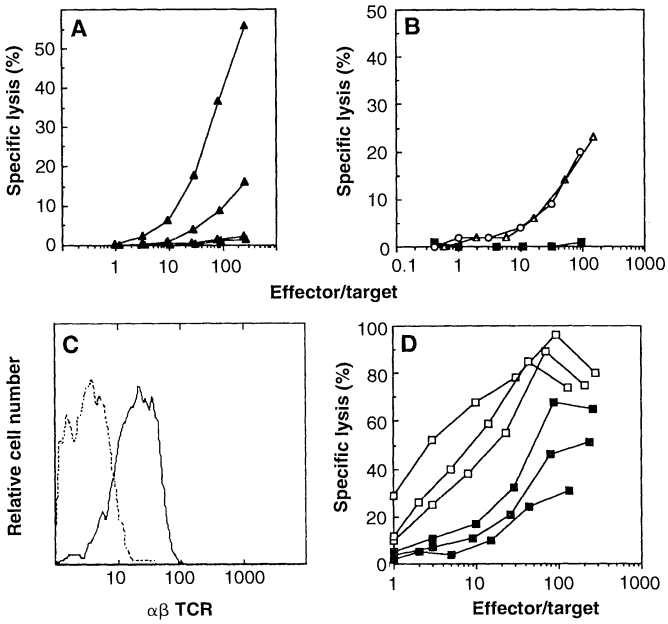

Fig. 1. The effect of oral administration of OVA on the CTL response. (**A**) Feeding OVA induces CTLs. Four B6 mice were fed 20 mg of whole OVA in 0.5 ml of phosphate-buffered saline (PBS) by intubation under light anesthesia (*3*). After 14 days, spleen cells were restimulated in vitro and then assessed for the generation of OVA-specific CTLs (*21*). Each line represents an individual mouse. Lysis of the OVA-expressing EL4 transfectant E.G7 is shown. Lysis of EL4 cells was less than 8% for the strongest response and less than 5% for all other responses. (**B**) OVA-specific CTLs induced by feeding are $CD8^+$. CTLs generated as in (A) were treated with CD8-specific monoclonal antibody (mAb) (squares), CD4-specific mAb (circles), or medium alone (triangles), and then exposed to rabbit complement. These cells were then assessed for OVA-specific lysis of E.G7 targets. Lysis of EL4 targets was less than 5%. (**C**) $CD8^+$ cells induced by feeding OVA express the αβ TCR. Untreated effector cells used in (B) were analyzed by flow cytometry (*12*) for the expression of CD8 and the αβ TCR. Samples were gated on $CD8^+$ cells and αβ expression is shown (solid line). The antibodies used were CD8-specific phycoerthrin (PE)–conjugated mAb 53.6.7 (Pharmingen), mouse αβ TCR–specific biotinylated mAb H57.597.1 (Pharmingen), and Tricolor-conjugated avidin (Caltag). Dashed line indicates no αβ TCR–specific mAb. (**D**) Feeding OVA reduced the CTL response induced by conventional priming. B6 mice were fed either 0.5 ml of PBS alone (open symbols) or 20 mg of whole OVA (closed symbols). To assess tolerance status, mice were primed 7 days later by intravenous injection of 25×10^6 15 grays OVA-loaded B6 spleen cells (*8*). After a further 7 days, spleen cells were restimulated in vitro and then assessed for OVA-specific cytotoxicity with the use of E.G7 targets. Lysis of EL4 cells was less than 5% at all effector/target ratios.

genic cells. This generated RIP-OVA chimeras that produced large numbers of OT-I $CD8^+$ T cells (Fig. 4A) but rarely became diabetic (1 out of 60 mice followed for 10 weeks after bone marrow reconstitution developed diabetes). When analyzed for islet infiltration, however, four out of eight chimeric RIP-OVA mice showed mild islet infiltration, with a mean of 22% of islets infiltrated 15 to 21 weeks after reconstitution. These chimeras provided a model for studying the effect of oral administration of autoantigen, because humans that are likely to be candidates for such treatment would be those characterized as "high risk" individuals, who are as yet nondiabetic but are most likely undergoing chronic islet infiltration and damage (*17*), as seen in the RIP-OVA chimeras. The predominance of $CD8^+$ T cells in the insulitis lesions of newly diagnosed insulin-dependent diabetes mellitus patients (*18*) also supports the use of the RIP-OVA model where autoreactive $CD8^+$ T cells are involved.

To examine the induction of autoimmunity, chimeric RIP-OVA mice were fed 60 mg of OVA or the control antigen bovine serum albumin (BSA) or were left unfed, and then were tested for glucosuria (Fig. 4B). Fourteen days after feeding OVA, 8 out of 18 chimeric RIP-OVA mice, but none of 8 chimeric B6 controls, became diabetic. All 13 BSA-fed and 9 unfed RIP-OVA chimeras remained normoglycemic. Histological examination of the pancreas 7 days after feed-

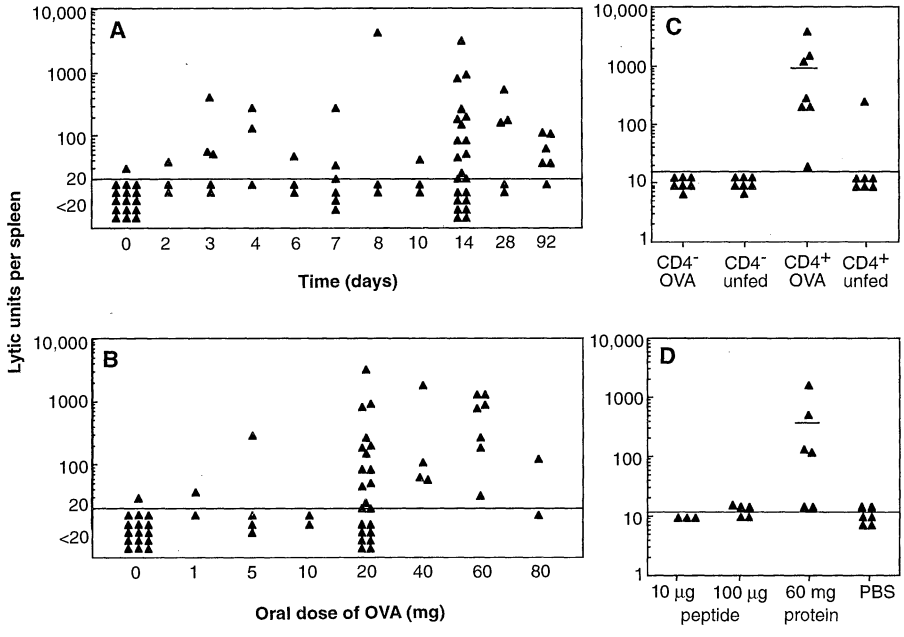

Fig. 2. Analysis of the kinetics and dose response for the induction of CTLs by feeding OVA. (**A**) Kinetics of the induction of CTLs by feeding OVA. In several experiments, B6 mice were fed 20 mg of OVA and then assessed at different time intervals for the induction of OVA-specific CTLs in their spleens as in Fig. 1A. Each point represents an individual mouse. Lytic units were calculated by determining the minimum number of effectors required to generate 10% OVA-specific lysis (in this case, background nonspecific lysis of EL4 targets was subtracted from that of the OVA-expressing E.G7 targets) and then dividing this into the total number of effectors generated from each responder spleen. As a guide to the values represented by lytic units, the two mice responding in Fig. 1A were calculated as 20 and 180 lytic units, respectively. Twenty lytic units was the minimum detectable response. Nonresponders are represented by points below the line drawn at 20 lytic units. (**B**) Dose response for the induction of OVA-specific CTLs by feeding. In several experiments, B6 mice were fed different doses of OVA, and 14 days later their spleens were assessed for the induction of OVA-specific CTLs as in Fig. 1A. (**C**) Oral induction of CTLs is dependent on $CD4^+$ T cells. B6 mice were thymectomized at 6 to 8 weeks, and then some mice were depleted of $CD4^+$ T cells by two injections of 100 μl of GK1.5 ascites, 4 days apart. Two to three weeks later, mice were fed 60 mg of OVA protein in 0.5 ml of PBS or were left unfed, and 14 to 23 days later, the CTL responses of these mice were assessed as in Fig. 1A. These data represent the combination of two independent experiments. It is not known why one unfed mouse responded to OVA. (**D**) Feeding peptide does not induce CTLs. B6 mice were fed 0.5 ml of PBS alone or containing 10 or 100 μg of $OVA_{257\text{-}264}$ peptide or 60 mg of OVA protein. Fourteen days later, their spleens were removed and cultured in vitro to stimulate CTL production as described in Fig. 1A.

ing revealed that feeding OVA, but not BSA, specifically induced infiltration in RIP-OVA chimeras and that some of the infiltrating cells were $CD8^+$ T cells (*16*).

Although some encouraging results have been obtained with the use of oral delivery of autoantigen for the treatment of autoimmune diseases (*2, 6, 7*), our results indicate that diabetes may be less amenable to this approach. In apparent contrast to our findings, feeding insulin has been shown to delay the onset and decrease the incidence of diabetes in nonobese diabetic (NOD) mice (*7, 19, 20*). In the case of insulin, however, it is not known whether there is a class I–restricted epitope, so it is unclear whether feeding this antigen could influence the $CD8^+$ T cell response. In one report, feeding insulin to NOD mice induced $CD8^+$ T cells that enhanced the disease after adoptive transfer (*19*), which suggests the existence of a class I–restricted epitope and supports our claim that feeding antigen can activate autoaggressive CTLs. The induction of autoimmune diabetes by feeding suggests that caution should be applied when attempting oral administration of autoantigen to treat human diseases that may be affected by cytotoxic T cells.

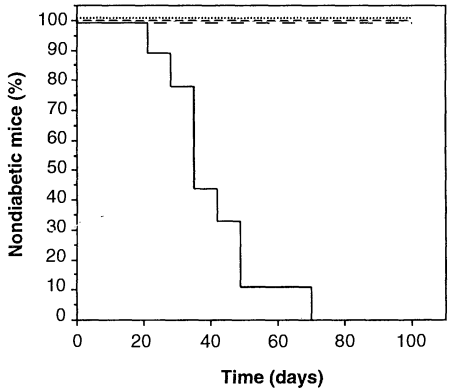

Fig. 3. RIP-OVA mice become spontaneously diabetic when crossed to the OT-I transgenic line. RIP-OVA mice on a B6 background were crossed to OT-I mice on a similar background, and offspring were analyzed for glucosuria. Mice were considered diabetic if urine glucose levels were ≥15 mM. Data include RIP-OVA mice (dotted line; $n = 28$), OT-I mice (small-dashed line; $n = 10$), RIP-OVA × OT-I mice (solid line; $n = 9$), and negative littermates (large-dashed line; $n = 26$). Five RIP-OVA × OT-I mice between the ages of 5 and 68 days were examined for islet infiltration. Histological staining with hematoxylin and eosin showed that islets in all five mice were heavily infiltrated, with an average of 82% of islets being infiltrated. Gomari's aldehyde fuchsin stain revealed that few β cells remained in the infiltrated islets. Spontaneous diabetes in double transgenic mice did not appear to be due to exogenous pathogenic bacteria or viruses because animals housed in germ-free conditions showed similar diabetes onset (*16*). RIP-OVA mice were generated with OVA cDNA driven by the rat insulin promoter by insertion of the complete OVA cDNA into the unique Hind III site of the pBlueRIP vector (*22*). Vector sequences were excised by digestion with Apa I and Not I. DNA was microinjected into pronuclei of B6 fertilized eggs as described (*23*).

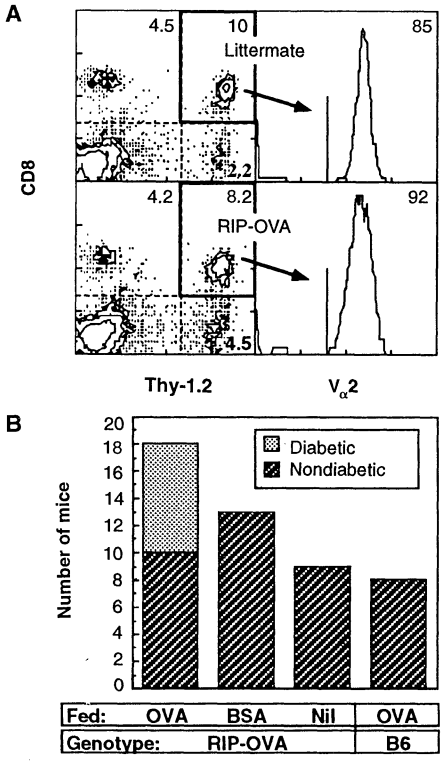

Fig. 4. Oral administration of OVA induces diabetes in chimeric RIP-OVA mice. (**A**) OVA-specific $CD8^+$ T cells were detected in the blood of chimeric RIP-OVA mice. Blood cells from chimeric RIP-OVA mice and their negative littermates, both of which were reconstituted with a 1:4 ratio of T cell–depleted OT-I and Thy-1.1 congenic B6 bone marrow, were analyzed for the expression of Thy-1.2, CD8, and $V_\alpha 2$ by flow cytometry. $Thy\text{-}1.2^+CD8^-$ cells and $Thy\text{-}1.2^+CD8^+V_\alpha 2^-$ cells represent radioresistant host cells. $V_\alpha 2$ expression by $Thy\text{-}1.2^+CD8^+$ cells is shown in the right panels. Mice were left undisturbed for 9 weeks before analysis by flow cytometry (*12*). (**B**) RIP-OVA chimeras and similarly treated negative littermates were fed 60 mg of OVA or 60 mg of BSA or were left unfed (Nil). Diabetes was assessed by glucosuria on day 14 after feeding. Chimeric RIP-OVA mice and chimeric negative littermate controls were generated by reconstitution of lethally irradiated (9 grays) mice with a mixture of 5×10^6 bone marrow cells from Thy-1.1 congenic B6 mice and 1.25×10^6 bone marrow cells from Rag-1–deficient OT-I mice ($Thy\text{-}1.2^+$). All mice were injected intraperitoneally with 100 ml of T24 ascites (Thy-1–specific) 1 day after bone marrow reconstitution to eliminate radioresistant host T cells. Mice were fed 9 weeks after reconstitution. No increase in diabetes incidence was observed after 14 days. In a separate experiment, glucosuria on day 14 was confirmed by blood glucose determination (*16*). Priming chimeric RIP-OVA mice with a nonspecific stimulus such as complete Freund's adjuvant did not result in diabetes (*16*).

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21. To generate CTLs, spleens were removed and all nucleated spleen cells from individual mice were cultured with 10^8 15 grays OVA-loaded B6 spleen cells in 30 ml of RPMI 1640 with 10% fetal bovine serum and 5×10^{-5} M 2-mercaptoethanol for 6 days. Their cytotoxicity was then assessed in a conventional ^{51}Cr-release assay using the $H\text{-}2^b$ cell line EL4 and its OVA-expressing transfectant E.G7 as targets. Cultures (30 ml) were concentrated to 1.2 ml, titrated in threefold dilutions, and enumerated to determine effector/target ratios.
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24. The care of all animals used in these studies was in accordance with institutional guidelines. We thank D. Beck, F. Karamalis, J. Falso, T. Banjanin, and M. Ekberg for technical assistance; P. Gleeson and L. Roman for providing the pBlueRIP vector; J. Howard for construction of the RIP-OVA vector; and S. Bennett for her contribution of unpublished findings. Supported by grants from the National Health and Medical Research Council of Australia, the Australian Research Council, and NIH (grant AI-29385).

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