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In mice, gluten in maternal diet primes systemic immune responses to gliadin in offspring

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SUMMARY

We have demonstrated recently immunological tolerance to gliadin in mice maintained on a diet that contains gluten. The aim of this study was to investigate whether oral tolerance is recreated in each generation by the ingestion of dietary gluten at weaning, or whether it is transferred from mother to young (as immune status or via passage of antigen) before birth or during lactation. Surprisingly, instead of transfer of tolerance we found priming of the immune response to gliadin in young mice. Mice born to mothers from normal, gluten-containing diet colonies had significantly greater systemic immune responses to gliadin after parenteral immunization than mice born to mothers from a gluten-free diet colony. Furthermore, feeding mothers gluten-containing diet for defined periods before and during pregnancy and during lactation also resulted in priming of the specific systemic immune responses of the offspring. These findings indicate that, in mice, sensitization to maternal dietary antigens readily occurs *in utero* or shortly after birth. This animal model should allow investigation of the immunological mechanisms concerned.

INTRODUCTION

Induction of systemic immunological hyporesponsiveness is the most frequent consequence of feeding soluble protein antigen (Mowat, 1987); nevertheless, in some circumstances priming may occur. This is particularly evident when antigen is ingested by infant mice. For example, a single feed of ovalbumin on the day before or on the day of birth leads to priming for subsequent specific antibody and cellular immunity, although this priming effect can be converted into one of tolerance if ovalbumin feeds continue to be given for the first few days of life (Strobel & Ferguson, 1984).

We are currently investigating the immunological properties of gliadin when presented via the gut, in the light of its etiological role in coeliac disease. By using the facility of a colony of mice maintained for several generations on a gluten-free diet, we have already shown that feeding gliadin produces oral tolerance in mice. Furthermore, by comparing gluten-free and normal diet animals we have demonstrated that animals from conventionally reared colonies are immunologically tolerant to gliadin (Troncone & Ferguson, 1988).

The aim of the present study was to investigate whether oral tolerance to gliadin is recreated in each generation by the ingestion of gluten-containing food after weaning, or whether it is induced by events that occur before birth or during lactation. Therefore, we have compared the systemic immune responses to gliadin of young mice, never exposed to this antigen, with those of mice that have encountered the antigen from the mother's diet (at least theoretically) via the placenta and/or milk.

On the basis of a previous report (Johnson *et al.*, 1985), we anticipated that the phenomenon of oral tolerance would already be established prior to weaning, and as a preliminary attempt to evaluate whether this is due to transmission of antigen or passive transfer of immune components from mother to young we switched animals between gluten-free and normal diets so that groups of mothers encountered dietary gliadin only prior to pregnancy, before and during pregnancy, or during lactation alone.

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MATERIALS AND METHODS

Animals and diet

Mice from two colonies of BALB/c mice were used. These are referred to below as normal diet (ND) and gluten-free diet (GFD) mice. ND mice were maintained on a standard rodent diet, which contains 2·8% gluten [CRM(X), Labsure Ltd, Poole, Dorset]. A gluten-free colony was established in 1985; these animals were fed a gluten-free (GF) diet (Special Diets Service Ltd, Witham, Essex); only third- or later-generation mice were used for these experiments.

Female mice from the ND and GFD colonies were subjected to various dietary manipulations before and during pregnancy and during lactation, as detailed in the experimental protocols

below. All offspring were weaned onto the GF diet at the age of 21 days, but to avoid those born to ND mothers nibbling gluten contained in the ND, GFD was supplied to all the mothers from 14 days after delivery. The immune status of offspring, tolerant or sensitized to gliadin, was assessed by measuring their systemic immune responses after parenteral immunization with gliadin in CFA.

Antigen

Wheat gluten is a mixture of glutenin, which confers elasticity to dough, and the storage protein gliadin. Purified gliadin used for immunization, ELISA plate coating and skin tests in these experiments was a kind gift of Dr H. Weiser (DFA fur Lebensmittelchemie, Garching, FRG). Details of the preparation and characterization of this material have been published elsewhere (Weise *et al.*, 1987). Briefly, defatted wheat flour (variation Kolibri) was extracted step-wise three times with sodium chloride 0·4 M/sodium potassium phosphate 0·067 M, pH 7·6, and three times with 70% aqueous ethanol; each suspension was centrifuged and the supernatants pooled, dialysed against water and acetic acid 0·01 M and finally freeze-dried.

Evaluation of immune status

At the age of 4–6 weeks, age and sex-matched groups of six to eight mice were immunized into one rear foot pad with 50 μg of gliadin in distilled water emulsified in 50 μl Freund's complete adjuvant (CFA) (Bacto-H37 Ra, Difco Ltd, West Molesey, Surrey). Control mice received water in CFA.

Mice were bled from the retro-orbital plexus, under light ether anaesthesia, 3 weeks after parenteral immunization and sera were tested for IgG antibodies to gliadin by an ELISA technique. Microtitre plates (Dynatech Ltd, Billinghurst, Sussex) were coated by overnight incubation at 4° with a solution containing 1 μg/ml gliadin. Test samples were diluted 1:2000 with 0·15 M saline containing 1% rabbit serum, 0·05% Tween 20 and 0·1% sodium azide. These were added to the plate, which was then incubated for 2·5 hr at room temperature. Affinity-purified goat anti-mouse IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories Ltd, West Grove, PA) was added at 1/5000 dilution and the plates incubated for a further 3 hr at room temperature. *p*-Nitrophenyl phosphate (Sigma, Poole, Dorset) was added as substrate, and the absorbance read at 405 nm using an automatic ELISA reader (Dynatech Ltd). Between each incubation step, plates were washed three times with 0·15 M saline containing 1% bovine serum albumin (Sigma) and 0·05% Tween 20 (Sigma).

Three weeks after immunization mice were also tested for delayed-type hypersensitivity (DTH). Footpad thickness was measured by skinfold calipers (POCOTEST-A, Carobronze Ltd, London) immediately before and 24 hr after an intradermal injection of 25 μg of antigen in 0·05 ml water.

Statistics

DTH results were compared by Student's *t*-test. ELISA results were compared by Wilcoxon's rank sum test, as non-parametric distribution of these data could not be ruled out.

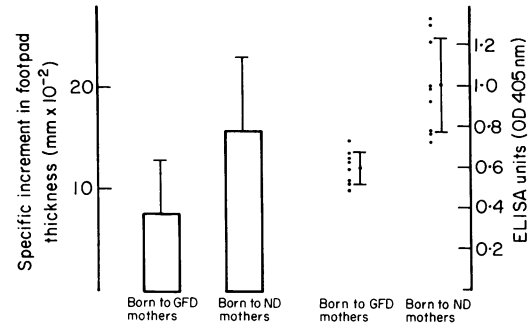

Figure 1. Systemic immune response to gliadin in mice born to mothers reared on normal diet or on gluten-free diet. DTH (left) and antibody (right) responses 3 weeks after parenteral immunization with gliadin in CFA. Mice were born to mothers reared on gluten-containing diet (ND mothers), and to mothers reared on gluten-free diet (GFD mothers). All were weaned on GFD and immunized at the age of 4 weeks. DTH results are shown as mean specific increment in footpad thickness + 1 SD. Antibody results are shown as individual OD_{405} readings in ELISA. Bars indicate mean + 1 SD.

RESULTS

Is oral tolerance to gliadin transmitted from mother to young?

Mothers from the ND colony were maintained on the ND during pregnancy and for 2 weeks after parturition. They were switched to GFD from Day 15–21 in order to prevent the young nibbling gluten. At 21 days the babies were removed and maintained on GFD, and their immune status subsequently tested. Another group of mothers, from the GFD colony, were maintained on the GFD throughout pregnancy and lactation. Their young were weaned on the GFD and tested in parallel with the young of ND mice. All the babies received gliadin in CFA at the age of 4–6 weeks, and their specific immune responses were measured as described above.

Mice born to mothers maintained on ND showed a DTH response to gliadin significantly higher ($P < 0·05$) than mice born to mothers maintained throughout on GFD. This result was consistently obtained in three different experiments; one of these is shown in Fig. 1. The effect of maternal diet on humoral responses of the infants was less consistent. Young mice were not tolerant; in two experiments no net effect of maternal diet was noted, and in one experiment there was priming of the antibody response in the litter born to mothers from the ND colony (Fig. 1).

Effect of feeding mothers ND during pregnancy and/or lactation on the immune responses of their offspring

All mice that entered this experiment were from the GFD colony. Control mice were maintained throughout on GFD (group A). The other mothers (except group F) were switched to the ND 3 weeks before mating and returned to a GFD at different times (Fig. 2). Group B returned to GFD at the time of mating; group C in mid-pregnancy; group D at the end of pregnancy; and group E after 2 weeks of lactation. The last group of mothers (group F) were fed ND for 2 weeks after parturition. All babies were weaned on the GFD. They received gliadin in CFA at the age of 4 weeks, and their specific immune

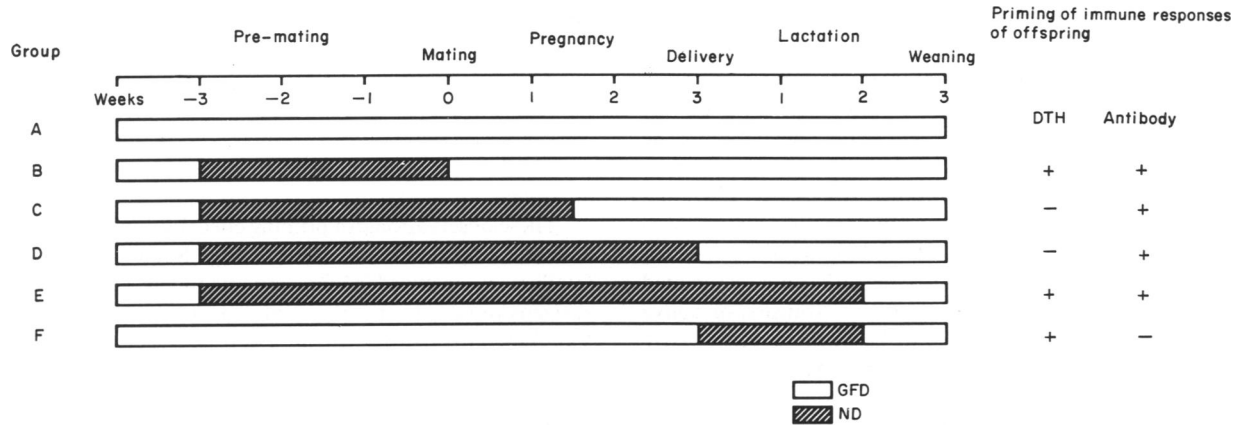

Figure 2. Effect of feeding mothers gluten-containing diet (ND) during pregnancy and/or lactation on the immune response of their offspring. Control mothers (group A) were maintained throughout on GFD. Mothers from the gluten-free diet (GFD) colony were switched to the gluten-containing diet (ND) 3 weeks before mating and returned to the GFD at different times (groups B–E). Group F mice were fed ND only for the first 2 weeks of lactation. All baby mice were weaned on GFD and parenterally immunized with gliadin at the age of 4 weeks. The systemic immune response was assessed 3 weeks later. Signs + and − indicate presence or absence of priming for DTH and/or antibody responses in the offspring.

Table 1. DTH and antibody response after parenteral immunization with gliadin in CFA, of mice born to mothers fed gluten-containing diet before and during pregnancy and/or lactation (see also Fig 2)

Group	Maternal diet	No. of offspring	DTH (mm)	IgG antibody (OD_{405})
A	GFD throughout	8	0·075+0·053	0·613 (0·502–0·744)
B	ND 3 weeks before conception	8	0·162+0·081*	0·927† (0·696–1·202)
C	ND until mid-pregnancy	8	0·091+0·036	0·909* (0·553–1·363)
D	ND until delivery	8	0·091+0·055	0·989† (0·837–1·284)
E	ND until 2 weeks after delivery	6	0·148+0·034*	0·848† (0·689–0·984)
F	ND only during lactation	8	0·126+0·040‡	0·711 (0·402–0·922)

Serum IgG antibody response was measured by ELISA 3 weeks after parenteral immunization and the results are expressed as mean and range of individual OD_{405} readings. Systemic DTH response was also assessed 3 weeks after immunization, and the results shown are mean specific increment in footpad thickness (mm+1 SD).

* $P<0·02$.

† P 0·001.

‡ P 0·05.

responses were measured as described above. The results are summarized in Fig. 2 and in Table 1.

Mice born to mothers fed ND from 3 weeks before mating, throughout the pregnancy and until 2 weeks after delivery (group E) had higher cellular and humoral systemic immune responses to gliadin than the control mice (group A). Feeding mothers ND for only 3 weeks before conception (group B) gave the same result. Mice whose mothers were fed ND from 3 weeks before mating until mid-pregnancy or until delivery (groups C and D) also showed priming of immune responses; both DTH and antibody responses were higher than in group A offspring, but only the humoral response was significantly different ($P<0·02$) compared with control mice. Finally, when mothers were fed ND only for the first 2 weeks of lactation (group F), their offspring showed priming of the systemic cellular immune response to gliadin.

DISCUSSION

This study was designed to investigate whether the state of immunological tolerance to dietary gliadin in mice was acquired anew in each generation at weaning or was transmitted from mother to young by events occurring before birth or during lactation. However, although in many experiments we have found that adult mice reared on a normal diet have unequivocal tolerance to gliadin, the work described above shows that the offspring of such animals exhibit priming of both cellular and humoral immune responses.

Several patterns of manipulation of the maternal diet have been used; all produced priming of the offspring, although in some cases only the humoral or cellular limb of the immune response was affected significantly. It seems likely that different immunological mechanisms will be implicated in the various

situations studied. Most difficult to explain is the priming observed in mice born to mothers whose dietary exposure to gluten was limited to the 3 weeks before mating. We have found that 1 week of ND tolerizes adult mice from the GFD colony (Troncone & Ferguson, 1988), and so by the time of mating the mothers in the present experiments should also have been tolerant. But it is conceivable that, only 3 weeks after introduction of a new dietary antigen, their overall immune status could still be evolving with a mixture of help and suppression, to which are then added the immunological perturbations associated with pregnancy. In this unusual situation, transfer of active maternal immunity to the young could be either via sensitized T cells, or via anti-idiotype antibodies mimicking the structure of antigen (Stein & Soderstrom, 1984).

As priming, rather than tolerance, was the outcome when the mothers had a gluten-containing diet during pregnancy, direct immunization of the fetus by transplacental antigen cannot be excluded. In rodents, proteins fed to pregnant animals have been detected, by immunoprecipitation, in amniotic fluid and fetal blood (Dahl *et al.*, 1984). Transfer of antigen via the milk could also explain the priming of mice born to mothers from group F, fed gluten only during lactation. Anti-idiotypic antibodies transferred in milk modulate immune responses of the new-born (Hanson *et al.*, 1985). Furthermore, dietary antigens have been demonstrated in the milk, the pattern of such a transfer varying with different antigens studied (Harmatz *et al.*, 1986b). The passage of gliadin into milk has not been investigated in rodents, but minute amounts of gliadin are present in human milk (Troncone *et al.*, 1987). Circulating maternal antibody has been found to limit the transfer of specific protein antigen from mother to nursing new-born (Harmatz *et al.*, 1986a). Whether such antibodies can interfere with the priming observed in our experiments is an issue that could be investigated readily.

If the sensitizing effect observed is due to antigen transferred via milk, the question arises why, in this circumstance, feeding antigen does not have the usual effect of tolerance induction. Age may be the critical factor. A single feed of ovalbumin during the first 24 hr after birth results in priming of both humoral (Hanson, 1981), and cellular (Strobel & Ferguson, 1984) immune responses; on the other hand, daily ovalbumin feeds for the first week or more, analogous to the situation of antigen transfer by lactating mice, resulted in tolerance (Strobel & Ferguson, 1984). The discrepancy between the latter data and our present findings may reflect properties or doses of the antigens; unfortunately, feeding of neonates with defined doses of gliadin is rendered difficult by the relative insolubility of gliadin in water or saline. The mode and frequency of presentation are other factors to be considered. Theoretically, antigen derived from the maternal diet, presumably transferred via the bloodstream into milk, will already have been 'gut-processed' by the mother and would be expected to produce tolerance, at least for DTH (Bruce & Ferguson, 1986), although the capacity of the hyperplastic intestine of lactating animals to process antigen has not been examined directly. However, we consider that the most likely explanation for the priming effect is the low dose reaching the infant mice. Feeding a low dose of ovalbumin (400 ng) has been shown to cause priming rather than tolerance in adult mice (Mowat, 1987).

In conclusion, gluten in the maternal diet of mice specifically primes the offsprings' systemic immune responses, although adult mice from a 'normal diet' colony are tolerant, and young animals from a 'gluten-free' colony are rendered tolerant to gliadin by exposure for 1 week after weaning, to a normal diet (R. Troncone and A. Ferguson, unpublished observations). This series of observations emphasizes the crucial role of diet around the time of weaning for the subsequent immune status of an animal.

These observations, of priming effect by maternal diet, could have great relevance in clinical allergy. Some infants of atopic families are sensitized *in utero* or immediately after birth to food antigens present in the maternal diet (Matsumura *et al.*, 1975; Van Asperen, Kemp & Mellis, 1983), and there is dispute as to what dietary advice should be given to atopic mothers. Although caution is mandatory before animal data is extrapolated directly into the clinical situation, further work with this animal model should contribute to the elucidation of mechanisms that lead to sensitization before birth.

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