Mucosal Antigen Primes Diabetogenic Cytotoxic T-Lymphocytes Regardless of Dose or Delivery Route

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Administration of antigens via mucosal routes, such as orally or intranasally, can induce specific immunological tolerance and has been used as a rational basis for the treatment of autoimmune diseases, including type 1 diabetes. Recently, however, orally delivered antigens were shown to induce CD8 cytotoxic T-lymphocytes (CTLs) capable of causing autoimmune diabetes. In this report, we have examined several mucosal routes for their ability to induce CTLs and autoimmune diabetes, with the aim of identifying approaches that would maximize tolerance and minimize CTL generation. In normal C57BL/6 mice, ovalbumin (OVA) delivered by either the oral or nasal routes or by aerosol inhalation was able to prime CTL immunity in both high- and low-dose regimens. To address the relevance of these CTLs to autoimmune disease, OVA was given to mice that transgenically expressed this antigen in their pancreatic β-cells. Irrespective of antigen dose or the route of delivery, mucosal OVA triggered diabetes, particularly after intranasal administration. These findings suggest that CTL immunity is likely to be a consequence of mucosal antigen delivery, regardless of the regimen, and should be considered in the clinical application of mucosal tolerance to autoimmune disease prevention. ***Diabetes*** **50:771–775, 2001**

Feeding a protein antigen suppresses subsequent priming to the same antigen. This phenomenon is referred to as "oral tolerance" and also occurs after antigen delivery via nasorespiratory and other mucosal routes (rev. in 1,2). Induction of mucosa-mediated tolerance to autoantigens is a potential means of preventing autoimmune disease, including type 1 diabetes. Several mechanisms are thought to account for mucosal tolerance, with antigen dose determining the dominant mechanism. High doses have been shown to preferentially delete antigen-reactive T-cells locally in Peyer's patches and possibly systemically after absorption, whereas low doses activate mucosal T-cells producing antiinflammatory Th2-type cytokines interleukin (IL)-4 and IL-10, or transforming growth factor-β, that may circulate as regulatory T-cells (1,2). The particular mucosal site to which antigen is targeted may also determine the nature of the immune response. It has been suggested that tolerance induction is more effective when antigen is delivered intranasally (3,4) or as inhaled aerosol (5) rather than by the oral route. In the nonobese diabetic (NOD) mouse, intranasal insulin B-chain peptide 9–23 (6), GAD peptides (7), or inhaled insulin aerosol (8) induce tolerance, which, as with repeated low-dose oral insulin (9–11), is transferable by regulatory T-cells into nontolerized recipients. However, in experimental autoimmune encephalomyelitis, Li et al. (12) found that Th2-type immunity occurred only after low-dose not high-dose intranasal antigen. Whether differences between mucosal sites reflect intrinsic differences in the nature of responses or in antigen dose-response relationships, degradation, or bioavailability is not clear.

In the NOD mouse, cytotoxic T-lymphocytes (CTLs) play a key role in β-cell destruction (13), and a CTL clone that recognizes an epitope in the insulin B-chain can transfer diabetes (14). Despite the promise of mucosal tolerance for preventing autoimmune disease, it remains possible that mucosal antigens containing epitopes for CD8 T-cells could also induce pathogenic CTLs. Garside et al. (15) were not able to detect CTLs in C57BL/6 mice in response to a single high dose (25 mg) of oral ovalbumin (OVA), but Blanas et al. (16) showed that a single high dose (20 mg) of oral OVA could induce CTLs in normal C57BL/6 mice and cause autoimmune diabetes in mice bearing OVA-specific transgenic T-cell receptors (TCRs) and expressing OVA transgenically in their β-cells. Lefrancois et al. (17) have used OVA-specific TCR mice to show that oral OVA activates CD8 T-cells in the intestinal mucosa. If these findings in a transgenic system can be extrapolated, they indicate that pathogenic CTL could be primed by oral antigen, which may explain why oral autoantigens sometimes exacerbate experimental autoimmune disease in rodents (18,19), including diabetes in BB rats (20,21). Clearly, attempts to prevent autoimmune disease by mucosal delivery of autoantigen should be based on protocols that do not induce CTL immunity. With the aim of optimizing mucosal tolerance, we set out to determine the effect of different mucosal delivery routes and antigen dose regimens on priming of CTL and development of diabetes in the OVA model.

RESEARCH DESIGN AND METHODS

Mice. Mice were bred and maintained at the Walter and Eliza Hall Institute of Medical Research. C57BL/6 mice were used at 6–8 weeks age. OT-I/recombinase activating gene (RAG)−/− (22), OT-II (23), and rat insulin promoter (RIP)-OVA^{lo} (16) transgenic mice were used at 6–12 weeks age. OT-I mice

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APC, antigen-presenting cell; CFSE, carboxy-succinimidyl-fluorescein-ester; CTL, cytotoxic T-lymphocyte; IL, interleukin; LN, lymph node; OVA, ovalbumin; PBS, phosphate-buffered saline; TCR, T-cell receptor.

carry a transgenic CD8 T-cell receptor (TCR) for the MHC class I–restricted OVA peptide aa 257–264; OT-II mice carry a transgenic CD4 TCR specific for the MHC class II–restricted OVA peptide aa 323–339. Attempts to express OT-II on the RAG−/− background have not been successful.

Mucosal delivery of OVA. Female C57BL/6 mice were given OVA (Grade VII; Sigma, St Louis, MO) in mouse tonicity phosphate-buffered saline (PBS) (320 mOsm/l) or PBS alone via three mucosal routes. The OVA dose schedules corresponded to those in the literature reported to induce mucosal tolerance (see below). The endotoxin concentration in a 10 mg/ml OVA solution measured in the Limulus lysate assay (BioWhittaker, Walkersville, MD) was 0.5 ng/ml. Oral OVA was given by intragastric intubation under light Penthrane anesthesia at either 0.5 mg for five doses or 20 mg for three doses, on alternate days, or as a single high (60 mg) dose, corresponding to schedules that induce low- or high-dose oral tolerance (1). Aerosol was given as previously described (8). Briefly, semisealed boxes of mice were each aerosolized by connection to a standard patient electric pump (Maymed Aerosol MKV; Anesthetic Supplies, Sydney, Australia) and an Aeroflo nebulizer (Waite 38, Sydney, Australia). Regimens were either 10 min (0.1, 1.0, or 10 mg/ml) daily for 5 days or 20 min (1.0 or 10 mg/ml) daily for 10 days. Intranasal OVA was given in 20 μl mouse tonicity–PBS under light Penthrane anesthesia in a dose of 0.1 mg daily for 3 days, a schedule reported to induce delayed-type hypersensitivity tolerance (24) and suppression of IgE-production (25), and in a larger dose of 0.5 mg daily for 5 days. OVA solutions were prepared under sterile conditions with precautions to avoid endotoxin contamination and were checked for solubility by nephelometry and ultracentrifugation.

CTL priming and measurement of CTL activity. To deliberately generate OVA-specific CTL, C57BL/6 mice were intravenously primed systemically with 20×10^6 OVA-coated H-2K^{bm-1} splenocytes, a mode that requires CD4 T-cell help. OVA-coated splenocytes were prepared by incubating cells in HEPES Eagle's medium containing 2.5% fetal calf serum and 10 mg/ml OVA for 10 min at 37°C, before washing and irradiation with 1,500 cGy. After 7 days, mice were killed, and their splenocytes stimulated in vitro with $OVA_{257–264}$ peptide-coated irradiated splenocytes for 6 more days.

Cytotoxicity was measured in a standard 4-h ^{51}Cr release assay from EL-4 mouse lymphoma target-cells (ATCC, Rockville, MD) pulsed with 1 μg/ml $OVA_{257–264}$ during ^{51}Cr labeling and from unpulsed labeled EL-4 cells (control). Results were expressed as percent OVA-specific lysis, obtained by subtracting nonspecific lysis (of unpulsed EL-4 cells) from OVA-specific lysis.

Cell transfers and flow cytometry. For cell transfers, splenocytes and lymph node (LN) cells were prepared from OT-I/RAG−/− or OT-II mice. Typically, 50% of cells from OT-I/RAG−/− mice and 20% of cells from OT-II mice were transgenic, as determined by flow cytometric analysis of Vα2- and Vβ5-positive CD8 and CD4 cells, respectively. Labeling of OT-II cells with carboxy-succinimidyl-fluorescein-ester (CFSE; Molecular Probes, Eugene, OR) and subsequent flow cytometric analysis (FACScan; Becton Dickinson, San Jose, CA) for in vivo proliferation have been described (26). For this purpose, 3×10^6 CFSE-labeled OT-II cells were transferred intravenously via tail vein.

Induction of diabetes by mucosal delivery of OVA in RIP-OVA^{lo} mice. After determining the numbers of OT-I and OT-II cells required for adoptive transfer to induce diabetes in RIP-OVA^{lo} mice subsequently primed with OVA-coated H2K^{bm-1} splenocytes, these numbers (0.3×10^6 OT-II and 0.2×10^6 OT-I cells) were injected intravenously into each mouse. Recipient mice were then exposed to OVA via different mucosal routes in dose regimens as used above to induce CTL immunity in normal nontransgenic C57BL/6 mice. Mice were bled from the retro-orbital venous plexus 10 and 17 days after the last dose of OVA. Diabetes was defined by a confirmed blood glucose value >12 mmol/l.

RESULTS

Oral, aerosol, or intranasal OVA prime CTL immunity. To determine whether different mucosal routes of delivery induced CTL immunity, C57BL/6 mice were given oral, aerosol, or intranasal OVA, and 14 days after the last dose, splenic CTLs were assayed after restimulation with $OVA_{257–264}$ peptide-coated irradiated splenotypes in vitro. Repeated low-dose oral OVA primed CTLs in most mice tested (Fig. 1). High-dose oral OVA, either 20 mg × 3 or 60 mg × 1 (Fig. 1), was associated with a range of CTL responses. The shorter exposure to 10 mg/ml aerosol OVA (10 min daily for 5 days) did not appear to prime CTL immunity, but longer exposure (20 min daily for 10 days) to the same dose or even the lower 1 mg/ml dose primed

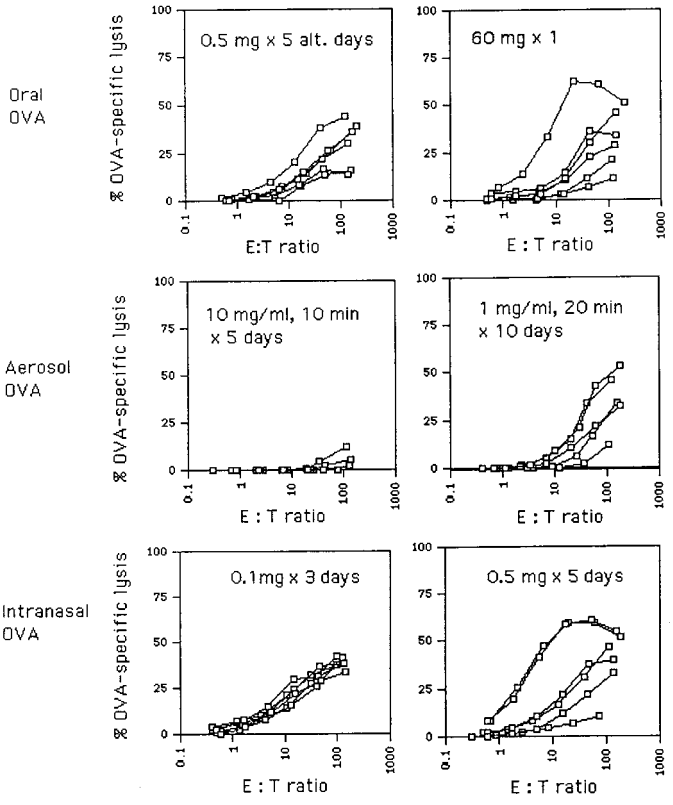

FIG. 1. Oral, aerosol, or intranasal OVA induce CTL immunity. Groups of six mice were exposed to oral, aerosol, or intranasal OVA in regimens similar to those reported to induce tolerance. Mice were killed after 14 days and their splenocytes stimulated in vitro for an additional 6 days before measuring CTL activity. Each curve is the result for an individual mouse. E:T, effector cell to target cell ratio.

CTLs (Fig. 1). Both low- and high-dose intranasal OVA primed CTLs (Fig. 1).

To confirm that OVA was delivered authentically into distinct mucosal compartments and to exclude any carryover, particularly of nasorespiratory OVA into the gut, CFSE-labeled OVA-reactive OT-II (CD4) T-cells were transferred into mice and monitored in different lymphoid tissues after exposure to OVA. In preliminary experiments, proliferation after a single dose of intranasal OVA was first observed in the anterior cervical LNs at around 60 h and for 5–6 days thereafter. Therefore, to compare the three routes of delivery, analysis was performed 72 h after a single exposure to antigen (Fig. 2). The number of peaks to the right of the major peak of fluorescently labeled cells is a measure of cell divisions after injection (26). Oral (20 mg) OVA stimulated cell proliferation selectively in gut-associated lymphoid tissues, i.e., Peyer's patches and mesenteric LNs. Aerosol OVA (10 mg/ml for 10 min) stimulated proliferation selectively in mediastinal LNs. Intranasal OVA (0.5 mg) stimulated proliferation in both upper (anterior cervical) and lower (mediastinal) respiratory tract LNs. In addition, small numbers of proliferating cells were detected in Peyer's patches and mesenteric LNs after intranasal OVA, and cells that had undergone four to six divisions were present in the spleen. A single low (0.5 mg) dose of oral OVA or a 10-min exposure to 0.1 mg/ml or 1 mg/ml aerosol OVA did not stimulate detectable proliferation (data not shown).

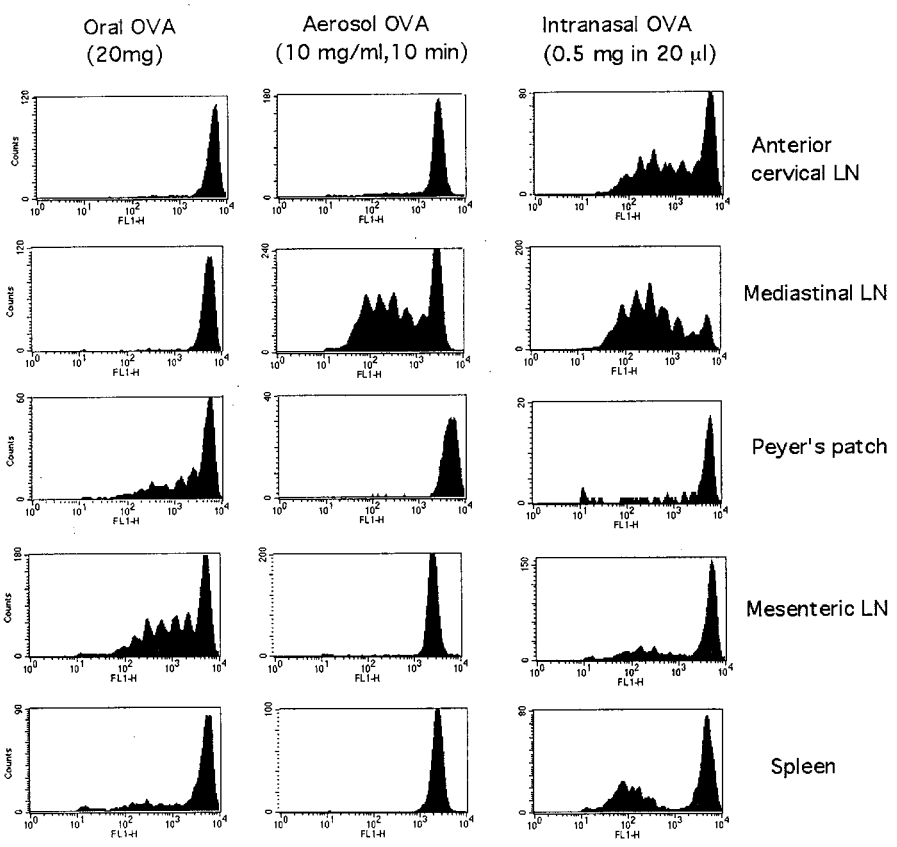

FIG. 2. Proliferation of OVA-specific CD4 (OT-II) T-cells in different lymphoid tissues in response to oral, aerosol, or intranasal OVA. Groups of three mice were exposed once to oral (60 mg), aerosol (10 mg/ml for 10 min), or intranasal (0.5 mg) OVA 4 h after receiving 3 × 10^6 CFSE-labeled OT-II cells intravenously. Mice were killed 3 days later and cells prepared from the indicated tissues, stained for Vβ5-positive (OT-II) T-cells, and analyzed by flow cytometry. Histograms represent cells gated for FL-2–positivity (Vβ5-positive cells). Dead cells were stained with propidium iodide and gated in the FL-1 channel. The experiment was performed twice, and individual mice from one experiment are shown.

Development of diabetes in RIP-OVA^{lo} mice after mucosal OVA. To determine whether CTL primed by oral, aerosol, or intranasal OVA could target OVA-expressing cells and cause tissue damage in vivo, transgenic RIP-OVA^{lo} mice expressing OVA in insulin-producing pancreatic β-cells were reconstituted with naive, potentially autoreactive CD8 CTL precursors (OT-I cells) and CD4 T-cells (OT-II cells) and exposed to OVA via the three mucosal routes. Reconstitution of cells was required because RIP-OVA^{lo} mice have leaky expression of OVA in the thymus, which deletes OVA-reactive T-cells from their repertoire. Diabetes development was monitored as the index of β-cell destruction. In preliminary experiments, we found that the minimum numbers of transferred OT-I cells and OT-II cells required for diabetes development in the majority of recipient mice when they were primed systemically in the conventional manner with intravenous OVA–coated splenocytes were 0.2×10^6 and 0.3×10^6, respectively (data not shown). These numbers of cells were therefore transferred into RIP-OVA^{lo} mice 3–4 h before administration of mucosal OVA in different dose schedules. Blood glucose was measured 10 and 17 days later. Diabetes developed in a proportion of mice after oral or aerosol OVA and in all mice after intranasal OVA, in either of two doses (Fig. 3): in 33% (2 of 6) and 29% (5 of 17) after low- and high-dose oral OVA; in 25% (2 of 8) and 44% (7 of 16) after low- and high-dose aerosol OVA; and in 100% (8 of 8) after either low- or high-dose intranasal OVA. The frequency of diabetes was identical at both points, and in other mice followed for up to 100 days after oral OVA, the incidence of diabetes did not increase after 14 days. Diabetes could not be induced by mucosal OVA in RIP-OVA^{lo} mice given up to 0.5×10^6 OT-I cells alone or 4×10^6 OT-II cells alone.

DISCUSSION

Given that a high dose of oral antigen primed autoreactive CTLs that mediated diabetes in a transgenic model (16), our aim was to determine whether different mucosal routes

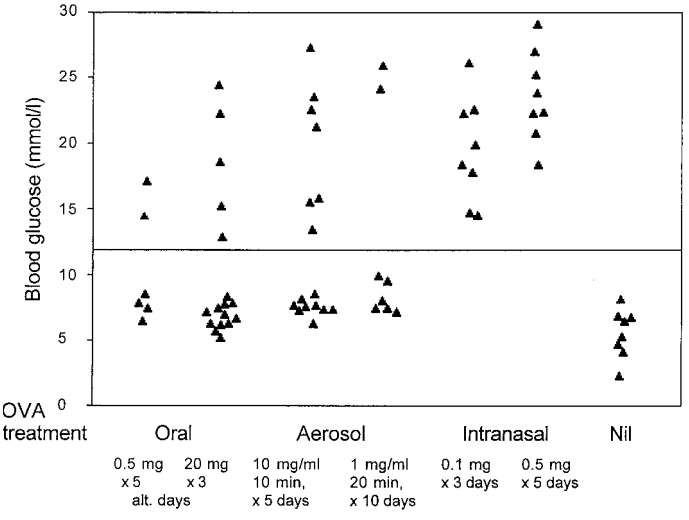

FIG. 3. Development of diabetes in RIP-OVA^{lo} mice that received mucosal OVA. Mice were adoptively transferred with CD8 CTL precursors (0.2×10^6 OT-I cells) and CD4 T-cells (0.3×10^6 OT-II cells) and exposed to oral, aerosol, or intranasal OVA in different dose schedules. Blood glucose was measured 10 days (shown) and 17 days later. The frequency of diabetes was the same at both time points. The horizontal line (12 mmol/l glucose) is the diagnostic cutoff for diabetes.

of antigen administration and/or lower doses of antigen would avoid CTL priming. Clearly, they did not. Irrespective of the mucosal route, both low- and high-dose OVA primed CTL in normal C57BL/6 mice. CTLs were detected after low- as well as high-dose oral or intranasal OVA, and only the short exposure to aerosol OVA (10 min daily for 5 days) failed to prime CTLs as detected by restimulation assay ex vivo. CTLs induced by mucosal OVA were conventional CD8 T-cells because they recognized the same MHC class I–restricted immunodominant epitope, $OVA_{257–264}$, as CTLs induced by systemic priming. To establish that mucosal OVA-induced CTLs were pathogenic, experiments were then performed in RIP-OVA^{lo} mice reconstituted with OVA-reactive transgenic T-cells. In these mice, CTL immunity to mucosal OVA was associated with the development of diabetes. Priming of pathogenic CTLs is therefore not just restricted to the oral route, but occurs when OVA is delivered as an aerosol or intranasally, and cannot be avoided by low doses reported to induce conventional oral tolerance.

Diabetes developed in 100% of RIP-OVA^{lo} mice after either low- or high-dose intranasal OVA. The strong proliferative response to intranasal OVA in mediastinal LNs is consistent with carryover of the nasal dose into the bronchi and might be avoided by reducing the delivery volume. The small numbers of proliferating cells in Peyer's patches and mesenteric LNs could also have resulted from carryover of the intranasal dose. However, the presence of cells in the spleen that had undergone proliferation indicates either absorption of intranasal antigen or trafficking of antigen-presenting cells (APCs) or of activated T-cells from the nasorespiratory tract. Tsitoura et al. (27) have recently shown that intranasal OVA is rapidly absorbed and activates OVA-specific CD4 T-cells in secondary lymphoid organs. In addition, APCs in nasal-associated lymphoid tissue may migrate readily to other sites such as the spleen (28). The intranasal route was the only one associated with evidence of carryover of administered OVA into other mucosal compartments. This may be due to relative antigen excess, but it is difficult to accurately equate dose-responses between mucosal sites. At face value, the intranasal route appeared to be the most efficient for priming CTLs to OVA, but further studies are required to understand the reason for this. Nevertheless, CTLs could still be primed in normal mice when the initial response to OVA was restricted to mucosal tissues, as with oral or aerosol delivery.

How are these findings reconciled with the considerable body of evidence for a tolerogenic and protective effect of mucosal autoantigen in experimental autoimmune disease? We suggest that the outcome of mucosal antigen administration represents a balance between tolerance and immunity, determined by a range of factors including antigen form and dose and precursor T-cell frequency (2). Clearly, CTLs are induced in normal B6 mice by low doses of mucosal OVA. Although the consequence of this for autoimmune diabetes has so far been documented only in transgenic mice with a high precursor frequency of OVA-reactive T-cells, this is not to say that such CTLs do not have pathogenic potential in normal animals. There are reports of autoimmune disease, including diabetes, being exacerbated by mucosal antigen (18–21). Furthermore, protection from experimental autoimmune disease after mucosal autoantigen is hardly ever complete, and the application of mucosal tolerance to humans has yet to meet with clinical success (1,2). Induction of CTL immunity could undermine the efficacy and safety of mucosal tolerance and explain its inefficiency and indeed failure in some reports. Our findings underscore the need to consider and monitor concomitant CTL induction after mucosal antigen. This may be particularly relevant to proinsulin as an autoantigen in type 1 diabetes, because peptides from insulin B-chain (6) and proinsulin (29) that induce regulatory T-cells, but incomplete protection from diabetes when administered intranasally to NOD mice, contain epitopes for CTLs (14,29).

The present findings in an experimental system suggest that CTL immunity will be difficult to avoid by manipulating antigen dose schedules or routes of delivery and may need to be averted by other strategies in clinical applications. One strategy is to select tolerogenic peptides lacking CTL epitopes; another is to use mucosal adjuvants such as cholera toxin B-chain to promote tolerance, as demonstrated by Ploix et al. (30) for the protective effect of oral insulin in NOD mice. Lefrancois et al. (17) found that OVA-specific OT-I CD8 T-cells were primed in the mucosa by a mechanism involving ligation of CD40 ligand directly on CD8 T-cells. Therefore, blocking CD40-CD40 ligand at the time of mucosal antigen administration could also prevent priming of CTLs.

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