0031-3998/89/2605-0486$02.00/0
PEDIATRIC RESEARCH
Copyright © 1989 International Pediatric Research Foundation, Inc.
Vol. 26, No. 5, 1989
*Printed in U.S.A.*

# Failure to Induce Oral Tolerance to Protein Antigens in Neonatal Mice Can Be Corrected by Transfer of Adult Spleen Cells

H.-J. PENG,[1] M. W. TURNER, AND S. STROBEL

*Department of Immunology, Institute of Child Health, London, U.K.*

**ABSTRACT. We have examined the mechanisms that prevent the induction of oral tolerance to protein antigens in neonatal mice. Serum collected from adult mice 1 h after feeding ovalbumin (1 mg/g body wt) was adoptively transferred to mice aged 1, 3, and 42 d (40 μL/g body wt). Whereas delayed-type hypersensitivity was significantly suppressed in adult recipients relative to control groups, no suppression of systemic delayed-type hypersensitivity was found in neonatal recipients. In attempts to identify the immunologic deficiency that prevents mature reactivity to protein antigens in neonates, adult splenocytes were transferred intraperitoneally ($10^8$ cells/recipient) 24 h before a feed of OVA (1 mg/g body wt) to neonates. Significant suppression of their systemic DTH response, but not of their anti-ovalbumin IgG antibody response was observed, indicating that spleen cell transfer only partially confers adult-type reactivity. Similar results were obtained using a second protein antigen, BSA. Our observations suggest that the failure to induce oral tolerance to protein antigens in neonatal mice is not simply due to immature antigen processing by the gut, but probably reflects cellular and/or antigen handling immaturity of the neonatal immune system. (*Pediatr Res* 26:486–490, 1989)**

**Abbreviations**

**DTH, delayed-type hypersensitivity**
**i.p., intraperitoneal**
**OVA, ovalbumin**

Oral tolerance is the state of systemic immunologic hyporesponsiveness induced after feeding protein antigens to naive adult animals (1). As an immunologic defense mechanism, the induction of oral tolerance to foreign proteins is probably important because the intestinal tract is repeatedly exposed to a large variety of food antigens. In experimental animal models it has been shown that the breakdown of oral tolerance can be associated with systemic and/or local DTH reactions and serum antibody responses (2–6).

Although the pathogenesis of clinical food hypersensitivity is basically unknown, a regulatory defect in the induction of oral tolerance has been postulated (1–12). Inasmuch as food hypersensitivity is most common in infancy (7–11) and gut permeability to food antigens is increased in infants (13) and newborn animals (5), it has been suggested that the early postnatal period is one of particular vulnerability. A delay in the maturation of antigen handling or in the induction of antigen specific suppressor T cells may be responsible (1, 12, 14). Indeed, such proposals are supported by recent reports that have shown that oral tolerance cannot be induced in neonatal mice fed with OVA at a wt-related dosage known to produce tolerance in adult mice (2, 5, 14).

We and others (15–18) have previously shown that adoptive transfer of serum collected after feeding OVA induces antigen-specific suppression of systemic DTH in adult mice. In our study we have used two experimental approaches to investigate the possible role of gut processing and immaturity of the neonatal immune system on the induction of oral tolerance. First, we transferred "gut-processed" tolerogenic OVA from adult mice to both mature and neonatal recipients, and second, we attempted to "educate" the immune system of the neonate with adult spleen cells before antigen administration to investigate whether this procedure would permit the induction of oral tolerance in neonates.

## MATERIALS AND METHODS

*General experimental protocol.* The general protocol for all the experiments performed is outlined in Table 1.

*Animals.* Adult (8 to 10 wk old) BALB/c mice, purchased from Charles River U.K. Ltd. (Margate, Kent), were used as breeding stock for a new colony in the Animal House, Institute of Child Health, London. Close to the expected date of delivery, pregnant mice were checked twice per day (1000–1800 h) and the offspring were treated within the first 24 h of life. Mice were maintained on a standard laboratory diet (CRM (X); Labsure Ltd., Manea, Cambridgeshire, U.K.) containing neither OVA nor bovine albumin. All colony-bred mice were weaned onto this standard diet at 21 d of age. Young (6 to 8 wk old) female BALB/c mice (Charles River, U.K. Ltd.) were used either as adult controls or as donors for both serum and spleen cell transfer experiments.

*Antigens.* OVA (grade V) and BSA Fraction V) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

*Oral administration of antigen.* Both OVA and BSA were dissolved in 0.15 M saline and prepared at two different concentrations: *1*) 100 mg/mL (1 mg/g body wt of adult mice): 0.25 mL/feed; *2*) 30 mg/mL (1 mg/g body wt of neonatal mice): 0.05 mL/feed.

Neonatal mice were fed by gentle gavage using an intravenous tube (Code 00; Portex Ltd., Hythe, Kent, U.K.) attached to a 28-gauge needle stock. To minimize trauma, the end of the feeding tube was blunted by heating and checked for smoothness under a light microscope. Adult mice were also fed by gavage using an 18-gauge feeding tube with a rounded tip (Fine Science Tools Inc., North Vancouver, B.C., Canada). Mice in the experimental groups were fed with OVA or BSA, and those in the control groups were fed with saline only. Animals that showed signs of regurgitation or bleeding after feeding were excluded.

Received February 23, 1989; accepted May 12, 1989.
Correspondence Dr. S. Strobel, Department of Immunology, Institute of Child Health, 30, Guilford Street, London, WC1N 1EH, U.K.
[1] Supported by the Ministry of Education, Taiwan.

Table 1. *Experimental protocol*

| | Neonate | Treatment | Adult |
|---|---|---|---|
| Protocol A | D 0 | i.p. injection | D 0 |
| Serum transfer | D 28 | Immunization | D 7 |
| | D 49/40 | Test (Ab,DTH) | D 28/29 |
| Protocol B | D 0 | i.p. injection of spleen cells | |
| Spleen cell transfer | D 1 | (B1) serum transfer | |
| | | (B2) OVA, BSA feed | |
| | D 28 | Immunization | |
| | D 49/40 | Test (Ab,DTH) | |

*Adoptive transfer of serum.* Donor mice were fed with 25 mg OVA dissolved in 0.25 mL saline or with 0.25 mL saline only. Under halothane anaesthesia, the mice were exsanguinated by cardiac puncture 1 h after feeding. The sera of animals in the OVA-fed or saline-fed groups were pooled separately and injected i.p. into recipient animals at a dosage of 40 μL/g body wt (15–18). Two groups of control animals received injections of either saline alone or pooled normal mouse serum "spiked" with OVA (70 ng/mL) and incubated under continuous rotation for 1 h at 37°C. The latter concentration was derived from preliminary experiments (Peng H-J, Turner MW, and Strobel S, unpublished observations).

Animals in each of the four treatment groups were subsequently immunized as described below.

*Adoptive transfer of spleen cells.* Under halothane anaesthesia, spleens were removed from naive adult mice and placed into RPMI 1640 (GIBCO, Paisley, Scotland). Spleen cells were liberated from the splenic capsule using RPMI 1640 in a 1-mL syringe with a 25-gauge needle. Thereafter, spleen cells were washed three times and injected i.p. into neonatal mice within 24 h of birth ($10^8$ viable cells in 0.1 mL RPMI 1640 per recipient).

*Systemic immunization.* Mice were parenterally immunized in the left hind footpad with 100 μg OVA or BSA emulsified in 0.05 mL complete Freund's adjuvant (Bacto H37Ra, Difco Labs., West Molesey, Surrey, U.K.). Neonatal mice were not immunized until 4 wk old, whereas adult animals were immunized 7 d after the transfer of serum.

*Assessment of specific IgG antibody responses.* Mice were bled from the tail 20 d after immunization. The separated serum was stored frozen at −20°C until required. Serum anti-OVA IgG antibodies were assayed by an ELISA (19). In brief, flat-bottomed microtitre plates (Linbro, Flow Labs. Ltd., Rickmansworth, Herts., U.K.) were coated with OVA (100 μg/mL) dissolved in 0.05 M carbonate buffer pH 9.6 (100 μL/well) overnight at 4°C and free sites on the plates were blocked with 1% goat serum. After six washes, serum samples (1/1000) and standards (affinity-purified mouse anti-OVA IgG), both diluted in saline-Tween containing 1% goat serum, were incubated for 1.5 h at 37°C. After washing, 100 μL of a 1/5000 dilution of alkaline phosphatase conjugated goat antimouse IgG (Fc-specific; Jackson Immunoresearch Labs., West Grove, PA) were added and the plates incubated for 1.5 h at 37°C. After the final washes, *p*-nitrophenylphosphate (1 mg/mL; Sigma) was added and the absorbance read at 405 nm in a Titertek Multiskan ELISA Reader (Flow Labs.).

Anti-BSA IgG antibodies were assessed in a similar ELISA. Briefly, plates were coated with BSA (100 μg/mL) overnight at room temperature. Free sites on the plates were blocked with OVA Tween, serum samples (1/1000), and standards (0–2000 ng/mL affinity-purified mouse anti-BSA IgG), diluted in saline-Tween, were incubated in duplicate in the wells for 1.5 h at 37°C. All the other steps in the procedure were the same as those in the ELISA for IgG anti-OVA antibodies.

*Measurement of systemic DTH responses.* Three wk after systemic immunization, the systemic DTH response was determined by a footpad swelling test. Mice received injections into the right hind footpad with 100 μg BSA or heat-aggregated OVA in 0.05 mL sterile saline. The footpad thickness was measured using a dial thickness gauge (Mitutoyo, MFG Co., Tokyo, Japan) both before and 24 h after challenge. The differences between these two measurements were used in the group comparisons.

*Statistics.* Both IgG antibody and systemic DTH responses were expressed as means ± 1 SD and all group comparisons were made using unpaired Student's *t* tests.

## RESULTS

*Immunologic effects of adoptive transfer of "gut-processed" OVA.* Groups of neonatal or adult recipients received i.p. injections with either *1*) saline, *2*) serum from saline-fed animals, *3*) serum "spiked" with OVA, or *4*) serum from OVA-fed animals (see Table 1, protocol A). Three weeks after this systemic immunization, serum anti-OVA IgG antibody and systemic DTH responses were assessed and the results are shown in Figure 1 and 2.

The serum anti-OVA IgG antibody responses of both neonatal and adult mice receiving serum from OVA-fed adult donors did not differ significantly from the responses of neonatal and adult

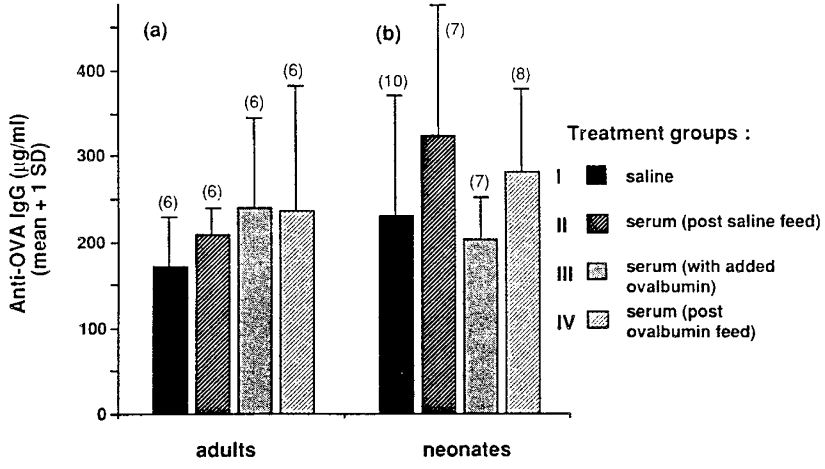


Fig. 1. Groups of adult (*a*) or neonatal (*b*) mice received i.p. injections with serum from either saline-fed treatment II or OVA-fed treatment IV animals (40 μL/g body wt). Two groups of control animals received injections of either saline alone (treatment I, or pooled normal, mouse serum containing added OVA (*ca* 70 ng/mL) (treatment III). One week later the adult recipients were immunized with 100 μg OVA in CFA. Neonatal recipients were immunized at 4 wk of age. IgG anti-OVA antibodies were measured in serum obtained 3 wk after this systemic immunization. The error bars represent 1 SD. The numbers of animals in the groups are shown in parentheses. Neither treatment group differed significantly from either control group (for both adults and neonates).

mice receiving saline or any of the serum preparations ($p > 0.05$; (Fig. 1).

Adult recipients receiving serum collected from OVA-fed donors had significantly lower systemic DTH responses than mice receiving serum from saline-fed donors or mice receiving serum spiked with OVA ($p < 0.005$ for both comparisons; Fig. 2). In addition, there was a significantly reduced DTH response compared to mice receiving saline injections ($p < 0.05$). In contrast, there were no differences between the systemic DTH responses of the four neonatal experimental groups ($p > 0.05$ for all comparisons; Fig. 2). This suggests that adoptive transfer of "gut-processed" OVA from adult mice to neonatal mice does not confer systemic DTH tolerance. Neonatal animals that had received adult spleen cells before transfer of the "tolerogenic" serum (see Table 1, protocol B1) did not develop suppression of either antibodies or DTH (data not shown).

*Effects of transfer of adult spleen cells on induction of oral tolerance in neonatal mice.* Neonatal mice, which had received either $10^8$ adult spleen cells or culture medium on the first day of life, were fed the next day with either saline or OVA (Table 1, protocol B2). Four weeks later they were systemically immunized with OVA in CFA. Three weeks after immunization their serum IgG anti-OVA antibody and DTH responses were assessed (Fig. 3).

There was no significant difference between any two groups in the levels of specific IgG antibody to OVA, although feeding a protein antigen to newborn mice might be expected to prime the animals.

In contrast, the neonatal mice receiving adult splenocytes on d 1 and a feed of OVA on d 2 showed a lower DTH response than any other experimental group ($p < 0.001$–$<0.02$; Fig. 3*b*).

Neonatal mice, which had received either $10^8$ adult spleen cells or 0.1 mL RPMI 1640 on the first day of life, were fed the next day with either saline or BSA. Four wk later they were systemically immunized with BSA in CFA. Three weeks after immunization their serum IgG anti-BSA antibody and DTH responses were measured (Fig. 4).

The mice receiving $10^8$ adult spleen cells on d 1 and a feed of BSA on d 2 showed a significantly lower DTH response than mice in the other treatment groups ($p < 0.001$–$<0.05$; Fig. 4*b*). However, the specific IgG antibody responses of these groups of animals did not differ ($p > 0.05$; Fig. 4*a*).

## DISCUSSION

Previous studies (2, 5, 15) have shown that oral antigen administration to neonatal mice does not lead to oral tolerance and we have speculated that this might be due to the immaturity of the antigen-processing capacity of the neonatal gut. The experiments reported here, however, show that transfer to neonates of serum containing tolerogenic ovalbumin moieties generated by the adult gastrointestinal tract does not induce systemic hyporesponsiveness of DTH in neonatal mice, whereas it is immunologically active in mature animals. In contrast, antibody responses are not affected in either mature or immature recipients.

Adoptive transfer of spleen cells from adult to neonatal mice permits the recipients to develop systemic hyporesponsiveness for DTH but not for antibody responses to orally administered

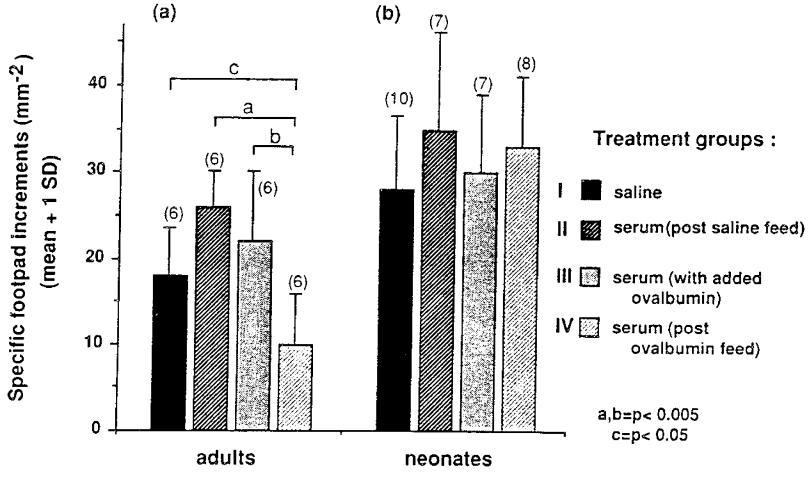


Fig. 2. Systemic DTH responses measured in adult (*a*) and neonatal (*b*) mice treated as described in the legend to Figure 1. DTH responses were evaluated by a foot-pad swelling test 3 wk after systemic immunization. The error bars represent 1 SD. The numbers of animals in the groups are shown in parentheses. The letters *a* and *b* indicate significant suppression of the DTH response.

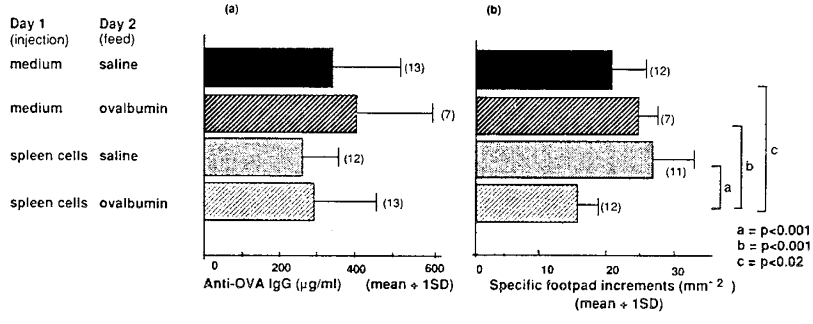


Fig. 3. Effects of transfer of adult spleen cells on the induction of oral tolerance to OVA in neonatal mice. Neonatal mice received either adult spleen cells or culture medium on the first day of life and were fed the next day with either saline or OVA. Then 4 wk later they were systemically immunized with OVA in CFA. Three wk after immunization their serum IgG anti-OVA antibody (*a*) and DTH responses (*b*) were assessed. The error bars represent 1 SD. The numbers of animals in the groups are shown in parentheses. The letters *a* and *b* indicate significant suppression of the DTH response.

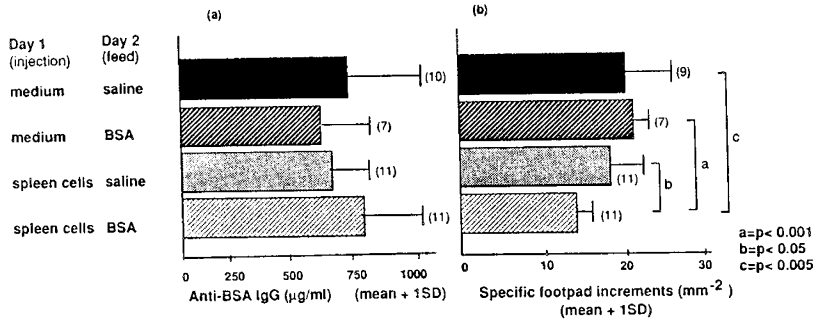


Fig. 4. Effects of transfer of adult spleen cells on the induction of oral tolerance to BSA in neonatal mice. Animals were treated as indicated and described in the legend to Figure 3 (substituting BSA for OVA). Three wk after systemic immunization with BSA in CFA, serum IgG anti-BSA antibody (*a*) and DTH responses (*b*) were assessed. The error bars represent 1 SD. The numbers of animals in the groups are shown in parentheses. The letters *a* and *b* indicate significant suppression of the DTH response.

proteins. Complete oral tolerance would require suppression of both limbs of the systemic immune response. The study of two different proteins suggests that the results reported here are representative for thymus-dependent protein antigens.

DTH tolerance can be transferred to adult naive mice with serum collected 1 h after feeding OVA (15–18). The amount of immunoreactive OVA circulating 1 h after feeding 1 mg/g body wt varies considerably (approximate range 60–100 ng/mL (17) (Peng H-J, Turner MW Strobel S, unpublished observations). Addition of such amounts of native OVA to normal mouse serum followed by incubation for 1 h at 37°C does not confer tolerogenic activity to the serum and does not lead to suppression of cell-mediated immunity. This indicates that the tolerogenicity of the serum is not directly related to the amount of immunoreactive antigen detected by ELISA.

Several reports suggest that antigen processing by the neonatal intestine is immature and both inefficient luminal proteolysis and increased gut permeability have been described (5, 13, 20). We have therefore transferred adult gut processed proteins to both adult and neonatal mice. Suppression of DTH responses was always observed in adult recipients but was never seen in neonatal recipients. These results suggest that immaturity of intestinal antigen processing is not the sole factor preventing the induction of oral tolerance in neonatal mice; other host factors possibly associated with the ontogeny of the immune system are of greater importance. Although the nature of intestinal antigen processing is unknown, the production of gut processed tolerogenic OVA has been shown to be abrogated in lethally irradiated mice (18). However, those mice have a variety of mucosal and immunologic abnormalities that include substantial T lymphocyte depletion of their lymphoid tissues. In a previous report (18), the capacity of the gut to create "tolerogens" could be restored by injection of normal spleen cells. This suggests that cells residing in the spleen may also be involved in the phenomenon of "antigen processing" or antigen presentation. In a different experimental system evaluating IgE responses after antigen inhalation, Holt *et al.* (21) recently reported a similar effect. These authors were unable to induce inhalation tolerance in neonatal rats but the transfer of adult spleen cells restored the capacity to induce antigen-specific inhalation tolerance.

Our results support the view that the immature immune system of the neonate can be partially modified to a mature pattern of reactivity 24 h after transfer of adult spleen cells. It is feasible that neonatal mice lack a specific suppressor T cell network otherwise activated by orally administered antigens or by adoptive transfer of gut-processed antigen. In addition, other factors such as natural suppressor cells or suppressor factors (22), high levels of $\alpha$-fetoprotein (23), a deficiency in antigen presentation (24), or deficient production of cytokines (25, 26), may play a role in the prevention of neonatal induction of oral tolerance.

In mature animals both limbs of the systemic immune system are generally suppressed after a protein feed; the transfer of spleen cells to neonates, however, only restores mature reactivity to DTH responses. The reasons for this apparent discrepancy remain unclear. Differences in the characteristics of induction and duration of the suppression of both limbs of the immune response have been reported (1, 19, 27). An additional finding was that even high dose spleen cell transfer failed to induce tolerance of systemic DTH after adoptive serum transfer. Further studies with graded doses of purified T lymphocytes or accessory cells are needed to examine whether these phenomena are due to a deficiency in antigen presentation.

Further insight into the immunochemical and cellular modulation of these immunoregulatory pathways is of potential clinical importance in the prophylaxis and/or therapy of hypersensitivity diseases and in the development of oral vaccines.

*Acknowledgments.* The authors thank the staff of the Institute of Child Health Animal House for their help and Miss Faith Hanstater for preparing the manuscript.

## REFERENCES

1. Mowat AMcI 1987 The regulation of immune responses to dietary protein antigens. Immunol Today 8:93–98
2. Strobel S, Ferguson A 1986 Modulation of intestinal and systemic immune responses to a fed protein antigen in mice. Gut 27:829–837
3. Mowat AMcI, Strobel S, Drummond HE, Ferguson A 1982 Immunological responses to fed protein antigens in mice. 1. Reversal of oral tolerance to ovalbumin by cyclophosphamide. Immunology 45:105–113
4. Mowat AMcI, Parrott DV 1983 Immunological response to fed protein antigens in mice. IV. Effects of stimulating the reticuloendothelial system on oral tolerance and intestinal immunity to ovalbumin. Immunology 50:547–554
5. Hanson DG 1981 Ontogeny of orally induced tolerance to soluble proteins in mice. 1. Priming and tolerance in newborns. J Immunol 127:1518–1524
6. Mowat AMcI 1986 Depletion of suppressor T cells by 2'deoxyguanosine abrogates tolerance in mice fed ovalbumin and permits the induction of intestinal delayed-type hypersensitivity. Immunology 58:179–184
7. Kuitunen P, Visakorpi JK, Savilahti E, Pelkonen P 1975 Malabsorption syndrome with cow's milk intolerance: clinical findings and cause in 54 cases. Arch Dis Child 50:351–356
8. Kuitunen P, Rapola J, Savilahti E, Visakorpi JK 1973 Response of jejunal mucosa to cow's milk in the malabsorption syndrome with cow's milk intolerance. Acta Paediatr Scand 62:585–595
9. Maluenda C, Phillips AD, Briddon A, Walker-Smith JA 1984 Quantitative analysis of small intestinal mucosa in cow's milk-sensitive enteropathy. J Pediatr Gastroenterol Nutr 3:349–356
10. Dannaeus A, Inganas M 1981 A follow-up study of children with food allergy. Clinical course in relation to serum IgE- and IgG-antibody levels to milk, egg and fish. Clin Allergy 11:533–539
11. Bjorksten B, Ahlstedt S, Bjorksten F, Carlson B, Fallstrom SP, Juntunen K, Kajosaari M, Kober A 1983 Immunoglobulin E and immunoglobulin G4 antibodies to cow's milk in children with cow's milk allergy. Allergy 38:119–124
12. Revillard JP, Lafont S, Kaiserlian D 1988 Regulation of mucosal immunity: an overview with special emphasis on secretory IgA production and oral tolerance. In: Reinhardt D, Schmidt D (eds) Food Allergy, Nestle Nutrition Workshop Series, Vol 17. Raven Press, New York, pp 35–50
13. Israel EJ, Walker WA 1988 Host defense development in gut and related disorders. Ped Clin North Am 35:1–15

14. Strobel S, Ferguson A 1984 Immune responses to fed protein antigens in mice. 3. Systemic tolerance or priming is related to age at which antigen is first encountered. Pediatr Res 18:588–594
15. Strobel S, Mowat AMcI, Pickering MG, Ferguson A 1983 Immunological responses to fed proteins in mice. 2. Oral tolerance for CMI is due to activation of cyclophosphamide sensitive cells by gut processed antigen. Immunology 49:451–456
16. Bruce MG, Ferguson A 1986 Oral tolerance to ovalbumin in mice. Studies of chemically modified and of "biologically filtered" antigen. Immunology 57:627–630
17. Bruce MG, Ferguson A 1986 The influence of intestinal processing on the immunogenicity and molecular size of absorbed, circulating ovalbumin in mice. Immunology 59:295–300
18. Bruce MG, Strobel S, Hanson DG, Ferguson A 1987 Irradiated mice lose the capacity to "process" fed antigen for systemic tolerance of delayed-type hypersensitivity. Clin Exp Immunol 70:611–618
19. Peng H-J, Turner MW, Strobel S 1989 The kinetics of oral hyposensitization to a protein antigen are determined by immune status and the timing, dose and frequency of antigen administration. Immunology (in press)
20. Walker WA 1988 Transmucosal passage of antigens. In: Reinhardt D, Schmidt E (eds) Food Allergy, Nestle Nutrition Workshop Series, Vol 17. Raven Press, New York, pp 15–34
21. Holt PG, Vines J, Britten D 1988 Suppression of IgE responses by antigen inhalation: failure of tolerance mechanism(s) in newborn rats. Immunology 63:591–593
22. Maier T, Hold A, Claman HN 1986 Natural suppressor cells. Member of the LGL regulatory family. Immunol Today 7:312–315
23. Crainie M, Semeluk A, Lee KC, Wegmann T 1989 Regulation of constitutive and lymphokine-induced Ia expression by murine alpha-fetoprotein. Cell Immunol 118:41–52
24. Lu CY, Calamai EG, Unanue ER 1979 A defect in the antigen-presenting function of macrophages from neonatal mice. Nature 282:327–329
25. Ishizaka ST, Stutman O 1983 Analysis by limiting dilution of interleukin 2-producing T cells in murine ontogeny. Eur J Immunol 13:936–942
26. Wakasugi N, Virelizier J-L 1985 Defective INF-$\gamma$ production in the human neonate. 1. Dysregulation rather than intrinsic abnormality. J Immunol 134:167–171
27. Strobel S, Ferguson A 1987 Persistence of oral tolerance in mice fed ovalbumin is different for humoral and cell-mediated immune responses. Immunology 60:317–318.