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Immune response in mice to ingested soya protein: antibody production, oral tolerance and maternal transfer

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While allergic reactions to soya are increasingly investigated, the normal immune response to ingested soya is scarcely described. In the present study, we wanted to characterise the soya-specific immune response in healthy mice ingesting soya protein. Mice fed a soya-containing diet (F0) and mice of the first (F1) and second (F2) offspring generation bred on a soya protein-free diet were used either directly or were transferred between the soya-containing and soya protein-free diet during pregnancy or neonatal life. The mice were compared as to levels of naturally occurring specific antibodies analysed by ELISA, and to the presence of oral tolerance detected as a suppressed antibody and cell-proliferation response upon immunisation with soya protein. F0 mice generated soya-specific antibodies, while oral tolerance to the same soya proteins was also clearly induced. When F0 dams were transferred to soya protein-free feed before mating, the F1 and F2 offspring generations showed no significantly different response, indicating that soya-specific immune components were not maternally transmitted. However, the ingestion of dietary soya protein by F1 mice during late pregnancy and lactation caused a lasting antibody response in the offspring, but in this case in the absence of oral tolerance. This indicates that, under certain conditions, factors involved in spontaneous antibody production can be transmitted from mother to offspring. Understanding the immune response to soya protein ingested under healthy conditions is important in the assessment of adverse effects of soya protein and in the use of animal allergy models. The present results add to this understanding.

Soya protein: Oral immunogenicity: Antibodies to dietary antigens: Oral tolerance

Human consumers and animals throughout the world ingest soya protein through a variety of soya-derived products. Unfortunately, soya protein frequently causes food allergy especially in childhood (Herian *et al.* 1993; Helm *et al.* 2000; Hiemori *et al.* 2000). To improve their nutritional, functional and cultivation properties, soya beans are objects for genetic manipulation introducing new proteins. Such compositional changes naturally hold the potential of influencing the undesirable allergenic property of soya, as allergens from a food known to be allergenic can be transferred to soya beans by genetic engineering (Nordlee *et al.* 1996). These facts raise a great need for understanding the causative factors involved in soya-protein allergenicity for which purpose experimental allergy animal models are being used (Atherton *et al.* 2002; Knippels & Penninks, 2002). For the proper assessment of the adverse immune reactions to soya protein, it is, however, of great importance also to know how the immune system responds under normal conditions to ingested soya protein, which has gained only sparse attention. In some of our preliminary studies on the immune response to dietary soya protein, we found that pre-immune serum from normal experimental mice fed a commercial rodent chow containing soya protein showed a comparatively high 'background' response when testing for antibodies in ELISA against a soya-protein extract. This prompted us to study whether this response was due to specific antibody production and, in general, how the healthy immune system responds to ingested soya protein.

In general, when ingesting a food antigen, a small amount of the antigen escapes digestion and is absorbed as the intact antigen (Husby *et al.* 1986). Upon encounter by the immune system, such antigens initiate mechanisms that under normal conditions mediate oral tolerance (Mowat, 1987), but which, under immunopathological conditions, can lead to allergic reactions. Although oral tolerance induction implies a down regulation of the immune response, the ingestion of some proteins still tends to induce a comparatively weak antibody response as a seemingly normal physiological event (Coombs *et al.* 1983; Barnes *et al.* 1988; Husby, 2000). However, the properties of soya protein as to oral tolerance induction and spontaneous antibody production have not yet been characterised in detail.

There is another important aspect to take into consideration when characterising the normal immune response to soya and when using experimental animals for studying soya-specific immune reactions. This important aspect is that components involved in the antigen-specific immune response, i.e. antigen, antigen-specific antibodies or even lymphocytes, are in some cases transmitted from mother to offspring both prenatally via the placenta and postnatally

Abbreviations: F0, mice bred on soya-containing feed; F1, first offspring generation of mice bred on soya protein-free feed; F2, second offspring generation of mice bred on soya protein-free feed; KSTI, Kunitz soya trypsin inhibitor; OVA, ovalbumin; PBS-T, PBS containing Triton X-100.

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via maternal milk (Arvola *et al.* 2000; Bednar-Tantscher *et al.* 2001; Hanson *et al.* 2003). In the Brown Norway rat strain, known to be a high IgE-responder and thus used as an allergy model, Knippels *et al.* (1998*a*) found that immunity to ingested soya protein was maternally transmitted, as soya-specific antibodies were detected in the first offspring generation fed a soya-free diet even at the age of 1 year.

In order to investigate the issues as regards soya protein, the aim of the present study was to characterise the immune response in healthy mice fed processed soya protein, including the evaluation of spontaneous antibody production, oral tolerance induction and transfer of soya-specific immune components from mother to offspring.

Materials and methods

Breeding of mice

BALB/c mice (M&B, Ry, Denmark) were fed a standard chow for rodents containing approximately 10 % (w/w) defatted soyabean flakes originating from oil-extraction processing (Altromin 1324; Brogaarden, Gentofte, Denmark); these were the mice bred on soya-containing feed (F0). Offspring generations not exposed to soya protein were bred by transferring F0 mice (8–10 weeks) to a laboratory-produced semi-synthetic feed free of soya protein (Table 1) 14 d before mating and onwards. The first offspring generation mice (F1), kept solely on the soya protein-free feed, were then used for breeding the second generation (F2), while new colonies of F0 and F1 mice were bred synchronously to ensure experimental comparability among the generations. By the use of an ELISA with high detectability (see later; p. 727), the amount of the soya protein Kunitz soya trypsin inhibitor (KSTI) was measured in feed extracts as a marker for the content of soya protein. No KSTI could be detected in the soya protein-free feed.

All mice were kept under standard animal housing with feed and water *ad libitum*. The guidelines formulated in 'The Council of Europe Convention for the Protection of Vertebrate: Animals used for Experimental and other Scientific Purposes' were followed. All animal studies were approved by The Danish Animal Experiments Inspectorate.

Design of experiments with unprimed mice

Age-matched F0, F1 and F2 male mice were allocated upon weaning to groups of ten to fifteen mice/group with siblings distributed equally between groups. At age 6, 7 and 8 weeks, blood samples were collected, whereupon the mice were killed and spleens were subjected to a cell-proliferation assay as described later (p. 728). Blood samples (50 μl) from all experiments were collected from the retro-orbital plexus. The samples were immediately diluted 1:16 in PBS containing 1 g Triton X-100/(PBS-T) and stored at −20°C until antibody titre analysis as described later.

Table 1. Composition of experimental diet free of soya protein (g/kg)

Component	Content
Caseinate (approximately 890 g protein/kg)	180
Sucrose	34
Yellow dextrin	34
Maize starch	306
Potato starch	306
Mineral mixture	28
Vitamin B mixture	12
Soya oil* with fat-soluble vitamins	50
Cellulose	50

* Free of protein residues.

Design of experiments with primed mice

Age-matched F0, F1 and F2 male mice were allocated upon weaning to groups of eight to ten mice/group. At age 8 weeks, the mice were intraperitonally immunised twice 14 d apart. They were immunised with a mixture of 100 μg soya-protein extract, 10 μg KSTI (Sigma-Aldrich, St Louis, MO, USA), 10 μg β-conglycinin (kindly provided by Dr M. Hessing, TNO, Zeist, Holland) and 10 μg ovalbumin (OVA; Sigma-Aldrich; control antigen) in 0·1 ml PBS (0·01 M, pH 7·4) mixed with 0·1 ml Freund's incomplete adjuvant. At 1 week after the second immunisation, blood samples were collected and the cell-proliferation assay was performed.

Design of experiments with experimentally induced oral tolerance

F0 and F2 male mice were each allocated upon weaning to two groups of eight to ten mice/group (four groups in total). At age 7 weeks, one group from each generation was supplemented for 5 d with soya-protein extract (2 g/l) and KSTI (2 g/l) in the drinking water. At 14 d after the supplementation was begun, all of the mice were immunised and treated as above.

Design of experiments with transfer of mice at different ages between soya-containing and soya protein-free feed

The study included seven groups (eight to ten mice/group) transferred from one feed to the other at different ages as illustrated in Fig. 1 (a) and outlined later (pp. 727–728). Group one was F2 mice kept on soya protein-free feed (negative controls); group two was F0 mice kept on soya feed (positive controls). Groups three, four and five were F2 mice transferred to soya feed 1 d before birth (transfer of pregnant F1 animals), 1 week after birth and at weaning (age 3 weeks), respectively. Group six was offspring from F0 mice transferred to soya protein-free feed the day of birth; group seven was F2 mice transferred to soya feed 1 week before birth (transfer of pregnant F1 animals) and back to the soya protein-free feed at weaning. Blood samples were collected at 6 and 8 weeks of age whereupon all mice were immunised as described above, but using 100 μg soya-protein extract and 10 μg KSTI. Blood samples were collected again 1 week after the last immunisation.

Preparation of soya-protein extract and glycinin fraction

Defatted soyabean flakes of the same source as used in the soya feed (kindly provided by Brogaarden, Gentofte,

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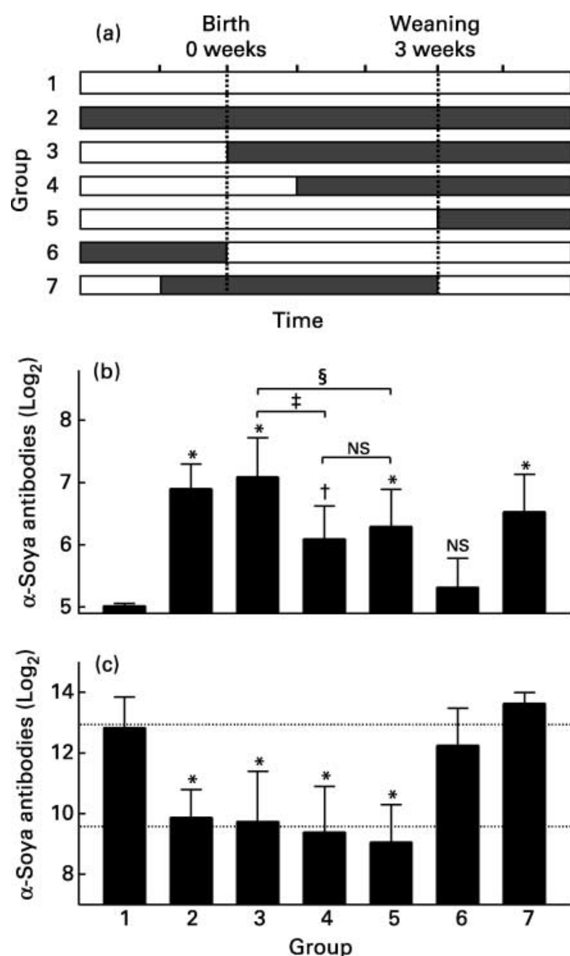

Fig. 1. Antibody response of mice transferred between soya protein-free and soya-containing feed at different ages as outlined in (a). Group 1, F2 mice kept on soya protein-free feed (□) (negative controls); group 2, F0 mice kept on soya feed (■) (positive controls); groups 3, 4 and 5, F2 mice transferred to soya feed 1 d before birth, 1 week after birth and at weaning (age 3 weeks), respectively; group 6, offspring from F0 mice transferred to soya protein-free feed the day of birth; group 7, F2 mice transferred to soya feed 1 week before birth (transfer of pregnant F1 animals) and back to the soya protein-free feed at weaning (*n* 8–10 per group). Soya-specific antibody response was measured in blood samples collected at the age of (b) 8 weeks (before immunisation), and (c) 11 weeks (after two-time immunisation with soya-protein extract and Kunitz soya trypsin inhibitor). Mean values are shown, with their standard errors represented by vertical bars. Mean values were significantly different (one-way ANOVA with Bonferroni's *post hoc* test of selected pairs): $^{*}P<0.001$, $^{\dagger}P<0.01$, $^{\ddagger}P=0.0098$, $^{\S}P=0.021$. Other differences were NS ($P\geq0.05$). Where no horizontal bars appear, the mean values are compared with group 1. For details of mice and procedures, see p. 726.

Denmark) were finely milled. This powder (300 mg), suspended in 5 ml ammonium hydrogen carbonate buffer (0·1 M, pH 8·5), was left on an ultrasound bath for 10 min and then incubated for 30 min at room temperature while shaking. The suspension was centrifuged (15 min, 1300 ***g***), the pellet was then re-suspended in 3 ml distilled water and then centrifuged again. One more time, the pellet was re-suspended in 5 ml water, incubated overnight at 4°C with shaking and then centrifuged. The three supernatant fractions were pooled and filtered (0·45 μm sterile filter). The protein concentration was determined by amino acid analysis as described elsewhere (Barkholt & Jensen, 1989).

A soya-protein fraction enriched in glycinin was prepared using a modified procedure of Moreira *et al.* (1979). Milled soyabean flakes were suspended (100 g/l) in extraction buffer (0·4 M-NaCl, 0·035 M-KH_2PO_4, 0·01 M-β-mercaptoethanol; pH 7·6) and incubated for 1 h at room temperature. After centrifugation (30 min, 1500 ***g***), the precipitate was discarded and 70 ml supernatant fraction was dialysed against two times 1 litre of dialysis buffer (0·035 M-KH_2PO_4, 0·01 M-dithiothreitol; pH 6·6) at 4°C for 2 d. The dialysed material was centrifuged (30 min, 1500 ***g***) and the pellet, containing a glycinin-enriched precipitate, was re-suspended in 70 ml extraction buffer and stored at −20°C until use. The protein concentration was determined by absorbance measurement at 280 nm using an extinction coefficient of 8·1 (10 g/l solution; 10 mm light path). An immunoblot of the soya-protein extract and the glycinin fraction obtained by this protocol has been published elsewhere (Christensen *et al.* 2003).

Determination of Kunitz soya trypsin inhibitor in feed extracts

To prepare feed extracts for ELISA analysis, samples of 600 mg finely milled feed were extracted first with 0·1 M-NH_4HCO_3 (10 ml) for 1 h, then with 6 ml water for 5 min, and finally with 10 ml water overnight at 4°C; all times with shaking. The KSTI content in the feed extracts was determined using a sandwich ELISA based on monoclonal antibodies produced in our laboratory. Between each of the following steps, the plates were washed in ten times diluted PBS-T. Microtitre plate wells (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with anti-KSTI antibody in carbonate buffer (0·05 M, pH 9·6) and then the wells were incubated for 1 h at room temperature with KSTI standard or samples two-fold serially diluted in PBS-T. Thereafter, the wells were incubated with biotinylated anti-KSTI antibody in PBS-T followed by incubation with horseradish peroxidase-conjugated streptavidin (Dako; Glostrup, Denmark) diluted 1:5000 in PBS-T. Plates were developed by incubating with a 3,3′,5,5′-tetramethylbenzidine–peroxide solution and the reaction was stopped after 10 min by 2 M-phosphorous acid. Optical density was measured at 450 nm with 630 nm as reference. Concentrations in samples were calculated from the standard curves. The assay detection limit was 15 ng KSTI/l.

Determination of antigen-specific antibody titre by ELISA

For the detection of antigen-specific antibodies in blood samples, microtitre plate wells were coated overnight at 4°C in carbonate buffer (0·05 M; pH 9·6) with either soya-protein extract, or glycinin fraction at 5 mg protein/l, β-conglycinin, or KSTI at 1 mg protein/l, or OVA at 1 g protein/l. After washing (PBS-T diluted 1:10), 100 μl serially diluted blood sample/well (starting dilutions of 1:32 and 1:512 for blood from unprimed and primed mice, respectively) was added and the plate was incubated for 1 h at room temperature. Thereafter, the plate was

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washed and incubated 1 h further at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse Ig (1:1000 in PBS-T; Dako). Plates were developed and measured as described earlier (p. 727). Results were determined as titres, defined as the sample dilution giving an absorbance of 0·2. Positive controls from antigen-immunised mice were included in all runs.

In vitro *spleen-cell-proliferation assay*

At 1 week after the second immunisation, mice were killed by cervical dislocation. Single cell suspensions of spleens from each mouse were prepared aseptically by mechanical means and centrifuged for 10 min at 300 ***g***. Erythrocytes were removed from spleen-cell suspensions by treatment with ammonium chloride (8·3 g/l; 5 min on ice) followed by washing two times in Dulbecco's modified eagle medium (BioWhittaker Europe, Verviers, Belgium) supplemented with penicillin (100 μg/ml) and streptomycin (100 IU/ml). The cells were finally re-suspended in serum-free medium (X-vivo 10; BioWhittaker). This medium was supplemented with 2 mM-L-glutamine, 100 μg penicillin/ml and 100 IU streptomycin/ml and cultured as 4×10^5 cells/225 μl per well in quadruplicate in a ninety-six-well flat-bottomed tissue culture plate (Nunc MaxiSorp, Roskilde, Denmark) with 0 (control) or 10 μg soya-protein extract, KSTI or OVA/ml. Upon incubation at 37°C in 5 % (v/v) CO_2 for 3 d, the cells were pulsed for another 20 h with [^{3}H]thymidine (1 μCi/ml; Amersham Biosciences, Buckinghamshire, UK) and then harvested onto glass-fibre filter mats. Cell proliferation was determined by measuring [^{3}H]thymidine incorporation using liquid scintillation counting (Tri-Carp; Packard, Meriden, NJ, USA). Results were calculated as the means of controls subtracted from the means of stimulated samples.

Statistics

Data were subjected to one-way ANOVA and, if significant, the analyses were followed by Bonferroni's test to compare the three generations of mice (GraphPad, version 3.02; GraphPad Software, San Diego, CA, USA). For the study of the mice fed soya-protein extract, Bonferroni's test was performed only for pre-selected pairs: extract-fed *v.* control-fed F0; extract-fed *v.* control-fed F2 mice; extract-fed F2 *v.* control-fed F0 mice. Likewise, for the study where groups of mice were transferred from one type of feed to the other, Bonferroni's test was performed only for the pre-selected pairs: group 2–7 *v.* the control group (group 1) and combinations between group 3, 4 and 5 to test the effect of age of first exposure to soya protein-containing feed. $P<0·05$ was considered significant.

Results

Specific immune response to ingested soya protein (unprimed mice)

F0 mice ingesting soya protein were found to have a significantly elevated soya protein-specific antibody response in comparison with the offspring generations F1 and F2, fed solely the soya protein-free feed ($P<0·001$; Fig. 2 (a)). The F1 and F2 mice showed no significantly different response ($P>0·05$), indicating that soya-specific immune components were not maternally transmitted from the F0 to the F1 mice under the breeding conditions used. The response patterns of the mice at age 6, 7 and 8 weeks were not significantly different for F0 mice, while a clear increase of the unspecific background of the F1 and F2 mice was evident with increasing age (data not shown). When testing against the control antigen OVA, which was not present in any of the diets, no difference between the mice fed the soya protein-containing and the soya protein-free feed was observed (Fig. 2 (b)).

In vitro antigen-stimulated spleen-cell proliferation was also performed for the F0, F1 and F2 mice. As expected for cells from animals not systemically antigen-primed, no significant response to soya-protein extract could be detected (data not shown).

Induction of oral tolerance to ingested soya protein (primed mice)

To evaluate the presence of oral tolerance to soya proteins, antibody levels were measured in F0, F1 and F2 mice systemically primed with a mixture of soya-protein extract, KSTI, β-conglycinin, and OVA. Compared with F1 and F2 mice, the F0 mice displayed a clearly suppressed antibody response towards all of the tested soya proteins, demonstrating that oral tolerance had been established to individual proteins of the ingested soya protein ($P<0·001$; Fig. 3). However, whilst the responses to glycinin and β-conglycinin were substantially suppressed, the response to KSTI was suppressed to a lesser degree. Of note, KSTI is a minor soya protein and the content of KSTI in the soya-containing feed was determined with ELISA to be only 31 (SD 1) mg/kg, corresponding to a daily dose of 100–200 μg for the F0 mice. For all of the tested antigens, the F1 and F2 mice displayed an equally

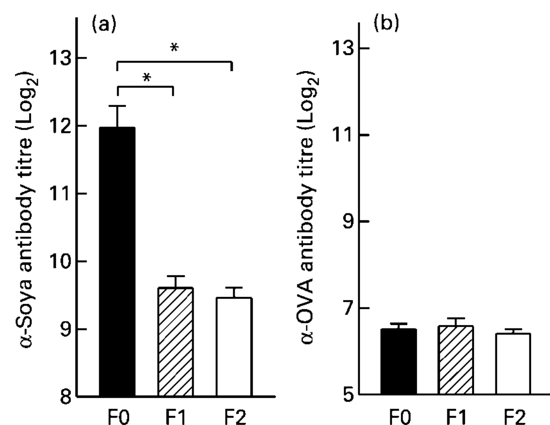

Fig. 2. Antibody response to (a) soya-protein extract or to (b) ovalbumin (OVA) control antigen (unspecific background) measured by ELISA in blood samples from unprimed F0, F1 and F2 mice at the age of 8 weeks (*n* 10–15 per group). Mean values are shown, with their standard errors represented by vertical bars. Mean values were significantly different for soya-protein extract (one-way ANOVA, Bonferroni's *post hoc* test): $^*P<0·001$. Other differences were NS ($P\geq 0·05$). For details of mice and procedures, see p. 726.

high response and thus suppression of the antibody production seemed only to be evident in F0 mice. No difference was found between the mice in their antibody response towards the control antigen OVA.

The proliferative response upon *in vitro* stimulation of spleen cells with soya-protein extract was statistically significantly suppressed in the F0 mice compared with the F2 mice ($P=0{\cdot}0094$; Fig. 4), but not compared with the F1 mice ($P=0{\cdot}098$). Repeatedly, we observed that the response of the F1 mice deviated from that of both F0 and F2 mice. The difference, however, did not reach a statistically significant level (F1 *v.* F2; $P=0{\cdot}15$). The same pattern was seen for the cellular response to KSTI stimulation; F0 mice were significantly suppressed only in comparison with F2 mice ($P=0{\cdot}024$). No difference occurred between the responses to the control antigen OVA.

The efficacy to induce tolerance of soya-protein extract supplied via the drinking water *v.* the soya protein in the feed was also examined because experimental animal models used to study food antigens are often based on the induction of oral tolerance by supplementing mice for a short time with the antigen via the drinking water. Soya-protein extract (10 mg) ingested via the drinking water was shown to be weak in suppressing the soya protein-specific antibody response of F2 mice ($P=0{\cdot}039$; Fig. 5 (a)). This in fact was much less efficient than soya protein ingested throughout life via the soya protein-containing diet by the F0 mice (response of soya-protein extract-fed F2 *v.* F0, $P<0{\cdot}001$; Fig. 5 (a)). The same pattern was evident when testing for the antibody responses to glycinin and β-conglycinin (data not shown). The cell-proliferation response was, however, efficiently suppressed in F2 mice fed soya-protein extract and was not significantly different from that of F0 mice (Fig. 5 (b)). Feeding soya-protein extract did not significantly affect the prior induced suppression of the soya protein-specific response of F0 mice. In contrast, the KSTI-specific antibody and cell-proliferation response of the soya-protein extract-fed mice (Figs. 5 (c) and (d), respectively) was very efficiently suppressed in F2 mice and, here, the existent suppression of the F0 mice was markedly enhanced reaching the same low level as that of fed F2 mice.

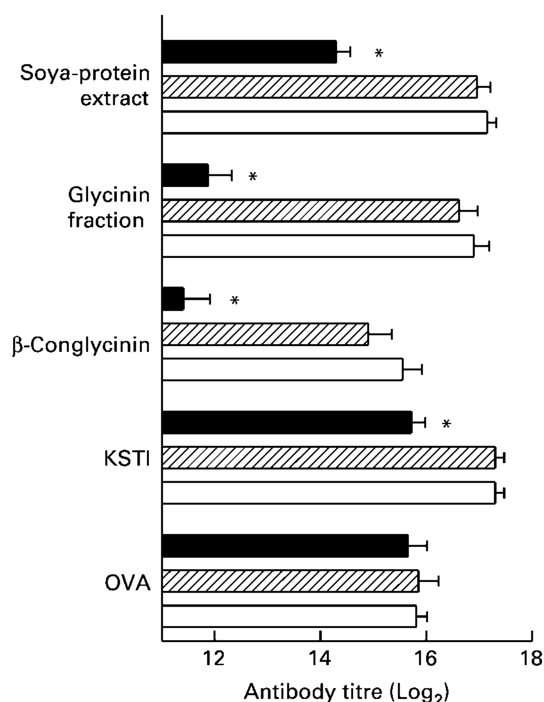

Fig. 3. Antibody response to various soya proteins and ovalbumin (OVA) control antigen in blood samples from F0 (■), F1 (▨) and F2 (□) mice immunised twice at the ages of 8 and 10 weeks, respectively, with a mixture of soya-protein extract, Kunitz soya trypsin inhibitor (KSTI), β-conglycinin and OVA (*n* 8–10 per group). Mean values are shown, with their standard errors represented by horizontal bars. Within each of the proteins, soya-protein extract, glycinin, β-conglycinin and KSTI, mean values were significantly different when compared with both F1 and F2 mice (one-way ANOVA, Bonferroni's *post hoc* test): $^*P<0{\cdot}001$. Other differences were NS ($P\geqq0{\cdot}05$). For details of mice and procedures, see p. 726.

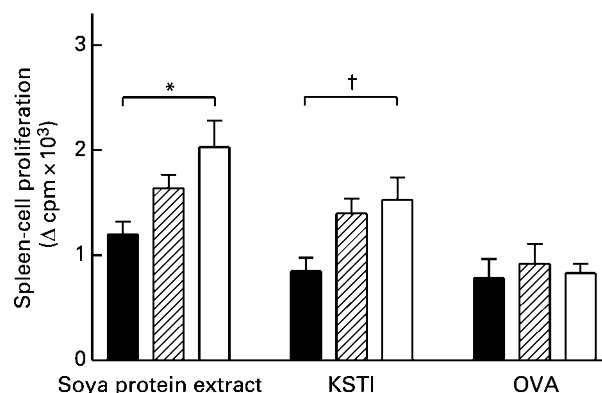

Fig. 4. Spleen-cell proliferation of F0 (■), F1 (▨) and F2 (□) mice immunised twice at the ages of 8 and 10 weeks, respectively, with a mixture of soya-protein extract, Kunitz soya trypsin inhibitor (KSTI), β-conglycinin and ovalbumin (OVA) (*n* 8–10 per group). Cells were stimulated *in vitro* either with PBS (background), soya-protein extract, KSTI or OVA (10 μg protein/ml) for 3 d before pulsing for 20 h with [^{3}H]thymidine. Mean values in delta counts/min (Δ cpm) are shown, with their standard errors represented by vertical bars. Mean values were significantly different for soya-protein extract and for KSTI stimulation (one-way ANOVA, Bonferroni's *post hoc* test): $^*P=0{\cdot}0094$, $^\dagger P=0{\cdot}024$. Other differences were NS ($P\geq0{\cdot}05$). For details of mice and procedures, see p. 726.

The effect of age of the first exposure to soya protein

We furthermore evaluated the importance of the age at which soya protein is ingested for the first time on the induction of antibody production and oral tolerance. To this end, groups of mice were transferred at different ages between soya protein-free and soya-containing feed as outlined in Fig. 1 (a). When F2 mice were transferred to the soya protein-containing feed either 1 d before birth (transfer of pregnant F1 mice), 1 week after birth or at weaning (groups 3–5, respectively), all mice generated a significant antibody response and induced oral tolerance as seen for F0 mice (Figs. 1 (b) and (c)). The antibody response tended, however, to be weaker when soya protein was not introduced in very early life (group 3 *v.* 4, $P=0{\cdot}0098$; group 3 *v.* 5, $P=0{\cdot}021$; group 4 *v.* 5, $P>0{\cdot}05$). Interestingly, when F2 mice were exposed to soya-protein feed from 1 week before birth (pregnant F1 mice) and until weaning (group 7), and thus exposed only indirectly through the dam and its milk, the mice showed an antibody response in adulthood, which, however, appeared in the absence of oral tolerance (Figs. 1 (b) and (c)).

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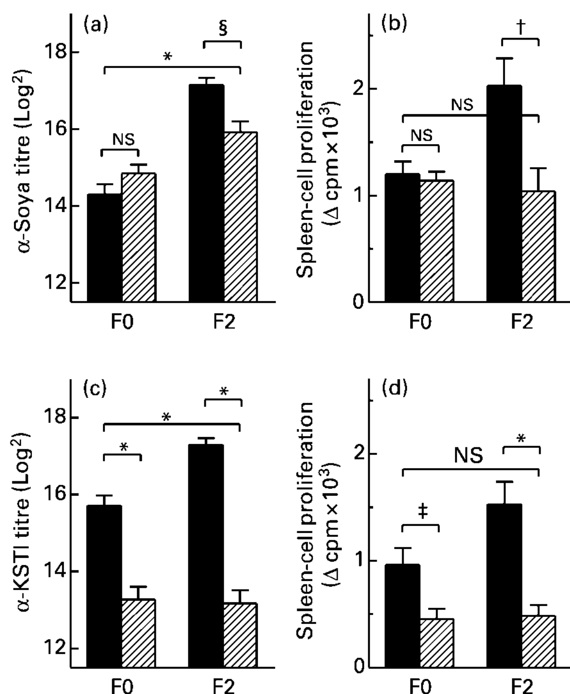

Fig. 5. Serum antibody response to (a) soya-protein extract or to (c) Kunitz soya trypsin inhibitor (KSTI), and spleen-cell-proliferation response to *in vitro* stimulation with (b) soya-protein extract or with (d) KSTI of F0 and F2 mice fed via the drinking water with either pure water (■) (control) or a mixture of soya-protein extract and KSTI (▨) for 5 d beginning at the age of 8 weeks. Mice were subsequently immunised twice 2 weeks apart with a mixture of soya-protein extract, KSTI, β-conglycinin and ovalbumin (OVA) (*n* 8–10 per group). Blood samples were analysed by ELISA. Spleen cells were stimulated *in vitro* with either PBS (background), soya-protein extract or KSTI (10 μg protein/ml) for 3 d before pulsing for 20 h with [^{3}H]thymidine, and the results represent delta counts/min (Δ cpm). For all results, mean values are shown, with their standard errors represented by vertical bars. Mean values were significantly different (one-way ANOVA with Bonferroni's *post hoc* test of selected pairs: extract-fed *v.* control-fed F0 mice, extract-fed *v.* control-fed F2 mice and extract-fed F2 *v.* control-fed F0 mice): $^*P<0.001$, †$P=0.0076$, ‡$P=0.037$, §$P=0.039$. Other differences were NS ($P\geq0.05$). For details of mice and procedures, see p. 726.

Offspring from F0 mice transferred to soya protein-free feed on the day of delivery (group 6) did not develop a significant antibody response (although a fraction of the mice developed a weak antibody response) and neither did the offspring exhibit tolerance induction. The response to pure KSTI-immunised mice in general resembled closely the anti-soya protein response (data not shown).

Discussion

Based on comparison with two offspring generations of mice bred on a soya protein-free diet (F1 and F2), we found that F0 mice, which were raised on the feed containing soya protein, generated a significant soya protein-specific antibody response. When immunising the animals, we found that, despite this active antibody production, F0 mice, in contrast to F1 and F2 mice, had clearly established oral tolerance to the soya protein observed as both a suppressed antibody and cellular response.

The immunological outcome of ingesting antigens can range from the induction of tolerance, the induction of systemic priming to the induction of a mucosal secretory response; the two former responses generally considered to be mutually exclusive (Strobel & Mowat, 1998). However, as observed for both human volunteers (Husby, 2000) and animals (Coombs *et al.* 1983), dietary antigens may in certain cases induce a comparatively weak antibody response in the absence of signs of true systemic priming. Compatible with this, the present study shows that the ingestion of soya protein by mice induces an antibody response along with oral tolerance and that these responses occur as a seemingly normal event against ingested soya protein. In line with these results, Bailey *et al.* (1993) found that feeding piglets soya protein from weaning resulted in an active antibody response but also the development of tolerance. Moreover, healthy human subjects have been found to have soya-specific antibodies (Hvatum *et al.* 1992), although such data suffer from the lack of proper controls (soya-unexposed individuals) to fully rule out the possibility that such response is due to cross-reacting antibodies generated against other antigens (gut flora, infections, etc). In a further study, we investigated the antigenic specificity of the soya-reactive antibodies (Christensen *et al.* 2003). We found that the antibodies were mostly reactive to glycinin and β-conglycinin through which we obtained direct evidence that an antibody response and oral tolerance towards an ingested antigen can co-exist.

Antibody titres to dietary antigens have been reported to decline with age, reaching negligible levels in human adults (Ahmed *et al.* 1997; Husby, 2000). The present results showed that F0 mice, within the age range of 6 weeks (premature) to 8 weeks (mature), exhibited an unchanged antibody response magnitude. It would, however, be of interest also to measure the response magnitude at later ages. Instead, we found that the age of introducing for the first time soya protein in the feed influenced the response magnitude; the introduction of soya protein to F1 dams from the day before delivery resulted in a higher antibody response in the F2 offspring than when postponing the switch to the soya-protein feed until 1 week after birth. This observation could relate to a higher uptake of maternal milk-derived soya antigen by the immature gut of newborn F2 mice, perhaps in combination with a comparatively high amount of antigen in the colostral milk of the F1 dams. This could be due to a lack of antigen-eliminating antibodies, as the dams were naive to soya protein by the time of feeding the soya-protein feed (Wilson *et al.* 1989).

The two generations of mice raised on the soya protein-free feed were bred not only to provide a negative reference, but also to study the occurrence of maternal transmission of soya-specific immune components. As the F2 mice are considered fully naive to soya protein, the fact that the ELISA response level of both unprimed and primed F1 mice equalled that of the F2 mice suggests that the spontaneously induced soya-specific antibody response and the oral tolerance in the F0 mice was not transmitted at a detectable level to the offspring. Knippels *et al.* (1998*a*) found that the ingestion of soya protein by rats induced a soya-specific antibody response. In contrast to the present results, however, they found soya-specific antibodies to be present also

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in the first offspring generation bred on a soya protein-free diet. The discrepancy between their data and the present data could be linked to the use by Knippels *et al.* (1998*a*) of Brown Norway rats, which are Th2-skewed high antibody-responder rats easily sensitised to food antigens (Knippels *et al.* 1998*b*). In contrast to conventional animals, Brown Norway rats might be easily primed by antigens or specific immune cells transmitted maternally. Maternal transmission either transplacentally or through the milk has been found to occur in other experimental animals (Arvola *et al.* 2000; Szepfalusi *et al.* 2000*a*,*b*; Hanson *et al.* 2003). Therefore, despite the present study's observation of a lack of detectable differences between the F1 and F2 mice, we cannot exclude the possibility that the F1 generation might be affected in a manner not seen in our experimental setting due to insufficient methods for detection. Supportive of this possibility is the observation that the cell-proliferation response of the immunised F1 mice tended, although not statistically significant, to deviate from that of both the F0 and the F2 mice. Accordingly, the F1 mice might still bear soya-specific immune components rendering these animals inappropriate for studying sensitisation properties of soya protein, and from a more experimental point of view, breeding for at least two generations using a diet free of soya protein is therefore to be recommended.

In the work with maternal transmission of specific immune components, we furthermore found that when pregnant F1 mice were fed soya protein during late gestation and throughout lactation, the F2 offspring exhibited at the age of 8 weeks an antibody response, which occurred in the absence of oral tolerance. Arvola *et al.* (2000) and others have demonstrated in mice that long-lived IgG-secreting plasma cells can be spontaneously transmitted from mother to offspring via milk. Hence, one plausible mechanism to explain the present findings is that upon switching to soya-protein feed, F1 animals induce soya-specific antibody-secreting B cells that are transmitted to the F2 offspring and there generate an antibody response in the absence of oral tolerance induction. On the other hand, pups begin at an early age to nibble on the feed provided for the dams during lactation and maternally ingested antigens are found to be secreted in the milk. Hence another possibility is that such ingestion of low amounts of soya protein might prime the offspring for antibody production and potentially also for oral tolerance induction. The latter statement relies on very recent studies, where we found that neonatally induced oral tolerance is detectable at the age of 6 weeks but not 8 weeks if the antigen is not present in the post-weaning diet (S Brix, HR Christensen, V Barkholt and H Frøkiaer, unpublished results), as is the case in the present study, where the F2 offspring were weaned onto soya protein-free feed.

As already mentioned, a similar transmission of components inducing a soya-specific response from F0 mice to F1 offspring seemed not to take place, as the F1 and F2 mice showed indistinguishable ELISA responses. The F0 breeding mice were transferred to soya protein-free feed from 14 d before mating, which might prevent the maternal transmission of antigen. In agreement with this is the observation that when the transfer of F0 mice to the soya protein-free feed is postponed until the day of delivery (Fig. 1; group 6), a fraction of the offspring (three out of nine) showed a weak antibody response. However, the mean of the group as a whole was not statistically significant from that of the F2 mice. In summary, the mode of exposure to dietary soya protein affects the transmission of specific immune components to the offspring.

In general for studies of oral tolerance induction to dietary antigens, the protocols involve supplementation for 1 to 5 d, via the drinking water or by oral administration, of a solution of milligram amounts (1–20 mg/feeding) of the protein (Gregerson *et al.* 1993; Friedman & Weiner, 1994; Faria *et al.* 2003). Thus, the capacity of soluble soya proteins to induce oral tolerance using a common feeding regimen was evaluated in the present study. The administration of 10 mg soya-protein extract/d for 5 d in the drinking water was found to be relatively weak in suppressing the soya-protein extract-specific antibody response and, in fact, significantly less efficient than the soya-protein feed. Conversely, the cell-proliferation response was equally effectively suppressed by the soya-protein extract in the drinking water and the soya-protein feed. The same picture was evident when looking at the individual antibody responses to the major soya-protein components glycinin and β-conglycinin. Previously, we have shown that our soya-protein extract consists mainly of glycinin and β-conglycinin and, thus, these proteins are provided in milligram amounts by feeding soya-protein extract (Christensen *et al.* 2003). Importantly, long-term feeding of antigen has been reported to suppress the antibody response more effectively than single feedings, whereas the suppression of the cellular response remains the same (Faria *et al.* 2003). In terms of oral tolerance induction, the amounts ingested by the soya protein feed-fed (approximately 500 mg/d) *v.* extract-fed (10 mg/d) are both to be considered as high-dose exposure (Strobel & Mowat, 1998) Thus, the duration of feeding, more than the different amounts of ingested soya protein, probably explains the present observation that the soya-protein extract was less efficient in suppressing the antibody response than soya protein ingested via the feed since the cell-proliferation response was equally suppressed.

Quite the reverse applied to KSTI; supplementation in the drinking water effectively suppressed both the antibody and the cell-proliferation response of F2 mice and, in fact, also enhanced the suppression of the responses of F0 mice, demonstrating that F0 mice ingesting the soya-containing feed have not established full suppression of the KSTI-specific response. F0 mice fed the soya-protein feed ingest only a small amount of KSTI through their feed (approximately 100–200 μg/d). Other studies of ours showed that for long-term administration of the egg protein ovomucoid, the efficacy of tolerance induction changed markedly in the interval of 10 to 1000 μg/d with enhanced suppression for higher doses of antigen (Kjaer & Frokiaer, 2002). It is thus indicated that the KSTI alike ovomucoid can induce variable degrees of oral tolerance depending on dose (and time), with a threshold below 100 μg/d, and that an already-established suppression can be enhanced by feeding higher doses.

In conclusion, soya protein ingested by healthy mice is recognised and responded to by the immune system

through a spontaneous antibody response coinciding with the induction of oral tolerance. Understanding the immune responses taking place toward soya ingested under healthy conditions is important in the assessment of adverse effects of soya protein and for the appropriate use of animal allergy models.

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