

Adjuvant Costimulation during Secondary Antigen Challenge Directs Qualitative Aspects of Oral Tolerance Induction, Particularly during the Neonatal Period

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In this report we demonstrate that although passive feeding of specific Ag to mice as neonates or adults can induce oral tolerance in both the cellular and humoral arms of the immune response, quantitative and, in particular, qualitative aspects of the tolerance process are determined by the nature of the inflammatory costimuli provided at the time of secondary Ag challenge. Moreover, this dependency upon nonspecific costimulation is more profound in Ag-fed neonates than in their adult counterparts. Thus, administration of Ag in the Th1-selective adjuvant CFA to prefed animals resulted in significant inhibition of IgG2a, IL-2, and IFN- γ responses, whereas IL-5 responses were increased. In contrast, rechallenge with Ag in the Th2-selective adjuvant aluminum hydroxide resulted in significant inhibition of IgG1, IgE, IL-2, and IL-5 responses, whereas IFN- γ responses were increased. Additionally, although soluble Ag challenge of prefed adults revealed marginal tolerogenic effects, the same challenge protocol in animals prefed as neonates elicited enhanced Th2-dependent IgG1 production. These results suggest that inflammatory stimulation at the time of Ag challenge is obligatory to trigger oral tolerance mechanisms, particularly in animals fed as neonates and also that the type of adjuvant used at the time of challenge selects for the type of Th cell population to be inhibited. *The Journal of Immunology*, 2004, 172: 2274–2285.

Oral tolerance is considered an important physiological mechanism that protects against hypersensitivity to food Ags and Ags from the microflora of the gastrointestinal tract (reviewed in Ref. 1). This form of tolerance can be induced to a wide range of Ags, and a number of host factors, such as age, genetic background, and nutritional status, influence the induction and maintenance of oral tolerance (reviewed in Ref. 1). Of particular interest to this report, oral tolerance has been found to be defective in early infancy in a number of experimental animal models (2–5). Oral Ag administration to neonatal rodents during the first 7–10 days of life may even prime for later systemic immunity rather than inducing unresponsiveness (2–5). This may explain findings from human seroepidemiological studies showing that food allergies are relatively common in infancy (6).

In the past decade, evidence has accumulated indicating that immune function in the early postnatal period is typically biased toward Th2-type responses. This stems from several lines of investigation. Firstly, it was discovered that pregnancy is a Th2-associated phenomenon, and it has been suggested that this serves to protect the placenta against the toxic effects of Th1-type cytokines, in particular IFN- γ (7). Ab responses during pregnancy were found to be associated with the Th2 cytokine pattern, and thus the Th2-biased environment in utero could also suppress development of IFN- γ ex utero (7, 8). This finding was further extended in murine leishmaniasis, where it was shown that Th2-type

cytokine expression during pregnancy impaired the resistance of C57BL/6J mice to *Leishmania major* infection (9). Secondly, in vitro experiments with murine neonatal T cells have demonstrated the propensity of these cells to produce low levels of IL-2 and IFN- γ and proliferate poorly in response to anti-CD3 mAb stimulation, yet produce high levels of IL-4 (10, 11). Thirdly, neonatal transplantation tolerance has been shown to be associated with preferential skewing toward Th2-type cytokine production (12, 13). Finally, for responses to protein, peptide, or vaccine Ags, it has been shown that Th2-type secondary responses were preferentially induced over Th1-type responses (14–16). This finding has important implications for vaccinology, because it implies that the Th1-type responses required for the resolution of intracellular infections could be impaired during early life. In relation to allergic diseases, it implies the existence of a temporal window in the early postnatal period when children may have an enhanced risk for primary Th2-polarized immunological memory against environmental allergens, a possibility supported by a wide body of epidemiological evidence (17–19).

The majority of published studies on neonatal Th1/Th2 regulation have used a systemic route of Ag exposure. The studies below sought to determine the relevance of these findings to regulation of oral tolerance during the early postnatal period in a murine model. We report that oral tolerance can be induced in neonatal animals, and more importantly, qualitative aspects of the oral tolerance process are markedly influenced by the type of adjuvant used for systemic challenge.

Materials and Methods

Animals

Specific pathogen-free C57BL/6J and BALB/c mice from the Animal Resource Center (Murdoch University, Murdoch, Western Australia, Australia) were housed under barrier conditions. Animals were fed a diet of acidified water and autoclaved, OVA-free food pellets. Advanced pregnant females were monitored daily at 0900 and 1700 h for the date of delivery. Birth day was designated day 0. Neonatal animals were defined as ≤ 24 h

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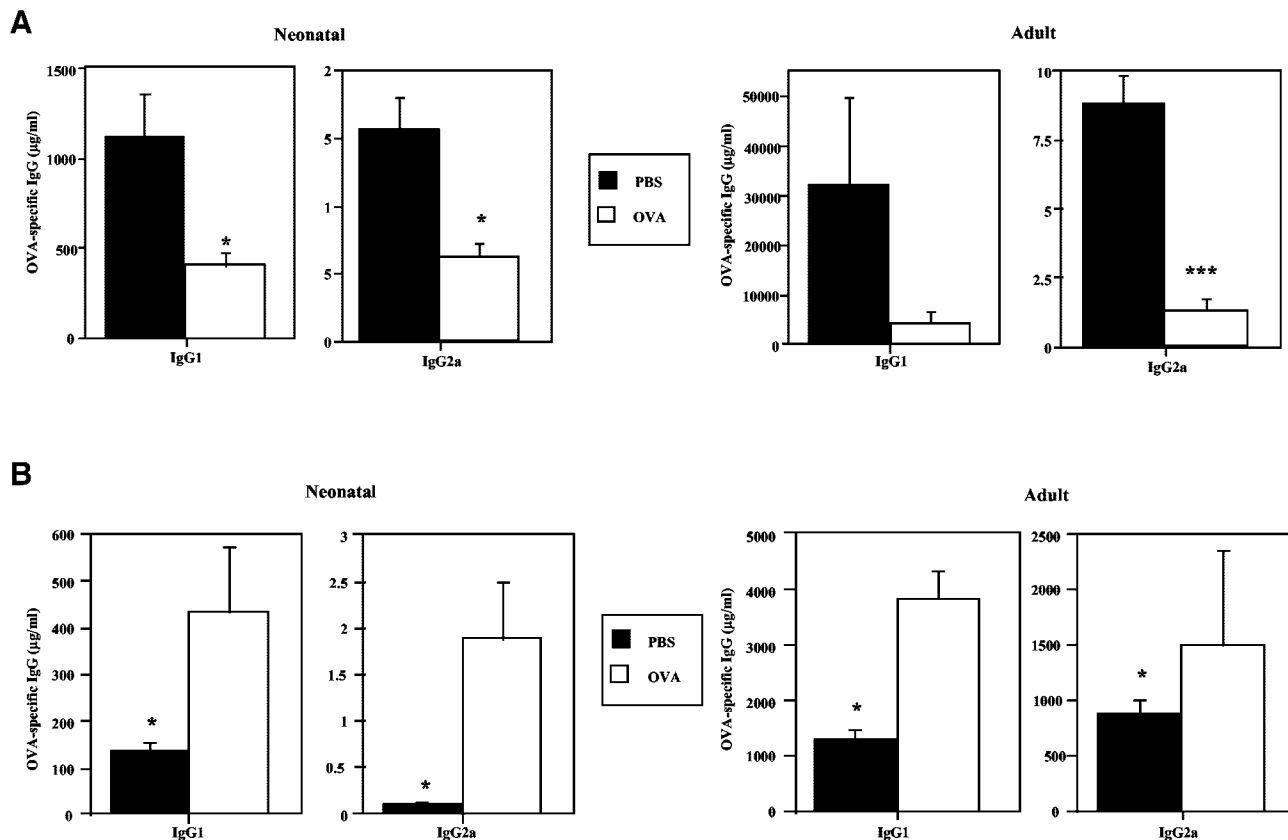


FIGURE 1. Effects of different routes of Ag exposure on secondary Ag-specific Ab responses in relation to initial age of Ag exposure. Neonatal (1-day-old) and adult (6- to 8-wk-old) mice were given a single feeding of OVA at 1 mg/g body weight (A) or were immunized with 10 μ g of OVA in IFA (B). Controls were fed PBS or immunized with PBS in IFA. Mice that received oral Ag exposure were challenged 4 wk after feeding with 100 μ g of OVA in CFA. Mice that received systemic Ag exposure were challenged 6 wk after injection with 10 μ g of OVA in CFA. Four weeks after challenge, serum was collected and analyzed for OVA-specific IgG1 and IgG2a by ELISA. Results are representative of two separate experiments, and each bar represents the group mean ($n = 5-7$ mice) $\pm$ SEM. *, $p < 0.05$, by Student's t test.

old. Adults were used at 6–8 wk of age. All animal experimentation was approved by the institute's animal ethics and experimentation committee.

Ag administration

OVA (grade V; Sigma-Aldrich, St. Louis, MO) was dissolved in PBS at a concentration of 100 mg/ml. For adult mice, OVA was administered by gastric intubation. Neonatal mice were fed using a pipette delivering a total volume of 10 μ l of OVA in PBS into the oral cavity. Four weeks after the last OVA feeding, the treated mice were challenged with 100 μ g of OVA emulsified in CFA (Difco, Detroit, MI) in a total of 100 μ l divided equally at the base of the tail. Alternatively, the mice were challenge i.p. with 100 μ g of OVA in 4 mg of aluminum hydroxide (alum, Amphogel; Wyeth Pharmaceuticals, Parramatta, Australia). In the experiment shown in Fig. 1B, neonates received i.p. injections of 10 μ g of OVA in CFA with 27-gauge needle whereas adults were injected s.c. Six weeks after primary immunization, the animals were challenged s.c. with 10 μ g of OVA in IFA.

Measurement of OVA-specific serum IgG isotype by ELISA

OVA-specific IgG Abs were measured by ELISA, using biotinylated murine anti-IgG1 (1/10,000) or anti-IgG2a (1/5000; both from Southern Biotechnology Associates, Birmingham, AL) and streptavidin-HRP (Life Technologies, Gaithersburg, MD; 1/1,000). Analysis of the data was performed using the universal assay software Assayzap program (version 2.51; Biosoft, Cambridge, U.K.). The limits of detection for both IgG isotypes are 1 ng/ml.

Cell preparation

Cell suspensions were prepared from lymph node (LN)² and spleens in Dulbecco's A and B salt solution supplemented with 0.2% BSA. RBC were

lysed with sterile 0.83% ammonium chloride. Cell debris and clumps were removed by filtering through cotton wool columns, and resulting cell suspensions were made up in RPMI 1640 (Life Technologies) containing glutamine and supplemented with 5% FCS, 2 g/L sodium bicarbonate, 20 μ g/ml gentamicin, 50 μ M 2-ME (Sigma-Aldrich), and 1% (v/v) MEM nonessential amino acids (ICN Biochemicals, Seven Hills, Australia) for cell culture.

For cytokine secretion, 3×10^6 cells/ml splenocytes or LN cells were cultured in 96-well, round-bottom, tissue culture plates (Falcon; BD Biosciences, Franklin Lakes, NJ) in aliquots of 200 μ l/well with or without 1 mg/ml OVA. Con A (Pharmacia Biotech, Uppsala, Sweden) was used as a positive control at 5 μ g/ml. The plates were then incubated at 37°C with 5% CO₂. Culture supernatants were collected at a range of time intervals after culture initiation to determine cytokine secretion. Preliminary experiments were performed to ascertain the time of peak production for each cytokine, and the production is reported at these times as follows: 24 h after culture for IL-2, 48 h for IL-10, and 72 h for IFN- γ and IL-5.

Cytokine assays

IFN- γ , IL-5, and IL-10 concentrations were determined by capture ELISA according to the manufacturer's instructions (all from BD Pharmingen, San Diego, CA). The concentrations of cytokine in the culture supernatant were interpolated from the linear portion of the standard curve with known amounts of recombinant cytokines using Assayzap universal calculator software (Biosoft, Cambridge, U.K.). The results are expressed as picograms per milliliter, and the sensitivity of the ELISA was 15 pg/ml for IFN- γ , 40 pg/ml for IL-5, and 70 pg/ml for IL-10.

Levels of IL-2 in supernatants were measured using the CTLL-2 bioassay adapted from Kelso (20), the final readout being proliferation of IL-2-sensitive CTLL-2 cells measured by [³H]thymidine incorporation. Data are presented as the mean counts per minute per culture after subtraction of background controls.

² Abbreviations used in this paper: LN, lymph node; DC, dendritic cell; MLN, mesenteric LN; PCA, passive cutaneous anaphylaxis; TLR, Toll-like receptor.

Adoptive transfer experiments

Splenocyte suspensions from PBS- or OVA-fed mice were incubated on nylon wool columns (type 200L; Robbins Scientific, Sunnyvale, CA) to remove the adherent cell population, including B cells and macrophages. Enriched T cell suspensions were eluted from the columns by washing with 20 ml of medium. Cells (5×10^6) were adoptively transferred into the peritoneal cavities of unirradiated syngeneic adult mice or 1×10^7 cells i.v. in the neonatal alum experiment. Immediately after transfer, the recipient mice were immunized at the base of the tail with 100 μ g of OVA in CFA or alum.

Determination of OVA-specific IgE by passive cutaneous anaphylaxis (PCA)

OVA-specific IgE Ab titers were determined by PCA (21). Serum samples were serially diluted in PBS and injected intradermally in 50- μ l aliquots into the dorsal skin of male WAG rats. Twenty-four hours later, the rats were injected i.v. with 4 mg/ml OVA in PBS containing 1% Evans Blue dye. Fifteen minutes later, the skin was examined for the development of blue lesions. The reciprocal of the highest serum dilution giving a blue lesion 5 mm in diameter was taken as the PCA titer. Serum collected from mice that were given multiple injections of OVA in alum was used as a positive control for the assay. PBS was used as a negative control.

Statistics

Results are expressed as the mean $\pm$ SEM and were compared using unpaired Student's *t* test. The results were analyzed using the Instat software program, version 2 (GraphPad, San Diego, CA) for MacIntosh computers. Differences were considered significant at *p* < 0.05.

Results

Contrasting immunological outcomes after oral vs parenteral Ag exposure of neonatal mice

Neonatal and adult C57BL/6J mice were fed OVA in PBS (1 mg/g body weight) or PBS alone in a single dose as described previously (2, 3). Four weeks after feeding, the mice were challenged s.c. with 100 μ g of OVA in CFA and were bled 4 wk later, and sera were analyzed for OVA-specific IgG1 (Th2-dependent) and IgG2a Ab (Th1-dependent) by ELISA (Fig. 1A). In contrast to previous reports (2, 3), we found that oral administration of Ag during the neonatal period induced significant suppression of Ag-specific secondary Ab responses elicited subsequently in adulthood (Fig. 1A). Comparable suppression was also achieved in BALB/c mice (data not shown). Parenteral immunization of neonates resulted in significant priming of both IgG1 and IgG2a Ab isotypes as in adults, but the magnitude of the Ab response was lower in the younger animals (Fig. 1B).

Effects of Ag dose and frequency of feeding on the degree of Ab suppression

Different Ag feeding regimens were initially studied to optimize the protocols (Fig. 2). Neonatal or adult mice were fed OVA in a single dose or over a period of 3 consecutive days (Fig. 2A). Four weeks after feeding, the animals were challenged s.c. with OVA in

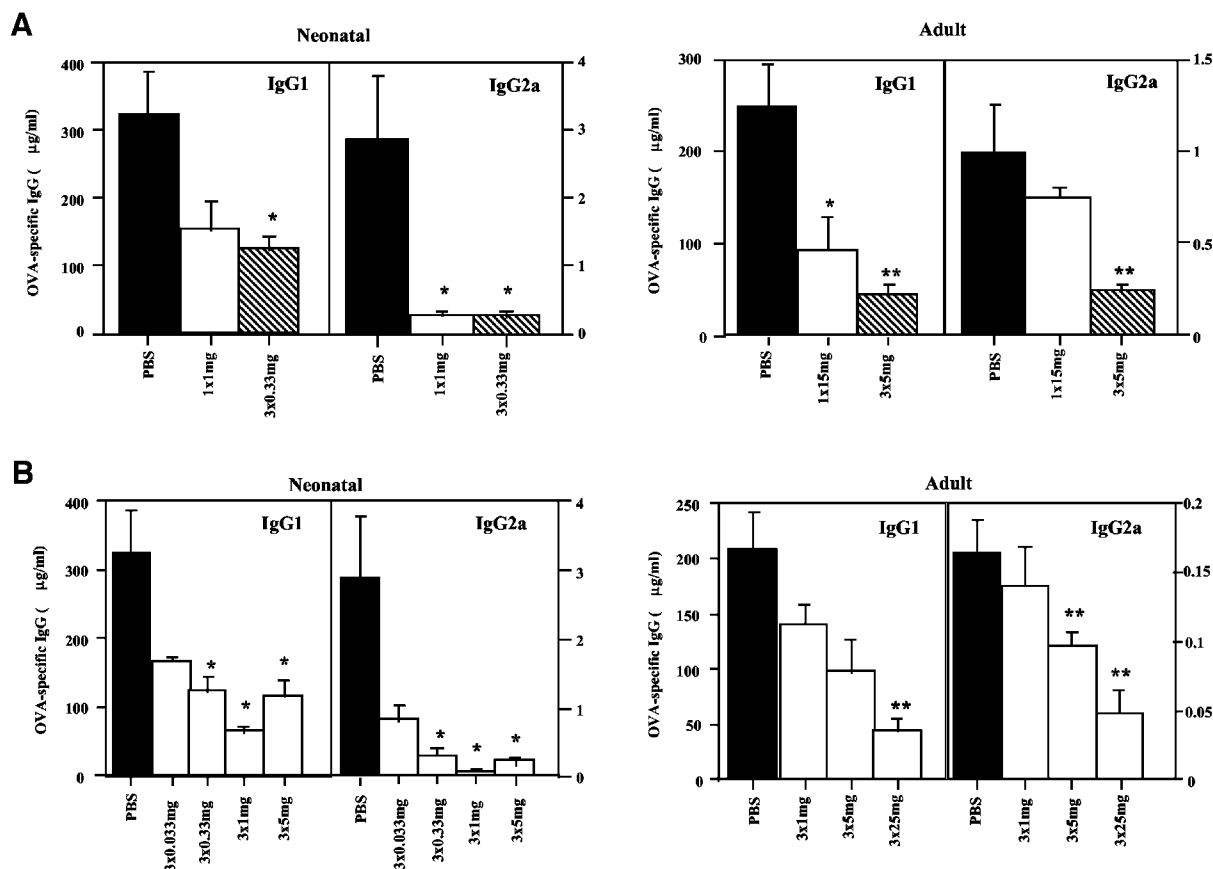


FIGURE 2. Impact of feeding frequencies and Ag dose on oral tolerance in relation to age at initial Ag exposure after OVA in CFA challenge. **A**, The degree of Ab suppression was greater with the multiple feeding regimen than with single feeding. Neonatal mice were fed a total of 1 mg of OVA in PBS, either in a single feeding or distributed over 3 days, and adult mice were fed either a single feeding of 15 mg of OVA in PBS or 3 consecutive days of 5 mg of OVA/day. Age-matched controls were fed PBS. Four weeks after the last feeding, all animals were challenged s.c. with 100 μ g of OVA in CFA. **B**, Dose dependence of Ag and degree of Ab suppression. Neonatal mice were fed OVA in PBS at doses ranging from 0–5 mg for 3 consecutive days, whereas adult mice were fed increasing doses of OVA in PBS ranging from 0–25 mg for 3 consecutive days. Four weeks after the last feeding, all animals were challenged s.c. with 100 μ g of OVA in CFA. **A** and **B**, Sera were collected 3 wk after challenge and measured for OVA-specific IgG1 and IgG2a by ELISA. Data shown are presented as the group mean $\pm$ SEM (*n* = 5–6 mice/group) and are representative of two experiments. *, *p* < 0.05; **, *p* < 0.01 (by Student's *t* test).

CFA, and serum IgG1 and IgG2a production was measured 3 wk after challenge. Single feeding in neonatal animals induced a significant suppression of OVA-specific IgG2a ($p < 0.05$), whereas multiple feedings resulted in significant suppression of both IgG1 and IgG2a Abs ($p < 0.05$) compared with neonatally fed PBS controls. This pattern was also detected at subsequent time points (data not shown). In adults, multiple feeds markedly inhibited IgG1 ($p < 0.01$) and IgG2a ($p < 0.001$) titers, whereas a single feed only inhibited IgG1 ($p < 0.05$) compared with PBS controls. The multiple feeding regimen was then used to test the effect of dose on the induction of tolerance (Fig. 2B). Neonatal mice were fed graded doses of OVA from 0.033–5 mg/day, whereas adults were fed 1–25 mg/day for 3 consecutive days and challenged s.c. 4 wk later with OVA in CFA. For neonates, doses >0.333 mg reduced both IgG1 and IgG2a titers ($p < 0.05$). The greatest inhibition was observed with the 1-mg dose. In adults, multiple feedings of 25 mg of OVA induced the greatest inhibition of both IgG1 and IgG2a Abs ($p < 0.01$). Note that the starting doses for the adult and neonatal experiments were 15-fold different so as to be in proportion to the body weights of the mice.

Influence of adjuvants on qualitative aspects of oral tolerance

Studies were undertaken to elucidate aspects of the Th cell component of the oral tolerance process. These involved the measurement of Th1- and Th2-type cytokine production in *in vitro* recall responses to OVA. IL-2 and IFN- γ were used as markers of Th1-

type reactivity, and the measurement of IL-5 was used as an index of Th2 reactivity. Additionally, IL-10 production was measured, because it has been reported to play a key role in the induction of oral tolerance (22, 23). Neonatal and adult mice were fed multiple doses of OVA or PBS and challenged s.c. with OVA in CFA 4 wk postfeeding. Eleven days after challenge, the draining LN cells were stimulated *in vitro* in the presence of 1 mg/ml OVA for measurement of peak cytokine responses. As shown in Fig. 3A, OVA feeding inhibited IL-2 responses in both neonatal- and adult-fed animals compared with their PBS controls ($p < 0.05$). IFN- γ production was also significantly reduced in both neonatal- and adult-fed groups ($p < 0.05$). In contrast, IL-5 responses were markedly elevated, and IL-10 production was only slightly reduced. Ab responses measured in neonatal-fed animals indicated that multiple feeding of OVA led to significant inhibition of OVA-specific IgG1 and IgG2a, in the latter case lasting for at least 13 wk after s.c. challenge (Fig. 3B). For the neonatal-fed animals, the IgG1 response was delayed in the fed group, and IgG2a was markedly inhibited over the entire time course (Fig. 3B). Ab suppression induced in OVA-fed adults (Fig. 3C) lasted for at least 16 wk, although the control IgG1 responses began to wane 8 wk after challenge, and the IgG2a response declined after 12 wk.

In the experiments described above, the Th1-stimulating adjuvant CFA was used during systemic Ag challenge after oral tolerance induction. To determine whether the adjuvant can modulate

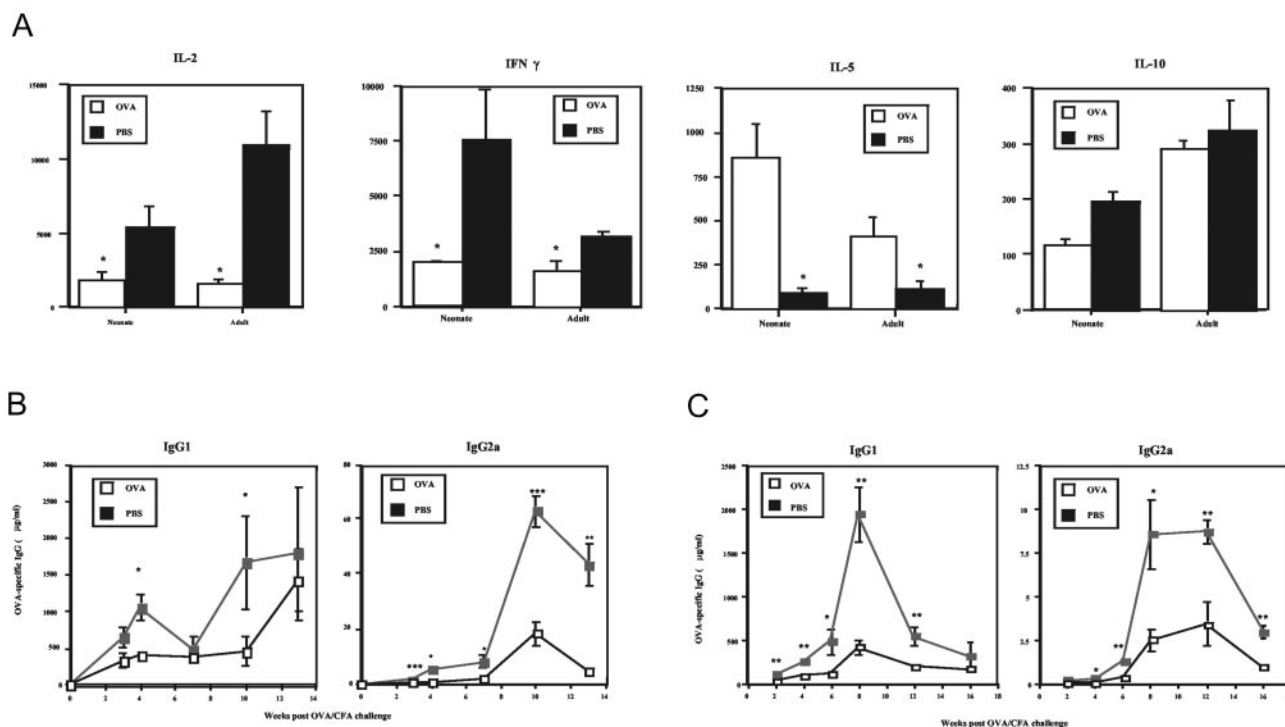


FIGURE 3. Immune responses elicited after OVA in CFA challenge in neonatal- and adult-fed mice. **A**, OVA-induced cytokine responses after OVA feeding and OVA in CFA challenge. For cytokine measurements, the draining LNs were collected 11 days after challenge, and the single-cell suspensions derived were stimulated *in vitro* with 1 mg/ml OVA. Culture supernatants were assayed at previously determined times of peak cytokine production. The limits of detection of each cytokine assays are indicated by a broken line in each graph. Data are represented as the mean value of five or six mice per group $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (by Student's *t* test). Data are representative of three independent experiments. **B**, Ab responses of neonatal-fed mice after OVA in CFA challenge. Neonatal mice were fed three doses of 1 mg of OVA in PBS. Age-matched controls were fed PBS only. Four weeks after the last feeding, the animals were challenged s.c. with 100 μ g of OVA in CFA. Sera were collected at specified time intervals for IgG1 and IgG2a analysis by ELISA. Data are presented as the mean $\pm$ SEM of five or six animals per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (by Student's *t* test). One experiment typical of three independent experiments is depicted. **C**, Suppression of IgG1 and IgG2a responses in adult-fed mice after OVA in CFA challenge. The experimental protocol is as described in **B**, except that adult mice were fed three doses of 25 mg of OVA. The data shown are representative of three experiments.

the tolerance process, an alternative protocol was examined involving i.p. challenge of fed animals with OVA in alum. The cytokine data from alum-challenged animals are presented in Fig. 4A. IL-2 and IL-10 production were not detected in LN cells from the neonatal OVA-fed group compared with control cells. In adult-fed animals, cells from the OVA-fed group produced significantly less IL-2 than control cells, but IL-10 production was only reduced by 20%, and this was not statistically significant. However, in contrast to the cytokine profiles following CFA challenge (Fig. 3A), IFN- γ production by LN cells of OVA-fed groups (neonatal- and adult-fed) was increased ($p < 0.05$), whereas IL-5 levels were markedly reduced ($p < 0.05$). The use of alum in systemic challenge after oral Ag administration also revealed preferential suppression of IgG1 isotype and not IgG2a in neonatally- and adult-fed OVA groups compared with PBS controls (Fig. 4, B and C). In addition, the titers for IgG2a were low compared with the titers obtained with OVA in CFA challenge. An increase in the IgG2a response was observed 16 wk after challenge, but this has not been investigated further. Significant suppression of OVA-specific IgE was also observed in both neonatal and adult OVA-fed groups compared with their respective controls (data not shown).

Collectively, these data suggest that when a Th1-selective adjuvant (such as CFA) is used with Ag to elicit recall responses in previously fed (putatively tolerized) animals, the resulting response displayed selective inhibition of the Th1 component (IFN- γ) of the immune response. In contrast, identically treated animals challenged with Ag in a Th2-selective adjuvant (alum) displayed a selective inhibition of Th2 responses.

Exclusion of adjuvant from systemic challenge protocols after oral administration of Ag

An additional series of experiments was performed in which no adjuvants were used. When soluble OVA (OVA/PBS) was used to challenge animals that had previously been fed OVA as neonates, the IgG1 responses were increased rather than suppressed (Fig. 5A), but IgG2a responses were not detected at any time point. However, the IgG1 titers were several hundred-fold lower than when an adjuvant was used. IFN- γ production was not detected in LN cell responses from these challenged animals; however, the levels of IFN- γ produced by spleen cells from the same animals were markedly elevated ($p < 0.01$). The IL-5 responses from LN cells in the OVA-fed group were slightly higher than those of PBS

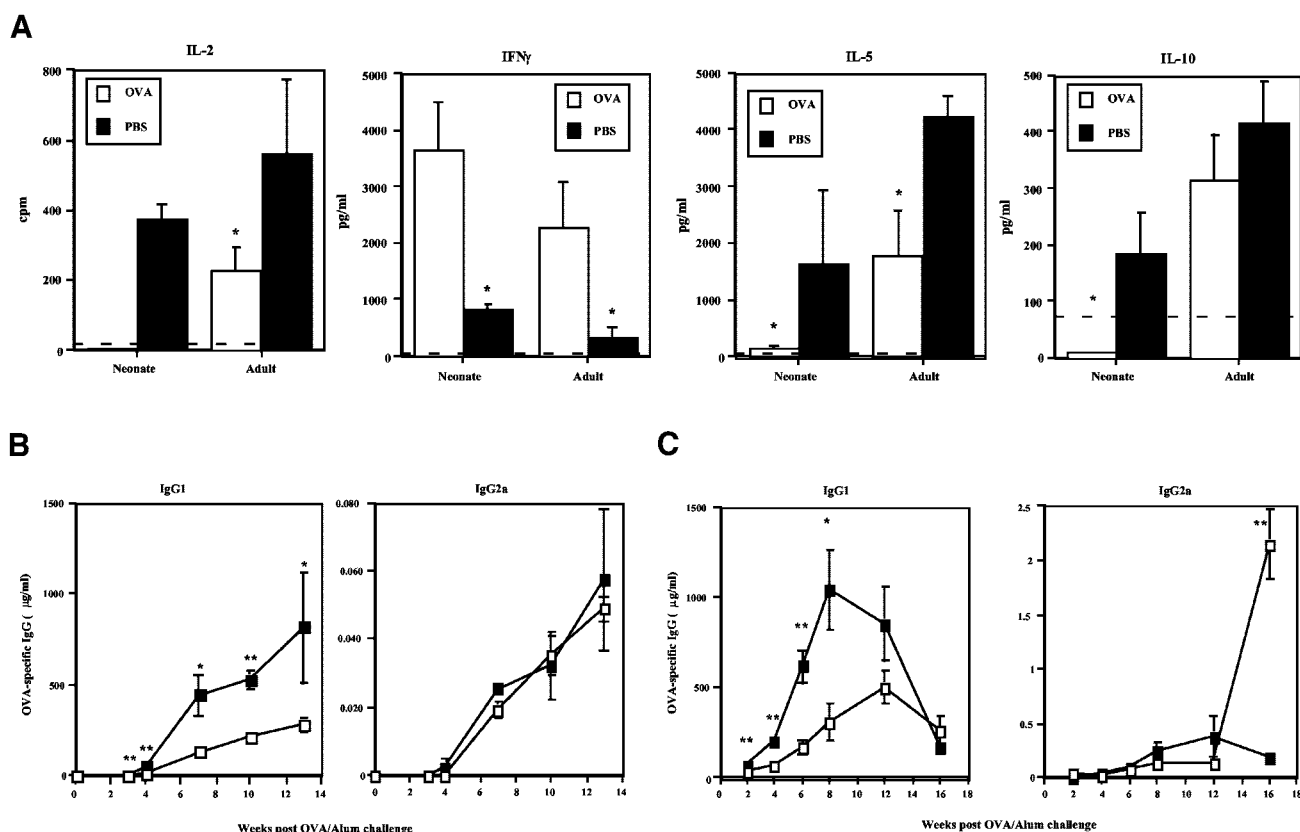


FIGURE 4. Th2-type responses were suppressed after OVA in alum challenge in neonatal- and adult-fed mice. **A**, Cytokine production induced in LN cells after feeding and OVA in alum challenge in both neonatal- and adult-fed animals. Neonatal mice were fed three doses of 1 mg of OVA or PBS, and adult mice were fed three doses of 25 mg of OVA or PBS. Four weeks after the last feeding, the animals were challenged i.p. with 100 μ g of OVA in alum. Eleven days after challenge, cells from draining LNs were stimulated in vitro with OVA, and cytokine production was assayed as described in Fig. 3. Background cytokine production in the absence of OVA was undetectable for all cytokines tested. Data are expressed as the mean value of five or six mice per group $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$ (by Student's t test) and are representative of three independent experiments. **B**, Ab responses of neonatally-fed animals after OVA in alum challenge. The feeding protocol is as described in A. Four weeks after the last feeding, all animals were challenged i.p. with 100 μ g of OVA in alum. Sera were collected at various time intervals and measured for the presence of OVA-specific IgG1 and IgG2a by ELISA. Data are presented as the mean value of five mice per group $\pm$ SEM, representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ (by Student's t test). **C**, IgG1 suppression in adult-fed animals after OVA in alum challenge. The feeding protocol is described in A. The animals were challenged i.p. with 100 μ g of OVA in alum, and serum IgG isotype responses were measured over a period of time by ELISA. Data are presented as the mean value of five mice per group $\pm$ SEM and are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ (by Student's t test).

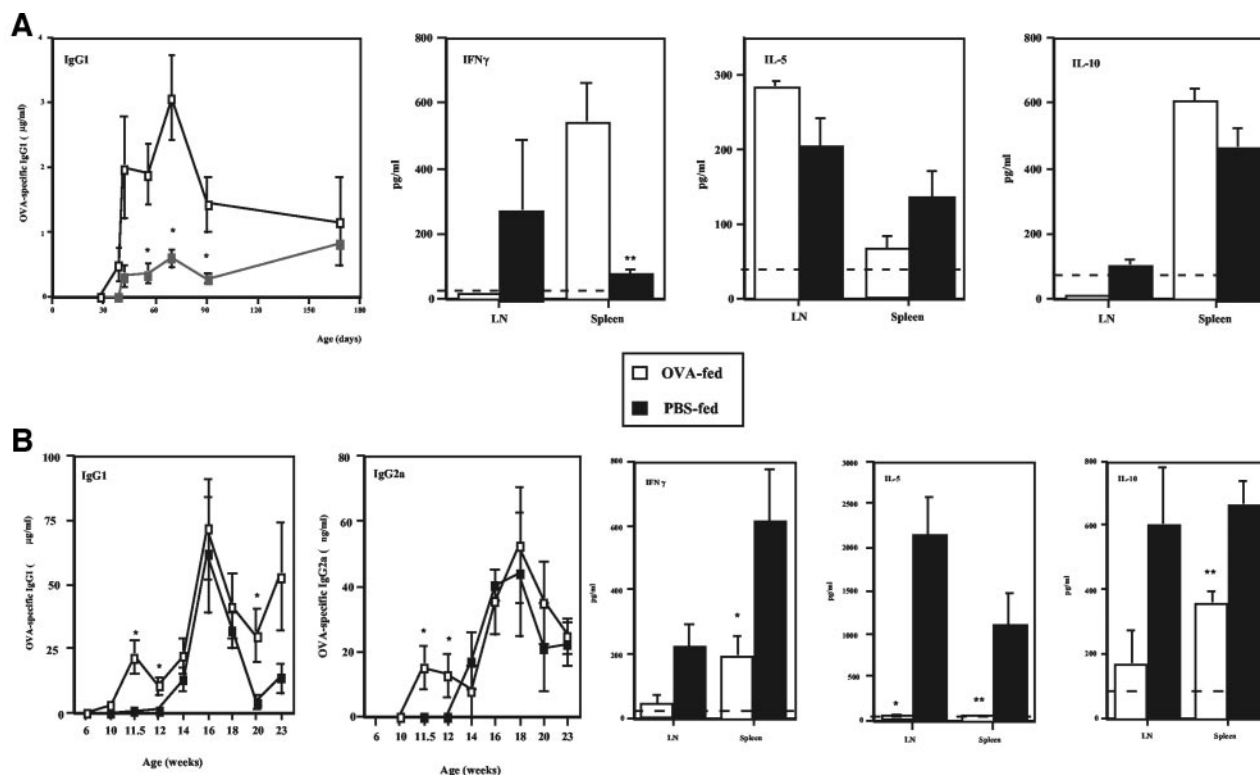


FIGURE 5. Differential effect of soluble Ag challenge in neonatal- and adult-fed animals. *A*, Immune responses in neonatally-fed animals after OVA in PBS challenge. Neonatal mice were fed 1 mg for 3 consecutive days; age-matched controls were fed PBS only. Four weeks after the last feeding, all animals were challenged with 100 µg of OVA in PBS and were given a booster challenge 2 wk later. Sera were collected at various time intervals for analysis of IgG1 and IgG2a by ELISA. Draining LN cells and spleen cells were harvested 11 days after challenge and were cultured in vitro with OVA, and cytokine production was assayed as described in Fig. 3. Ab data are shown as the mean of five mice per group $\pm$ SEM and are representative of two independent experiments; cytokine data are represented as the mean of two separate experiments $\pm$ SEM. The dashed line represents the sensitivity level of each cytokine assay. *, $p < 0.05$; **, $p < 0.01$ (by Student's *t* test). *B*, Immune responses induced in adult-fed animals after OVA in PBS challenge. The experimental protocol is described in *A*.

controls, and the reverse profile was observed in the splenic response. IL-10 levels were undetectable in LN cells from the OVA-fed group, and there was no significant difference between the two groups in the levels from spleen cells. Thus, challenge (in adulthood) of neonatal-fed mice with soluble Ag in the absence of adjuvant stimulated the production of IFN- γ in spleen cells, whereas IL-5 and IL-10 responses remained generally unaffected. Priming of the IgG1 Ab response was observed, instead of suppression.

Mice fed as adults and challenged with soluble OVA did not show the same degree of stimulation as that produced by neonatal feeding, but no suppression was observed, and the Ab titers of fed mice were higher at early and late time points. In contrast to the cytokine profile of the neonatal-fed group, a substantial decrease in all cytokines, especially IL-5, was found in the postchallenge responses of the adult OVA-fed group in both the LN and spleen cell cultures. IL-2 was not detected in any group, which contrasts with the readily detectable IL-2 response found after challenge with an adjuvant such as CFA or alum.

T cell activation precedes oral tolerance

To investigate the events associated with the induction of oral tolerance, mice were fed OVA in PBS or PBS alone and in vitro recall cytokine responses of mesenteric LN (MLN) cells were examined 3 and 8 days later.

Within 3 days of OVA feeding, MLN cells from neonatal-fed animals (Fig. 6A) were able to respond to in vitro OVA challenge,

inducing elevated IFN- γ , IL-5 and IL-10 (Fig. 6A). By 8 days after feeding, the IL-5 response became undetectable, whereas the IFN- γ and IL-10 responses from MLN cells of OVA-fed animals remained elevated (Fig. 6A). In adult-fed animals (Fig. 6B), there was an increase in the level of IFN- γ produced in MLN cells of OVA-fed group on day 3 and a slight increase in the IL-5 response (Fig. 6B). However, in contrast to findings in the animals fed as neonates, IL-10 production in the OVA-fed adults group was negligible. Also in contrast to the neonatal fed group, the IFN- γ component of the initial response in the adults was transient, and production decreased rapidly to control levels by day 8 (Fig. 6B). In addition, IL-5 and IL-10 responses were undetectable. IL-2 was not detected in the culture supernatants of either group of mice.

Cell transfer studies

Splenocytes from OVA- and PBS-fed donors were passed through nylon wool columns to generate a T cell-enriched population of ~80–85% purity. The resulting suspensions were transferred to naive syngeneic recipients, which were then challenged with OVA in either CFA (Fig. 7) or alum (Fig. 8).

Mice previously fed OVA as neonates displayed a significantly suppressed IgG2a response (Fig. 7A, donor responses in the right panel; $p < 0.001$) after OVA in CFA challenge, but only marginally decreased IgG1 responses (Fig. 7A, left panel). The same pattern of selective inhibition of IgG2a responses was observed in the recipient mice after cell transfer of splenocytes from comparable

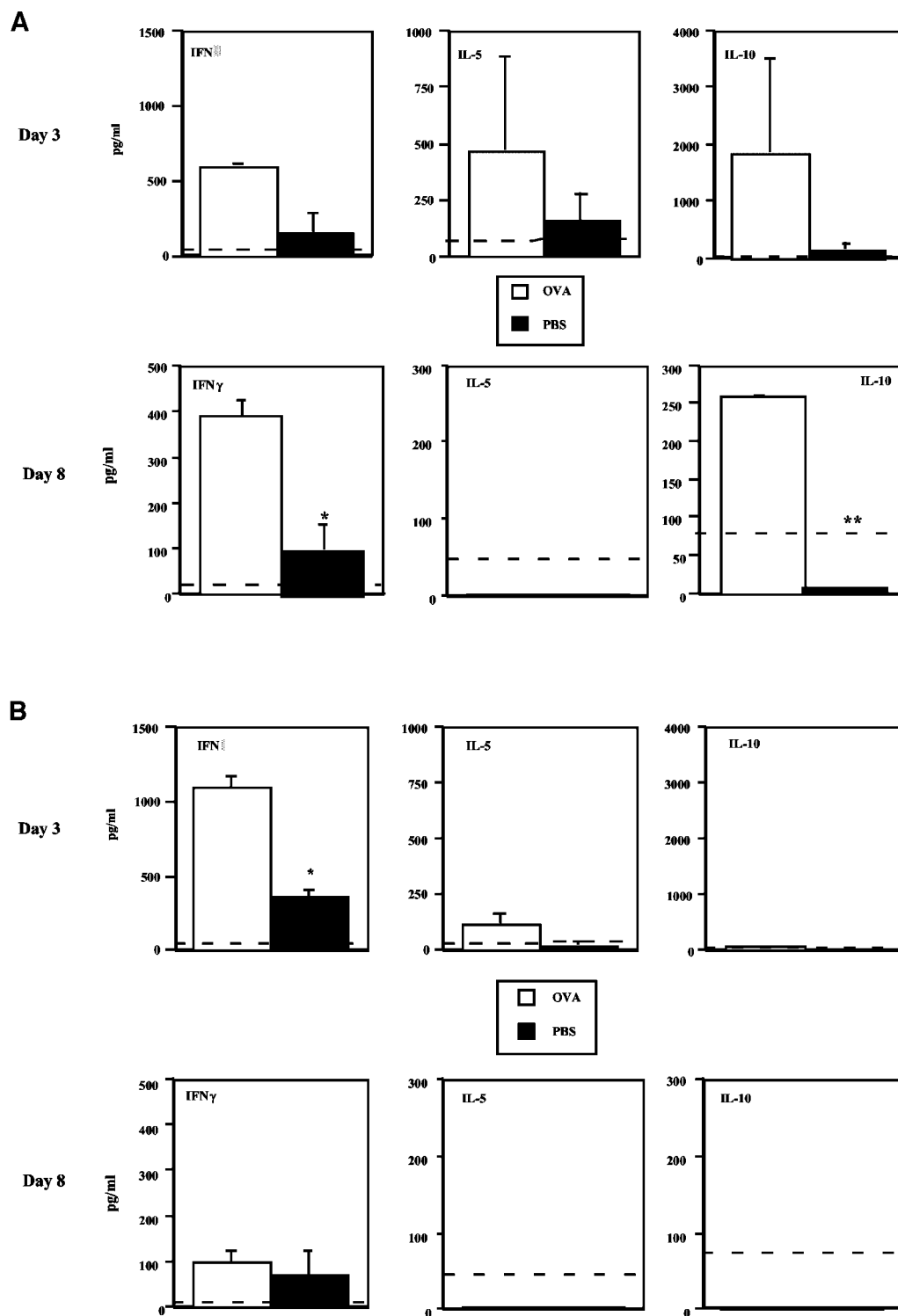
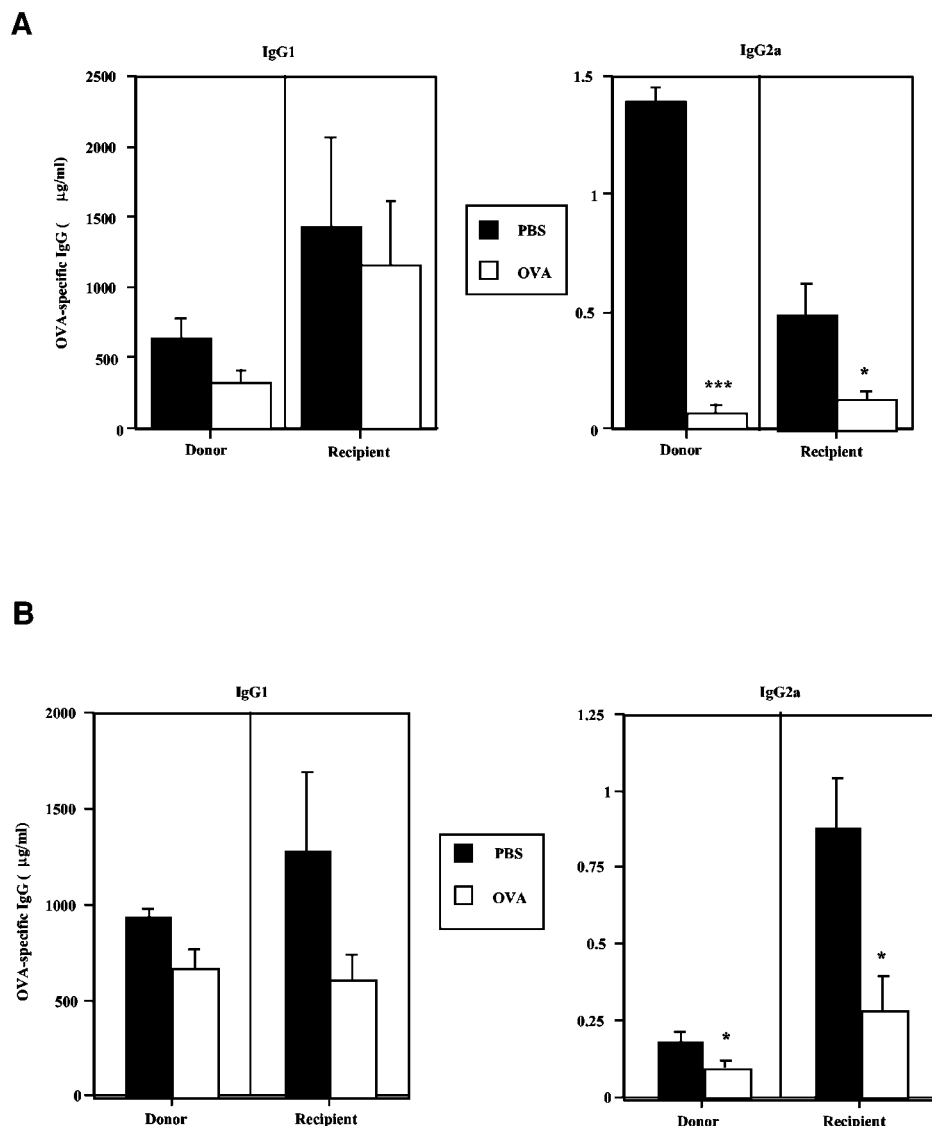


FIGURE 6. Activation precedes tolerance. *A*, Cytokine production induced to orally administered OVA in the MLN cells of neonatal mice. Neonatal mice were fed 1 mg of OVA in PBS for 3 consecutive days. Three and 7 days later, MLN were processed, cells were stimulated *in vitro* with OVA, and cytokine production was assayed as described in Fig. 3. No IL-2 was detected in either the OVA- or PBS-fed group. Data are presented as the mean of two independent experiments $\pm$ SEM ($n = 40$ –50 animals/group/experiment). The dashed line represents the sensitivity of each assay. *, $p < 0.05$ (by Student's *t* test). *B*, Cytokine production induced after OVA feeding in MLN cells of adult mice. Adult mice were fed three doses of 25 mg of OVA in PBS or PBS alone as controls. The experimental protocol is described in *A*. Data are presented as the mean $\pm$ SEM ($n = 5$ mice/group) and are representative of three separate experiments. The dashed line represents the sensitivity of each assay. *, $p < 0.05$ (by Student's *t* test).

neonatal-fed donors (Fig. 7A, right panel). No suppression was observed for the IgG1. An identical pattern of selective transfer of IgG2a suppression was observed in parallel experiments involving animals fed OVA as adults (Fig. 7B).

When these experiments were repeated substituting alum for CFA at the time of systemic challenge, no suppression was observed (Fig. 8). Adult mice given multiple feedings of 25 mg of OVA and subsequently challenged with OVA in alum showed

FIGURE 7. Adoptive transfer studies with CFA as adjuvant during challenge. **A**, Adoptive transfer of splenocytes from mice fed OVA as neonates. Neonatal mice were fed 1 mg of OVA or PBS for 3 consecutive days. At 4 wk after feeding, 5×10^6 unfractionated T cells were collected from pooled spleens and injected i.p. into naive adult syngeneic animals (recipients). Concomitantly, the recipient groups were challenged s.c. with 100 μ g of OVA in CFA. In parallel, other groups of animals that had previously been fed OVA or PBS were also challenged with 100 μ g of OVA in CFA without transfer (donors). OVA-specific IgG1 and IgG2a serum responses were measured at 3 wk. Data are presented as the mean IgG titer of five or six animals $\pm$ SEM. *, $p < 0.05$; ***, $p < 0.001$ (by Student's *t* test). Data are representative of two experiments. **B**, Adoptive transfer of splenocytes from mice fed OVA as adults. Adult mice were fed 25 mg of OVA or PBS for 3 consecutive days. Four weeks later, splenocytes were tested for suppressive activity as described in **A**. Additional donor animals were also challenged with OVA in CFA as described in **A**. OVA-specific IgG1 and IgG2a serum responses were measured at 3 wk. Data are presented as the mean IgG isotype titer of five or six animals $\pm$ SEM and are representative of two experiments. *, $p < 0.05$ (by Student's *t* test).



significant IgG1 suppression compared with PBS controls (Fig. 8A). However, in contrast to the experiments shown in Fig. 7, this suppression was not adoptively transferable (Fig. 8B). Moreover, increasing the number of cells transferred and injecting the cells i.v. rather than i.p. did not induce IgG1 suppression in recipients (data not shown).

The IgE response of OVA-fed adult animals after OVA in alum challenge was significantly suppressed (Fig. 9A; donor responses). However, similar to IgG1 data, this suppression could not be adoptively transferred (Fig. 9A; recipient responses). This pattern was also found for IgE responses of neonatal fed animals. The IgE response of OVA-fed donors was significantly suppressed (Fig. 9B; donor responses), but the response of recipients was not (Fig. 9B; recipient responses).

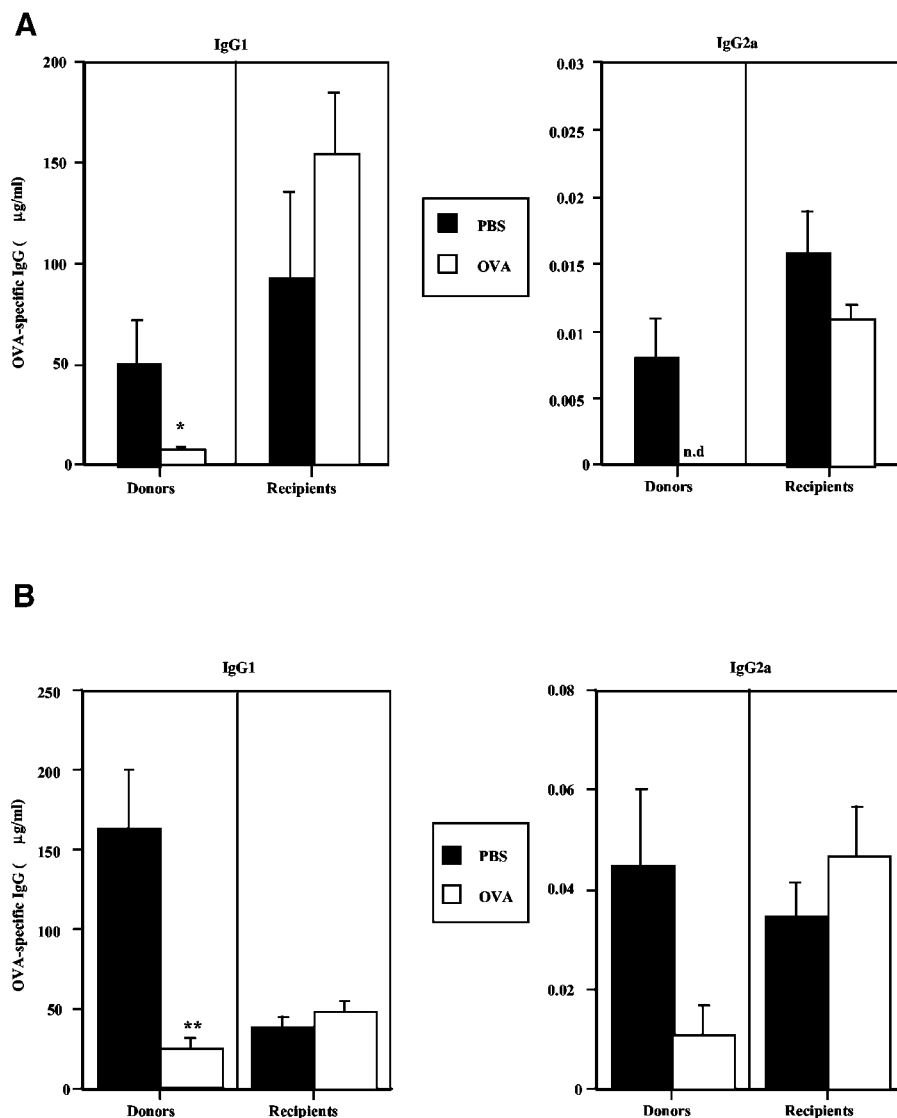
Discussion

The principal aim of this study was to investigate how oral Ag administration during the neonatal period affects Th1/Th2 responses in light of the reports that Th1 and Th2 cells are regulated differently during neonatal exposure to parenteral Ag (14, 15). Previous studies have shown that unlike the situation in adults, oral

administration of Ag during the first 3 days of life can prime Ab responses rather than induce tolerance (2, 3). The mechanism(s) for these apparent age-dependent differences have not been clearly defined, but have been suggested to involve deficiencies in APC functions in early life (2, 24, 25). These studies, however, did not examine the role of individual Th cell subsets in the priming process.

In contrast, our present results clearly demonstrate that neonatal mice do develop oral tolerance provided that appropriate conditions of Ag challenge are used. Notably, it was found that a single feeding of OVA at a dose/body weight equivalent to that used for adults resulted in significant suppression of Ab responses to OVA when the challenge protocol included adjuvant (Fig. 1A). Increasing the frequency of feedings induced further inhibition of both IgG1 and IgG2a isotypes (Fig. 2A) and dose-response experiments demonstrated that inhibition of Ab responses could be induced over a range of Ag doses (0.033–5 mg OVA; Fig. 2B). Furthermore, oral exposure of neonatal animals to high doses of Ag followed by parenteral challenge of OVA in adjuvant resulted in tolerance, as measured by a profound inhibition of IL-2 like that in adult-fed animals.

FIGURE 8. Adoptive transfer studies with alum adjuvant during challenge. *A*, Adoptive transfer of splenocytes from mice fed OVA as adults. The protocol is identical with that described in Fig. 7*B*, except that alum was substituted for CFA in the challenge. Data are shown as the mean Ab titer $\pm$ SEM ($n = 5$). **, $p < 0.01$ (by Student's *t* test). Data are representative of two independent experiments. *B*, Adoptive transfer of splenocytes from mice fed OVA as neonates. Neonatal mice were fed for 3 consecutive days with 1 mg of OVA or PBS as controls. Four weeks after feeding, spleen cells were harvested and transferred as described in Fig. 7*A*, except that 1×10^7 cells were transferred i.v., and alum was used rather than CFA for challenge. At 3 wk after challenge, OVA-specific serum IgG1 and IgG2a were measured by ELISA. Data are shown as the mean Ab titer $\pm$ SEM ($n = 5$) and are representative of two independent experiments. *, $p < 0.05$ (by Student's *t* test).



One difference between the protocol used in the previous studies and that used in the earlier studies cited above was the method of delivery of Ag to the infant animals. The method used in this study was via introduction into the mouth, in contrast to direct intragastric gavage (2, 3). Gavage was deliberately avoided in our studies to avoid possible physical trauma to the esophageal wall, which could lead to leakage of the Ag directly into the bloodstream. Hence, the route of Ag exposure in the present model would provide access of APC populations in the oropharyngeal mucosa to Ag, a possibility that is negated in models in which intragastric gavage was used for exposure (2, 3). In this context, an elaborate network of dendritic cells (DC) has been documented in the epithelium of the oral mucosa, and sublingual administration of the contact-sensitizing Ag, oxazolone or PCI, has been shown to induce tolerance (26). Sublingual allergen administration has also been shown to result in suppression of allergen-specific IgE in adult rats (27). Similarly, Eriksson and colleagues (28) have demonstrated that DC in the buccal epithelium can sequester Ags and migrate to the draining LNs, where they present processed Ag in an MHC class II-restricted manner to T cells. Thus, different populations of DC may have been stimulated by the different methods

of feeding, which, in turn, may have an important impact on the initiation of oral tolerance. It is pertinent to note also that Ag transfer to infant animals via maternal milk has been shown to induce tolerance (29, 30), which further reinforces the view that oral mucosal exposure may be important in the tolerogenic process.

DC have been implicated as the most likely APC candidate involved in the induction of oral tolerance in adult animals (31, 32), and these cells are functionally and numerically deficient in all (including mucosal) tissues during the neonatal period. In this context, earlier studies in this laboratory comparing tissue sites throughout the respiratory tract of infant animals indicates a relationship between the proximity of individual mucosal sites to the outside environment, and hence the frequency/intensity of local Ag exposure, and the rate of postnatal maturation of local mucosal DC populations (33). In particular, DC populations in the nasopharyngeal and tracheal mucosa mature postnatally much faster than those deeper in the respiratory tree (33). A similar situation may be operative within the gastrointestinal tract, resulting in more rapid postnatal maturation of oral mucosal DC populations relative to their counterparts in the lower gastrointestinal tract due to a higher

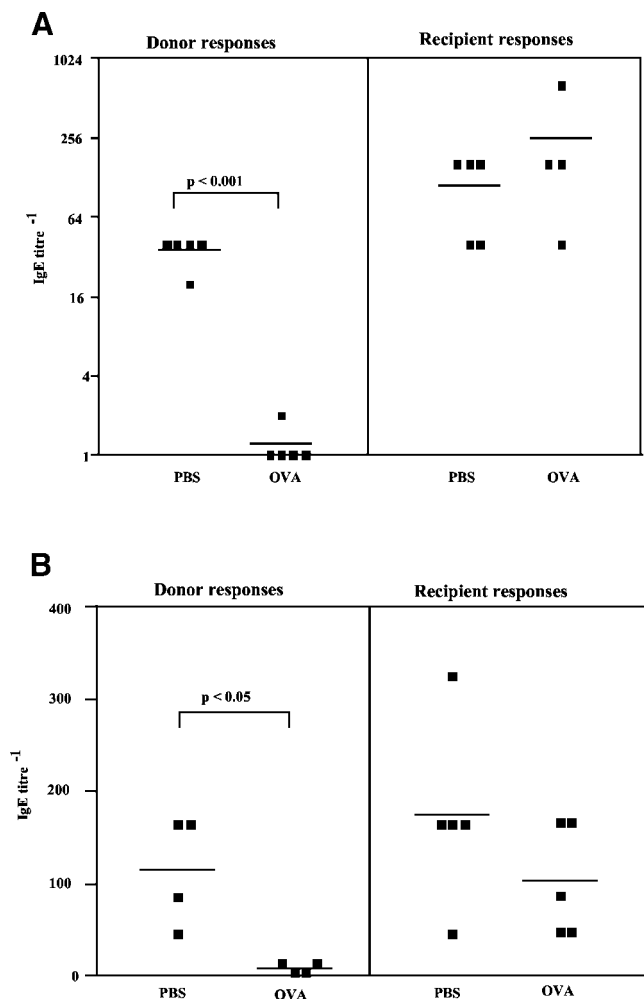


FIGURE 9. IgE responses of neonatal- and adult-fed animals in adoptive transfer experiments with OVA in alum challenge. **A**, Adoptive transfer of splenocytes from mice fed OVA as adults. The experimental protocol is as described in Fig. 8, except that IgE was measured 3 wk after OVA in alum challenge by PCA. Data represent IgE titers of individual animals and are representative of two experiments; the mean value of the groups is indicated by the bar. $p < 0.001$ (by Student's t test). **B**, Adoptive transfer of splenocytes from mice fed as neonates. The experimental protocol is as described in Fig. 8B, except that OVA-specific IgE was measured 3 wk after challenge by PCA. $p < 0.05$ (by Student's t test). Data are representative of two experiments.

intensity response to exogenous inflammatory and antigenic stimuli. If so, then oral exposure per se may be an important requirement for the efficient induction of oral tolerance in neonates.

The standard protocols used to demonstrate tolerance in Ag-fed animals involve challenge with Ag in adjuvant. However, the contribution of these costimuli to tolerogenesis has not previously been investigated, and elucidation of their role in the process was a key aim of this study. Systemic challenge with OVA in CFA after OVA feeding demonstrated that the feeding resulted in marked inhibition of IgG1 and IgG2a Abs (Fig. 3, *A* and *B*). Ag-specific IL-2 and IFN- γ production was also inhibited, but IL-5 production increased (Fig. 3C). Immunization of OVA in alum after feeding revealed a marked inhibition of IgG1 (Fig. 4, *A* and *B*), IgE (Fig. 9), and IL-5, whereas IFN- γ was increased (Fig. 4C). The IgG2a responses were too low to analyze. Taken together, this shows that challenge of fed animals with Ag in a Th1-type adjuvant such as CFA reveals selectively suppressed Th1-associated responses, whereas challenge with Ag plus a Th2-type adjuvant

such as alum demonstrates selectively suppressed Th2-type responses. This was observed in both neonatal- and adult-fed animals.

This latter finding is consistent with reports by others showing that in adults both Th1 and Th2 cells are susceptible to oral tolerance (34, 35). Moreover, it suggests further that the tolerance process may involve feedback mechanisms that operate to prevent overexpansion of whichever particular type of Th cell becomes dominant in specific secondary responses in challenged animals. One possible example of such feedback is that recently proposed by Skok et al. (36) involving regulation of Th1 development via IL-12. They demonstrated that IL-12 can enhance the production of IL-6 and IL-10 message and, moreover, is crucial for optimal induction of IL-4 expression in differentiating T cells, which, if triggered, would inhibit further Th1 development (36). Additional possibilities include mechanisms in which activated T cells become targets for elimination via the expression of activation-associated receptors, such as in the Fas/Fas ligand pathway (37). Although the precise mechanisms remain to be clarified, the results in this study may explain some of the confusion in the literature concerning the relative susceptibility of the Th1 and Th2 pathways to tolerance induction.

Although IL-10 has been implicated as a regulatory cytokine in oral tolerance, the data in this study do not show a consistent increase. It should be noted that previous studies involved models of experimental autoimmune uveitis (22) or measured IL-10 production from Peyer's patches in mice undergoing oral tolerance induction (23), whereas in the model described in this study, IL-10 secretion was measured in LN cells.

From soluble challenge experiments it was found that the IgG1 response was primed, instead of inhibited, in neonatal-fed animals (Fig. 5A), and no IgG2a response was detected. This suggests that an inflammatory stimulus (in the form of adjuvant) is needed to produce Ab suppression after oral exposure of Ag. Humoral responses in the adult-fed animals were similar to those in the neonatally-fed groups, but the priming response was not as marked, and IgG2a responses were detected (Fig. 5B). It should be noted that the magnitude of the IgG1 titers induced in neonatal-fed OVA animals were at least 20-fold lower than those in adults, whereas if an adjuvant was used for challenge, the IgG1 responses of both age groups were similar.

Although soluble Ag challenge after oral exposure did not induce Ab suppression, inhibition of all cytokines tested was observed in adult-fed animals (Fig. 5B). In contrast, significant priming of IFN- γ in spleen cultures (although not in LN cultures) was observed in neonatal-fed animals (Fig. 5A). Why there is a difference in IFN- γ production between LN and spleen in neonatal-fed animals after soluble Ag immunization is unclear. Differences in the local microenvironment and lymphocyte trafficking between the two organs may be responsible. Adkins et al. (16) have reported that one of the major differences between newborn and adult responses was the relative contributions of spleen and LN to secondary responses, and they speculated that the recirculation of memory cells may be different during the neonatal period and adulthood. Taken together, these findings suggest that Ag/adjuvant challenge is important to achieve Ab inhibition in prefed animals (especially neonates), but it has differential impacts on cytokine production. Additionally, soluble Ag challenge did not selectively inhibit Th1- or Th2-type cytokine responses.

Tolerance induced by feeding was found to be preceded by T cell activation characterized by heightened Th1- and Th2-type cytokine responses (Fig. 6). Neonatal oral Ag exposure primed for large recall responses, characterized by enhanced production of IFN- γ , IL-5, and IL-10. This shows that Ag exposure by the oral route is a very effective way to induce T cell activation/priming

during the neonatal period, which is a novel finding of this report. Transient activation was also found for IFN- γ and IL-5 in adults. T cell activation has been shown to precede oral tolerance in a number of other model systems. Feeding tolerogenic doses of Ag to TCR transgenic mice, recipients of TCR transgenic cells, or normal mice induces an early production of IFN- γ (38–44). This early phase of T cell activation can be seen in peripheral LN and spleens of Ag-fed mice as well as MLN and PP, although to a lesser extent.

A marked difference between neonates and adults in these experiments was that IFN- γ priming by Ag feeding in adults was transient, whereas T cells from neonatal-fed animals displayed a prolonged production of IFN- γ and IL-10. This implies differential regulation or lack of negative feedback in neonatal animals. It is of interest that a subset of regulatory T cells producing both IFN- γ and IL-10 has recently been implicated in the persistence of *L. major* infection (45).

High doses of Ag have been reported to induce deletion and/or anergy of the Ag-specific T cells, whereas low doses produced immune deviation or induction of regulatory cells (reviewed in Ref. 46). The data from the adoptive transfer experiments in this study show transfer of oral tolerance, but only in the Th1 compartment (IgG2a), when the recipients were immunized with Ag in CFA. This was evident in both neonatal- and adult-fed animals. The reasons for the lack of transfer of suppression of responses elicited with alum immunization remain to be investigated. It is possible that Th1-type cells are intrinsically more susceptible to suppression than Th2-type cells, as has been demonstrated with Th clones (47, 48). It has also been shown that Th1 cells may be more susceptible to Fas/Fas ligand-mediated cell death compared with Th2 cells (37). The lack of suppression of IgE by the adoptive transfer contrasts with the marked suppression of this isotype in the donor animals. Although the transfer of such suppression has been previously reported (49), there has been surprisingly little work published on the subject. Although the experiments showed convincing suppression with Peyer's patch cells, similar suppression could only be achieved with a very high doses (10^8) of spleen cells (49).

An explanation for the different suppression found with CFA and alum could be that their mechanisms of action are dependent on different interactions with the APC and downstream regulatory mechanisms. For example, CFA has been shown to preferentially enhance the presentation of Ag by macrophages and to activate NK cells (50–52), whereas alum activates the complement cascade (53, 54). Furthermore, the composition of the adjuvants may also have differential effects on their mode of action. CpG motifs derived from mycobacterial components present in CFA have been shown to be recognized by members of the Toll-like receptor (TLR) family, whereas alum does not act via TLR because it can act via MyD-88-independent and, hence, TLR-independent signaling pathway (54, 55).

In conclusion, these studies demonstrate that oral exposure to Ag during the neonatal period induces tolerance, as measured by the ability to mount a response to systemic Ag challenge. The initial stages of tolerance induction appear to involve the priming of cytokine production, most notably IFN- γ and IL-10, by lymphocytes in MLN. The choice of adjuvants used for systemic immunization in the challenge phase of oral tolerance induction markedly influenced subsequent cytokine responses. Notably, systemic challenge of tolerant animals with CFA or alum after initial oral Ag exposure led to a preferential inhibition of Th1- or Th2-type responses, respectively. Challenge with soluble Ag alone had little impact on the polarization and, moreover, revealed covert priming as opposed to tolerance in animals prefed as neonates. It

is possible, therefore, that environmental costimulation during Ag rechallenge may be an obligatory component of the oral tolerance process, particularly during early life.

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